Abstracts of papers presented at the
LX Cold Spring Harbor Symposium
on Quantitative Biology

PROTEIN KINESIS: The Dynamics of Protein Trafficking and Stability

May 31–June 7, 1995

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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PROTEIN KINESIS: The Dynamics of Protein Trafficking and Stability

May 31–June 7, 1995

Arranged by
Bruce Stillman, *Cold Spring Harbor Laboratory*

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

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PROGRAM

WEDNESDAY, May 31—7:30 PM

Welcoming Remarks: Bruce Stillman

SESSION 1 INTRODUCTION

Chairperson: **D. Sabatini,** New York University School of Medicine

THURSDAY, June 1—9:00 AM

SESSION 2 PROTEIN FOLDING AND MODIFICATION IN THE ER

Chairperson: **H. Pelham,** Medical Research Council, Cambridge, United Kingdom

THURSDAY, June 1 2:00—4:00 PM

SESSION 3 POSTER SESSION

THURSDAY, June 1 4:30—5:30 PM

Wine and Cheese Party

THURSDAY, June 1—7:30 PM

Reginald G. Harris Lecture

THURSDAY, June 1—8:15 PM

SESSION 4 PROTEIN TRAFFICKING

Chairperson: **A. Helenius,** Yale University School of Medicine

FRIDAY, June 2 3:00—5:00 PM

SESSION 6 POSTER SESSION

FRIDAY, June 2—7:30 PM

SESSION 7 LATER STAGES OF PROTEIN TRAFFICKING

Chairperson: **T. Südhof,** University of Texas Southwestern Medical Center

SATURDAY, June 3—9:00 AM

SESSION 8 PROTEIN TRANSLOCATION AND FOLDING

Chairperson: **S. Gottesman,** NCI, National Institutes of Health

SATURDAY, June 3—3:00 PM

PICNIC

SATURDAY, June 3—7:30 PM

SESSION 9 PROTEIN DEGRADATION

Chairperson: **M.-J. Gething,** University of Melbourne, Australia

SUNDAY, June 4—9:00 AM

SESSION 10 PROTEIN TRAFFICKING AT THE CELL SURFACE

Chairperson: **M. Farquhar,** University of California, San Diego

SUNDAY, June 4—2:00 PM

SESSION 11 PEPTIDE AND PROTEIN TRANSLOCATION

Chairperson: I. Mellman, Yale University School of Medicine

SUNDAY, June 4—5:00 PM

DORCAS CUMMINGS LECTURE

Gunter Blobel
Howard Hughes Medical Institute,
Rockefeller University

"How Proteins Find Their Addresses in the Cell"

SESSION 12 POLARITY

Chairperson: **J. White,** University of Virginia, Charlottesville

SESSION 13 POSTER SESSION

MONDAY, June 5—7:30 PM

SESSION 14 NUCLEAR TRAFFICKING

Chairperson: **G. Schatz,** Biozentrum, University of Basel, Switzerland

TUESDAY, June 6—9:00 AM

SESSION 15 MEMBRANE DYNAMICS

Chairperson: **S.L. Schmid,** Scripps Research Institute, La Jolla, California

TUESDAY, June 6—2:30 PM

SESSION 16 PROTEIN IMPORT INTO ORGANELLES

Chairperson: **J. Beckwith,** Harvard Medical School

TUESDAY, June 6

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

WEDNESDAY, June 7—9:00 AM

SESSION 17 MOLECULAR MOTORS

Chairperson: B. Stillman, Cold Spring Harbor Laboratory

Summary: George Palade, University of California, San Diego

AUTHOR INDEX

PROTEIN TRANSLOCATION ACROSS CELLULAR MEMBRANES

Günter Blobel, Laboratory of Cell Biology, Howard Hughes Medical Institute, Rockefeller University, New York, N. Y. 10021

Most of the protein translocation systems of cellular membranes operate in a unidirectional mode: proteins are translocated from the "cis" side to the "trans" side of the membrane. The endoplasmic reticulum's protein translocation system is among the best understood of these unidirectional protein translocation systems and in many ways paradigmatic. A signal sequence of the protein to be translocated is recognized by a soluble factor on the cis side of the membrane. This soluble factor also functions in targeting to a cognate receptor on the cis side of the membrane. After its dissociation from the soluble factor the signal sequence then serves as a ligand to open a cognate protein conducting channel to an estimated diameter of ~ 2 nm. For partial translocation (integral membrane protein) a second topogenic sequence, referred to as stop transfer sequence can open the channel laterally, in the plane of the membrane. Translocation to the trans side is likely to proceed by a thermal ratchet mechanism.

In contrast, translocation of proteins across the nuclear pore complex (NPC) of the nuclear envelope operates bidirectionally, with the nuclear localization sequence (NLS) apparently able to specify translocation into or out of the nucleus. The apparent diameter of the NPC opening is ~ 25 nm. Besides proteins, RNPs and DNPs are transportable, in either direction. The NLS is recognized and docked to the NPC by a two subunit complex, referred to as karyopherin (the alpha subunit recognizes the NLS, the beta subunit docks to the NPC). Docking is at multiple sites on both the nucleoplasmic and cytoplasmic sides of the NPC. The repetitive peptide domain of several nucleoporins serve as docking sites. The small GTPase Ran and several Ran interactive proteins are proposed to function as molecular switches. Via multiple rounds of docking and release they allow transport across the NPC by guided diffusion.

MOLECULAR MECHANISMS OF INTRACELLULAR PROTEIN TRANSPORT

James E. Rothman

Cellular Biochemistry and Biophysics Program
Memorial Sloan-Kettering Cancer Center, 1275 York Avenue
New York, New York, 10021.

Reconstitution of intracellular protein transport in cell-free systems has led to insights into the mechanisms responsible for vectorial transport by carrier vesicles. Transport between Golgi cisterna is mediated by COP-coated vesicles whose principal subunits are a complex of Coatomers and a small GTP-binding protein, ADP-Ribosylation Factor (ARF). Coatomer is composed of seven distinct subunits. The concomitant budding of the transport vesicle occurs in a series of steps. GTP-dependent binding of ARF to the membrane in a nucleotide exchange reaction is followed by binding of coatomer and assembly into buds. Fatty-acyl-CoA is required for the buds to pinch off. Coated vesicles then uncoat when the ARF-bound GTP is hydrolyzed. Uncoating exposes a vesicle-born targetting molecule (termed v-SNARE) that docks the vesicle as it pairs with its cognate t-SNARE at the target membrane. The general fusion machinery, involving NSF and SNAP proteins, then assembles on the SNARE complex and fusion is initiated upon ATP hydrolysis by NSF. This mechanism is of great generality, as yeast temperature-sensitive mutants defective in transport and fusion encode homologues of coatomer and fusion components discovered in animal cells, and similar components operate in neurons at synapses.

INTRACELLULAR TRANSPORT OF MHC MOLECULES AND PEPTIDES.

Michael R. Jackson, Klaus Früh, Lars Karlsson, Luc Teyton, Young Yang and Per A. Peterson, The R.W. Johnson Pharmaceutical Research Institute, San Diego, CA 92121

Due to the different life-styles of pathogens the immune system has evolved molecules, which present peptides to T-cells both from intra- and extracellular organisms. The MHC class I molecules acquire peptides from proteins synthesized by the cell and display such peptides at the cell surface. In contrast, MHC class II molecules obtain peptides derived from proteins internalized by cells.

Peptides acquired by class I molecules are mainly generated by the proteasome, a cytosolic multicatalytic proteinase. Peptides consisting of 8 to 10 amino acid residues are transported into the endoplasmic reticulum (ER), where nascent class I molecules associated with a heterodimeric transporter protein bind peptides with certain sequence characteristics. The peptide-binding releases the class I molecules from the transporter protein such that they can emerge on the cell surface.

Class II molecules associate with the invariant chain (Ii) in the ER. Ii has two functions. First, it prevents class II molecules from binding nascent proteins in the ER, which have not yet completed their folding. Second, Ii contains sequence motifs which brings it and associated class II molecules to a subcompartment of the endosomal system. A highly specialized class II molecule, H-2M, reaches the same compartment without assistance from Ii, H-2M facilitates peptide loading onto conventional class II molecules by a process which is as yet poorly understood.

SINGLE MYOSIN MOLECULE MECHANICS:
HOW MOLECULAR MOTORS WORK

James A. Spudich, Dept of Biochemistry, Stanford University

Our research interest is the molecular basis of molecular motor function and cell motility. Three specific foci are, the molecular basis of energy transduction that leads to ATP-driven myosin movement on actin, the biochemical basis of the regulation of actin and myosin interaction and their assembly states, and the roles these proteins play *in vivo,* in cell movement and changes in cell shape. We use two experimental systems. Skeletal muscle has the most highly organized contractile apparatus of any cell type, and the chemistry, biochemistry and structure of muscle actin and myosin are well characterized. *Dictyostelium* exhibits all of the behavior of nonmuscle mammalian cells and, unlike many other eukaryotic cells, can be grown in large amounts for biochemical work. Furthermore, we discovered efficient gene targeting by homologous recombination in this organism and first applied this approach to the myosin gene and thereby created myosin null strains. These experiments provided genetic proof that this myosin participates in cytokinesis, development and directed cell movement. Many mutagenized forms of this myosin have been constructed and characterized biochemically after expression in *Dictyostelium* as a host. In addition, we probe the effects of each altered myosin form on the phenotype of the cell. To understand the mechano-chemical coupling of myosin, we have designed a number of *in vitro* assays for ATP-dependent movement of purified myosin on filaments reconstituted from purified actin. These assays allow us to examine mutant myosin molecules for altered function. We have recently developed laser trap technology to measure unitary displacements and unitary forces generated by single myosin molecules pulling on a single actin filament. All of these approaches are being combined with crystallographic approaches to disclose the relationship between the enzyme's structure and its function both at the atomic and cellular levels.

THE PROTEIN-CONDUCTING CHANNEL IN THE MEMBRANE OF THE ENDOPLASMIC RETICULUM IS OPEN LATERALLY TOWARD THE LIPID BILAYER

Martoglio B., Hofmann M.W., Brunner* J., and Dobberstein B.
ZMBH, Heidelberg, D-69120; *ETH, Zürich, CH-8092

Entry of a nascent polypeptide into the membrane of the endoplasmic reticulum involves the formation of a loop structure, with the N-terminus remaining in the cytoplasm and the growing C-terminal part being continuously translocated across the membrane. Lipids and proteins were found to contact a nascent type II membrane protein, as well as a nascent secretory protein, during their insertion into the membrane of the endoplasmic reticulum. This suggests that the protein-conducting channel is open laterally toward the lipid bilayer during an early stage of protein insertion. Using site-specific photocrosslinking, we found that contact with lipids was confined to the hydrophobic core region of the respective signal or signal anchor sequence. Thus the nascent polypeptide is positioned in the translocation complex such that the signal or signal anchor sequence faces the lipid bilayer, whereas the hydrophilic, translocating portion is in proteinaceous environment.

NASCENT-POLYPEPTIDE ASSOCIATED COMPLEX (NAC): A BRIDGE BETWEEN RIBOSOME AND CYTOSOL

Lauring, B.*, Wang, S., Sakai, H., Davis, T. A., Wiedmann, B., Kreibich, G.*, and Wiedmann, M.
Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021; * Department of Cell Biology, New York University School of Medicine, New York, NY 10016

After synthesis, polypeptides have to be folded correctly and then many of them must be transported to specific organelles or secreted from the cell. The first steps in these processes can occur during translation.

While comparably much is understood about the sorting and folding machineries, little is known about proteins which interact with growing polypeptide-chains near the peptidyl transferase center on the ribosome.

We searched for novel proteins which interact exclusively with nascent-chains, because by acting at the interface of ribosome and cytosol, they may functionally connect the nascent-chain with proteins involved in folding and/or translocation. We have purified such proteins: Nascent-polypeptide Associated Complex (NAC). We show *in vitro* that removal of cytosolic factors such as NAC from nascent-chains results in several inappropriate protein-protein interactions of the growing polypeptides (accessibility to proteases, mistargeting to and mistranslocation into the Endoplasmic Reticulum). Because NAC restores specificity to these interactions, we believe that NAC may serve a general function to ensure the fidelity of protein-protein interactions at the ribosome-cytosol interface.

INTEGRATION OF MEMBRANE PROTEINS INTO THE ER

Angela Borel*, Martin Friedlander+ and Sanford Simon*
* Lab of cellular biophysics, Rockefeller University
+ Scripps Research Institute

The biogenesis of integral membrane proteins starts on ribosomes bound to the endoplasmic reticulum (ER) using much of the same machinery utilized to translocate secretory proteins across the ER membrane. We have characterized the biogenesis of two different membrane proteins: P-glycoprotein, which has its amino terminal on the cytosolic side, and opsin, which has its amino terminal in the ER lumen. Our studies examine the involvement of transmembrane segments in targeting to the ER, translocation across the membrane, interactions with protein-conducting channels and integration into the bilayer. Translocation does not initiate until at least half of the first transmembrane segment has emerged from the ribosome. Each transmembrane segment sequentially translocates across the membrane as it emerges from the ribosome. However, none immediately emerge from the protein-conducting channels and integrate into the bilayer. These observations suggest transmembrane domains are stabilized across the membrane until protein-synthesis is complete and the ribosomes dissociate from the membrane, allowing the protein-conducting channels to close. We have started to characterize some of the mutations in opsin that are found in retinitis pigmentosa and observed that they affect the kinetics of opsin biogenesis in the ER.

THE ENVIRONMENT OF NASCENT SECRETORY AND MEMBRANE PROTEINS AT THE ER MEMBRANE DURING TRANSLOCATION AND INTEGRATION.

Arthur E. Johnson, Shuren Liao, Jialing Lin, Hung Do, Brian Hamman, and David W. Andrews,† Texas A&M University Health Science Center, College Station, TX, and †McMaster University, Hamilton, Ontario, Canada.

The environment of a nascent secretory protein during translocation through, or of a nascent membrane protein during integration into, the membrane of the endoplasmic reticulum (ER) has been examined directly by a novel spectroscopic approach. Fluorescent probes have been incorporated into the nascent polypeptides by *in vitro* translation of various full-length or truncated mRNAs in the presence of εNBD-Lys-tRNA, an analog of Lys-tRNA that has an NBD dye covalently attached to the side-chain amino group of the lysine. Since the NBD fluorescence lifetime is short (~ 1 nsec) in an aqueous medium and long (~ 8 nsec) in the hydrophobic interior of the bilayer, the polarity of the environment of a probe in a nascent chain can be determined directly by analyzing the fluorescence signal. Probes positioned in a secretory protein traversing the ER bilayer each had a short lifetime, thereby indicating that secretory proteins occupy an aqueous pore that spans the entire phospholipid bilayer during translocation. This topography was confirmed when each probe in the bilayer was found to be collisionally quenched by iodide ions from the lumenal side of the ER membrane (iodide ions cannot pass through the nonpolar core of the membrane). The selection and movement of a nonpolar transmembrane sequence of a nascent membrane protein out of the aqueous pore and into the hydrophobic bilayer has now been examined by constructing a single-spanning membrane protein that contains only a single lysine codon in the middle of the transmembrane segment. The insertion of this stop-transfer segment into a nonpolar environment is detected by a substantial increase in the NBD fluorescence lifetime.

GLYCOPROTEIN FOLDING IN THE ER. *A. Helenius*, D. Hebert, U. Tatu, J. Simons, J. Peterson, Wei Chen, B. Foellmer, A. Ora. *Dept. of Cell Biology, Yale School of Medicine*, New Haven 06510, Ct.

Our studies in live tissue culture cells, in *S. cerevisiae* and in isolated microsomes focus on the folding, oligomeric assembly and quality control processes that glycoproteins undergo in the ER. For most of them, the rate and efficiency of folding and oligomeric assembly determines how efficiently, how fast, and in which form they reach their target organelles or are secreted.

We have shown that two homologous ER chaperones, **calnexin** (a membrane protein) and **calreticulin** (a soluble lumenal protein) bind to growing nascent chains and to newly synthesized glycoproteins. Both are lectins with specificity for **monoglucosylated core oligosaccharide** moieties that occur as intermediates in the glycan trimming. Although the ligand specificity's of the two lectins are identical, the protein substrates are overlapping but nonidentical suggesting that other factors such as glycan number and position may influence selectivity. The two lectins constitute part of a sophisticated machinery that links the trimming of the N-linked oligosaccharides functionally to the conformational maturation of glycoproteins, thus explaining many of the processing events that take place in the ER. Other components of the pathway include **glucosidases I and II** which remove three glucoses from the core oligosaccharides, and a **UDP-glucose:glycoprotein glucosyl transferase.** The glucosidases are necessary both for binding and release of substrates from the lectins, and the transferase serves as a folding sensor.

Using influenza hemagglutinin and vesicular stomatitis virus G protein as models, we have demonstrated that calnexin/calreticulin increases the efficiency but decreases the rate of folding, delays oligomerization, prevents degradation and blocks premature ER to Golgi transport. Its main function is to increase the efficiency and fidelity of glucoprotein expression. Nevertheless, the pathway is dispensable for many glycoproteins, and necessary only when other chaperones, such as BiP, are compromised.

THE YEAST OLIGOSACCHARYLTRANSFERASE: ANALYSIS OF *OST1*, *OST2* AND *OST3* MUTANTS

Susana Silberstein, Denise Karaoglu, Paula Collins, Peter Rapiejko, Dan Kelleher and Reid Gilmore
Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School; Worcester, MA 01655

The oligosaccharyltransferase (OST) catalyzes the transfer of high mannose oligosaccharide from a dolichol-linked donor (dolichol-P-P-$GlcNAc_2Man_9Glc_3$) to glycosylation acceptor sites (NXS/T) in newly translocated polypeptides within the lumen of the rough endoplasmic reticulum. For many glycoproteins, N-linked glycosylation is a prerequisite for the folding and oligomeric assembly reactions that occur in the lumen of the endoplasmic reticulum. The oligosaccharyltransferase from *Saccharomyces cerevisiae* has now been purified as a protein complex composed of six nonidentical subunits (α–ζ). The β and δ subunits of the yeast OST complex (Wbp1p and Swp1p) were initially identified by genetic methods by Marcus Aebi and colleagues, and were found to be homologous to the OST48 and ribophorin II subunits of the mammalian oligosaccharyltransferase.

Yeast genes (*OST1*, *OST2* and *OST3*) that encode the α, γ and ε subunits of the OST complex have now been isolated and sequenced. The protein encoded by the *OST1* gene is 28% identical to ribophorin I, a subunit of the mammalian oligosaccharyltransferase. Disruption of the *OST1* gene is lethal in haploid yeast. Temperature sensitive *ost1* mutants underglycosylate proteins in vivo and show reduced in vitro transfer of high mannose oligosaccharides to an acceptor tripeptide substrate.

The *OST2* gene encodes a 130 residue integral membrane protein. Recently, homologues of the Ost2 protein have been identified in several vertebrate organisms. The *OST2* gene is an essential yeast gene. Temperature sensitive *ost2* mutants show in vivo and in vitro defects in N-linked glycosylation. Overexpression of the Ost2 protein will rescue the temperature sensitive *wbp1-2* mutant. Phenotypic rescue of *wbp1-2* yeast by overexpression of Ost2p is accompanied by enhanced stability of the Wbp1p and Swp1p subunits of the OST complex, and increased in vivo and in vitro oligosaccharyltransferase activity.

The *OST3* gene encodes a 34 kD integral membrane protein. Unlike the *OST1*, *OST2*, *WBP1* and *SWP1* genes, disruption of the *OST3* gene is not lethal. However, yeast lacking the Ost3 protein show mild underglycosylation of several soluble yeast glycoproteins. Surprisingly, more severe glycosylation defects were apparent for several integral membrane proteins. These observations suggest that the Ost3 protein may influence acceptor site utilization.

A VIRAL INHIBITOR OF PEPTIDE TRANSPORTERS FOR ANTIGEN PRESENTATION

Kwangseog Ahn, Klaus Früh, Hakim Djaballah, Pascal Sempé, Per A. Peterson, and Young Yang
The R. W. Johnson Pharmaceutical Research Institute at The Scripps Research Institute, Department of Immunology, 10666 North Torrey Pines Road, La Jolla, CA, 92037, USA

Cytotoxic T-lymphocytes lyse target cells after T cell receptor-mediated recognition of class I major histocompatibility complex molecules presenting peptides. Antigenic peptides are generated in the cytoplasm by proteasomes and translocated into the lumen of the endoplasmic reticulum (ER) by peptide transporters (TAP). Herpes simplex virus express a cytoplasmic protein, ICP47, which seems to interfere with such immune surveillance by mediating retention of class I molecules in the ER. By expressing ICP47 in HeLa cells under the control of an inducible promoter, we show that ICP47 efficiently inhibits peptide transport across the ER membrane such that nascent class I molecules fail to acquire antigenic peptides. This interference was overcome by transfecting murine TAP. Furthermore, we demonstrate that ICP47 colocalizes and physically associates with TAP within the cell. Possible sites of interference might be ATP-binding or hydrolysis. Alternatively ICP47 might interfere with peptide binding or translocation as well as yet to be defined TAP functions. Inhibition of TAP-dependent peptide translocation by a viral protein indicates a previously undocumented mechanism for viral immune evasion. We are currently investigating which step is specifically interfered by ICP47.

Small synaptic vesicle-analogues in neuroendocrine cells: functional and biochemical analysis using mammalian tumor cell lines.

Gudrun Ahnert-Hilger, Adele Stadtbäumer, M. John, Rostislav Bychkov, Hans Scherübl, Gaby von Blankenfeld#, H. Kettenmann#, Ernst-Otto Riecken, Bertram Wiedenmann.

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Max-Delbrück-Centrum für Molekulare Medizin Berlin-Buch, Abt. Zelluläre Neurobiologie.

In neuroendocrine cells regulated secretion is mediated by two types of secretory vesicles, peptide hormone storing large dense core vesicles (LDCVs) and neuron-like, low molecular weight transmitter containing, small synaptic vesicle-analogues (SSV-analogues). Whereas secretion by LDCVs has been investigated in neuroendocrine cell lines quite well, functional sudies with SSVs are restricted to neuronal cells in primary culture. Here we present functional and morphological aspects of SSV-analogues using various neuroendocrine cell lines as model systems.

The tumor cell lines AR42J, BON, RIN, and INR take up GABA by a low affinity plasma membrane transporter, different from the neuronal type. After permeabilization of the plasma membrane, an ATP-sensitive GABA uptake into an intracellular compartment is observed. The stored GABA is released upon stimulation with either 50 mM potassium or the calcium ionophore A23187 depending on extracellular calcium (1). This calcium-dependent secretion could be mediated by voltage dependent calcium channels expressed by the cells. All cell lines contain membrane proteins of small synaptic vesicles, such as protein SV2, synaptophysin and synaptobrevin. Proteins of the docking fusion complex involved in final exocytosis, such as syntaxin and SNAP25 have been also found to be highly expressed. By an electrophysiological approach and using RT-PCR, $GABA_A$-receptors could be detected in the tumor cell lines AR42J and RIN (2).

The data support the idea that SSV-analogues in neuroendocrine tumor cells store GABA. Its secretion occurs at least in parts by regulated exocytosis. The GABA released may stimulate $GABA_A$-receptors via an autocrine loop.

References

(1) Ahnert-Hilger, G., Wiedenmann, B. FEBS Lett. 314, 41-44 (1992)
(2) von Blankenfeld, G. Turner J., Ahnert-Hilger, G., John, M., Kettenmann, H., Wiedenmann, B. Eur. J. Physiol. 429, in press (1995).

FUNCTIONAL INTERACTIONS BETWEEN THE YEAST NUCLEAR PORE-MEMBRANE PROTEIN POM152P AND A FAMILY OF CONSERVED NUCLEOPORINS.

John D. Aitchison[1], Michael P. Rout[1], Marcello Marelli[2], Günter Blobel[1] and Richard W. Wozniak[2]. 1. HHMI, The Rockefeller University, New York, NY. 2. Dept. of Cell Biology and Anatomy, University of Alberta, Edmonton, AB, Canada.

To further our understanding of the mechanism by which the nuclear pore complex (NPC) translocates macromolecules across the nuclear envelope we have focused on defining the composition and molecular organization of the yeast NPC. We have begun to explore the interactions between a major integral pore-membrane protein, POM152p, and constituents of the NPC (nucleoporins) using biochemical and genetic approaches. We have identified two yeast nucleoporins, termed NUP170p and NUP156p, which are homologous to the mammalian nucleoporin rNUP155p. NUP170p was identified by a synthetic lethal screen designed to identify proteins which genetically interact with the yeast pore-membrane protein POM152p and by peptide sequence analysis of a highly enriched yeast nuclear pore complex (NPC) fraction. NUP156p was identified by additional peptide sequence analysis of this fraction. In addition to NUP170p, the genetic screen yielded at least three other complementation groups, one of which was rescued by the gene encoding a previously identified nucleoporin, NIC96p. A second group is complemented by a novel unrelated nucleoporin, NUP188p, and the third group remains to be characterized. Deletion/disruption of NUP170 reveals that, like POM152 and NUP188, the null haploids are viable. However, double null mutants are synthetically lethal in any combination suggesting that NUP170p, POM152p, and NUP188p all genetically interact. Interestingly, the mutants which can be rescued by NUP170p can also be complemented by the expression of mammalian rNUP155p demonstrating the functional homology between these proteins. Quantitative gel scans of yeast NPC proteins indicate that NUP170p, NUP156p, POM152p, NUP188p, and NIC96p comprise a significant portion of the mass of the isolated yeast NPC. Finally, we have examined the morphological consequences of varying the levels of NUP170p expression. Overexpression of NUP170p in the absence of POM152p leads to the formation of intranuclear annulate lamellae. Depletion of NUP170p revealed dramatic distortions of the nuclear envelope, including massive invaginations and projections suggesting the NPC has a role in maintaining nuclear structure.

INTRA-CELLULAR FOLDING, GLYCOSYLATION AND SECRETION OF tPA.

Simon Allen & Neil J. Bulleid, School of Biological Sciences, Stopford Building, University of Manchester, Oxford Road, Manchester, M13 9PT, U.K.

Tissue-type plasminogen activator (tPA) contains three potential sites for N-linked glycosylation at asparagines 117, 184 and 448, and is synthesized as a mixture of two glycoforms that differ in their extent of glycosylation. Type I tPA is glycosylated at all three sites, whereas type II tPA lacks glycosylation at Asn-184. To investigate whether intra-cellular protein folding underlies this variability in glycosylation, we have expressed tPA in CHO cells in the presence of mild concentrations of the reducing agent dithiothreitol (DTT). Co-translational disulphide bond formation of tPA was prevented and only the type I glycoform was synthesized. When tPA was removed from the cells, this over-glycosylated tPA folded, formed disulphide bonds then was secreted. Prolonged exposure of the cells to DTT resulted in the sequestration of tPA within the endoplasmic reticulum. We also show that tPA present within cells is more susceptible to reduction with low concentrations of dithiothreitol than secreted tPA.

MAPPING THE GOLGI RETENTION SIGNAL IN THE CYTOPLASMIC TAIL OF THE UUKUNIEMI VIRUS G1 GLYCOPROTEIN.

Agneta Andersson, Lars Melin and Ralf F. Pettersson. Ludwig Institute for Cancer Research, Stockholm Branch, Karolinska Institute, Box 240, S-17177 Stockholm,Sweden.

We are using the spike proteins G1 and G2 of Uukuniemi virus, a phlebovirus (Bunyaviridae), as a model system to study intracellular transport to and retention of proteins in the Golgi complex (GC). Previous work in our laboratory has shown that G1 and G2 are transported as heterodimers from the ER to the Golgi, where they accumulate during virus infection. Our results indicate that the cytoplasmic tail of G1 is important both for ER to GC transport and Golgi retention whereas the transmembrane domain contributes little to the Golgi localization. To map the retention signal in more detail, we have constructed mutants with progressive deletions of the G1 tail and expressed them in BHK-21 cells using the Semliki Forest virus expression system. The results show that increasing deletions of the G1 tail resulted in progressively decreased Golgi retention and increased transport of the mutants to the cell surface. Since wt G1, as well as the tail mutants, expressed in the absence of G2, exit the ER inefficiently, we are currently studying chimeric proteins with the ectodomain and the transmembrane domain of CD4 fused to the G1 tail deletion mutants. With these constructs we hope to be able to fine-map the Golgi retention signal within the 100 amino acids-long G1 tail. The role of the G1 TM domain in retention is being analyzed by the construction of additional CD4 chimeric proteins in which the length of the TM region has been modified.

"IDENTIFICATION OF A NOVEL MEMBER OF THE RAB FAMILY FROM THE RAT BASOPHILIC LEUKAEMIA CELL LINE RBL.2H3."

John T Armstrong, Jane H Squire, Nicola Thompson and Roberto Solari

Department of Cellular Science, Glaxo Research and Development, Greenford Road, Greenford, Middlesex UB6 OHE.

We describe the cloning of a cDNA from the rat basophilic leukaemia cell line (RBL.2H3) encoding a novel member of the Rab family of small GTP binding proteins. The novel clone, which we call Rab 8b, is most highly related to the Rab 8 family with substantial divergence in the variable C-terminal domain. Northern blot analysis reveals highest levels of expression of Rab 8b in the spleen, testis and brain, which is in marked contrast to the tissue distribution of Rab 8. The Rab 8b cDNA was modified to introduce a c-myc epitope tag at the extreme N-terminus of the protein, and transient transfection studies were performed to analyse the intracellular localization of Rab 8b by confocal microscopy. Transient expression of the c-myc/Rab 8b fusion protein in both PC12 and RBL.2H3 cells shows staining of both the plasma membrane and vesicular structures, and in the case of RBL.2H3 cells appears to induce striking outgrowths of the plasma membrane.

THE RIBOSOME IS THE GUANINE NUCLEOTIDE EXCHANGE FACTOR FOR THE SIGNAL RECOGNITION PARTICLE

Gerald Bacher, Henrich Lütcke, Berit Jungnickel[1], Tom A. Rapoport[1] and Bernhard Dobberstein

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The signal recognition particle (SRP) is a ribonucleoprotein consisting of 7S-RNA and six different protein subunits. The 54 kDa subunit (SRP54) can be divided in two domains: a G-domain (GTP-binding domain) and a M-domain (Methionine-rich) which interacts with signal sequences when they emerge from the ribosome. After contact of SRP with the SRP-receptor in the membrane of the endoplasmatic reticulum the signal sequence is released from SRP and inserts into the ER-membrane.

Requirements for GTP-binding to SRP54 were investigated. We found that the ribosome increases the affinity of SRP54 for GTP. Furthermore, the GTP-bound state of SRP54 is a prerequisite for the functional interaction of SRP with its receptor. We propose that the transition from the GDP-bound "inactive" state to the GTP-bound "active" state of SRP is catalysed by the ribosome.

POSITIONAL CLONING OF THE MOUSE *beige* MUTATION, A LOCUS CONTROLLING PROTEIN KINESIS TO LATE ENDOSOMES AND LYSOSOMES.

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An understanding of the mechanisms regulating protein kinesis has been greatly aided by molecular characterization of yeast mutants with defective vesicular transport. In an effort to examine control of vesicular traffiking to lysosomes in mammals, we have undertaken a positional cloning approach to determine the genetic defect in the mouse mutation *beige*. *beige* homozygotes display a variety of protein sorting defects, and cells from affected mice exhibit abnormal, large perinuclear vesicles with protein markers characteristic of lysosomes and late endosomes. Using two interspecific and one intraspecific mouse backcrosses which segregate for the *beige* mutation, we have generated a high resolution linkage map of proximal mouse chromosome 13. DNA from over 650 *beige* backcross mice has been typed for 24 genetic markers, distributed over 10 ordered loci. The *beige* locus has been localized to a 0.26±0.18 cM interval. 5 markers (*Nid, D13Mit44, D13Mit114, D13Mit134,* and *D13Mit207*) cosegregated with *beige* in over 650 meiotic events. Mouse YAC clones corresponding to genetic markers in the vicinity of *beige* have been identified, and a YAC contig of the *beige* critical region is being assembled. Preliminary physical mapping studies using pulsed field gel electrophoresis suggest the *beige* critical region to be over 1000-kb in length. These studies provide a starting point for the molecular definition of a mammalian locus that controls protein kinesis to late endosomes and lysosomes.

THE GENERAL FUSION FACTOR TAP/P115/USO1P IS REQUIRED FOR BINDING OF VESICLES TO TARGET MEMBRANES IN EXO- AND ENDOCYTIC MEMBRANE TRAFFIC. M. Barroso and E. Sztul, Dept. Molecular Biology, Princeton University, Princeton, NJ 08544

We have previously described a cell-free fusion assay that reconstitutes the final stage of transcytosis, fusion of transcytotic vesicles with the plasma membrane. We have shown that a 108kD peripheral membrane protein named TAP (Transcytosis Associated Protein), found on transcytotic vesicles, is required for the fusion. Biochemical and electron microscopic analyses indicate that TAP is an extended parallel homo-dimer, similar to myosin II dimers. The TAP dimer contains two globular "heads" (each 9nm in diameter) and an elongated "tail" (45nm in length). Sequence analysis of the open reading frame predicts a 107kD cytosolic protein composed of three structural domains: an N-terminal 72kD globular domain; an internal 32kD dimerization domain containing 4 distinct coiled-coil regions each flanked by helix-breaking prolines and glycines; and a C-terminal 3kD domain containing a preponderance of acidic residues. The predicted domain organization of TAP fits well with the dimensions of the "head" and "tail" domains as defined by EM. TAP is identical to rat p115 and 90% identical to bovine p115. p115 is a recently described cytosolic protein required in conjunction with NSF, SNAPs and other transport factors for *cis* to *medial* Golgi transport of VSV G protein *in vitro*. TAP is also related to Uso1p, a *S. cerevisiae* protein required for endoplasmic reticulum to Golgi transport *in vivo*. The "head-tail-acidic" domain organization of Uso1p and the sizes of the "head" and the acidic regions are similar to those of TAP but the Uso1p "tail" is almost four times the length of TAP's tail. Although the overall homology between TAP and Uso1p is limited, there are three regions of extreme amino acid conservation which appear to represent functional domains of the proteins. We are examining them by mutational analysis. Since TAP is identical to p115 and homologous to Uso1p, we suggest that TAP is a general fusion factor, probably performing an identical function in exocytic and endocytic membrane traffic. This is supported by the distribution of TAP in mammalian cells where TAP is found associated with transcytotic vesicles, secretory vesicles derived from the TGN, within the Golgi complex, and on endosomal compartments. Vesicle targeting and fusion are dependent on ordered reversible interactions between membrane receptors (v-SNAREs and t-SNAREs) and soluble factors (NSF, SNAPs, rabs and various SEC gene products). Where does TAP function? To define the molecular mechanism of TAP action, we modified our *in vitro* transcytotic fusion assay in order to separate the overall reaction into: 1) binding of vesicles to the acceptor membrane and 2) membrane fusion. Using this partial assay we determined that TAP is required for stable binding of vesicles to the target membrane. We propose that TAP might act as a vesicular "anchor" by interacting with the target membrane and holding the vesicular and target membranes in close proximity. Interestingly, other proteins involved in vesicular traffic also contain coiled-coil domains. The transmembrane v-SNARE (VAMP) and t-SNARE (syntaxin and its yeast homolog SED5) and the soluble α- and β-SNAPs contain coiled-coil domains and might form protein-protein interfaces with the coiled-coil regions of TAP. Analyses of such interactions are currently in progress.

Specialized Role for Clathrin in Drosophila Spermatogenesis. C. W. Bazinet[1], J.J. Fabrizio[1], S.K. Lemmon[2], and A. P. Mahowald[3] 1) St. John's University, Jamaica, NY 11439 2) Case Western Reserve University School of Medicine, Cleveland, OH 44106 3) The University of Chicago, Chicago, IL.

In order to identify specialized roles of vesicular transport machinery in development and in the specialized cell types of differentiated complex tissues, we have undertaken a genetic analysis of the clathrin heavy chain gene of *Drosophila melanogaster*. One mutant allele, Chc^4, allows the survival of flies to adulthood, but invariably results in male sterility. This argues that some aspect of spermatogenesis requires a specialized function of the clathrin heavy chain, or that some part of the spermatogenic pathway requires a clathrin function used in other tissues at extraordinarily high levels. Examination of developing mutant spermatids by phase-contrast and electron microscopy reveals an apparent defect in the sperm individualization process, where spermatids are cleaved out of the large syncytium in which they have been assembled. This process requires extensive membrane remodeling, which may be driven in part by a specialized actin cytoskeletal structure that translocates the length of the cyst during the individualization process. Treatment of fixed permeabilized Drosophila testis with rhodamine-conjugated phalloidin reveals the presence of intensely-staining microfilament bundles at discrete sites in the developing cysts. In mutant testis, this actin fiber-based structure is observed at considerably lower frequencies than in wild-type testis, and then only in an altered, apparently disrupted state. Proper assembly or maintenance of this microfilament-based cytoskeletal structure may.depend on its coassembly with membrane machinery that requires clathrin.

HUMAN ß-COP INTERACTS WITH THE HIV-1 NEF PROTEIN IN HIV-1 INFECTED CELLS.

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Nef is a 27 kD myristylated protein conserved in most HIV-1, HIV-2 and SIV isolates. In macaques, the SIV Nef protein plays an essential role in natural infection and is required for high viral load and pathological effects. In cultured cells, the effect of Nef on viral replication is still controversial; however, Nef has been consistently shown to down-regulate the cell surface expression of CD4. This functional interaction may be mediated by proteins required for cell-surface expression of CD4. We used the yeast two-hybrid system to identify cellular proteins that would interact with Nef from the HIV-1 LAI isolate. A cDNA was isolated which encodes a C-terminal fragment of human ß-COP, a major coat component of non-clathrin-coated vesicles. We showed that the Nef-ß-COP interaction is specific. ß-COP was found to interact with Nef from HIV-1 related viruses such as HIV-2 and SIV. Using recombinant proteins, we confirmed that Nef and ß-COP interact *in vitro*. ß-COP was also co-immunoprecipitated with Nef from lysates of HIV-1 infected cells, by anti-Nef antibodies. HIV-1 Nef point mutants which have lost the ability to interact with ß-COP are presently under characterization. These observations indicate that ß-COP is physically associated with Nef and suggest that ß-COP could be one of the cellular mediators of Nef function in HIV-1 infected cells.

RAB GDI ISOFORMS IN 3T3-L1 ADIPOCYTES: A PUTATIVE DOWNSTREAM SITE OF INSULIN REGULATION

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Small GTPases of the Rab family play a crucial, though ill-defined, role in vesicular transport. Guanine-nucleotide dissociation inhibitors (GDIs) inhibit GDP release from Rabs and solubilize membrane-associated Rabs. Within the past year several GDI isoforms have been reported from mouse and rat, but it remains unclear whether these isoforms are redundant or subserve distinct regulatory functions.

We have cloned two full-length Rab GDI isoforms from a 3T3-L1 adipocyte cDNA library. Clone 1 likely represents the murine homologue of rat Rab GDIα, and Clone 2 likely represents the murine homologue of rat Rab GDIβ. The nucleotide and predicted amino acid sequences of Clone 2 differ from those of mGDI-2, a murine Rab GDI cloned from skeletal muscle that also is thought to be expressed in 3T3-L1 adipocytes. Therefore, it appears that at least three Rab GDI isoforms are expressed in 3T3-L1 adipocytes. By northern blot analysis Clone 1 hybridizes to two transcripts: a 2.7 kb transcript that is induced approximately threefold from day 0 to day 8 of 3T3-L1 adipocyte differentiation and a 3.3 kb transcript that is expressed equally on day 0 and day 8. Clone 2 hybridizes to a single 2.4 kb transcript that also is expressed at constant levels from day 0 to day 8.

We have generated anti-carboxy terminal polyclonal antisera based upon the predicted amino acid sequences of Clones 1 and 2. These antisera recognize proteins in 3T3-L1 adipocyte postnuclear extracts of approximately 55 kD and 46 kD respectively. Because Rab proteins have been implicated in insulin-stimulated glucose transport, we have investigated the effect of insulin stimulation on Rab GDI's in 3T3-L1 adipocytes by two-dimensional gel electrophoresis. Preliminary data suggest that the isoelectric point of the Rab GDIα homologue (Clone 1) shifts to a more acidic level by 6 minutes of insulin stimulation, while the isoelectric point of the Rab GDIβ homologue (Clone 2) remains unchanged. These data suggest that Rab GDIα may be phosphorylated in response to insulin in 3T3-L1 adipocytes. Futher experiments to confirm and characterize the insulin-induced acidic shift in Rab GDIα are underway. The differential effect of insulin on the isoelectric points of the two Rab GDI's that we report supports the hypothesis that Rab GDI isoforms are not merely redundant but rather may perform distinct regulatory roles.

ANALYSIS OF CELLULAR TARGETTING OF THE SRC-LIKE TYROSINE KINASE P56*lck*.

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P56*lck* (Lck) is a member of the Src family of tyrosine kinases that is specifically expressed in T lymphocytes. Lck is essential for the maturation of CD4-CD8- cells into CD4+CD8+ cells and for the activation of mature cells. T cell activation requires at least the interaction of the N-terminal ("unique") domain of Lck with the cytoplasmic tail of the T cell coreceptor CD4 (in T helper cells) or CD8 (in cytotoxic T cells).

In accordance with Lck's function in T cell activation, most of the kinase is found at the plasma membrane, a cellular distribution that is distinct from that of other members of the Src family, such as c-Src, v-Src and Fyn. We are aiming to understand how Lck, which is synthesized on cytosolic ribosomes, is targetted to its site of action, the plasma membrane. More specifically we wish to understand: 1) the role of CD4 , 2) the role of palmitoylation and myristoylation of Lck and 3) the role of the individual domains of Lck in its targetting.

In T cells and in transfected non-lymphoid cells, we find that interaction with CD4 is not absolutely essential for deposition of Lck at the plasma mebrane. In T cell lines that either lack CD4 or express a tailless form of CD4, the majority of Lck is found at the plasma membrane, as assessed by immunofluorescence. Furthermore, dissociation of Lck from CD4, which can be induced by phorbolester, and leads to the subsequent endocytosis of CD4, does not change plasma membrane localization of Lck. In 3T3 fibroblasts, where more detailed localization analysis is possible, transfected Lck is found at the plasma membrane in the presence of CD4, and both at the plasma membrane and associated with the Golgi in the absence of CD4. Thus, while Lck is targetted to the plasma membrane in the absence of CD4, its transfer to this site is aided by CD4. Palmitoylation was found to be essential for membrane association, even when myristoylation had occurred.

EFFECT OF PHOSPHORYLATION ON GTPase ACTIVITY OF DYNAMIN

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Dynamin, an enzyme which catalyzes the hydrolysis of GTP, is involved in synaptic vesicle recycling and in the early steps of endocytosis. Analysis of dynamin mutants has revealed that the GTPase activity of dynamin is essential for unimpaired endocytosis . Several in vitro activators of dynamin GTPase activity have been identified, including microtubules, negatively-charged liposomes and the SH3-domain-containing protein, Grb2 and it has been demonstrated that increased GTPase activity is coupled to dynamin self-association. We have observed that aggregates of dynamin formed during purification or storage have activities approaching those of microtubule- or lipid-activated enzyme.

All known GTPase activators of dynamin bind to a very basic, proline-rich C-terminal region of the dynamin molecule that contains several potential phosphorylation sites. Also, it has been shown that dynamin is a good substrate for protein kinase C (PKC). Therefore, we expected that phosphorylation of dynamin, particularly in this region, would modulate the interaction of dynamin with its GTPase activators. Our results indicate that phosphorylation by PKC does not affect the intrinsic GTPase activity of dynamin, but abolishes its microtubule-activated GTPase activity. This inhibition is accompanied by a corresponding reduction in microtubule binding. Neither phospholipid- nor Grb2-activated GTPase activity of dynamin are affected by phosphorylation by PKC.

We have recently found that dynamin can be phosphorylated by Mitogen-activated protein kinase (MAP kinase) within its proline-rich C-terminal domain. The effects of this modification are currently being investigated in our laboratory.

PHAGOSOME MOTILITY ALONG MICROTUBULES *IN VIVO* AND *IN VITRO*.

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Using video microscopy of intact cells, we show that internalized latex beads move on linear tracks within cells. This movement is abolished by nocodazole, indicating that it is microtubule-mediated. To better understand this process, we purified phagosomes from J774 macrophages using floatation on a sucrose step gradient, and reconstituted the motility of these organelles along microtubules *in vitro*. For this purpose we have used fluorescent polarity-marked microtubules stabilized with taxol, and fluorescent latex beads. The binding of phagosomes to microtubules requires macrophage cytosol and phagosome membrane proteins (see abstract of Burkhardt *et al.*). Phagosome motility is ATP dependent and occurs 70% towards the minus end of microtubules. The average maximal speed of minus end-directed movement is 1um/sec, that of plus end-directed movement 0.8um/sec. Approximately 10% of phagosomes move bidirectionally. We are investigating the relative roles of known microtubule motors and accessory factors in controlling the polarity of movement. Since it allows complementary analysis of microtubule binding and motility, this model system provides a simple and practical means to study organelle-microtubule interactions in general.

NORADRENALINE RELEASE FROM STREPTOLYSIN-O PERMEATED RAT CORTICAL SYNAPTOSOMES: ENHANCEMENT OF THE CA^{2+}-DEPENDENT SIGNAL AND EFFECTS OF N-ETHYLMALEIMIDE

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We examined [^{3}H]-noradrenaline (^{3}[H]-NA) release from rat brain cortical synaptosomes permeated with streptolysin-O (SLO) under a variety of conditions. Three temperatures (20°C, 25°C and 30°C) were tested at different times of permeation. Lowering the incubation temperature to 20°C decreased basal release, but Ca^{2+}-induced [^{3}H]-NA release increased slightly, so the Ca^{2+}-dependent component increased. Also, the incubation time where the maximal signal-to-noise ratio was achieved shifted to longer times with decreasing incubation temperature. If the synaptosomes were permeated with SLO before release was triggered, similar results were observed. Permeation at 20°C allowed [γ-^{32}P] ATP and cAMP-dependent protein kinase (PKA) catalytic subunit to rapidly enter the synaptosomes to phosphorylate synapsins. Lactate dehydrogenase (LDH) efflux was time- and SLO-concentration dependent and generally slower than entry of the smaller PKA catalytic subunit. The fact that 0.1 mM Cd^{2+} did not inhibit [^{3}H]-NA release from permeabilized synaptosomes indicated that permeabilization by SLO was complete under this condition. This also suggests that the Ca^{2+} trigger involved in release after Ca^{2+} entry is not sensitive to Cd^{2+}. One mM N-ethylmaleimide (NEM) inhibited the Ca^{2+}-sensitive component of [^{3}H]-NA release from SLO-permeated synaptosomes after 5-25 min incubations at 20°C. This suggests that an NEM-inhibitable component is involved in [^{3}H]-NA release.

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THE REGULATORY DOMAIN OF THE EGF RECEPTOR CYTOPLASMIC TAIL IS REQUIRED FOR SELECTIVE BINDING TO THE CLATHRIN-ASSOCIATED COMPLEX AP-2.

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Clathrin-associated protein complexes (APs) link the entrapment of selected receptors in coated pits to the formation of the clathrin-coat. Activated EGF receptors (EGFR), which are down-regulated in response to EGF, are removed from the plasma membrane by internalization via clathrin-coated pits and coated vesicles and associate at the plasma membrane directly with the clathrin-associated complex AP-2. Efficient internalization of EGFR requires receptor kinase activity and presence of endocytic sequences located in the regulatory domain of its cytosolic tail. Using a kinase dead mutant and a truncated mutant of the EGFR, we have found that the regulatory domain is essential for the recruitment of AP-2s by EGFR *in vivo*. We then show that the regulatory domain is also required for the association between EGFR captured on the surface of a BIAcore sensor chip and purified AP-2s. Finally we find that tyrosine auto-phosphorylation is not an essential pre-condition for AP-2 recruitment both *in vivo* and *in vitro*.
We conclude that the EGFR binds selectively to AP-2s, and the regulatory domain of its cytoplasmic tail is required for this interaction. These results support the view that activation of the EGFR kinase stimulates endocytosis by phosphorylation of a factor distinct from EGFR itself.

INFECTIOUS HIV IS TRANSCYTOSED ACROSS HUMAN EPITHELIA

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The mechanism by which human immunodeficiency virus (HIV) is sexually transmitted accross genital remains unclear. *In vitro* infection of human epithelia has been reported as well as HIV transport accross M cells of rabbit (which cannot be infected because of their non human origin).
We have developed an *in vitro* model that may mimick how HIV interacts with and is transmitted through the intestinal epithelium. Human intestinal epithelia are cultivated as tight and polarized monolayer on permeable support. These conditions allow basolateral (serosal) feeding as *in vivo*, and give experimental access to both the apical and basolateral side of the monolayer, independantly. Addition of HIV infected lymphocytes or macrophages, the two major HIV carrier in secrtions, to the apical (luminal) side of the monolayer trigger a massiv budding of HIV particles at the contact site.
We show for the first time that the newly budded virus is transported across a human cell monolayer to the basolateral (serosal) side by transcytosis. Transcytosed HIV is detected as soon as 40 min after apical contact much earlier than infection which can be detected only several days latter. However, transcystosis of previously isolated HIV particles could not be observed. Transcytosis of newly budded HIV was inhibited at 4°C or by pretreating the epithelia with colchicin (an agent known to block transcystosis). More importantly, the transcytosed HIV was still infectious as it could productively infect potential in vivo targets: lymphocytes or macrophages (from PBL) placed at the basolateral side of the monolayer.
Molecules implcated in HIV transcytosis are gp120-HIV-envelope, the epithelial galactosyl ceramide (described as a CD4 alternative receptor to gp120). In contrast, transcytosis remains insensitive to mAbs anti-CD4. CD26/ Dipeptidyl Peptidase IV appears to be implicated in transcytosis.
We therefore propose that HIV transcystosis accross epithelia is an efficient mechanism by which HIV gains access to blood stream without any mucosal lesion. Transcytosis may be a relevant mechanism for HIV transmission across the human mucosa *in vivo*.

THE ROLE OF PROTEIN PROCESSING IN GENETIC DISEASE

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The rough endoplasmic reticulum is the site at which polypeptides are translated from messenger RNA and ribosomes into the intracellular vacuole system. Within the lumen of the endoplasmic reticulum, newly synthesized pre-protein undergoes folding, post-translational modification and subunit assembly. However the folding process is not 100% efficient and a component of the endoplasmic reticulum processing machinery is thought to ensure that each protein molecule is correctly folded or processed, specifically binding and retaining incorrectly folded protein for subsequent degradation. It is postulated that any mutant gene product which has a structural change will be recognised and retained for degradation by the endoplasmic reticulum. This removal process is postulated to occur, even in cases where the function of the protein has only been partially impaired or for fully functional protein, resulting in a substantial contribution to a particular pathological condition.

The lysosomal storage disease, Mucopolysaccharidosis type VI (MPS VI or Maroteaux-Lamy Syndrome) is an autosomal recessively inherited genetic disease and has been used as a model system to investigate the intracellular removal of aberrant protein. The lowered intracellular content of 4-sulphatase protein and its altered polypeptide conformation have been demonstrated in both MPS VI patient fibroblasts and in expression cells, using a panel of conformation sensitive monoclonal antibodies (Brooks et al., 1991 ; Am. J. Hum. Genet. **48**, 710-719). The removal of structurally altered 4-sulphatase in an early vacuolar compartment has been characterised in a mutant 4-sulphatase expression system (Arlt et al., 1994 ; J. Biol. Chem. **269**, 9638-9643). In a C91T 4-sulphatase expression cell line (Brooks et al., 1995 ; B.J. **307**, 457-463), the rapid removal of aberrant 4-sulphatase has also been demonstrated. However, as may be expected, in some other mutant 4-sulphatase expression cell lines evidence for alternative mechanisms for reduced 4-sulphatase protein levels are apparent.

IDENTIFICATION OF AN ANNEXIN-BINDING PROTEIN

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The annexins are a family of proteins that are able to bind lipid membranes in a Ca^{+2} dependent manner. Members of the family are able to cause chromaffin granule aggregation and it has been proposed that the annexins may be involved in exocytosis by mediating membrane contact in the presence of Ca^{+2}. Proteins that are able to bind to certain members of the annexin family have been identified. Annexin I has been shown to bind S100C, annexin II binds p11, and annexin XI binds calcyclin. It has been proposed that protein binding to the annexins may modify not only the physical state of the annexin, but may also alter its biochemical properties. Thus, identifying additional proteins that specifically interact with members of the annexin family may help to clarify the functions of the annexins *in vivo*.

In order to identify proteins that interact with the annexins, several methods were used. Annexin affinity chromatography, where purified annexin was bound to CNBr Activated Sepharose 4B, was used to determine the capacity of proteins found in cytosolic fractions of bovine adrenal medulla to bind to the immobilized annexin in a Ca^{+2} dependent manner. Several proteins were eluted from an annexin I affinity column in the presence of 2mM EGTA including, protein kinase C (PKC) and a 26kD protein which appeared as a prominent band on SDS polyacrylamide gels. The identity of PKC was confirmed by Western blotting. The 26kD protein was purified on a Mono Q column and determined to be apolipoprotein AI (ApoAI) by peptide sequencing. Far Westerns using ApoAI immobilized on nitrocellulose and annexin I in solution with detection using an anti-annexin I antibody were performed to verify ApoAI-annexin I interactions. Additionally, the ability of biotin-labeled ApoAI in solution to bind to several purified annexins immobilized on nitrocellulose was determined by detection with HRP labeled avidin. Using these methods, it was shown that both annexin I and annexin VII bind to bovine apolipoprotein AI in a Ca^{+2} dependent manner. Other annexins, such as annexin IV and annexin VI do not exhibit this binding. The results suggest that certain annexins may function as extracellular binding sites for plasma proteins.

BIOSYNTHETIC PROCESSING OF *NEU* DIFFERENTIATION FACTOR (NDF): GLYCOSYLATION, TRAFFICKING, AND REGULATED CLEAVAGE FROM THE CELL-SURFACE

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Neu differentiation factor (NDF), also known as Heregulin, is structurally related to the EGF family of growth factors; it stimulates tyrosine phosphorylation of the Neu/HER-2 oncogene and causes differentiation of certain human breast cancer cell lines. Stimulation of *neu*/HER-2 phosphorylation appears to be caused indirectly by NDF/Heregulin binding to the related tyrosine kinase receptors, HER-3 or HER-4, through the formation of homo- and heterodimeric receptor molecules. Alternative splicing of a single gene gives rise to multiple isoforms of NDF/Heregulin, as well as the neuronal homologues, ARIA (Acetylcholine Receptor Inducing Activity) and GGF (Glial Growth Factor)--at least 15 structural variants are known. All but two of the NDF/Heregulin cDNAs are predicted to encode transmembrane, glycosylated precursors of soluble NDF.

We have characterized the biosynthetic processing of different NDF isoforms in stably transfected Chinese Hamster Ovary cells (*CHO*) expressing individual NDF isoforms, and in the native cell line Rat 1-EJ which expresses at least 6 different NDF isoforms. We found that the precursors for NDF undergo typical glycosylation and trafficking. Some of the nascent molecules are proteolytically cleaved intracellularly leading to the constitutive secretion of soluble, mature NDF into the culture media. However, a significant portion of the NDF precursor molecules escape intracellular cleavage and are transported to the cell surface of both transfected, and native cells, where they reside as full-length, transmembrane proteins. Finally we show that these full-length, transmembrane NDF molecules can undergo phorbol ester regulated cleavage from the membrane, releasing the soluble growth factor into the medium.

MAP-DEPENDENT BINDING OF PURIFIED PHAGOSOMES TO MICROTUBULES IN VITRO.

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In parallel with our studies of phagosome motility (see abstract by Blocker, et al.), we have investigated the binding of phagosomes to microtubules in a cell-free system. J774 macrophages were allowed to internalize rhodamine latex beads, and bead-containing phagosomes were purified by floatation in a sucrose gradient. Binding of phagosomes to taxol-stabilized rhodamine microtubules was then tested by light microscopy. Binding is stimulated 5-10 fold by incubation in the presence of 2mg/ml macrophage cytosol. At higher concentrations, phagosome binding is inhibited. Order of addition experiments indicate that the binding factor can associate independently with either the membranes or the microtubules. The latter result suggests that the factor is a MAP. Indeed, the activity is lost upon depletion of MAPs from the cytosol, and is detected in a MAP-enriched preparation. Binding is not supported by a preparation of microtubule motor proteins. In addition to cytosolic factors, binding requires the activity of one or more proteins on the phagosome surface. Uninternalized latex beads do not bind, nor do phagosomes which have been treated with certain proteases. Importantly, this microtubule binding machinery appears to be progressively lost as the phagosome becomes more lysosome-like.

DEVELOPMENT OF MODEL SYSTEM TO STUDY TURNOVER OF MUTANT PYRUVATE DEHYDROGENASE E1a POLYPEPTIDE IN VITRO

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Pyruvate dehydrogenase (PDH) deficiency is a childhood disorder presenting encephalopathy and lactic acidemia. Almost all cases appear to be defects in the X-linked E1a subunit of the massive (~$6x10^6$D), polymeric PDH complex. We have identified a variety of PDH E1a subunit missense and insertion mutations in which enzyme activity is reduced largely due to increased rates of turnover of the mutant polypeptide. In some rare cases, there is clinical evidence for variations of enzyme activity between tissues of the same patient suggesting tissue-specific variations in turnover rates.

We have been developing two cell culture systems to study these problems. The first will allow us to compare the turnover rates of mutant and wild-type PDH E1a polypeptides against identical genetic backgrounds. This is done using fibroblast cell lines from females heterozygous for a PDH E1a mutation, transfecting them with a papilloma virus-derived $HZIP_K$ plasmid which extends the primary culture lifespan, and subsequently cloning and selecting of cell lines which differ only in their X-inactivation state, and hence PDH E1a allele expressed (either mutant *or* wild-type). The second cell system should help us examine differences in turnover determined by cell differentiation state: human wild-type or mutant PDH E1a cDNA is stably transfection into pluripotent mouse 10T1/2 cells which, when treated with 5-azacytidine, differentiate into myoblasts, chondrocytes, and adipocytes.

Throughout these experiments, we will be exploring the possible roles of chaperone proteins and oxidative respiratory rates as factors regulating turnover.

REGULATION OF E-CADHERIN TRAFFICKING IN POLARIZED EPITHELIAL CELLS AND FUNCTION IN INDUCTION OF CELL SURFACE POLARIZATION

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E-cadherin mediates Ca^{++}-dependent cell-cell adhesion and is an instructive inducer of polarized distribution of other membrane proteins in epithelial cells. To gain insights into this later role, we have attempted to divert E-cadherin, a lateral domain-specific protein, into the apical membrane of MDCK cells, and then induce E-cadherin homotypic interactions in the apical membrane in order to examine the effects on the trafficking of other membrane proteins and the organization of cytoskeleton. Basolateral sorting signals with a sequence motif Y-XXXX -$(E/D)_3$ were previously identified in the LDL receptor and shown to be tyrosine-dependent; deletion of these signals results in transport of the LDL receptor to the apical membrane of MDCK cells. E-cadherin cytoplasmic domain contains two motifs with striking sequence and positional similarity to these sorting signals of the LDL receptor. We have created epitope-tagged Y-> A mutants in the potential sorting signals of canine E-cadherin. These mutant E-cadherin can interact with catenins. We have introduced these E-cadherin mutants into two MDCK cell lines which show different Na/K-ATPase trafficking pathways. We are currently analyzing the steady-state distribution and trafficking of E-cadherin mutants in these two MDCK cell lines, and the effects on cell surface polarity of Na/K-ATPase.

To study mechanisms involved in trafficking E-cadherin to cell-cell contacts, we have sought to observe E-cadherin distribution in living cells. A chimeric cDNA comprising a full-length canine E-cadherin fused, at its C-terminus, to the jellyfish green fluorescent protein as a fluorescent tag (Cad-GFP), was constructed, and then expressed in HEK293 cell. The function of Cad-GFP as an adhesion molecule is demonstrated by: (1) interaction with cadherin-binding proteins, catenins; (2) resistance to detergent extraction (0.5% Triton X-100) at cell-cell contact sites, which indicates interaction with the cytoskeleton; and (3) induction of clusters of cuboidal cells with distinct lateral cell-cell contacts. Cad-GFP was observed in live HEK293 cells with a laser scanning confocal microscope (excitation wavelength = 488 nm) or a two-photon excitation microscope (excitation wavelength = 800 nm). Two-photon excitation resulted in better signals and less photo-bleaching. HEK293 cells transiently transfected with Cad-GFP show highly dynamic movements of fluorescent vesicles, a fluorescent perinuclear structure reminiscent of the Golgi apparatus, and fluorescent staining of the plasma membrane. Transfer of Cad-GFP from Golgi apparatus, in vesicles, to the cell surface was observed.

Together, these studies are providing novel insight into the regulation of E-cadherin trafficking in polarized epithelial cells, and mechanisms of induction of cell surface polarity.

MULTIPLE SIGNAL PEPTIDE/SIGNAL ANCHOR PATHWAYS FOR PROTEIN TRANSPORT INTO THYLAKOID MEMBRANES OF CHLOROPLASTS. K. Cline, R. Henry, X. Li*, N. E. Hoffman*, and Jianguo Yuan. Horticultural Sciences and Plant Molecular and Cellular Biology, University of Florida and *Department of Plant Biology, Carnegie Institute of Washington.

The thylakoid membrane is an internal membrane in plant chloroplasts, separated from the inner and outer envelope membranes by the aqueous stroma. Because many thylakoid proteins are synthesized in the cytosol, they have to cross up to three membranes en route to their functional locations. This occurs by sequential translocation events, first across both envelope membranes and second across or into the thylakoid membrane. Analysis of energy and soluble protein factor requirements for transport, as well as the use of *in organello* precursor competition assays, indicate that there are at least three different precursor-specific pathways for thylakoid protein transport. Commitment to a particular pathway is determined by subtle differences in targeting peptides. Chimeric proteins consisting of targeting peptides and mature proteins from different pathways are translocated on the pathway dictated by the targeting peptide. These targeting peptides resemble the signal peptides that direct proteins across bacterial cytoplasmic membranes and across the endoplasmic reticulum. One thylakoid pathway requires ATP and a soluble protein and is stimulated by a transmembrane ΔpH. We recently purified the soluble protein and showed it to be a chloroplast homologue of the bacterial SecA protein. A second pathway, i.e. for integration of a thylakoid membrane protein, requires GTP, a stromal protein, and is stimulated by ΔpH. Collaborative studies with Neil Hoffman (Carnegie Institute) has identified the soluble component as a homologue of the signal recognition particle (SRP) subunit SRP54. Unlike other known SRPs, the chloroplast SRP54 homologue can bind its substrate post-translationally. A third pathway is unique in that it relies solely on a trans-thylakoidal pH gradient for translocation. Current studies are involved in identifying the pathway-specific elements of each targeting peptide, in reconstituting binding of targeting peptides with pathway-specific components, and in identifying other putative components of the chloroplast SRP system.

SECRETORY GRANULE CONTENT AND MEMBRANE PROTEINS AGGREGATE *IN VITRO* AT MILDLY ACIDIC PH: IMPLICATIONS FOR TARGETING OF PROTEINS TO THE REGULATED SECRETORY PATHWAY.

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A major unresolved issue in the field of secretory granule biogenesis is the extent to which the aggregation of granule content proteins is responsible for the sorting of regulated from constitutively secreted proteins. The aggregation process is postulated to take place in the *trans* Golgi network (TGN) and immature secretory granules (ISG) as the proteins encounter mildly acidic pH and high calcium concentrations. We have developed *in vitro* assays that reconstitute the precipitation out of solution of secretory granule content proteins of anterior pituitary gland and adrenal medulla. In the assays, all of the major granule content polypeptides form a precipitate as the pH is titrated below 6.5, and this precipitate can be recovered in the pellet fraction after centrifugation. Addition of calcium is required for the aggregation of chromaffin granule content. In contrast to the proteins secreted by the regulated pathway, the constitutively secreted proteins IgG, albumin, and angiotensinogen, when added to the assays, remain predominantly in the supernatant. Among the individual proteins tested, prolactin is found to aggregate homotypically under these conditions and can drive the co-aggregation of other proteins, such as the chromogranins. Soluble forms of granule membrane proteins, including dopamine β-hydroxylase and peptidyl glycine α-amidating enzyme also co-aggregated with granule content proteins. The results provide strong evidence favoring the hypothesis that spontaneous aggregation of secretory granule content proteins is involved in their preferred targeting to storage granules. They further support the idea that co-precipitation of membrane proteins with content proteins in the TGN and immature secretory granules plays a seminal role in the segregation of granule membrane proteins from those transported directly to the cell surface. Such membrane-content protein interactions would also ensure the acquisition, by the aggregating mass of content proteins, of an appropriate membrane envelope during granule formation. Supported by grants from the N.I.H. and the American Heart Association, NYC affiliate.

THE DYNAMIN-LIKE GTPASE VPS1P IS REQUIRED FOR THE FORMATION OF PREVACUOLE-BOUND VESICLES FROM THE LATE GOLGI IN YEAST

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The Vps1p protein of *Saccharomyces cerevisiae* is an 80 kD GTPase that shows 66% identity to mammalian dynamin in the N-terminal GTP-binding domain, and 46% identity overall. Whereas dynamin is thought to mediate the pinching off of clathrin coated vesicles at the plasma membrane, Vps1p is peripherally associated with the Golgi apparatus and is required for the sorting of soluble vacuolar proteins to the vacuole. We have found that Vps1p is also required for the correct localization of the Golgi membrane proteins DPAP A and Kex2p, as well as the Golgi-localized hybrid membrane protein A-ALP. These proteins are thought to be maintained in the Golgi through a process of retrieval from a post-Golgi, prevacuolar compartment, and do not traffic through the cell surface. However, they are rapidly transported to the vacuole upon Vps1p inactivation. Vacuolar transport of A-ALP is blocked in *vps1 sec4* and *vps1 end4* double mutants, indicating that Golgi membrane proteins are diverted to the cell surface in the absence of functional Vps1p. Moreover, delivery of the vacuolar membrane protein alkaline phosphatase is also blocked in these strains, and both proteins expressed in *vps1Δ end4-ts* cells at the elevated temperature can be detected on the plasma membrane by a protease digestion assay. These data strongly suggest that Vps1p is required for the formation of vesicles from the late Golgi apparatus that carry both vacuolar and Golgi membrane proteins bound for the prevacuolar compartment, and that in the absence of Vps1p all membrane traffic from the Golgi is redirected to the cell surface.

Cells containing null alleles of both *vps1* and *end4* are inviable. In order to identify other gene products that act together with Vps1p, we are screening for mutants that exhibit synthetic lethality with *end4Δ*, and also mislocalize soluble vacuolar proteins. Analysis of these mutants should allow us to identify other proteins involved in the formation of transport vesicles at the late Golgi.

YEAST SYNAPTOBREVIN HOMOLOGS (SNC1 AND SNC2) ARE MODIFIED POST-TRANSLATIONALLY BY PALMITATE ADDITION.

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Yeast have two homologs of the synaptobrevin family of vesicle-associated membrane proteins that function in membrane recognition and vesicle fusion. We have demonstrated that these proteins, Snc1 and Snc2, localize to secretory vesicles and are required for constitutive exocytosis. We have also shown that they form a tight physical complex with a plasma membrane protein, Sec9, and that formation of the Snc-Sec9 complex is necessary for vesicle docking and fusion to occur *in vivo*. Thus, functional interactions between these putative SNARE proteins have been evolutionarily conserved.

Here we show that Snc proteins undergo a single post-translational modification with the addition of a palmitate moiety to cysteine^{95}. Modification of this cysteine, which is located proximal to the transmembrane domain, results in formation of mature Snc protein. Snc protein palmitoylation is rapid; occurs in the endoplasmic reticulum, and appears necessary for Snc protein stability. This is the first experimental evidence that proteins of the synaptobrevin family are modified post-translationally. Moreover, we predict that other members of the family which possess conserved cysteines may also be modified. Thus, fatty acylation may be common to these essential vesicular components of the exocytic machinery.

INTERACTION OF KINESIN AND NCD WITH MICROTUBULES

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Kinesin and non claret disjunctional are closely related molecular motors which move in opposite directions along microtubules (MTs). We have used recombinant single headed and double headed constructs of both rat kinesin heavy chain and non claret disjunctional to investigate the interactions of these motor proteins with microtubules. Using pelleting assays the stoichiometry of binding for ncd and kinesin to MTs is one molecule (single or double headed) per tubulin heterodimer. In the absence of added nucleotide, addition of increasing amounts of one motor results in the competitive displacement of the other motor from the MTs. This effect is apparent also in the presence of the nucleotide analogue 5'-adenylimidodiphosphate (AMPPNP) which tightens the binding of both kinesin and ncd. Competition for binding sites is also apparent under conditions of steady state ATP turnover. We conclude that despite their opposite directionality kinesin and ncd compete for overlapping or identical binding sites on the MT surface.

These motors walk along microtubules via an ATP-driven cycle of conformational changes. We have measured the affinity of various intermediates of ATP turnover for the MTs, and find only two modes of binding, a weak (K_d ~10 μM) mode (stabilised by ATPγS, ADP.AlF_4, and ADP) which traps nucleotide(K_{off} >>0.007 s^{-1}), and a strong (Kd ~0.1 μM) mode (stabilised by AMPPNP and apyrase) which rapidly exchanges nucleotide (K_{off} >>0.6 s^{-1}). The large number of chemical intermediates of ATP turnover by kinesin and ncd thus correspond to only two basic states, a weak state which traps nucleotide, and a strong state which exchanges nucleotide.

Together these data indicate that kinesin and its oppositely-directed homologue ncd walk (bind via one head at a time) using common footprint along MTs, and that ATP turnover drives the heads to alternate between strong (force holding; K_d ~0.1 μM) and weak (detachable; K_d ~10 μM) modes.

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ARF GTPase ACTIVATING PROTEIN: PRIMARY STRUCTURE AND ANALYSIS OF FUNCTIONAL DOMAINS.

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The small GTP binding protein ARF is a key regulator of coat protein association with Golgi cisterna and with the TGN. Coat protein binding is triggered by ARF in its GTP-bound state, and is terminated following GTP hydrolysis. The latter reaction depends upon the interaction of ARF with a GTPase activating protein (GAP). We have recently achieved the purification of a cytosolic ARF GAP from rat liver (Makler et al, J. Biol. Chem., March 1995). Here we present the primary structure of the GAP as predicted by its cloned cDNA. The GAP possesses a single N-terminal Zn finger that apparently confers a high stability on the protein. Truncation and point mutation analysis shows that the GAP function is segregated within the amino terminal 35% of the protein, and depends on an intact Zn finger structure. Two alternatively spliced variants were identified in library clones, one of which encodes a truncated protein possessing GAP activity. The role of the GAP and its variants in membrane traffic is currently under investigation.

ACTIVITY-INDUCED INTERNALIZATION OF VOLTAGE DEPENDENT Na^+CHANNELS IN DEVELOPING NEURONS.

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Control of the density of voltage-dependent Na^+ channels is a potential regulatory mechanism for neuronal excitability. We have previously shown that activation of Na^+ channels in fetal rat brain neurons induced their rapid but partial disappearance from the cell surface. Experiments using radioiodinated α-scorpion toxin (^{125}I-α-ScTx) as both a Na^+ channel activator and a surface channel probe show that a fraction of bound toxin was internalized since it was not releasable by acidic washing. Internalization was inhibited by tetrodotoxin, a channel blocker, and by substitution of Na^+ by choline ions. This observation was confirmed by a quantitative analysis of electron microscopy autoradiography. ^{125}I-α-ScTx labeling associated with vesicular structures in neuron processes was twofold higher than in control cells after a 60 min incubation at 37°C. The internalization of Na^+ channels was directly followed using a photoactivable derivative of a ^{125}I-α-ScTx, showing a degradation of the covalently linked α-ScTx-Na^+ channel complex. Furthermore, the recycling of internalized Na^+ channels back to the cell surface was almost negligible and pretreatment of the cells with chloroquine prevented internalization, indicating that an endosomial/lysosomial compartment is involved in activity-induced Na^+ internalization.

Internalization is not mediated by protein kinase C, cAMP- and cGMP-dependent kinases and is not blocked by tyrosine kinase inhibitors. A rise in cytosolic Ca^{++} induced either by thapsigargin or by a Ca^{++} ionophore was unable to provoke internalization. However, a partial inhibition was observed in presence of BAPTA-AM, a cell permeant Ca^{++} chelator, indicating a requirement for Ca^{++}. Since the Na^+ ionophore amphotericin B was able to provoke internalization, Na^+ channel internalization is not provoked by the occupancy of toxin binding sites in contrast to ligand-induced internalization but is triggered by Na^+ influx into neurons.

Na^+ channel internalization is specific to a particular stage of neuronal development because it only occurred in immature neuronal tissue, either cultured fetal neurons, or postnatal hippocampal slices but was absent in adult brain. This study describes the first example of voltage sensitive ion channel internalization. Activivity-induced internalization points out the signalling role for sodium in neurons and could play a key role in the acquisition of electrical excitability.

Dargent *et al.* , Neuron, 1994, 13, 683-690; Dargent *et al.*, J.Neurochem, 1995, in press.

SELECTIVE DEGRADATION OF TROPOELASTIN BY A CYSTEINE PROTEASE IN THE ENDOPLASMIC RETICULUM IS INDUCED FOLLOWING BREFELDIN A TREATMENT OF ELASTOGENIC CELLS. Elaine C. Davis and Robert P. Mecham. Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110.

Elastin is secreted as soluble tropoelastin monomers which are then crosslinked in the presence of extracellular microfibrils to form insoluble elastic fibers. Although the secretion of tropoelastin is thought to be mediated and targeted by an intracellular chaperon complex, the intracellular route taken by this protein and the role of such a chaperon complex remain undefined. In the present study, the specific pathway of tropoelastin secretion was investigated in fetal calf ligamentum nuchae (FCL) cells using brefeldin A (BFA) to disrupt the secretory pathway. Consistent with the reported effects of BFA on intracellular organelles, electron microscopic studies of BFA-treated FCL cells showed extensive dilation of the endoplasmic reticulum (ER), no identifiable Golgi stacks and a perinuclear accumulation of tubulo-vesicular elements. FCL cells labeled for 4 hours with ^{3}H-leucine followed by immunoprecipitation with a tropoelastin antibody, SDS-PAGE and autoradiography revealed metabolically labeled tropoelastin in both the cell lysate and medium. When labeled in the presence of BFA, however, radiolabeled tropoelastin was not detected in the cell medium, nor was there an accumulation of intracellular radiolabeled tropoelastin. In contrast, another secreted protein, fibronectin, accumulated within the cell in the presence of BFA. Northern analysis of mRNA levels in FCL cells showed that the message for tropoelastin was unaffected by the BFA treatment thus suggesting that the newly synthesized tropoelastin was being degraded. To investigate the degradation of tropoelastin, FCL cells were pulsed for 1 hour in the presence of BFA and then chased in complete medium containing BFA. Within 3 hours of chase, no radiolabeled tropoelastin could be detected in the cell lysate, whereas, the amount of fibronectin remained unchanged. Including ammonium chloride, nocodazole, or cycloheximide in the chase had no affect on the degradation of tropoelastin indicating, respectively, that the degradation does not involve the endosome/lysosome pathway, movement via microtubules or a short-lived protein. When the cysteine protease inhibitor, ALLN, was included in the chase, the degradation of tropoelastin could be completely inhibited. Furthermore, incubation of FCL cells with BFA in the presence of ALLN allowed the protein to accumulate in the cells during a 4 hour pulse. These results provide evidence for selective degradation of a soluble secreted protein following retention of the protein in the ER. The involvement of a cysteine protease in the degradation process is consistent with membrane proteins that undergo regulated ER degradation.

DIFFERENTIAL PROCESSING AND SORTING OF EGF-LIKE GROWTH FACTOR PRECURSORS IN POLARIZED EPITHELIAL CELLS.

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In MDCK cells, we have shown previously that newly synthesized proTGFa is delivered directly to the basolateral surface where it is sequentially cleaved to release mature 5.6 kD TGFa into the basal medium. ProTGFa cytoplasmic tail is 39 amino acids in length, is highly conserved between species and does not contain any known basolateral sorting motifs except a dileucine motif. To investigate the role of the proTGFa cytoplasmic tail in basolateral sorting we have performed deletional and site-directed mutagenesis analysis. These studies have demonstrated that deletion of the last 31 amino acids from the C-terminus (TC31 mutant), which also removes the dileucine motif, still retains basolateral sorting. However, a TC38 mutant which deletes the entire cytoplasmic domain, except for one amino acid, demonstrates a loss of basolateral sorting as measured by levels of TGFa immunoreactivity released into the apical and basal conditioned media. These studies suggest a unique basolateral sorting motif of 7 amino acids is located in a juxtamembrane position within the cytoplasmic tail.

To investigate the molecular basis for trafficking of other EGF-like growth factor precursors in polarized epithelial cells, full-length cDNAs were introduced into MDCK cells. Under steady state conditions, two high molecular weight proEGF forms are detected at the cell surface, 175 and 180 kD respectively. ProEGF is cleaved at the cell surface to release a high MW 175 kD form into the medium. No mature EGF 6kD is detected in the medium. Based on pulse-chase combined with cell surface immunoprecipitation, proEGF is delivered equally to both apical and basolateral cell surfaces. Surprisingly, the kinetics of delivery of the two proEGF forms to the cell surface differ. In 5 min pulse-chase experiments, the major 175kD proEGF form is detected at 0 chase time at the cell surface and is Endo H sensitive. At longer chase times (45-60 min) the minor 180 kD form appears at the cell surface and is Endo H resistant. The differences in processing and sorting of proTGFa and proEGF will be discussed in relation to our preliminary results with amphiregulin and HB-EGF and their possible roles as autocrine and paracrine factors in polarized epithelial cells.

ANTI RHODOPSIN MAB INHIBITS RHODOPSIN-BEARING POST-GOLGI VESICLE FORMATION IN A RETINAL CELL-FREE SYSTEM.

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In retinal photoreceptors highly polarized organization of the light-sensitive organelle, the rod outer segment (ROS), is maintained by sorting of rhodopsin and its associated proteins into distinct post-Golgi vesicles that bud from the trans-Golgi network (TGN) and by their vectorial transport to the base of the organelle. These vesicles have a relatively simple protein composition. Small G-proteins rab6 and rab8 are tightly bound to rhodopsin-bearing vesicle membranes and may regulate their budding and/or targeting.

We have developed an assay that reconstitutes the formation of these vesicles in a retinal cell-free homogenate. Frog retinas are pulse labeled for 1 hour. After removal of the ROS and the neural retina, retinal homogenates enriched in photoreceptor-derived biosynthetic membranes are incubated in the presence of ATP and an ATP regenerating or an ATP depleting system for 2 hours of chase. Post-Golgi vesicle formation in this cell-free assay is ATP, GTP and cytosol dependent. In frog retinal photoreceptors, vesicle budding also proceeds at 0°C, both in vivo and in vitro. Vesicles formed in cell-free homogenates are indistinguishable from the vesicles formed in vivo by their buoyant density, protein composition, topology and morphology. In addition to the previously identified G-proteins, rhodopsin-bearing post-Golgi vesicles also contain rab11. Concurrently with vesicle budding resident proteins are retained in the TGN. Collectively these data suggest that rhodopsin and its associated proteins are sorted upon exit from the TGN in this cell-free system.

Removal of membrane-bound GTP-binding proteins of the rab family by rab GDP dissociation inhibitor (rab GDI) completely abolishes formation of these vesicles and results in the retention of rhodopsin in the Golgi. A monoclonal antibody to the cytoplasmic (carboxyl-terminal) domain of rhodopsin, and the Fab fragments of this antibody, also strongly inhibit vesicle formation and arrest newly synthesized rhodopsin in the TGN rather than the Golgi. Control antibodies have no effect. This suggests that sorting of rhodopsin-bearing vesicles as they exit from the TGN is mediated by the interaction of rhodopsin's cytoplasmic domain with the intracellular sorting machinery.

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PDGF STIMULATES AN INCREASE IN GTP-RAC VIA ACTIVATION OF PHOSPHOINOSITIDE 3-KINASE.

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We show here that platelet-derived growth factor (PDGF) can stimulate an increase in the level of GTP-Rac by at least two distinct mechanisms: firstly, by increased guanine nucleotide exchange and secondly, by inhibition of a Rac GTP-ase activity. The first of these mechanisms is the most important, and we show that it is dependent upon PDGF-stimulated synthesis of phosphatidylinositol (3,4,5)-trisphosphate. These results suggest that Rac activation lies downstream of PI-3-kinase activation on a PDGF-stimulated signalling pathway. Furthermore, as Rac has been implicated in at least two diverse cellular responses that are also thought to requiere activation of PI 3-kinase- a reorganization of the actin cytoskeleton known as membrane ruffling and the neutrophil oxidative burst- these results suggest that Rac may be a major effector protein for the PI 3-kinase pathway in many cell types.

CLONING AND CHARACTERIZATION OF THE cDNA FOR PEA CHLOROPLAST SEC-A: EVOLUTIONARY CONSERVATION OF THE BACTERIAL-TYPE PROTEIN TRANSPORT SYSTEM WITHIN CHLOROPLASTS.

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According to the endosymbiont hypothesis, plant chloroplasts may have derived from oxygen-evolving photosynthetic bacteria that resembled cyanobacteria. In prokaryotic cells, Sec proteins including SecA and SecY mediate protein transport across the cytoplasmic membrane. Cyanobacteria have, in addition to the cytoplasmic membrane, the internal thylakoid membrane network and contain SecY and SecA in both the cytoplasmic and thylakoid membranes. The membrane structures of chloroplasts resemble those of cyanobacteria. They are surrounded by the two-membrane envelope and have internal thylakoids. However, most of the chloroplast proteins are encoded by the genes in the nucleus, synthesized outside the chloroplasts, and then imported back into the chloroplasts. Once inside the chloroplasts, proteins are transported to their final destination such as thylakoids. Since this intraorganellar protein transport process in the chloroplasts resembles the intracellular protein transport process in cyanobacteria, it is interesting to ask if chloroplasts have cyanobacterial-type Sec proteins for protein transport within organelles. Indeed recently, a part of the *secA* homologous gene was cloned from pea cDNA and the antibodies against the partial pea SecA recognized a 110-kDa protein of the chloroplast stroma (1). The 110-kDa stromal protein appeared to mediate intraorganellar transport of some thylakoidal proteins, which are imported from the cytosol (1, 2).

Here we report the isolation of the entire gene for pea chloroplast SecA. Pea SecA encodes a polypeptide of 1,011 amino acids and shows high sequence similarity with cyanobacterial SecA. Pea SecA was synthesized as a larger precursor and was imported into isolated chloroplasts *in vitro*. The purified pea SecA, which was expressed in *E. coli* cells, stimulated the *in vitro* import of the 33-kDa protein of the oxygen-evolving complex into thylakoids. These results indicate that higher plant chloroplasts contain a bacterial-type SecA protein-dependent system for the intraorganellar protein transport into thylakoids.

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A YEAST KARYOPHERIN HETERODIMER THAT TARGETS PROTEINS TO NUCLEAR PORES.

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Protein import into the cell nucleus occurs through the nuclear pore complex (NPC). Cytosolic receptors termed karyopherins function to target proteins that contain nuclear location sequences to the NPC. Saccharomyces cerevisiae contains a structural homologue of mammalian karyopherin α, named Srp1/Kap60p. We replaced cellular Kap60p with a Kap60-Protein A chimera. A complex of Kap60-Protein A and a protein of ~95 kDa was isolated on Immunoglobulin-G Sepharose beads. Microsequence analysis identified p95 as a protein of unknown function in the yeast genome termed L8300.15. Because p95 is 63 % homologous to rat karyopherin β, we named it Kap95p. Kap60 and Kap95 proteins were expressed separately in E. Coli, and were purified to near homogeneity. Recombinant Kap60 and Kap95 proteins assembled stoichiometrically into a heterodimer, and promoted docking of a fluorescent import-substrate to the nuclear envelope of permeabilized mammalian cells. Neither Kap60p nor Kap95p displayed docking activity when added alone.

CHEMOATTRACTANT CONTROLLED ASSEMBLY OF ACTIN-ASSOCIATED PROTEINS FORMING A HIGHLY DYNAMIC COMPLEX AT THE LEADING EDGE OF *DICTYOSTELIUM* CELLS

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When aggregating cells of *D. discoideum* are locally stimulated by cAMP, a new front is protruded within seconds towards the attractant and polarity of the cells is changed. A front is generated by the recruitment of a selection of proteins from the cytosol, which become incorporated into the actin network of the leading edge. These proteins include talin (1), coronin (2) and members of the hsc70 and hsc90 families. These chaperones colocalize with F-actin in the cortical regions of the cells. One isoform of hsc70 has been shown to activate the 32/34 kDa heterodimeric F-actin capping protein, the *Dictyostelium* homolog of capZ (3).
Protein targeting to the leading edge can be monitored on-line in locomoting cells using fusions with green fluorescent protein (gfp). Local assembly and sorting of actin-associated proteins similar to that observed in chemotaxis occurs in the process of cytokinesis.

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AMINO ACID STARVATION HALTS THE CELL CYCLE IN YEAST BY INHIBITING *PRG1*–REGULATED UBIQUITIN–DEPENDENT PROTEIN DEGRADATION.

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Limitation of cells for amino acids causes them to stop proliferating. This could be caused directly by a decrease in translation, or starvation could be sensed by the cell inducing cell cycle arrest. We have used a molecular approach to identify the signal responsible for growth arrest of amino acid starved yeast cells. The results indicate that starvation induces cell cycle arrest by interfering with ubiquitin–dependent degradation of one or more target proteins.

Cells lacking the gene encoding the general activator of amino acid biosynthesis, *GCN4*, are phenotypically defective in arginine metabolism. Cells lacking *GCN4* grow extremely slowly without arginine; starved cultures accumulate cells exhibiting a phenotype associated with mitotic arrest. We identified high–copy suppressors which promoted normal growth in the absence of arginine. Among these were five clones of the *PRG1* gene, a subunit of the proteasome. Overexpression of three other subunits of the 20S proteasome had no effect. *PRG1* plasmids are bypass suppressors, since cells overexpressing *PRG1* are still phenotypically starved for arginine as judged by the fact that the stringent control system still inhibits expression of several target genes (ribosomal protein genes and Ty2 elements).

Stringent control depends on the phosphorylation state of the ubiquitous transcriptional activator Rap1. The *PRG1* promoter includes three consensus Rap1 binding sites which appear to cause stringent regulation of its transcription. *PRG1* is necessary for ubiquitin–dependent degradation of the mitotic cyclin Clb2 by the proteasome. Inability to destroy Clb2 causes arrest late in mitosis. This suggests that amino acid starvation causes cell cycle arrest by interfering with degradation of Clb2. Since other proteasome subunits (*PRE4, PTS1, PUP2A*) have no effect, the*PRG1* product may regulate Clb2 degradation by the proteasome. If the *PRG1* product targets the proteasome to degrade Clb2 it could be a molecule which receives the signal for cells to exit mitosis.

IMPLICATION OF ANNEXIN XIIIB IN THE VESICULAR TRAFFIC TO THE APICAL PLASMA MEMBRANE IN MDCK CELLS

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The sorting of apical and basolateral proteins into vesicular carriers takes place in the *trans*-Golgi network (TGN) in Madin-Darby canine kidney (MDCK) cells. We have previously analyzed the protein composition of immunoisolated apical and basolateral transport vesicles and have identified a component that is highly enriched in apical vesicles.

Isolation of the encoding cDNA revealed that this protein, annexin XIIIb[1], is a new isoform of the epithelial specific annexin XIII sub-family which includes the previously described intestine-specific annexin XIIIa[2]. Annexin XIIIb differs from annexin XIIIa in that it contains a unique insert of 41 amino acids in the N-terminus and is exclusively expressed in dog intestine and kidney. Immunofluorescence microscopy demonstrated that annexin XIIIb was localized to the apical plasma membrane and underlying punctate structures. Since annexins have been suggested to play a role in membrane-membrane interactions in exocytosis and endocytosis we investigated whether annexin XIIIb is involved in delivery to the apical cell surface. To this aim we used permeabilized MDCK cells and a cytosol dependent *in vitro* transport assay. Antibodies specific for annexin XIIIb significantly inhibited the transport of influenza virus hemagglutinin from the TGN to the apical plasma membrane while the transport of vesicular stomatitis virus-glycoprotein to the basolateral cell surface was unaffected[1].

In order to elucidate the exact role of annexin XIIIb in vesicular transport, we are currently identifying annexin XIIIb-binding proteins by several approaches, including cross-linking, affinity chromatography and the yeast two-hybrid system. Since recent results suggest that rab/NSF/SNAP/SNARE components are not involved in the apical pathway in MDCK cells[3], annexin XIIIb interacting molecules might provide important clues about the apical transport machinery.

1 Fiedler, K., Lafont, F., Parton, R. and K. Simons (1995) J.Cell Biol. *128*, in press.
2 Wice, B.M. and Gordon, J.I. (1992) J.Cell Biol. *116*, 405-422.
3 Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C. and K. Simons (1995) Cell, in press.

SEC3 GENETICALLY INTERACTS WITH *SEC4* AND *YPT1*, AND MAY FUNCTION AT MULTIPLE STAGES OF THE SECRETORY PATHWAY IN *SACCHAROMYCES CEREVISIAE*

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SEC3 is one of ten *SEC* genes required for targeting and/or fusion of post-Golgi secretory vesicles with the plasma membrane. We have isolated two new alleles of *SEC3* using a genetic screen for temperature-sensitive secretory mutants that are also synthetically lethal with *sec4-8*, a temperature-sensitive allele of *SEC4*. Sec4 is a rab protein that acts at the last stage of the secretory pathway.

The new *SEC3* alleles, *sec3-4* and *sec3-5*, were used to clone the *SEC3* gene by complementation. The *SEC3* gene is identical to *PSL1* (Profilin Synthetic Lethal 1), sequenced by B. Haarer and S. Brown. Antibodies made against a fusion protein of maltose binding protein with the 100 amino-terminal amino acids of Sec3 recognize two proteins of 205 and 120 kDa on Western blots. When the sole copy of the *SEC3* gene is under the control of the inducible galactose promoter, both proteins are present only under conditions of active transcription from this promoter, indicating that both proteins are products of the *SEC3* gene.

sec3 is synthetically lethal with all of the other late-acting *sec* mutants, as well as with *sec19-1* and *Δdss4*, which encode, respectively, the GDI and the GNRP for Sec4. The *sec3* alleles are also synthetically lethal with mutant alleles of *YPT1*, the rab family member involved in ER to Golgi transport, with *bet2-1* which encodes a component of the geranylgeranyl transferase that prenylates Sec4 and Ypt1, and with *bet3-1*. Double mutants for *sec3* and genes encoding the cytoskeletal proteins *myo2-66* and *pfy1-111* are inviable as well.

Electron microscopic examination of *sec3-4* and *sec3-5* cells reveals that at the permissive temperature, they accumulate ER, Golgi, and vesicular structures. At the restrictive temperature post-Golgi secretory vesicles are more abundant. The *sec3* mutants do not secrete invertase at the restrictive temperature. Transport of the vacuolar protease CPY is partially blocked in *sec3-4* and *sec3-5* cells at the restrictive temperature. These results indicate that Sec3 may act at multiple stages of the secretory pathway. We are employing genetic, molecular biological, and biochemical approaches to learn more about the role of Sec3 in exocytosis and the nature of its interactions with Sec4 and Ypt1.

PTI1P IS A SYNAPTOBREVIN-RELATED PROTEIN REQUIRED FOR MEMBRANE TRAFFIC FROM THE GOLGI TO THE YEAST VACUOLE. G. Fischer von Mollard, S.F. Nothwehr, and T.H. Stevens
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Transport of soluble vacuolar hydrolases from the Golgi apparatus to the vacuole requires over 40 gene products in yeast. One of these proteins, Vps10p/Pep1p, an integral membrane protein, is the sorting receptor for the vacuolar hydrolase carboxypeptidase Y (CPY). To identify proteins that regulate the membrane traffic pattern of Vps10p we screened for interacting proteins using the two-hybrid system with a fusion protein containing the cytoplasmic domain of Vps10p and the LexA DNA-binding domain as the bait. This led to the identification of *PTI1*, a gene encoding a protein of 217 amino acids with a C-terminal hydrophobic domain and sequences that are predicted to form coiled coils. *PTI1* shows low homology with the proteins of the synaptobrevin family and lower homology with proteins of the syntaxin family. Synaptobrevins and syntaxins are part of a protein complex necessary for fusion of transport vesicles with their target membranes in a variety of systems. Pti1p is required for CPY sorting to the vacuole. Disruption of *PTI1* is lethal in haploid yeast cells. In contrast, all known *VPS* genes are non-essential, presumably because yeast cells are viable without an assembled vacuole. Temperature-sensitive mutants were obtained which either missort CPY at elevated temperatures but don't show a growth defect or have a growth defect at elevated temperature but correctly target CPY clearly separating both functions. Pti1p was primarily detected in vesicles that are distinct from Golgi structures by immunofluorescence. Pti1p migrated at the same position as the syntaxin-homologue Pep12p/Vps6p in a sorbitol density gradient and was separated from Golgi proteins. Pti1p was localized to the prevacuolar/late endosomal compartment in *vps27*-mutants that accumulate this structure. These observations suggest that Pti1p is localized to a compartment involved in transport from the Golgi to the vacuole in wild type cells.

DEFINITION OF HSP70 BINDING SITES IN P53

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Mutations within conserved regions of the tumor suppressor protein, p53, result in oncogenic forms of the protein with altered tertiary structures. In most cases, the mutant p53 proteins are selectively recognised and bound by the molecular chaperone, hsp70, but the site(s) in p53 for this interaction has not been clearly defined.

Using synthetic peptides we have previously identified potential binding sites for hsp70 proteins in the N-terminal, central, and C-terminal domains of p53. In the present study we have screened a library of overlapping biotinylated peptides, spanning the entire human p53 sequence, for binding to the hsp70 proteins, hsc70 and DnaK. The results showed that most of the high affinity binding sites for these proteins were located in the central DNA binding domain where the majority of oncogenic p53 mutations are found. Although peptides corresponding to the C-terminal region of p53 also contained potential binding sites, p53 proteins with C-terminal deletions were capable of binding to hsp70, indicating that this region was not required for complex formation.

We propose that mutations in the p53 protein alter the tertiary structure of the central DNA binding domain, thus exposing high affinity hsp70 binding sites which are cryptic in the wild type molecule.

MOLECULAR CELL BIOLOGY OF PEROXISOME ASSEMBLY FACTOR-1, PAF-1

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Peroxisome assembly factor-1 (PAF-1), a 35-kDa peroxisomal integral membrane protein, restores the biogenesis of peroxisomes and complements the defect of peroxisomal functions in one, Z65, of three mutually distinct, peroxisome-deficient Chinese hamster ovary (CHO) cell mutants. PAF-1 also complements the peroxisomal dysfunctions in one complementation group of peroxisome assembly-defective human disorders such as cerebrohepato-renal Zellweger syndrome. The cause of the syndrome in the patients is a homozygous point mutation of a codon for ^{119}Arg that results in the premature termination of PAF-1. The homozygous patient inherited the mutation from the parents, each of whom was heterozygous for the mutation. Human PAF-1 gene is assigned to chromosome 8q21.1. Prenatal diagnosis of Zellweger syndrome is now available using DNA analysis. PAF-1 from three species, human, rat, and Chinese hamster, shows high sequence-homology and contains a conserved cysteine-rich sequence, RING finger, at the C-terminal region. Truncation in PAF-1 either of 19 amino acids from the N-terminus or 92 residues from the C-terminus maintained the peroxisome assembly-restoring activity when tested in both the Z65 and the fibroblasts from a Zellweger patient. In contrast, deletion of 27 or 102 residues from the N-or C-terminus eliminated the activity. PAF-1 is encoded by free polysomal RNA, consistent with a general rule for biogenesis of peroxisomal proteins including membrane polypep-tides, implying the posttranslational transport and integration of PAF-1 into peroxisomal membrane.

MEMBRANE MICRODOMAIN FORMATION AND APICAL SORTING IN INTESTINAL EPITHELIAL CELLS.

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Glycosyl-phosphatidyl inositol (GPI)-linked proteins are enriched in the apical membrane of many epithelial cell types. In an intestinal cell line, Caco-2, we have found that GPI-linked proteins became resistant to mild detergent extraction at 4°C during their transport to the apical cell surface. They were found in membrane microdomains containing Sucrase-Isomaltase, a transmembrane apical membrane protein, proteins from the src family, fimbrin, ezrin and actin. These structures are different from caveolae since they do not contain caveolin , a protein not expressed in Caco-2 cells and no caveolae-like structures could be observed in these cells at the electron microscopy level.

Sorting of membrane microdomains was perturbed when the cells were treated with NH_4Cl, to raise the pH of intracellular compartments, since alkaline phosphatase, a GPI-linked protein, was still included in these structures but not anymore delivered preferentially to the apical surface. Protein analysis of the treated membrane microdomains showed that some components were absent suggesting that they could be involved in sorting. We propose a mechanism for the apical sorting in Caco-2 cells that rely on interactions between the ectodomain of some GPI-anchored proteins and putative transmembrane proteins acting as sorters. Acidic pH could be necessary for a correct or transient recognition between sorters and cargo proteins.

THE hCG/LH RECEPTOR IS INVOLVED IN TRANSCYTOSIS OF THE HORMONE THROUGH THE MICROVASCULAR ENDOTHELIUM IN TARGET ORGANS.

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The transfer of human choriogonadotropin (hCG) through the endothelial cells of the rat testis was examined by electron microscopy and by analysing the transport of radioiodinated hormone and monoclonal anti-hCG/LH receptor antibodies.

To follow ultrastructurally the cellular route of hormone we perfused *in situ* the testicular vasculature with hCG-gold complex and collected testes after 5, 10, 15, and 20 min of perfusion. Electron microscopic analysis showed that the hormone crossed the microvascular endothelial barrier in several steps : binding to luminal plasma membrane, uptake via coated pits and vesicles, concentration into the endosomal compartment, and delivery by large vacuoles (derived from the endosomes) into the subendothelial space. The transport of both iodinated-and gold-tagged hormone was negligible at 4°C and suppressed by the presence of an excess of unlabeled hormone. Moreover, a monoclonal antibody against the hCG/LH receptor ectodomain coupled to colloidal gold followed the same route and its transport through the endothelial cells was apparently accelerated by administration of hormone. All these results suggest that the hormone cross the endothelial barrier through a transcytosis mechanism involving the hCG/LH receptor. No such transendothelial transport was observed in non-target tissues (i.e., heart, lung, epidydimal fat). The route followed by hormone-receptor complexes in endothelial cells differed from the endocytosis previously described in Leydig cells (Ghinea N., Vu Hai M.T., Groyer-Picard M.T., Houiller A., Schöevaërt D., and Milgrom E., 1992, J. Cell Biol., 118: 1348-1358). In the latter, the complexes are directed towards lysosomes. Thus, different sorting mechanisms take place in endosomes to orient the hormone-receptor complexes along different pathways.

In conclusion, we suggest that there is a specific step in protein hormone transport across the vascular barrier in target organs. New regulatory mechanisms may thus exist at this level and may also have importance for the practicability of drug targeting in gonads.

Preliminary evidences suggest that similar mechanisms exist for other protein hormones.

TRANSMEMBRANE DOMAIN OF HSV GLYCOPROTEINS IS SUFFICIENT FOR TARGETING TO NUCLEAR ENVELOPE.

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Herpes simplex virus (HSV) assembles in the inner membrane of the nucleus. Viral particles acquire their envelope by budding through a modified inner membrane of the nucleus containing HSV envelope glycoproteins. As a first step to understand the assembly and budding of HSV in the nuclear envelope (NE), we have studied the localization of HSV-1 glycoproteins gB and gD in the NE. Both gB and gD are essential for HSV entry and infectivity. To determine whether specific domains of gB and gD are involved in localization in the NE, we have used chimeric proteins constructed by replacing the domains of the cell surface glycoprotein G of vesicular stomatitis virus (VSV) with the corresponding domains of gB or gD. Localization of the chimeric proteins expressed in COS cells by immunoelectron microscopy showed that the transmembrane region of gB was sufficient for localization of the hybrid protein containing the ecto- and cytoplasmic domains of VSV G protein (J. Virol. 68, 2272 (1994). In the case of gD, however, both the transmembrane and the cytoplasmic domains were required for NE localization. The role of individual amino acids in the transmembrane region, which is conserved in the gB proteins of all α-herpes viruses, in NE localization is being studied by substitution mutagenesis. Supported by MRC Canada.

CHARACTERIZATION OF PXR1, THE HUMAN RECEPTOR FOR THE TYPE-1 PEROXISOMAL TARGETING SIGNAL (PTS1), AND DEMONSTRATION THAT PXR1 IS ALSO REQUIRED FOR IMPORT OF PROTEINS CONTAINING THE TYPE-2 PEROXISOMAL TARGETING SIGNAL (PTS2)

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PXR1 is the gene responsible for complementation group 2 (CG2) of the peroxisome biogenesis disorders (PBDs). This gene is highly similar to the yeast *P. pastoris PAS8* and *Saccharomyces cerevisiae PAS10* genes. Like these yeast genes, *PXR1* encodes the receptor for proteins containing the tripeptide peroxisomal targeting signal (PTS1). Two patients (PBD018 and PBD093) which exhibit a specific defect in import of PTS1-containing proteins into peroxisomes are homozygous for an inactivating missense mutation (N489K) in *PXR1*. Surprisingly, a third patient from complementation group 2 (PBD005) is unable to import PTS2-containing proteins. This patient is homozygous for a nonsense mutation (R390ter) in *PXR1* and has severely reduced levels of *PXR1* mRNA and undetectable levels of Pxr1 protein. We have identified two distinct forms of Pxr1p generated by alternative splicing. The short form of Pxr1p is the predominant species in the cell, is able to rescue the PTS1 import defects of all CG2 patients, but is unable to rescue the PTS2 import defect of PBD005. In contrast, the long form is the minor species of Pxr1p in normal cells but is able to rescue all peroxisomal protein import defects of all CG2 cell lines. Thus, the PTS1 receptor in humans appears to be essential for import of PTS2 proteins into peroxisomes. Regardless of whether the interaction between Pxr1p and PTS2 proteins is direct or indirect, our results indicate that it is mediated by a 37 amino acid-long segment generated by alternative splicing of the *PXR1* transcript. We have also found that Pxr1p is a predominantly cytoplasmic protein which only transiently associates with peroxisomes. We will present these and other findings on Pxr1p and discuss a model for Pxr1p as a component of a peroxisomal targeting signal recognition particle.

ANALYSIS OF THE SECRETORY PATHWAY IN RECOMBINANT NS/0 CELLS

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We have undertaken a study of a series of recombinant cell lines, derived from the mouse NS/0 cell line, to identify possible rate- or yield-limiting steps in protein secretion. The three cell lines represent a non-amplified transfectant, an amplified clone, and a single-cell derivative clone. The cell lines secrete varying levels of h1B4, a humanised monoclonal antibody. The secretion levels of h1B4 have been measured by ELISA and are 15-60pg/cell/day.

The recombinant lines and a series of wild-type, control lines have been analysed by western blotting using antibodies against the binding protein (BiP) and protein disulphide isomerase (PDI), the enzyme that catalyses protein folding. The activity of PDI has also been measured in both recombinant and wild-type lines. The results of the western blotting and PDI activity assays show no correlation with protein secretion level.

Pulse-chase experiments have been done on the high-secreting line to monitor protein synthesis, assembly and secretion. One- and two-dimensional SDS-PAGE under reducing and non-reducing conditions has shown various immunoglobulin assembly intermediates, including a heavy-light (HL) chain dimer, as well as the whole IgG_4 molecule (H_2L_2). These intermediates occur in both intracellular and secretory forms. Very short labelling experiments have suggested that this HL dimer is a newly-synthesised, unassembled form of IgG_4, rather than a degradation product.

Further studies are in progress on the synthesis, assembly and secretion of the recombinant antibody and the roles of BiP and PDI in these processes.

A NOVEL INTERMEDIATE ON THE IMPORT PATHWAY OF CYTOCHROME B_2 INTO MITOCHONDRIA : EVIDENCE FOR CONSERVATIVE SORTING

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Cytochrome b_2 is sorted into the intermembrane space of mitochondria by a bipartite N-terminal targeting and sorting presequence. In an approach to define the sorting pathway we have identified a so far unknown import intermediate. Cytochrome b_2-DHFR fusion proteins were arrested in the presence of methotrexate (MTX) so that the DHFR domain was at the surface of the outer membrane whilst the N-terminus reached into the intermembrane space where the sorting signal was removed. This membrane spanning, mature-size species was efficiently chased into the mitochondria upon removal of MTX. Thereby, an intermediate was generated which was exposed to the intermembrane space, but still associated with the inner membrane. This intermediate was also found upon direct import of cytochrome b_2 and derived fusion proteins. These membrane bound mature-size cytochrome b_2 species loop through the matrix as they could be recovered in a complex with mt-Hsp70 and the inner membrane MIM44/ISP45, a component of the inner membrane import apparatus.

This novel sorting intermediate can only be explained by a pathway in which cytochrome b_2 passes through the matrix. The existence of such an intermediate is inconsistent with a pathway by which entrance of the mature part of cytochrome b_2 into the matrix is stopped by the sorting sequence but is fully consistent with the conservative sorting pathway.

CHARACTERIZATION OF A TEMPERATURE-SENSITIVE ε-COP IN MUTANT CHO CELLS

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We have shown that ldlF, a temperature-sensitive CHO cell mutant, exhibits the following six distinguishing characteristics at 39.5 °C (nonpermissive temperature): (a) dramatically reduced LDL receptor activity; (b) instability of cell surface LDL receptors; (c) abnormal posttranslational processing of proteins in the secretory pathway; (d) drastically reduced protein secretion; (e) dissociation of the Golgi apparatus; and (f) death after prolonged incubation (>24 hr). Using a CHO cell cDNA expression library and complementation of the temperature-sensitive conditional lethality of ldlF cells, we isolated a cDNA which can correct all temperature-sensitive defects in ldlF cells. Sequence analysis of the cloned cDNA revealed that it encoded ε-COP. ε-COP is one of seven subunits of the coatomer complex that appears to play a key role in intracellular vesicular transport. We have recently identified a point mutation in the coding sequence of the ε-COP gene in ldlF cells. This mutant form of ε-COP ("ts-ε-COP") is the only ε-COP expressed in ldlF cells.

ε-COP in ldlF and CHO cells was examined by immunoblot and immunoprecipitation assays using an anti-ε-COP anti-peptide antibody. At the permissive temperature (34 °C), there was approximately half as much of the ts-ε-COP in ldlF cells as there was wild-type ε-COP in CHO cells (mRNA levels were also ~ 50% of wild-type). After shifting the ldlF cells to the nonpermissive temperature, ts-ε-COP became abnormally unstable (there was, however, no temperature-dependence of mRNA abundance). Most of the newly synthesized ts-ε-COP was degraded after a 4 hr incubation at 39.5 °C. These and other data suggest that the mutation in ε-COP is responsible for the temperature-sensitive phenotypes of ldlF cells. Further analysis of ts-ε-COP and ldlF cells should provide additional insights into the structure of ε-COP, its interactions with other COPs and the functions of coatomers.

SYNTHETIC LETHALITY CAUSED BY INTERMOLECULAR DISULFIDE BONDS IDENTIFIES POINTS OF CONTACT BETWEEN SecE AND SecY

Chris! Harris and Thomas J. Silhavy
Princeton University

Certain mutations in *secE* and *secY* are functional in isolation but toxic in combination. This toxicity is highly allele-specific, and is termed synthetic lethality. Intriguingly, the synthetic lethal pairs are juxtaposed topologically. One pair of mutations mapped to periplasmic portions of SecY and SecE, whereas three other pairs mapped to positions within the membraneous portions of the two proteins. One explanation for these data posits that these positions are sites of close contact between the two proteins. If true, these examples of synthetic lethality can be explained at a molecular level: by altering amino acids at points of contact, the two proteins either fail to interact, interact nonproductively, or interact too strongly.

To test this hypothesis, we have introduced cysteine residues within SecY's first periplasmic loop and SecE's second periplasmic loop, at specific positions revealed by the synthetic lethal screen. If the residues are in close contact, a disulfide bond may form, covalently linking the two proteins.

As was the case for the previously-described pair of mutations, the cysteine-containing mutant proteins are functional and non-toxic in isolation, but extremely toxic in combination. However, with the cysteine mutations, toxicity is relieved in a strain that lacks DsbA, the protein that catalyzes formation of disulfide bonds. These data indicate that a disulfide bond can form between the two cysteines. We conclude that these positions are sites of contact between two proteins.

By placing cysteines at other positions within these two loops, we have identified several pairs of cysteine mutations that are not toxic in combination. These likely represent positions of noncontact between SecY and SecE, and further illustrate that toxicity is allele-specific. Using toxicity as an assay, we have also identified a second position of apparent disulfide-bonding between the first periplasmic loop of SecY and the second periplasmic loop of SecE. Altogether, the positions of the toxic and nontoxic pairs of cysteine mutations has enabled us to formulate a strong prediction regarding the structure of these two loops: they appear to interact as antiparallel α-helices.

TOXICITY OF THE SEVENTH TRANSMEMBRANE HELIX OF SecY

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Princeton University

Two SecY protein fusions were found to be toxic upon expression by Escherichia coli: P4bla, in which ß-lactamase is fused C-terminal to the fourth periplasmic loop of SecY, and P4AP, in which alkaline phosphatase is fused at the same location. A series of deletions within the SecY moiety of P4bla revealed that the seventh transmembrane helix (TM7) of SecY was necessary to effect toxicity. Moreover, fusing ß-lactamase to TM7 alone created another toxic molecule, TM7bla. Therefore, TM7 is necessary and sufficient for the toxicity of the fusion proteins.

We believe that the toxicity of TM7 is relevant to the process of signal peptide recognition by SecY. TM7 is a hot-spot for mutations that convert wild-type SecY into a suppressor of mutant signal peptides. TM7 mutations capable of converting SecY into a suppressor of signal peptide mutations also relieve P4bla toxicity. Conversely, TM7 mutations that do not convert SecY into a signal peptide suppressor have little or no effect upon toxicity.

Osborne and Silhavy noted that the positions of signal peptide suppressor mutations in TM7 define one face of a coiled-coil structure, and suggested that TM7 is able to form a stable structure with signal peptides. As a preliminary test of this hypothesis, we introduced a signal peptide processing mutation into ß-lactamase, then examined whether this protein affected the toxicity of P4AP. The processing mutation is likely to increase the level of signal peptide in the membrane, which would then interact with and titrate TM7 of P4AP, reducing its toxicity. Indeed, the toxicity of P4AP is decreased by the presence of unprocessed ß-lactamase. This represents the first evidence that TM7 of SecY directly binds signal peptides.

A YEAST EARLY GOLGI PROTEIN IS LOCALIZED VIA RECYCLING

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Localization to the Golgi is thought to occur by a strict retention mechanism. To test if there is also a recycling component to localization, we have constructed a hybrid protein, OKS, which consists of Och1p fused to invertase with a Kex2 cleavage site between the two proteins. Och1p, a type II integral membrane protein residing in the early Golgi, is the α-1,6-mannosyltransferase that catalyzes the first mannose addition to the core N-linked oligosaccharide obtained in the ER. Secretion of the invertase moiety is required for utilization of sucrose as a carbon source. The Kex2 protease resides in a late Golgi compartment distinct from the Och1p-containing early Golgi. We reasoned that if localization occurs via retention, *suc2* deletion cells expressing a properly retained OKS fusion protein would remain Suc^- because invertase would be sequestered in the early Golgi. However, cells containing the OKS hybrid are Suc^+ suggesting that a portion of the fusion protein reaches the late Golgi. Growth on sucrose is dependent on Kex2 cleavage as wild-type cells containing an identical fusion construct without the cleavage site (OS) are unable to grow on sucrose. Exposure to Kex2 is very efficient as greater than 85% of the total invertase activity from the OKS fusion protein is secreted. Pulse-chase analysis indicates that cleavage of OKS is rapid; nearly all the fusion protein is cleaved within 10 min. After cleavage, the Och1p moiety persists with a half-life very similar to wild-type Och1p. Thus, Och1p that has reached the Kex2 compartment is not rapidly degraded in the vacuole. Finally, we have found that although the fusion proteins are exposed to the Kex2 compartment, they are still able to complement the *och1* null, suggesting that they are correctly localized. Together these data suggest a model in which the Och1 fusion proteins rapidly gain access to the late Golgi. The persistence of the cleaved Och1p combined with the ability to complement the null suggests that the Och1p may be recycled back to the early Golgi. Based on the half-time of cleavage and the half-life of the protein, several rounds of cycling from the early to the late Golgi and back again may occur before the protein is finally degraded.

NUCLEOLAR TRAFFICKING OF VIRAL AND HOST PROTEINS

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Human retroviruses, HTLV and HIV, code for proteins targeting the nucleolus regardless of the presence or absence of viral RNAs.

The arginine-rich region of Rex protein of HTLV was originally found as a nucleolar targeting signal NOS, which corresponded later with a binding site of viral Rex responsive element (RexRE). Rex is a phosophoprotein found predominantly in the nucleolus of the infected cells. The treatment of the HTLV infected cells with TPA auguments the phosophorylation of Rex at the 70th serine residue specifically which is blocked by the protein kinase C inhibitor H-7.

Mutations of NOS in Rex protein affect the partitioning and nuclear export of unspliced, partially spliced or fully spliced species of viral RNAs to the cytoplasm.

The molecular interaction between the NOS and a host nucleolar protein B23 was investigated. Two highly acidic regions of B23 were identified as acceptor regions for NOS. B23 may serve as a shuttle for the import of Rex from the cytoplasm to the nucleolus coupled to the partitioning and export of viral RNAs.

Tat and Rev, *trans*-acting regulatory proteins of HIV also contain the NOS, whose molecular and topological characterisitics have been studied. Tat-NOS and Rev-NOS indicate functions, interaction with host proteins and subcellular localization differently. Mutational analysis of the NOS in Tat, Rev and Rex, revealed that every NOS contained intrinsically nuclear localization signal NLS. NLS is sufficient for nuclear import but insufficient for nucleolar trafficking, which is fulfilled only by the NOS.

BINDING OF CLATHRIN ADAPTORS TO SORTING SIGNALS ANALYSED BY SURFACE PLASMON RESONANCE

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The clustering of membrane proteins into clathrin coated pits is presumed to be driven by the interaction of cytoplasmic sorting signals with clathrin adaptors. We have developed a novel assay to monitor this interaction in real time. The method is based on surface plasmon resonance. Synthetic peptides comprising sorting signals were immobilized to the surface of a BIAcore sensor chip. The modified surfaces were tested for the binding of clathrin adaptors purified from calf brain. As a model system we used peptides corresponding to the short cytoplasmic domains of wild-type influenza virus hemagglutinin (HA) and various mutants. Wild-type HA is a resident plasma membrane protein, the HA mutants were shown to yield internalization rates from 6 to 56 %/min in CV1 cells (Naim and Roth, JBC 269 (1994) 3929-3933). The association rates of the adaptors to the endocytosis signals as determined by surface plasmon resonance showed a clear correlation with the in vivo internalization rates of the respective HA. This finding indicates that the binding of the adaptors to the cytoplasmic sorting signal is the rate limiting step for internalization. For the peptide corresponding to the HA mutant with the highest internalization rate in vivo the apparent dissociation constant was measured to be approximately 10^{-6} M.

STABLE OR DYNAMIC INTERACTIONS OF INFLUENZA HEMAGGLUTININ MUTANTS WITH COATED PITS: DEPENDENCE ON INTERNALIZATION SIGNAL BUT NOT ON AGGREGATION

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Measurements of the lateral mobility of native and mutated membrane proteins, combined with treatments that alter clathrin lattice structure, are capable of detecting and characterizing the interactions of these proteins with coated pits in live cells (Fire, E., Zwart, D. E., Roth, M. G. and Henis, Y. I. (1991) J. Cell Biol. 115, 1585-1594). We have extended this approach to investigate the nature of the interactions between coated pits and several influenza hemagglutinin (HA) mutants, which differ in the internalization signals in their short cytoplasmic tails and which are endocytosed at different rates. The wt HA provides a natural control in this system since it is excluded from coated pits and has unrestricted lateral motion in the plasma membrane. Our results indicate that mutants that contain strong internalization signals and are endocytosed at fast rates (~50%/min) are entrapped in cell-surface coated pits for the entire duration of the lateral mobility measurement, suggesting stable association (slow dissociation) with coated pits. Mutants exhibiting slower internalization rates (~7-10%/min) show transient interactions with coated pits, reflected in a reduced lateral diffusion rate. Both types of interactions disappear or are significantly reduced upon disruption of the clathrin lattices. Unlike the dependence on the cytoplasmic internalization signal, the internalization rate and the interactions with coated pits did not depend on the aggregation state (measured by sucrose gradient centrifugation after solubilization in n-octylglucoside) of the mutants.

PROTEIN TRANSPORT IN MALARIA

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The malarial parasite extensively alters its host red blood cell; inserting parasite derived proteins into the plasma membrane of the red blood cell, establishing 'organelles' within the cytoplasm of the infected red blood cell and even secreting proteins from the infected red blood cell. This 'takeover' of the red blood cell is quite remarkable considering that mature human red blood cells completely lack intracellular organelles or any of the vesicular machinery for protein transport.

We have recently observed by laser scanning confocal microscopy a dynamic population of parasite derived vesicles in the host red blood cell cytoplasm. These vesicles are highly mobile, and are shown by following a single cell to be derived by budding from the parasite. These vesicles are most prominent some hours after the invasion, and become very rare at the trophozoite stage. Although we have not yet established the function of these vesicles, their initial appearance at the mid ring stage when major changes to the host red blood cell are initiated, suggests that they may be the vehicle by which malarial proteins are transported across the infected red blood cell cytoplasm.

Small macromolecules such as lucifer yellow are readily taken up into live malaria parasites and there is tantalising evidence that larger molecules such as antibodies directed against parasite proteins can be internalised. The proposal by Pouvelle et al (1991) that a 'duct' traverses the infected red blood cell in malaria has created considerable controversy. We have found that the small latex beads originally used in their experiments are not taken up into live parasites, but dye released from the beads stains the parasite and sometimes extensions of the parasite that reach out into the red blood cell cytoplasm. It would appear that an open 'duct' does not exist in malaria. This will now open the way for establishing the true nature of macromolecular uptake in malaria.

Pouvelle et al (1991) Nature **353**, 73-75.

THE HISTIDINE-RICH PROTEIN FROM *PLASMODIUM FALCIPARUM* IS TRANSPORTED FROM THE PARASITE TO THE RED BLOOD CELL MEMBRANE VIA A BREFELDIN A-INSENSITIVE PATHWAY

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As in other eukaryotic cells, many *Plasmodium falciparum* polypeptides are transported to different cellular compartments or are secreted. However, since the parasite develops within the host erythrocyte, those polypeptides which are exported to the red cell cytoplasm or to its surface must first cross the parasite and the parasitophorous vacuole membranes before reaching their destinations. The mechanisms and pathways implicated in the intracellular transport are poorly understood. To better characterise the *Plasmodium* secretory pathways, synchronised parasite cultures were treated with Brefeldin A (BFA). The cultures were reacted with antibodies specific for defined antigens, processed for immunofluorescence and analysed by confocal microscopy. In BFA-treated cultures malarial antigens known to be secreted into the external medium (HRPII) or targeted to the red cell membrane (Pf332, MESA) accumulated within the parasite, suggesting that these polypeptides are secreted through the endoplasmic reticulum-Golgi pathway. Most interestingly, we observed that the transport of the Histidine Rich Protein I (HRPI), an antigen that associates with the erythrocyte membrane, is not blocked by BFA. Furthermore, the transport of HRPI is not blocked by incubating the parasite cultures at either 15°C or 20°C and the *in vitro* translated HRPI antigen is not translocated into dog pancreas microsomes. These results suggest that HRPI is transported through an unknown BFA-insensitive pathway. In contrast to other polypeptides that bypass the ER-Golgi secretory pathway which do not have a signal peptide, the HRPI has a hydrophobic region located 21 amino acids from the N-terminus.

THE TRANSMEMBRANE DOMAINS OF MEMBRANE Ig HEAVY CHAINS PLAY A CRITICAL ROLE IN THE INTERACTION OF THESE PROTEINS WITH THE ER CHAPERONE CALNEXIN

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Molecular chaperones contribute to the general mechanism of "quality control" which ensures proper folding and assembly of proteins. One of these chaperones, calnexin, has been implicated in the retention of misfolded and incompletely assembled multimeric proteins within the endoplasmic reticulum. Calnexin also associates with secreted glycoproteins and recent evidence suggests that this tranmembrane ER chaperone may function as a lectin which exclusively binds monoglucosylated nascent chains and retains them until proper folding has been achieved. This model may, however, be applicable only to single chain glycoproteins. In the case of multimeric complexes, proper folding of individual chains would presumably not suffice to permit egress from the ER as each chain must oligomerize with its partners before exiting. We have analyzed membrane Ig heavy chain - calnexin interactions as a model system for calnexin-oligomeric membrane protein interactions. We wished to explore the hypothesis that calnexin mediates association with individual integral membrane protein components of multimeric complexes via structural motifs other than carbohydrate side chains.

In COS cells expressing transfected wild-type and mutant membrane IgG heavy chains, we only detect association of calnexin with the membrane form of the IgG heavy chain and not with a heavy chain protein lacking a transmembrane domain. This association does not depend on the presumed lectin function of calnexin, as castanospermine (an inhibitor of N-linked glycan trimming) and tunicamycin do not abolish the interaction of membrane IgG and membrane IgM heavy chains with calnexin. This demonstrates that calnexin associates with membrane Ig heavy chain proteins in a transmembrane domain dependent and glycan independent manner.

THE D136N MUTANT OF THE SMALL GTPASE RAB5 HAS REVERSED AFFINITY FOR GTP AND XTP WHILE MAINTAINING TOPOLOGY AND FUNCTIONAL ACTIVITY

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The small GTPase Rab5 regulates the fusion of early endosomes. Analysis of the molecular function of Rab5 is complicated by the presence of multiple distinct GTPases with putative activity in endosome fusion. A D136N Rab5 mutant, which was predicted to have specificity for xanthine nucleotides, was generated in an attempt to produce a reagent whose activity could be regulated independently of other GTPases. The mutant protein was insoluble when expressed in *E. coli*, and therefore was extracted and purified in urea. After refolding by dialysis against an XDP/CHAPS/Mg/Tris buffer, biochemical properties of Rab5 D136N were compared to those of wild type (WT) Rab5. The affinity of WT protein was ≈1,000-fold higher for GTP than XTP, and this differential affinity was essentially reversed for the mutant. The affinity of ITP was 50-fold higher for WT than mutant Rab5. The conformation of the mutant protein was assessed by its nucleotide-dependent resistance to trypsin degradation. The protection of Rab5 D136N from proteolysis in the presence of XTP and of a 20 kDa fragment in the presence of XDP was similar to the protection of Rab5 WT by GTP and GDP, respectively. WT and mutant proteins were geranylgeranylated only in the presence of cognate nucleotides, and both preferred diphosphate over triphosphate nucleotide. The D136N mutant thus appears to provide a specific reagent for biochemical assay of Rab5 activity in crude cellular fractions. This strategy should be useful for the analysis of other GTPase-regulated cytotic processes.

A NOVEL YEAST GENE, PAS9, ESSENTIAL FOR PEROXISOME BIOGENESIS

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Our laboratory is involved in a genetic dissection of the mechanism of peroxisome biogenesis in the yeast *Saccharomyces cerevisiae* [1, 2]. Here we will report on a novel component essential for peroxisome assembly, the PAS9 gene.

The gene was cloned by functional complementation of mutant pas9 with a *S. cerevisiae* YCp50 genomic library. Sequence analysis revealed an open reading frame of 597 bp which encodes a protein with a predicted molecular mass of 23 kD. Hydropathy analysis according to Kyte and Doolittle [3] and others indicated a putative membrane spanning region in the NH_2-terminal half of the Pas9 protein (Pas9p). Search of data bases showed no significant sequence similarity to any other known protein.

To gain first information on the function of the PAS9 gene product we have started to determine the intracellular localization of Pas9p. For this purpose a ß-lactamase-Pas9 chimera was constructed which rescued the pas9 null mutant and cofractionated with peroxisomes. In addition, specific antibodies against the Pas9 protein detected a polypeptide band with expected molecular weight in peroxisomal gradient fractions. Western blot analysis of high salt extracted peroxisomal membrane proteins indicate that Pas9p is a peripheral membrane protein. We are presently investigating the topology of the Pas9 protein.

To address the question whether the Pas9 protein interacts with other PAS gene products, the two-hybrid system is currently employed.

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[3] Kyte, J. and Doolittle, H. (1982) J. Mol. Biol. 157: 105-132

PROTEIN SORTING AND VESICLE BUDDING FROM THE ENDOPLASMIC RETICULUM

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We have purified three cytosolic Sec proteins that are required for the formation of transport vesicles from the endoplasmic reticulum. Sar1p is a small GTP binding protein that is targeted to the ER through transient interaction with Sec12p, a membrane glycoprotein that facilitates nucleotide exchange on Sar1p. Next a complex of Sec23p and Sec24p binds to ER membranes in a reaction that requires Sar1p and GTP or GMPPNP. Finally, another complex consisting of Sec13p and Sec31p binds only when the other components are present. A complete incubation produces vesicles that contain cargo molecules (α-factor precursor and a plasma membrane permease precursor) and v-SNARES (Sec22p, Bet1p, and Bos1p), but lack resident ER proteins (Sec61p and BiP). However, cargo packaging and vesicle budding are not obligately coupled; Sec22p continues to be packaged into vesicles produced by membranes isolated from cycloheximide-treated cells.

Vesicles formed with Sec proteins and GTP or GMPPNP contain a novel coat structure (COPII) whose assembly/disassembly resembles the process of coatomer/ARF-mediated vesicle (COPI) budding within the Golgi complex. COPII-mediated budding operates to produce vesicles directly from the ER. Isolated yeast nuclei are observed to form buds and vesicles from the outer nuclear membrane when all three Sec protein fractions and GTP (or GMPPNP) are present.

Nuclei mixed with coatomer/ARF and GTPγS produce buds and vesicles that morphologically resemble the COPII buds and vesicles produced by Sec proteins. However, α-factor precursor is captured only in COPII vesicles. A mixed incubation containing Sec proteins and coatomer/ARF produces distinct vesicles that contain non-overlapping coat subunits. Isolated COPI and COPII vesicles contain the same v-SNARES which presumably serve to target both vesicles to the Golgi complex. The distinctive cargo content and the absence of ER resident proteins in ER-derived COPI and COPII vesicles suggest that the coat proteins play an active role in protein sorting and cargo concentration early in the secretory pathway.

THE ERYTHROPOIETIN RECEPTOR: BIOGENESIS AND INTRACELLULAR SIGNALING

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Addition of erythropoietin (EPO) to responsive cells leads to dimerization of cell surface receptors (EPO-R's), followed by binding of the protein-tyrosine kinase JAK2 to the EPO-R, tyrosine-phosphorylation of JAK2 and the EPO-R, and activation of JAK2. The protein-tyrosine phosphatase SH-PTP1 associates with the EPO-R after EPO addition, an interaction mediated by the SH2 domains of SH-PTP1 binding to phosphorylated tyrosine 429 in the cytoplasmic domain of the EPO-R. When expressed in hematopoietic cells, EPO-Rs lacking Y429 are unable to bind SH-PTP1, EPO-induced autophosphorylation of JAK2 is prolonged, and the cells proliferate in one-tenth the concentration of EPO required for cells expressing the wild-type EPO-R. SH-PTP1 but not its close homologue SH-PTP2 specifically dephosphorylates and inactivates JAK2. A phosphotyrosine-containing peptide derived from residue Y429 of the EPO-R specifically activates SH-PTP1. Thus activation of SH-PTP1 by binding to phosphoY429 in the EPO-R leads to dephosphorylation of JAK2; this is important for down-modulation of signals generated by the activated erythropoietin receptor and plays a major role in terminating proliferative signals.

Both in transfected and normal hematopoietic cells the majority of newly-made EPO-R subunits are retained in the endoplasmic reticulum (ER), destined for degradation, while only a small fraction fold properly and exit the ER. We isolated a mutant EPO-R with a change in the middle residue of the conserved WSXWS motif, A234E, which was processed in the ER more efficiently than the wild type receptor and was expressed in elevated numbers at the cell surface. This is the first example of a mutant receptor polypeptide that folds more efficiently than the wild-type in the ER, and suggests that the inefficient processing of the wild-type EPO receptor is one mechanism for controlling the number of plasma membrane receptors.

Another is rapid endocytosis of cell surface EPO receptors, which are degraded rapidly with a half life of about an hour in lysosomes or other acidic intracellular vesicles. In both normal erythroid and transfected cells the majority of EPO-Rs are intracellular, with less than 2000 molecules on the cell surface.

PROTEINS THAT DIRECT TRAFFIC IN THE EARLY STAGES OF THE SECRETORY PATHWAY

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Secretion invoves the movement of proteins from the endoplasmic reticulum (ER) through several compartments in the Golgi complex to the cell surface. This traffic is bi-directional: resident ER proteins may move to the Golgi apparatus, but are then selectively retrieved. Movement occurs primarily in transport vesicles which pinch off from one compartment and fuse with the next, the specificity of the fusion reactions being determined by integral membrane proteins on the vesicles and target membranes, termed v-SNARES and t-SNARES respectively. In yeast, four v-SNARES involved in transport from ER to Golgi have been identified, as has the t-SNARE with which they interact (the Sed5 protein). Recent experiments suggest that Sed5p also acts as the t-SNARE for retrograde traffic from a later Golgi compartment, recognising a distinct v-SNARE that mediates this step. Sed5p seems to define the identity of the first post-ER compartment, and indeed when Sed5p or its mammalian homologue is overexpressed, this compartment enlarges. To understand the organisation of the secretory pathway, it will be necessary to identify the SNARES that specify forward and retrograde transport from each compartment to the next, and to determine the mechanisms that direct these proteins to their appropriate locations. Progress towards this end will be discussed.

RETENTION IN THE ENDOPLASMIC RETICULUM (ER) : ROLE OF COAT PROTEINS

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Dilysine motifs in cytoplasmic domains of transmembrane proteins are signals for their continuous retrieval from the Golgi back to the ER. We describe a system to assess retrieval to the ER in yeast cells. Whereas retrieval was unaffected in most mutants tested, a defect in retrieval was observed in previously characterized coatomer mutants, as well as in newly isolated retrieval mutants (*sec21-2*, *ret1-1*). *RET1* was cloned by complementation and found to encode the α subunit of coatomer. Coatomer from β'-COP and α-COP (*ret1-1*) mutants, but not from γ-COP mutants, had lost the ability to bind dilysine motifs *in vitro*. These results suggest that coatomer plays an essential role in retrograde Golgi-to-ER transport and retrieval of dilysine-tagged proteins back to the ER.

FACTORS MEDIATING THE LATE STAGES OF ER TO GOLGI TRANSPORT IN YEAST Lian, J. P., Stone, S., Jiang, Y., Lyons, P., Rossi, G., Mao, Y., Kolstad, K., Feliciano, E., Sacher, M., Singer-Krüger, B., and Ferro-Novick, S. Department of Cell Biology, Yale University, New Haven, CT

Proteins are transported from the ER to the cell surface through a series of membrane-bound compartments. In the case of ER to Golgi transport, specific carrier vesicles have been shown to mediate membrane traffic at this stage of the secretory pathway. Using a combined genetic and biochemical approach, we are studying the mechanism by which vesicles bind and fuse with their acceptor membrane in the yeast Saccharomyces cerevisiae.

The *BET* and *BOS* genes were initially identified through our efforts to define those genes whose products mediate the targeting and fusion of the ER to Golgi transport vesicles with their acceptor compartment. *BOS1* interacts genetically with *SEC22* and four other genes (*BET1*, *BET3*, *SEC21*, and *YPT1*) encoding factors that mediate ER to Golgi transport. *BOS1* and *SEC22* encode integral membrane proteins of the ER to Golgi transport vesicles that are homologous to the synaptic vesicle protein synaptobrevin and function in vesicle targetting and fusion. These findings have led to the proposal that Bos1p and Sec22p are v-SNAREs, or vesicle receptors for soluble fusion factors. Although these proteins are not associated with each other prior to vesicle budding, we have shown that they form a complex on isolated vesicles. Additional copies of Bos1p relieve the growth defect of a strain that lacks Sec22p, indicating that the overexpression of Bos1p compensates for the loss of Sec22p. Based on these and previous findings, we propose that Bos1p is a key component of a targeting/fusion complex that resides on the ER to Golgi transport vesicles, while Sec22p facilitates its activity on this compartment. The Ras-like GTP-binding protein Ypt1p appears to regulate these interactions.

THE FINAL STAGE OF THE YEAST SECRETORY PATHWAY

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The products of ten secretory (*SEC*) genes are required for the vectorial transport and localized fusion of Golgi-derived secretory vesicles with the plasma membrane. In addition, a synaptobrevin homolog on the vesicle membrane, Snc, and a syntaxin homolog on the plasma membrane, Sso, are also required. *SEC4* encodes a GTP binding protein that cycles through the cell. Sec4 is synthesized as a soluble protein, but rapidly associates with the cytoplasmic plasma membrane. A recycling pathway exists by which Sec4 can be released from the plasma membrane and reassociate with a new round of secretory vesicles. Genetic studies suggest that this cycle of localization may be obligatorily coupled to a cycle of binding and hydrolysing GTP. The cycle could be regulated at three sites: exchange of GDP for GTP; attachment of soluble Sec4 to the membrane; and hydrolysis of the bound GTP to GDP. Through suppressor analysis we have identified a gene, *DSS4*, which encodes a protein which increases the off rate of GDP. We have also cloned a gene, *GDI1*, that encodes an activity which allows the dissociation of Sec4 from membranes in a nucleotide specific fashion and we have detected an activity which stimulates the hydrolysis of GTP by Sec4.

Recent studies indicate that the Sec6, Sec8 and Sec15 proteins and 5 unidentified polypeptides comprise a stable complex that is associated with the plasma membrane at the site of vesicle fusion. Three other gene products, Sec3, Sec5 and Sec10, are required for the assembly of the Sec6/8/15 complex. We have also identified a complex containing Snc, Sso and Sec9. Formation of this complex may be a key step in the targeting of vesicles to the plasma membrane. Genetic studies have led to our working hypothesis that Sec4, by binding and then hydrolysing GTP, regulates the assembly and subsequent disassembly of this targeting complex and this regulation is mediated through the Sec6/8/15 complex. Studies from other laboratories indicate that proteins closely related to Sec4 are needed to regulate other vesicular transport events. Each class of vesicular carriers appears to be marked by a GTP binding protein as well as a distinct relative of synaptobrevin. These families of proteins may play an essential role in maintaining the specificity of vesicular transport in all eukaryotic cells.

Mutations in the *MYO2* gene, which encodes a non-conventional myosin, lead to the accumulation of vesicles in the mother cell, while, mutations in *SEC1* or *SEC6* lead to the accumulation of vesicles in the bud. This suggests that the Myo2 protein may be a motor which moves vesicles along actin fibers to the bud, while Sec1 and Sec6 proteins are required at a later step. However, our studies suggest that only a subset of secretory vesicles require Myo2.

GTPASES: MOLECULAR SENSORS REGULATING ENDOPLASMIC RETICULUM (ER) TO GOLGI TRANSPORT.

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COPI and COPII coats are involved in endoplasmic reticulum (ER) to Golgi transport. Both are required for transport of the same cargo molecule, vesicular stomatitis virus glycoprotein (VSV-G), from the ER to the Golgi stack. Using activated mutants of the small GTPases Sar1p and ARF1 which control the assembly of COPII and COPI coats, respectively, we find that the two coat complexes are recruited separately and sequentially to membranes. While Sar1 mediated COPII recruitment is responsible for export from the ER, mediating a vesicular transport step essential for the appearance of VSV-G in pre-Golgi intermediates, COPI is recruited onto pre-Golgi intermediates where it initiates segregation of anterograde and retrograde transported markers preceding transport of VSV-G to the Golgi stack. This sequential coupling between COPII and COPI coats is critical to facilitate sorting and concentration of protein during vesicle budding from the ER, a step specifically activated by the presence of cargo.

PROTEIN-PROTEIN INTERACTIONS GOVERNING MEMBRANE TRAFFIC AT THE SYNAPSE

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Significant progress has been made in recent years in the identification and characterization of synaptic proteins involved in synaptic vesicle exocytosis and recycling. The synapse differs from other membrane trafficking systems in the exquisite temporal and spatial regulation of synaptic vesicle exocytosis. At this point, candidate molecules for many steps in the synaptic vesicle pathway have been described, and the functions of selected proteins have been investigated. My laboratory has used a combination of three approaches to gain insight into the nature of some of the mechanisms underlying synaptic vesicle traffic: 1. Biochemical identification and characterization of components of the pathway and their interactions; 2. Studies on the mechanism of action of toxins that inhibit the pathway (botulinum and tetanus toxins) or accelerate it (α-latrotoxin)1; and 3. Characterization of mouse mutants in selected synaptic proteins. Although a concise understanding of synaptic membrane traffic is not likely to be achieved in the near future, key proteins that operate at defined points of the pathway have been identified and models for their mode of action have been generated. For example, a combination of biochemical and genetic studies has pinpointed a pivotal role for synaptotagmin in the Ca^{2+} dependent last step in exocytosis, and Ca^{2+} dependent protein-protein interactions for synaptotagmin with a Ca^{2+} dependency approaching that of exocytosis have been identified. I will discuss the most recent results emerging from these multidisciplinary studies.

MOLECULAR INSIGHTS INTO THE FORMATION OF NEUROSECRETORY VESICLES

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Three types of neurosecretory vesicles exist, (i) secretory granules which store neuropeptides, (ii) synaptic vesicles which contain classic neurotransmitters, and (iii) small dense core vesicles which contain biogenic amines.

Secretory granules originate from the trans-Golgi network (TGN). Sorting of secretory proteins to these vesicles involves their selective aggregation induced by the lumenal milieu of the TGN, and the recognition of signals by specific membrane components. The cytoplasmic machinery mediating secretory granule formation includes cytosolic phospho-proteins, classical heterotrimeric G proteins and a novel "extralarge" G protein, XLαs.

Synaptic-like microvesicles of neuroendocrine cells originate from early endosomes. The biogenesis of these vesicles is independent of that of secretory granules.

Small dense core vesicles are a hybrid of the synaptic vesicle and the secretory granule membranes.

Recent progress in the identification of the components and mechanisms involved in the biogenesis of these vesicles will be presented.

CONTROL OF THE EXOCYTOTIC FUSION APPARATUS IN NEURONS

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It is currently believed that exocytosis of synaptic vesicles is mediated by an exocytotic fusion complex that contains the synaptic vesicle protein synaptobrevin (VAMP) and the synaptic membrane proteins syntaxin 1 and SNAP-25. This complex interacts with the soluble fusion factors NSF and SNAPs. Strong support for this concept was provided by our finding that tetanus and botulinal neurotoxins act as metalloproteases that inhibit neurotransmitter release by cleaving each of these proteins. Each individual neurotoxin is highly selective for a single protein.

We have now studied the life cycle of these proteins in the synapse in order to identify regulatory mechanisms controlling their assembly and disassembly. First, we investigated whether the assembly of the fusion complex is controlled by other proteins. We focused on the synaptic vesicle protein synaptobrevin since this protein is highly concentrated in the vesicle membrane and since only a limited number of additional proteins are present on this organelle. We found that the ability of synaptobrevin to bind to syntaxin and SNAP-25 appears to be tightly controlled by synaptophysin, a major membrane protein of synaptic vesicles. Binding of synaptobrevin to synaptophysin prevented its association with SNAP-25 and syntaxin suggesting that synaptophysin may function as a regulator of exocytosis.

Second, the regions of SNAP-25 responsible for the interactions with syntaxin and synaptobrevin were mapped. We found that these proteins have the intrinsic property to associate with each other. Binding was assigned to distinct domains that have a high propensity to form coiled-coils, i.e. special forms of α-helices known to mediate intra- or intermolecular interactions. Shortening of the C-terminus of SNAP-25 by 9 amino acids, which corresponds to the fragment generated by botulinum neurotoxin A, does not affect its binding to syntaxin but weakens the interaction with synaptobrevin. In intact nerve terminals, poisoning by BoNT/A leads to the assembly of a complex that contains both intact and truncated forms of SNAP-25. Like the intact complex, this complex can be disassembled by NSF suggesting that NSF-driven disassembly may be necessary but is not sufficient to catalyze membrane fusion.

PROTEINS INVOLVED IN SYNAPTIC VESICLE DOCKING AND FUSION

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A large number of proteins have been identified in presynaptic terminals, but it has been difficult to elucidate the roles of these proteins in the exocytotic secretion of neurotransmitters. We have employed the squid "giant" synapse system to determine the function of a number of these presynaptic proteins. Our general approach has been to:

- clone the genes for these proteins from a squid cDNA library
- use the inferred primary sequences of these proteins to develop chemical probes - such as recombinant proteins, peptides, antibodies and toxins - that are specific for individual proteins
- screen for the functional activity of these probes by injecting them into living presynaptic terminals while measuring evoked transmitter release
- use the resultant perturbations to identify the presynaptic sites of action of the targetted proteins

With this approach, we have obtained evidence supporting roles for **synaptotagmin, synaptobrevin, syntaxin, SNAP** and **NSF** in neurotransmitter secretion. Based on the perturbations produced by our probes, we propose that:

- all of these proteins act downstream of Ca entry into the terminal or docking of synaptic vesicles
- synaptobrevin, syntaxin, SNAP and NSF put docked vesicles into a primed, pre-fusion state
- synaptotagmin is the Ca receptor that triggers neurotransmitter secretion by allowing primed vesicles to fuse with the presynaptic plasma membrane.

Unravelling the interactions among these proteins should help define the forces that produce exocytosis.

CALCIUM-DEPENDENT VESICULAR EXOCYTOSIS: FROM CONSTITUTIVE TO REGULATED SECRETION.

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Constitutive exocytosis is ubiquitous in all eukaryotic cells. It is responsible for the recycling and renewal of plasma membrane components and for the secretion of molecules into external space. In neuroendocrine cells a Ca^{2+}-regulated form of vesicular exocytosis is known to be responsible for the secretion of transmitters and peptides. The distinction and inter-relationship of these two pathways have attracted our recent attention because of the following findings. **First,** we found isolated myocytes exhibit spontaneous quantal acetylcholine (ACh) release after intracellular loading of ACh, reminiscent of that occurs at the presynaptic nerve terminal. Remarkably, this quantal secretion was elevated by raising intracellular Ca^{2+} and secretion can be evoked by a brief depolarization of myocyte membrane. **Second,** cultured fibroblasts showed similar quantal and evoked ACh secretion after cytoplasmic ACh loading. Evidence suggests that this ACh secretion from myocytes and fibroblasts results from exocytosis of vesicles of constitutive pathways that accumulated cytoplasmic ACh. In support of this notion, we found that similar spontaneous and evoked ACh secretion can be observed after ACh was loaded directly into endocytic recycling compartments by brief incubation of the fibroblasts in ACh-containing medium. **Third,** while myocyte and fibroblasts may exhibit Ca^{2+}-dependent exocytosis, the excitation-secretion coupling is relatively weak, in comparison to that of neuronal secretion. There is a higher failure rate and a longer delay-of-onset for the depolarization-evoked secretion in these non-neuronal cells. Thus the basic mechanism of vesicular exocytosis is Ca^{2+}-dependent and the distinction between constitutive and regulated exocytosis may reside in the subcellular distribution of the vesicles and in the presence of proteins that modulate the efficiency of calcium-dependent exocytotic process. **Fourth,** in support of the above notion, synaptotagmin 1-transfected CHO fibroblasts (provided by K. Buckley) showed a reduced spontaneous exocytosis of endosome-derived vesicles, but an increased Ca^{2+}-dependent spontaneous and evoked exocytosis. Thus, through stepwise introduction of neuron-specific proteins into non-neuronal cells, the role of neuronal proteins in the transition from constitutive to regulated secretion may be revealed.

MEMBRANE TRAFFICKING IN THE NERVE TERMINAL

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Three multimeric complexes of the protein syntaxin are important in neurotransmitter secretion: (1) syntaxin and n-sec1, (2) syntaxin, VAMP and SNAP-25 and (3) syntaxin, VAMP, SNAP-25, α-SNAP and NSF (20S complex). In this talk, it is demonstrated that unique, yet overlapping, domains of syntaxin are required to form these complexes. The formation of higher order heteromultimers has a distinct set of structural requirements from those required for the dimeric interactions. Dissociation of the 20S complex by NSF following ATP hydrolysis requires amino terminal regions of syntaxin which are outside of the binding domains for the 20S constituent proteins. These data are consistent with a model whereby conformational changes in syntaxin, resulting from protein-protein interactions and ATP hydrolysis by NSF, mediate vesicle docking and fusion.

GENETIC ANALYSIS OF DROSOPHILA SYNAPTIC MECHANISMS

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We have isolated mutations in the Drosophila genes encoding syntaxin (*syx-1*), and synaptotagmin (*syt*). Null alleles of *syx-1* and *syt* differ markedly in the severity of their phenotype: *syx-1* mutant embryos do not move and do not hatch, but *syt* mutants can hatch and crawl weakly. Physiological recordings from mutant *syt* neuromuscular junctions reveal that low levels of Ca^{++}-dependent synaptic transmission persists in *syt* nulls and that *syt* mutations cause modest increases in the spontaneous fusion of synaptic vesicles.

Ultrastructural changes in the vesicle population implicate synaptotagmin in several stages of the vesicle life cycle. There is a 75% decrease in the number of vesicles that are docked at release sites by morphological criteria. The appearance of some abnormal vesicles suggests that other stages of the vesicle life cycle are also altered. Moreover, we observe a genetic interaction of *syt* and *shibire*^{ts} (dynamin) mutants that implicates synaptotagmin in vesicle recycling as well. These diverse effects are consistent with the multitude of biochemical interactions of synaptotagmin with other nerve terminal components and indicate that docking and recycling may be central to synaptotagmin function.

In addition to an apparently essential role in synaptic transmission, *Syx-1* is implicated in non-neuronal membrane trafficking. It is expressed in early embryos, prior to cellularization, and the significance of this non-neuronal expression is currently under investigation.

IDENTIFICATION AND CHARACTERIZATION OF NUCLEAR PORE-TARGETING COMPLEX COMPONENTS

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The selective nuclear import of karyophilic proteins is directed by short amino acid sequences termed nuclear location signal (NLS). The process of mediated nuclear import is known to involve at least two steps, ATP-independent binding to the cytoplasmic face of nuclear pores, followed by translocation through the nuclear pore complex depending on hydrolysis of ATP. We recently found that a karyophilic protein forms a stable complex with cytoplasmic components prior to pore binding-step of transport. Such a complex, termed nuclear pore-targeting complex, contains two essential proteins with electrophoretically estimated molecular mass of 54 and 90kDa. We cloned full length of mouse cDNAs encoding these proteins. The recombinant 54 and 90kDa proteins, when added together, mediated NLS-dependent pore binding-step of import in the digitonin permeabilized cell-free transport assay. In reference to their calculated molecular mass of 58 and 97kDa, we designated these proteins PTAC58 and PTAC97 (nuclear pore-targeting complex components of 58 and 97kDa). Biochemical analysis using recombinant proteins revealed that the pore-targeting complex is formed through interactions of NLS with PTAC58, and PTAC58 with PTAC97. Using affinity purified antibodies raised against PTAC58, we further provide *in vivo* evidence that this targeting complex component is involved in nuclear protein import through association with a karyophile in the cytoplasm prior to nuclear pore-binding.

STRUCTURAL REQUIREMENT FOR RECOGNITION OF THE PRECURSOR PROTEINS BY THE MITOCHONDRIAL PROCESSING PEPTIDASE

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Mitochondrial processing peptidase (MPP) specifically recognizes a large variety of mitochondrial precursor proteins and correctly cleaves off the extension peptides. To determine the structure common to all the extension peptides that is required for specific recognition by MPP, we synthesized various oligopeptides of different chain lengths and amino acid sequences, based on the amino acid sequence of the extension peptide of pre-malate dehydrogenase* and determined kinetic parameters of the cleavage reactions.

Two sets of basic amino acids in the peptide, the proximal arginine residue at positions -2 (or -3) and the distal one at -10 relative to the cleavage site, were necessary for effective hydrolysis. The proximal arginine residue could not be replaced by a lysine, whereas replacement of the distal arginine by lysine had no effect on the cleavage. The amino acid at position -1 was variable, while MPP prefers aromatic and hydrophobic amino acids as the residue at position +1. The intervening sequence between the proximal and distal arginine residues was dispensable except flexible amino acid residues. These results suggest that the distal and proximal arginine residues are spatially close by the aid of the flexible sequence and form in combination with the amino acid at position +1 a structure cleavable at the correct site when accommodated in the binding pocket of MPP.

* MLSALARPVGAALRRS$^{\nabla}$FSTSAQNN-

SECY-SECE TRANSLOCATOR COMPLEX: AN AAA FAMILY ATPASE, FTSH, PARTICIPATES BOTH IN ASSEMBLY AND PROTEOLYTIC ELIMINATION OF SECY IN THE CELL

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SecY and SecE, two important integral membrane components of *E. coli* protein translocator, form a complex, thereby providing a putative channel-like path in the membrane for the passage of secretory proteins. We will first summarize our genetic analyses of SecY-SecE interaction (1), and then report our recent findings about the "quality control" of the complex.

The protein-translocating pathway should strictly be gated such that it does not allow diffusion of non specific solutes. When uncomplexed forms of SecY are produced in *E. coli* cells, they are rapidly eliminated by proteolysis. We have evidence that such a proteolytic mechanism is important for the survival of the cell and its protein export activity. We found that a membrane-bound ATPase, FtsH, is required, and is the rate-limiting factor, for the degradation of uncomplexed SecY molecules (2). The cytoplasmic domain of FtsH belongs to the "AAA family", widely distributed among eukaryotes and prokaryotes. By systematic isolation of SecY-stabilizing mutants we found two additional loci that can be mutated to be SecY degradation-defective. One class of these mutations fell into either the *hflX* or the *hflK* gene, previously shown to encode a protease involved in degradation of the cII gene product of phage λ.

The involvement of FtsH in the proteolytic activity should be reconciled with our own previous proposal that FtsH is somehow required for proper assembly of SecY and certain membrane proteins (3, 4). As our evidence indicates that the "stop transfer-defective" phenotype seen for a SecY-PhoA fusion protein in some *ftsH* mutants is not a consequence of its stabilization, we suggest that FtsH is a protein with multiple functions. This ATPase might possess chaperone-like activities by which it leads SecY to proper assembly (when its partner SecE is available), or to its degradation (when SecE is missing). We also present our analysis of the subunit structure of the FtsH complex, as well as our mutational analyses of the roles of its ATPase domains in the ATPase activity and binding/release of SecY. Additionally, a new protein that interacts with SecY and affects its stability/activity (5) will be reported.

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RECONSTITUTION OF PROTEIN TRANSLOCATION ACROSS THE MEMBRANE OF THE ENDOPLASMIC RETICULUM IN SEMI-INTACT CELLS

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We have reconstituted efficient translocation of neo-synthesized proteins across endoplasmic reticulum membranes of semi-intact cells. This model system could be used to overcome some technical limitations of studies performed with isolated microsomes. MelJuSo cells, grown in monolayers were treated in suspension with saponin in order to induce a differential permeabilization of their membranes (Wassler, M. *et al.*, 1987). Permeabilization was optimized through measurement of marker enzymes release. During preparation, treated cells lose their cytosol but retain ER structures. This was confirmed in microscopy after staining of ER with the fluorescent dye $DIOC_6(3)$ (Terasaki, *et al.*, 1984).

Once incubated in the presence of a wheat germ extract, the semi-intact cells allow efficient translation of an exogenous mRNA coding for a type II membrane protein (Invariant Chain). Furthermore, we demonstrate that a reconstituted translocation event is taking place as judged from glycosylation of the nascent polypeptide chain and from results of a protease protection assay. Translocation yield was compared to standard results obtained with dog pancreas microsomes and shown to be very similar. Site specific photo cross-linking experiments (High, S. *et al.*, 1993) were used to demonstrate directly interactions of the protein nascent chain with components of the translocation apparatus. These results indicate that during membrane insertion, the environment of the nascent polypeptide is similar to the one observed in canine pancreas microsomes.

We think that such a combination between the site specific photo cross-linking approach and the use of those semi-intact cells could be a valuable model system for investigations of protein-protein or protein-lipid interactions taking place downstream of the translocation complex.

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cAMP STIMULATES SIALYATION AND APICAL SECRETION OF ALPHA-1 ANTITRYPSIN BY COLONIC EPITHELIAL CELLS , Tamas Jilling and Kevin L. Kirk, Department of Physiology and Biophysics, UAB, Birmingham, AL

The secretion of proteins that do not accumulate in distinct storage vesicles is often referred to as constitutive or bulk flow secretion. The term "constitutive secretion" implies the lack of an acute regulation of secretion such as observed during the acute secretagogue-induced release of mucins from storage vesicles in intestinal goblet cells. We utilized pulse-chase metabolic labeling of filter-grown monolayers of HT29-CL19a colonic epithelial cells to study the regulation of the constitutive secretory pathway in polarized epithelial cells. The kinetics and regulation of secretion were investigated by analyzing the secreted products via TCA-precipitation followed by scintillation counting or via SDS PAGE analysis followed by phosphor imaging. We found that cAMP stimulates the apical but not the basolateral secretion of proteins from colonic cells. Based on it's characteristic appearance on two dimensional PAGE, alpha1-antitrypsin (AT) was identified as one of the secreted proteins; a result that was confirmed by immunoprecipitation. AT is secreted in both the apical and the basolateral directions, however, only the apical secretion is stimulated by cAMP. Since AT is a glycoprotein that undergoes posttranslational processing, we monitored it's processing in the secretory pathway by immunoprecipitation and one dimensional isoelectric focusing. The following results indicate that cAMP increases apical protein secretion by stimulating the constitutive secretory pathway; (i) a lack of accumulation of fully processed AT in the cells under unstimulated conditions, (ii) a substantial rate of secretion under unstimulated conditions, (iii) a linear time course of secretion under stimulated as well as stimulated conditions and (iv) the insensitivity of either the baseline or the stimulated secretion to changes in intracellular [Ca^{++}]. When the chase was performed at 23^{o}C (i.e., a non permissive temperature for TGN to cell surface transport) terminally sialyated AT accumulated in the cells at a rate that was stimulated by cAMP, suggesting an increased acidification in the TGN. Acidification of the TGN is carried out by the vacuolar proton ATP-ase and requires the presence of a Cl^- conductance to maintain electroneutrality. CFTR, a cAMP regulated Cl^- channel, provides the major Cl^- conducting pathway in the apical domain of HT29-CL19a cells and has been implicated in the acidification of the TGN (1). Our data are not proof for but are entirely consistent with the notion that the cAMP dependent opening of CFTR in the TGN facilitates acidification. Acidification in the TGN has been shown to be required for both the processing of secretory glycoproteins (2) and for the association of ARF to TGN membranes(3). A cAMP and CFTR-dependent acidification of the TGN therefore could stimulate the rate of secretion by both facilitating the processing of "cargo" and enhancing coatomer formation and secretory vesicle budding at the TGN.

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STATISTICAL ANALYSIS OF TRISKELION STRUCTURAL FEATURES: ESTIMATION OF ARM RIGIDITIES.

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The structural and energetic properties of clathrin triskelions influence many aspects of *receptor-mediated endocytosis* in functioning cells. Topological characteristics of the triskelions lead to constraints on the transformations which determine coated-pit invagination and coated-vesicle generation (Jin and Nossal, 1993). To extract more detailed properties of individual triskelions, we have recently developed a quantitative analytical scheme that systematically examines the distributions of, and correlations between, several triskelion shape parameters. When this methodology is applied to a set of electron micrographs supplied by Kocsis *et al.* (1991), we find that the three arms of individual triskelions exhibit independent and equivalent fluctuations in structure. Although the average conformation of a triskelion is determined to be that of a curved pin-wheel, sharp bends in arm curvature are not observed. We also infer that the fluctuations in curvature along the arm backbones are approximately uniform. Results support an energetic model in which triskelions have elastic-like deformation properties and analysis of the fluctuations of the triskelion arms indicates a persistence length for arm rigidity of about 35 nm. Concomitant numerical estimate of the bending modulus leads to the conclusion that the clathrin coat is somewhat more rigid than the plasma membrane component of coated pits, which may be of significance when analyzing detailed mechanisms by which coated vesicles bud from cell surfaces.

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A PROTEIN COAT ON THE ER REQUIRED FOR VESICLE BUDDING

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We have been investigating early events in the formation of transport vesicles that bud from the ER. Screens in *S. cerevisiae* for genes involved in vesicle assembly have identified three genes specifying ER proteins that appear to be important for seting the stage on the ER membrane for vesicle assembly.

SEC16 was identified by temperature-sensitive mutations that block vesicle budding from the ER *in vivo*. Sec16p is a 240 kD peripheral membrane protein that is both on the ER and on vesicles that have budded from the ER. The C-terminal domain of Sec16p binds Sec23p, one of the constituents of the vesicle coat indicating that Sec16p is an important structural element of vesicles. Sec16p tightly adheres to membranes and there is no soluble Sec16p in cell extracts. This implies that Sec16p on the ER membrane may be incorporated into vesicles as a scaffold onto which coat proteins such as Sec23p assemble.

An understanding of the events that trigger Sec16p incorporation into vesicles has come from analysis of *SED4*, a gene we isolated as a high copy suppressor of *sec16* mutations. Sed4p is an integral ER protein that is not incorporated into vesicles but does bind to the C-terminal domain of Sec16p. Genetic and phenotypic characterization of *sed4* deletions show that Sed4p is important but not essential for vesicle formation. Suppression and synthetic interactions between *SED4*, *SEC16* and *SAR1* (a small GTPase required for budding) indicate that these proteins function in close conjunction. *SED4* is homologous to *SEC12*, a nucleotide exchange factor for Sar1p suggesting that Sed4p may also be an exchange factor that catalyzes incorporation of Sar1p into the Sec16p membrane coat.

The gene *LST1* was identified in a screen for mutants that exacerbate the transport defects of the known budding genes. Chromosomal deletions of *LST1* are viable but have defects in vesicle budding and abnormal ER morphology. Lst1p is a 105 kD peripheral membrane protein that appears to be located exclusively on the ER. Lst1p binds to Sec16p and therefore appears to be a component of the ER coat. *LST1* is homologous to *SEC24*, a constituent of the vesicle coat. But unlike Sec24p, Lst1p is only associated with the ER and does not appear to be incorporated into vesicles. A simple explanation for these findings is that the Sec16p scaffold exists in two conformations: a flat, ER conformation in association with Lst1p and a curved, vesicle conformation associated with Sec24p. Possibly the action of Sed4p and Sar1p brings about the switch from one conformation to the other.

RECRUITMENT OF THE SIGNAL PEPTIDASE COMPLEX TO THE TRANSLOCATION SITE MAY INVOLVE ONE OF ITS TWO SUBUNITS WITH MAJOR CYTOSOLIC DOMAINS

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The signal peptidase complex of the mammalian endoplasmic reticulum (ER) membrane contains five subunits. Four have been cloned, and three are known to be single-spanning membrane proteins exposed to the lumen of the ER, where the active site of the enzyme is located. We have now determined the sequence of the remaining subunit (SP12). SP12 and SP25 each span the membrane twice with neighboring membrane anchors, and have only very small lumenal domains.

To understand the role of the cytosolic domains of the enzyme complex, we have carried out crosslinking experiments with bis-maleimides. The results demonstrate that SP25 is in close spatial proximity to the β-subunit of the Sec61p-complex, a key component of the protein translocation apparatus. The interaction is only observed if the translocation site is actively engaged in protein transport, suggesting that the cytosolic domain of SP25 is involved in recruiting the enzyme complex to the translocation site. As the appearance of a crosslink between the α- und β-subunits of the Sec61p-complex is also dependent on translocation, these data provide the first evidence for a change in the assembly of translocation components during protein translocation.

GLUT4-CONTAINING VESICLES:
SPECIALIZED ORGANELLES OR REGULAR ENDOSOMES?

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GLUT4 is a fat and muscle specific isoform of glucose transporter protein which is responsible for insulin-regulated glucose uptake. Under basal conditions it is localized in intracellular microsomal vesicles and is recruited to the plasma membrane upon insulin administration with corresponding increase in glucose uptake. We have purified GLUT4-containing vesicles from rat adipocytes to apparent homogeneity and demonstrated that they represent uniform 50-70 nm round-shaped particles with a sedimentation coefficient of 120 S and a buoyant density in sucrose of 1.12 gm/cm^3. GLUT4-vesicles from skeletal muscle are very similar if not identical to those from adipocytes. Using cell surface biotinylation as a criteria of protein translocation from an intracellular pool to plasma membrane we have demonstrated that three major component proteins of GLUT4-vesicles (gp230, gp160, and gp110) are recruited to the cell surface along with GLUT4 in an insulin-dependent fashion. Gp160 has been isolated, partially sequenced, and identified as a new member of aminopeptidase N family. With the help of anti-peptide antibodies prepared to the known portion of gp160 we have shown that gp160 is present only in GLUT4-containing vesicles and may be considered as a second (besides GLUT4 itself) marker protein for insulin-stimulated glucose transport. Gp230 has been identified as the IGF-II/Man-6-P receptor. Only 10-15% of the total receptor pool and >90% of the receptor molecules which can be biotinylated at the cell surface are associated with GLUT4-containing vesicles. So, these vesicles are the main or the only structures in adipocytes responsible for the recycling of the receptor to the plasma membrane. However, neither the insulin receptor, nor any other markers of the normal endocytotic pathway could be detected in the vesicles. We conclude, therefore, that GLUT4-containing vesicles may represent specialized structures different from regular endosomes in 1. insulin sensitivity 2. physico-chemical parameters 3. major component proteins and 4. pathways of intracellular trafficking.

THERMOSTABLE MUTATIONS OF HUMAN α_1-ANTITRYPSIN SUPPRESSING THE FOLDING DEFECT OF THE Z VARIANT RELIEVED THE SECRETION BLOCK OF THE VARIANT

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Human α_1-antitrypsin (α_1-AT), a major serine protease inhibitor in plasma, is synthesized predominantly in liver and secreted into blood. The Z variation (Glu342→Lys) of α_1-AT causes the α_1-AT deficiency in plasma due to the secretion block of the protein, leading to emphysema. Most of the Z variant proteins accumulate as soluble aggregates and eventually degraded in the endoplasmic reticulum of hepatocytes. In the *in vitro* folding analysis, we have previously showed that the Z type protein had the folding defect that was partially suppressed by a thermostable mutant (Phe51→Leu) at the hydrophobic of the protein. To investigate further the effect of thermostable mutations on the folding and secretion of the α_1-AT protein *in vivo*, we exploited the secretion system of yeast *Saccharomyces cerevisiae*. The normal M variant of human α_1-AT was efficiently secreted from yeast cells, while the Z variant failed to be secreted from yeast cells and underwent degradation rapidly inside cells, as observed in hepatocytes. The thermostable mutations suppressing the folding defect of Z variant *in vitro* relieved the intracellular degradation as well as the secretion defect of Z variant in yeast secretion system. Significantly, the extent of suppression in the secretion was proportional to the extent of suppression in the folding, which is related with the enhancement in thermal stability. The results demonstrate that the *in vivo* defect associated with the Z variant is derived from the folding defect and the defect can be corrected by other mutations that recover the folding efficiency of the variant.

INOSITOL POLYPHOSPHATES REGULATE BINDING OF A NOVEL G-PROTEIN-RELATED PROTEIN TO MICROSOMAL MEMBRANES

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We have identified a 100kDa protein (p100), that is immunologically related to the alpha subunits of the G-proteins G_i and transducin (1-3). We envision that p100 might be involved in the regulation of receptor trafficking because the region of similarity between p100 and G_i corresponds to the G-protein receptor binding site. p100 was found to colocalize to a receptor-enriched subfraction of endosomal vesicles (1). It cycles on and off the cytoplasmic face of the endosomal membrane by reversible binding to receptor proteins (2). The interaction of p100 with the membrane is regulated by both adenine and guanine triphosphate analogs (2).

Here, we present data that indicate that inositol polyphosphates (IPs) may also regulate the interaction of p100 with the microsomal membrane. The IPs release the membrane bound form of p100 into the cytosol and inhibit the binding of soluble p100 to the microsomal membrane. In addition, free Ca^{2+}, at micromolar concentrations, also releases membrane-bound p100. The effects of the IPs on the membrane interactions of p100 are mediated by their binding to microsomal protein(s) through a putative receptor, which selectively binds both (1,4,5)IP_3 and IP_6 with low affinity. It is presenty unknown whether p100, itself, is the IP-binding protein. Unlike the IP_3 receptor involved in calcium mobilization from intracellular stores, the IP binding protein responsible for regulating the membrane interaction of p100 can be extracted from the microsomal membrane by treatment with 0.5M Tris-HCl.

Stimulation of secretory RBL-2H3 cells, transfected with the M1 muscarinic receptor, with agonists that raise intracellular levels of IPs, results in increased levels of p100 in the cytosol. This effect is reversible; a decline in levels of IP_3 is associated with the rebinding of cytosolic p100 to the membrane. The concommitant release of p100 from microsomal membranes and the presence of elevated levels of signal transduction components supports the hypothesis that p100 may be involved in receptor recycling.

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POLYMERIZATION OF HSP70 BY YEAST DnaJ IN THE PRESENCE OF ATP C. King, E. Eisenberg, and L. Greene, Lab of Cell Biol., NHLBI, NIH, Bethesda, MD. 20892-0301

In *E. coli*, DnaJ has been found to be required for the interaction of DnaK, the *E. coli* hsp70 protein, with various substrates; in some cases DnaJ is thought to present these substrates to DnaK. In the present study, we investigated the interaction of the yeast DnaJ, YDJ1, with bovine brain hsp70. In the presence of ATP, where hsp70 is normally monomeric, YDJ1 induces almost all of the bovine brain hsp70 to form large polymers which are readily sedimentable. These polymers are much larger than the dimers and trimers of hsp70 which normally form in the presence of ADP. The polymerization occurs only in the presence of ATP, not ADP, and is completely reversible when either YDJ1 or ATP is removed. The extent of polymerization increases with an increase in either the hsp70 or YDJ1 concentration. However, complete polymerization occurs at substoichiometric ratios of YDJ1 to hsp70 showing that the YDJ1 is acting catalytically. At 25 °C both the rate of polymerization and the initial burst of ATP hydrolysis which accompanies polymerization are too fast to measure. However, at 4 °C, while the rate of polymerization is still too fast to measure, the initial burst of ATPase activity is slower demonstrating that hydrolysis of ATP follows polymerization of the hsp70 rather than preceeding or occuring simultaneously with polymerization. Following polymerization and the initial burst of ATPase activity, almost all of the nucleotide bound to the polymerized hsp70 is present as ADP, and the steady-state ATPase rate is equal to the rate of exchange of ATP with ADP on the polymer. Therefore ADP release is the rate-limiting step in the ATPase cycle. Exchange of monomeric hsp70 with polymeric hsp70 also occurs at this rate, suggesting that both polymerization and depolymerization of hsp70 require the presence of ATP on hsp70. These data suggest that, in the presence of ATP, YDJ1 may present one hsp70 to another just as under other conditions it presents protein substrates to hsp70. Furthermore, these data suggest that YDJ1 may only bind transiently to hsp70 during this presentation process.

ENDOSOMAL POOL OF RAPIDLY RECYCLABLE β_2-ADRENERGIC RECEPTORS MAINTAINED BY CHRONIC AGONIST EXPOSURE.

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The ligand-induced intracellular trafficking of G-protein-coupled receptors plays a key role in the regulation of signal transduction. We have studied the endocytosis, downregulation and recycling of an epitope-tagged β_2-adrenergic receptor (β_2AR) in transfected HEK293 cells (12B6 line). More than 60% of the cell-surface β_2ARs internalize by 10' after the addition of the β-agonist isoproterenol (ISO), without any detectable alterations in fluid phase endocytosis or surface distribution of transferrin receptor. Internalized β_2ARs colocalize extensively with Rab5, a ras-related regulatory GTPase. Rab5 has previously been localized to the early endosome, and appears to be rate-limiting for fluid-phase and transferrin receptor endocytosis. After removal of ISO, >75% of internalized β_2ARs recycle to the surface with rapid kinetics. Receptor downregulation (the loss of total radioligand-binding sites) was undetectable after 24 hr of ISO treatment. Consistent with this finding, trafficking of β_2ARs to a compartment marked by uptake of BSA-Texas Red (lysosomes and possibly late endosomes) could not be detected after chronic ISO treatment. Instead, β_2ARs remained colocalized with Rab5. Receptors in these chronically-treated cells recycled to the surface with kinetics similar to those observed after 10' of agonist exposure. These results indicate that in 12B6 cells, agonist maintains β_2ARs for prolonged periods of times within a rab5-containing compartment, in the absence of detectable downregulation.

TARGETING OF T-CADHERIN AND N-CADHERIN IN POLARIZED EPITHELIAL CELLS

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Calcium-dependent cadherin cell adhesion molecules play a crucial role in the polarization of epithelial cells by establishing adhesive junctions between adjacent cells. In polarized epithelial cells, classical cadherins containing the conserved cytoplasmic region are segregated to the basolateral membrane domain, while glycosylphosphatidyl-inositol (GPI)-linked membrane molecules are targeted apically. T-cadherin (T = truncated) is a functional member of the cadherin family that differs from classical cadherins by its membrane attachment through a GPI-moiety. To study the targeting of T-cadherin in polarized epithelial cells, Madin-Darby Canine Kidney (MDCK) cells were stably transfected with chick T-cadherin cDNA. Confocal- and electron microscopy revealed that T-cadherin is specifically targeted to the apical side in MDCK cells. In contrast, transfected chick N-cadherin is segregated to the basolateral domain. To determine which regions of T-cadherin and N-cadherin are responsible for differential targeting, mutants between T-cadherin and N-cadherin were constructed and examined for their segregation to specific membrane compartments. An N-cadherin mutant (N/TGPI) containing the extracellular portion of N-cadherin and part of the T-cadherin EC5 domain (including the GPI-anchor consensus sequence) was targeted to the apical membrane domain. In contrast, NΔcyt, a deletion mutant of N-cadherin in which all but three amino acids of the cytoplasmic domain are eliminated, was localized to the basolateral side. Thus, the cytoplasmic region of classsical cadherins which associates with the cytoskeleton via catenin-binding is not sufficient for basolateral targeting. Our results suggest that an amino acid stretch located within the carboxyterminal portion of EC5 and the hydrophobic domain is crucial in targeting T-cadherin and N-cadherin to distinct membrane domains.

IMMATURE β-SECRETORY GRANULES SERVE AS A MAJOR SORTING COMPARTMENT FOR NEWLY-SYNTHESIZED LYSOSOMAL ENZYMES. R. Kuliawat and P. Arvan Division of Endocrinology, Beth Israel Hospital and Harvard Medical School, Boston MA 02215

In pancreatic β-cells, C-peptide (C) and insulin (I), two stoichiometrically-generated products of proteolytic processing of the proinsulin precursor, are dominant proteins contained within storage granules destined for regulated exocytosis. Nevertheless, during the period in which newly-synthesized C and I are contained within immature secretory granules (IGs, the first compartment in the regulated secretory pathway), C is secreted in molar excess of I by constitutive-like secretion, i.e., a vesicle trafficking pathway that emerges from IGs. Thus, the IG is a protein sorting compartment. Two recent observations indicate that: 1) in INS-1 cells (a β-cell line), intragranular loss of C far exceeds that which can be accounted for by constitutive-like vesicle traffic from IGs (Neerman-Arbez and Halban, J. Biol. Chem. 1993 268: 16248-16252), and 2) in mouse islets, newly-synthesized lysosomal enzymes actually enter β-cell IGs before their removal from the regulated secretory pathway (Kuliawat and Arvan, J. Cell Biol. 1994 126: 77-86). Therefore, we have examined trafficking of two lysosomal proteases in INS cells. In these cells, Western blotting for cathepsin B showed a preponderance of mature enzyme, indicating that in the steady-state, most of this protease resides in lysosomes. Nevertheless, by pulse-chase of INS cells, newly-synthesized procathepsin B appears to enter the stimulus-dependent secretory pathway at high levels, with an efficiency at least half that of proinsulin. Within 2h after synthesis, newly-synthesized procathepsin B is fairly efficiently relocated to lysosomes and converted to the mature form, so that by 3-4h of chase, there is no longer any stimulus-dependent secretion of new cathepsin B from these cells. By contrast in cells pulse labeled after tunicamycin treatment, stimulated secretion of unglycosylated procathepsin B could still be observed after prolonged chase, suggesting carbohydrate (Man-6-P) recognition participates in removal of procathepsin B from secretory granules. When newly-synthesized procathepsin L was examined in INS cells, once again a major fraction demonstrated stimulus-dependent secretion, regardless whether glycosylated (control cells) or unglycosylated (tunicamycin-treated cells). However, despite normal glycosylation, stimulus-dependent secretion of the newly-synthesized hydrolase persisted even after prolonged chase, indicating failure of a large fraction of cathepsin L to relocate out of the regulated secretory pathway of control INS cells. Since lysosomal enzymes that fail to relocate from IGs are stranded (i.e., mis-sorted) and may autoactivate in the acidic granule milieu, these molecules are candidates that may explain the intragranular degradation of susceptible regulated secretory proteins such as C-peptide.

YRB1: A POSSIBLE LINK BETWEEN PLASMA MEMBRANE SIGNALING AND NUCLEAR EVENTS DURING YEAST MATING.

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Ran, a small Ras-like GTPase in mammals, and its homologs in other organisms (Gsp1 & Gsp2 in *Saccharomyces cerevisiae*), have been implicated in various nuclear processes, such as DNA replication, cell cycle control, RNA processing, nucleo-cytoplasmic transport of RNA and protein, and nuclear organization. We isolated the *S. cerevisiae YRB1* gene, which encodes a homolog of a mammalian Ran•GTP-binding protein, RanBP1, in a search for mutations that suppressed the mating defect of a *fus1* null mutant. The pheromone-induced *FUS1* gene product is an integral plasma membrane protein that localizes to the site of cell fusion and acts in an as yet unknown way to facilitate both the degradation and reorganization of the cell wall and the plasma membrane fusion that occur during yeast mating. The genetic interaction between *fus1* and *yrb1* mutations suggests a potential link between events that occur at the plasma membrane and processes in the nucleus. To understand the elements that mediate this interconnection, we have been investigating the subcellular localization of Yrb1 and identifying proteins that may physically interact with Yrb1.

Initial results using immunofluorescence and a functional derivative of Yrb1 tagged with a c-Myc epitope indicate that Yrb1p is a cytosolic protein and is localized in a fibrous cage-like structure around the nucleus. To confirm these results and to follow changes in the localization of Yrb1 in live cells in real time under different physiological conditions, we have constructed a *YRB1-GFP* gene fusion that is also functional, as judged by its ability to complement the temperature-sensitive lethality of a *yrb1^{ts}* allele. From a genetic screen for dosage suppressors of the same *yrb1^{ts}* mutation, we have isolated repeatedly two genes. Based on database searches for related sequences, one gene may encode a protein with microtubule-related functions and the other gene is related to a protein that is involved more directly in nucleo-cytoplasmic transport. Also, we noted that Yrb1 shares 30% amino acid sequence identity over nearly its entire length with the C-terminal domain of the yeast nucleoporin, Nup2. A *nup2Δ* mutation has no detectable phenotype; whereas, cells carrying the *yrb1-1^{ts}* mutation can grow at 30°C, but not at 34°C. In contrast, a *nup2Δ yrb1-1^{ts}* double mutant grows poorly at temperatures below 30°C and is inviable at 30°C. This genetic interaction ("synthetic lethality") suggests that Yrb1 has a function in nucleo-cytoplasmic transport, perhaps in conjunction with Nup2. We are currently using alternative methods to test the implications of these findings and to determine whether they represent actual protein-protein interactions.

TRANSPORT ROUTE FOR SYNAPTOBREVIN VIA A NOVEL PATHWAY OF INSERTION INTO THE ER MEMBRANE

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Synaptobrevin/VAMP, one of the SNARE proteins, is proposed to be a receptor protein in vesicles that provides specificity for their targeting and fusion with the plasma membrane. It belongs to a class of membrane proteins which lack a signal sequence and contain a single hydrophobic segment close to their C-terminus, leaving most of the polypeptide chain in the cytoplasm (tail-anchored). We show that in neuroendocrine PC12 cells, synaptobrevin is not directly incorporated into the target organelle, synaptic-like vesicles. Rather, it is first inserted into the endoplasmic reticulum (ER) membrane and is then transported via the Golgi apparatus. Its insertion into the ER membrane *in vitro* occurs post-translationally, is dependent on ATP and results in a trans-membrane orientation of the hydrophobic tail. Membrane integration requires ER protein(s) different from translocation components needed for proteins with signal sequences, thus suggesting a novel mechanism of insertion.

CLATHRIN BINDING AND ASSEMBLY ACTIVITIES OF EXPRESSED DOMAINS OF THE SYNAPSE-SPECIFIC CLATHRIN ASSEMBLY PROTEIN AP-3

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In order to contribute to an understanding of synaptic function, we cloned the synapse-specific phosphoprotein F1-20 [1]. F1-20 was then shown to be identical to the clathrin assembly protein AP-3 [2, 3]. Recently, it was shown that specific inositol polyphosphates bind to the 33 kD N-terminal region of AP-3 with high affinity, and inhibit clathrin assembly [4, 5]. In the study presented here, we separately expressed the 58 kD C-terminal, 42 kD middle, 16 kD C-terminal and 33 kD N-terminal region of AP-3, and determined their clathrin binding and assembly properties. The 58 kD C-terminal region of AP-3 is able to bind to clathrin triskelia and assemble them into a homogeneous population of clathrin cages, and will also bind to preassembled clathrin cages. The 42 kD central region of AP-3 can bind to both clathrin triskelia and to clathrin cages, but cannot assemble clathrin triskelia into clathrin cages. The 16 kD C-terminal region of AP-3 can bind to clathrin cages, but cannot bind to clathrin triskelia, or assemble clathrin triskelia into clathrin cages. The clathrin binding activities of the 42 kD central region and 16 kD C-terminal region are weaker than the corresponding activity of either the 58 kD C-terminal region or full-length AP-3. Previous efforts had mapped a clathrin binding site within the N-terminal 33 kD of AP-3 [3, 6]. However, while the N-terminal 33 kD of AP-3 is able to bind to clathrin triskelia [6, 7], it does not promote their assembly into clathrin cages [6, 7], or bind to preassembled clathrin cages [7]. It appears that the smallest functional unit that carries out all of the reported clathrin binding and assembly properties of AP-3, essentially as well as the full-length protein, is the 58 kD C-terminal region.

Citations: **1.** Zhou, S., Sousa, R., Tannery, N.H. and Lafer, E.M. (1992) *J Neurosci* 12, 2144-55; **2.** Zhou, S., Tannery, N.H., Yang, J., Puszkin, S. and Lafer, E.M. (1993) *J Biol Chem* 268, 12655-62; **3.** Morris, S.A., Schroder, S., Plessmann, U., Weber, K. and Ungewickell, E. (1993) *Embo J* 12, 667-75; **4.** Ye, W., Ali, N., Bembenek, M., Shears, S.B. and Lafer, E.M. (1995) *J. Biol. Chem.* 270, 1564-8; **5.** Norris, F.A., Ungewickell, E. and Majerus, P.W. (1995) *J Biol Chem* 270, 214-7; **6.** Murphy, J.E., Pleasure, I.T., Puszkin, S., Prasad, K. and Keen, J.H. (1991) *J Biol Chem* 266, 4401-8; **7.** Ye, W. and Lafer, E.M. (1995) *J. Neurosci. Res.* 41, 15-26.

MICROTUBULE MOTORS IMPLICATION IN BASOLATERAL AND APICAL EXOCYTOSIS IN MDCK CELLS

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The maintenance of a polarized cell surface requires vectorial transport of vesicles to the apical and the basolateral membrane domains. Transport of newly synthesized apical proteins and transcytosis from the basolateral to the apical surface have been demonstrated to depend on microtubules. On the other hand, movement of membrane proteins to the basolateral surface has been claimed to occur by diffusion and to be microtubule- and actin-independent.

We have reexamined the role of microtubules using a recently developed *in vitro* polarized transport assay in permeabilized Madin-Darby canine kidney cells (MDCK). We showed that both apical and basolateral transport are inhibited by nocodazole treatment (Lafont *et al.* (1994) Nature 372:801-803). Transport to the basolateral surface was inhibited by immunodepletion of cytosolic kinesin but not by dynein immunodepletion or photocleavage. In contrast, apical transport was inhibited by both dynein and kinesin depletion. Our data demonstrate that in epithelial cells microtubule motors are involved in the movement of apical and basolateral vesicles. Based on these results and previous descriptions of the cytoarchitectural organization of the cytoskeleton in MDCK cells, we propose that the differential requirement for microtubule-based motors is related to the microtubule organization.

To dissect more precisely the influence of the cytoskeleton on the polarized vesicular delivery in MDCK cells, we are now addressing the question of the roles of the dynein-associated dynactin complex, a component of which is the actin-related centractin, and of the actin network.

MOLECULAR CLONING AND EXPRESSION OF A 58 kDa *cis*-GOLGI PROTEIN

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A 58 kDa (p58) homodimeric and oligomeric nonglycosylated membrane protein has previously been biochemically characterized and localized to vesicular elements at the ER-Golgi interphase and the *cis*-Golgi cisternae. To clone a cDNA encoding p58, the protein was first purified to homogeneity by two-dimensional electrophoresis. Four peptides generated by protease digestions were purified and microsequenced. A rat pancreas cDNA library was screened with degenerate oligonucleotide probes and several positive clones were isolated. A full-length cDNA sequence was constructed from overlapping clones and the 5' region was isolated by using RACE PCR-amplification. The cDNA sequence encodes a 517 amino acid protein showing 89% identity (94% similarity) with the human intermediate compartment ERGIC-53 protein. The 12 residue-long cytoplasmic tail contains a double-lysine motif at positions -2 and -3, typical for proteins retained in the ER and recycling between the ER and the Golgi complex. Clones obtained from screening of a *Xenopus laevis* liver cDNA library revealed a high conservation of p58 between rat, human, and frog. Peptide antibodies against different domains of the protein have been generated and the membrane topology has been probed *in vitro* by translating the mRNA in the presence of microsomal membranes. To express p58 in cultured cells, cDNA, containing *myc*-epitope tag was cloned into a Semliki Forest virus-based vector (pSFV1-3) and BHK21-cells were transfected by lipofection. The results from these expression studies will be presented and discussed.

A TYROSINE KINASE SUBSTRATE(S) OTHER THAN THE EGF-R ITSELF IS REQUIRED FOR THE EFFICIENT RECRUITMENT OF ACTIVATED EGF-Rs INTO COATED PITS.

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The binding of EGF to its cell surface receptor triggers efficient receptor internalization and leads to a well-defined cascade of intracellular events that regulate cell proliferation and development. EGF-induced internalization results in the down-regulation of EGF-R, a critical first step in the attenuation of their cell proliferative signal. However, there exists a controversy as to whether ligand-induced activation of the EGF-R tyrosine kinase is required for internalization or for lysosomal targeting. In order to address the role of the EGF-R tyrosine kinase in endocytosis, we have used a cell-free system that reconstitutes the recruitment of EGF-R into coated pits. While wild-type EGF-R were efficiently recruited into coated pits in response to EGF, kinase-deficient receptors were recruited inefficiently at the same bulk rate of endocytosis of inactivated receptors or receptors lacking any cytoplasmic domain. The recruitment of a deletion mutant EGF-R lacking autophosphorylation sites is also dependent on its active tyrosine kinase. We have been able to specifically restore the efficient recruitment of kinase-deficient EGF-R into coated pits by addition of an active soluble EGF-R tyrosine kinase. We suggest that a tyrosine kinase substrate(s) other than the EGF-R itself is required for its efficient ligand-induced recruitment into coated pits. Our assay provides a powerful means to identify the tyrosine kinase substrate(s) regulating the efficient recruitment of activated EGF-R into coated pits. (Supported by Philippe Foundation, France and USA Army Program Project fellowship).

GPI-ANCHORED OR CHIMERIC DIPHTHERIA TOXIN RECEPTORS ALLOW MEMBRANE TRANSLOCATION AND MODIFY CHANNEL ACTIVITY

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Membrane translocation of diphtheria toxin is dependent on the presence of its high affinity receptor, which is the HB-EGF precursor. To investigate the role of the transmembrane- and cytoplasmic domains of the diphtheria toxin receptor in membrane insertion and translocation of the toxin, two mutants were constructed in which these domains were replaced either by a 37 aa sequence signalling membrane attachment via GPI-anchor (DTRGPI) or by the transmembrane- and cytoplasmic domains of the human EGF receptor (DTRHER).

Diphtheria toxin insensitive NIH-3T3 cells were transfected with these constructs and the wild type receptor. Transfected cells exhibited specific binding of diphtheria toxin and became toxin-sensitive. Cells transfected with wild type receptor and DTRHER displayed similar sensitivity as Vero cells, whereas DTRGPI cells were about 10 fold less sensitive. When the toxin was translocated at the plasma membrane by exposing cells with bound toxin shortly to low pH, all the cell lines were similarly sensitive, indicating that the less efficient intoxication in the case of DTRGPI was due to reduced internalization. Thus, both mutant receptors allowed membrane translocation of diphtheria toxin. However, with respect to the number of binding sites that were expressed in the transfectants, the membrane translocation was about six times less efficient in case of the two mutant receptors than in case of the wild type receptor.

Diphtheria toxin forms cation selective channels in Vero cell membranes at low pH. Toxin-induced $^{22}Na^{+}$-influx was efficient in case of cells expressing the wild type receptor, but was about 10 fold reduced in DTRHER cells and not detectable in DTRGPI cells. Hence, the transmembrane and/or cytoplasmic part of the receptor might be involved in or modify diphtheria toxin's channel activity.

AMINO TERMINAL B-HEXOSAMINIDASE SEQUENCE NECESSARY FOR ROUTING GLYCOPROTEINS TO THE LYSOSOMES OF *DICTYOSTELIUM DISCOIDEUM*

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During growth of *Dictyostelium discoideum*, b-hexosaminidase is localized in lysosomes. The sequence of this protein has previously been published. Two hydrophobic domains reside between amino acids 1-22 and 28-56. These are followed by a relatively hydrophilic serine / threonine rich region. The first hydrophobic domain serves as a trans-membrane signal sequence. To determine if the second hydrophobic domain is necessary in sorting, hybrid proteins were construcied. Four hybrid proteins, (Hex 22-INV, Hex 34-INV, Hex 56-Inv and Hex 70-INV), which contain either amino acids 1-22, 1-34, 1-56, or 1-70 respectively, fused to invertase were expressed in the axenic AX2 strain of *Dictyostelium discoideum.* Transformed Ax-2 lines were determined to contain the appropriate size constructs by PCR analysis. The distribution of b-hexosaminidase and acid phosphatase was normal in these transformants. Hex 22-INV was quantitatively secreted through the default pathway, as was Hex 34-INV (97%) and Hex 56-INV (89%). Thus the second hydrophobic domain is not critical for routing.

It might play a role in the transitory membrane association of the enzyme during its biosynthesis. We are in the midst of testing this. In pulse chase experiments an early form of native b-hexosaminidase behaves as an integral membrane protein. The early form of the protein remains bound to membranes after bicarbonate extraction and it is converted to a soluble form with a lower molecular weight. 10-15% of the intracellular Hex 70-INV also behaves as an integral membrane protein. This fraction remains in the membrane after exhaustive extraction with the detergent rich phase, after temperature-induced phase separation in Triton X-114. We have not detected a similar form of Hex 22-INV. We are currently measuring the phase distribution of the other constructs .

Since 40-60% of Hex 70-INV is retained within the cell, and is sorted to a prelysosomal organelle, amino acids 57-70 (ser-val-ser-met-asp-arg-tyr-thr-asp-leu-phe-phe-pro-phe) seem necessary for proper routing.

RIBOSOMES HAVE AN INTRINSIC ABILITY TO MEDIATE THE CO-TRANSLATIONAL TARGETING OF NASCENT POLYPEPTIDES TO THE ER WHICH IS REULATED BY NAC AND SRP

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Signal peptides direct the co-translational targeting of nascent polypeptides to the membrane of the endoplasmic reticulum (ER). It is currently believed that the Signal Recognition Particle (SRP) mediates this targeting by first binding to signal peptides and then by directing the ribosome/nascent chain/SRP complexes to the SRP receptor at the ER. Data are presented which suggest that ribosomes, rather than SRP, mediate targeting by directly binding to translocation sites. When purified away from cytosolic factors including SRP and Nascent polypeptide Associated Complex (NAC), in vitro assembled translation intermediates representing ribosome/nascent chain complexes efficiently bound to microsomal membranes, and their nascent polypeptides could subsequently be efficiently translocated if they contained signal peptides. Surprisingly, under the same conditions, an equivalent proportion of ribosomes containing signal-less polypeptides were targeted to the ER, and a fraction of these polypeptides were subsequently translocated.

Since removal of cytosolic factors from the ribosome/nascent chain complexes resulted in mis-targeting of nascent polypeptides, it was investigated whether the readdition of cytosolic factors such as NAC and SRP could restore fidelity to targeting. Addition of purified NAC to the ribosome/nascent chain complexes prevented all nascent chain-containing ribosomes from binding to the ER membrane. Furthermore, SRP prevented NAC from blocking ribosome-membrane association only when the nascent polypeptide contained a signal. Thus, NAC is a global ribosome binding prevention factor whose activity is blocked by signal peptide-directed SRP binding.

A model based on these findings is presented which postulates that ribosomes are the targeting vectors for delivering nascent polypeptides to translocation sites. SRP and NAC contribute to the specificity in this aspect of ribosomal function by regulating the exposure of a ribosomal membrane attachment site which binds to receptor proteins present in the ER membrane.

INVOLVEMENT OF THE MOLECULAR CHAPERONES (DNAJ-HOMOLOGS AND HSP70S) IN THE UBIQUITIN-DEPENDENT DEGRADATION OF SHORT-LIVED AND ABNORMAL PROTEINS IN YEAST

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The molecular chaperones, DnaJ and DnaK, are required not only for protein folding but also for the rapid degradation of certain abnormal polypeptides in E. coli. We demonstrate here that in yeast cytosol, DnaJ homologs (Ydj1 and Sis1) and Hsp70s (Ssa proteins) are also involved in degradation of such proteins by the ubiquitin-proteasome pathway. The ts mutant of YDJ (*ydj1-151*) and the double mutant of Hsp70 (*ssa1ssa2*) strongly inhibited the breakdown of short-lived proteins and of analog-containing abnormal polypeptides, but not of long-lived proteins. In particular, the *ydj1-151* ts mutation prevented the ubiquitin-dependent degradation of the fusion protein, Ub-P-β-gal, but not the degradation of N-end rule substrates, L-β-gal and R-β-gal. Furthermore, in the *ydj1-151* mutant, the total level of ubiquitinated proteins including multi-ubiquitinated Ub-P-β-gal was strongly reduced. In wild-type cells, Ydj1 (as well as Ssa1p and Ssa2p) is associated with Ub-P-β-gal but not with stable β-gal. The mutant of the other DnaJ-homolog, Sis1 (*sis1-85* ts mutant) also strongly inhibited the degradation of Ub-P-β-gal and Clb5-β-gal, but not of the N-end rule substrates. In this *sis* mutant, unlike the *ydj1-151* mutant, a large build-up of ubiquitinated proteins including the multi-ubiquitinated Ub-P-β-gal was observed, which suggests that protein ubiquitination in this strain is functional, but not the degradation of multi-ubiquitinated proteins by 26S proteasome. Thus, these two DnaJ-homologs (Ydj1 and Sis1) are involved in the selective degradation of certain abnormal and regulatory proteins by ubiquitin-proteasome pathway; Ydj1 appears necessary for the ubiquitination of substrates and Sis1 essential for the degradation of these ubiquitinated proteins by 26S proteasome.

NUCLEOCYTOPLASMIC TRAFFICKING OF THE GLUCOCORTICOID RECEPTOR: DISPLACEMENT OF STEROID RESPONSIVE RECEPTOR FROM CYTOPLASM TO NUCLEUS

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Glucocorticoid receptor (GR) is a transcription factor that cycles between a transcriptionally inert, steroid-sensitive cytoplasmic form, which is maintained in a heat shock protein complex, and a predominantly nuclear, free active form. Binding of glucocorticoid agonists to the cytoplasmic form produces the nuclear DNA-binding form of the receptor which activates transcription of glucocorticoid-responsive genes, while binding of the antagonist, RU486, produces a nuclear DNA-binding GR with impaired activation potential. Using a semi-quantitative indirect immuno-fluorescence assay we have examined nucleocytoplasmic trafficking during G_0. Retention of liganded GR in the nucleus was mediated by DNA binding, as DNA binding mutants of GR failed to redistribute completely to the nucleus in response to hormonal challenge. However, the rate of transfer was not affected. Following hormone withdrawal, the mutant GRs redistributed more rapidly to the cytoplasm. By contrast, RU486 treatment of wild type and mutant GRs resulted in receptors that were trapped indefinitely in the nucleus following withdrawal of antagonist regardless of the ability of the receptor to bind DNA. Remarkably this effect could be reproduced completely in cells withdrawn from agonist, by treatment with the protein kinase inhibitor H-7 at the time of hormone withdrawal. Conversely, phorbol ester treatment markedly enhanced the reappearance of complexed receptor in the cytoplasm following withdrawal of hormone. However, TPA treatment was unable to overcome the RU486-mediated block. Despite this block in subcellular redistribution, unliganded free nuclear GR retained the ability to shuttle between nucleus and cytoplasm in energy depletion and transient heterokaryon experiments, regardless of treatment. Importantly, RU486 withdrawn receptors were as effective in inducing transcription in response to hormone as cytoplasmic GR in naive cells. Thus, the ligand free nuclear form of GR does not require re-distribution to the cytoplasm in order to respond to a second hormone stimulus. Further, we conclude that nucleo-cytoplasmic trafficking of GR is controlled largely by retention and that return of GR to the cytoplasm following hormone withdrawal requires the action of a TPA/H-7 sensitive kinase.

TARGETING OF CHOLERA TOXIN AND *E. coli* LABILE TOXIN IN POLARIZED EPITHELIA: ROLE OF C-TERMINAL K(R)DEL. W.I. Lencer, T. Hirst, M. Jobling, J.L. Madara, and R. Holmes. Children's & Brigham&Women's Hospitals; Research Sch. of Biosciences, University of Kent,UK; Microbiology, Uniformed Services University of the Health Sciences; and Pediatrics & Pathology, Harvard Medical School, Boston.

Cholera (CT) and *E. coli* labile toxins (LT) elicit a secretory response from intestinal epithelia by binding apical receptors (ganglioside GM1) and subsequently activating basolateral effectors (adenylate cyclase). We proposed that signal transduction in polarized cells may occur by transcytosis of toxin-containing membranes (submitted). Targeting of CT into this pathway depends initially on binding of toxin B-subunits to GM1. The anatomical compartments in which subsequent steps of CT processing occur are less clearly defined. However, the enzymatically active A-subunit contains a C-terminal KDEL motif. KDEL is the sorting signal that allows lumenal endoplasmic reticulum proteins to be retrieved from post-ER and Golgi compartments. Thus if the KDEL sequence were required for normal CT trafficking, movement of CT from the Golgi to ER would be implied. To test this idea, recombinant wild type (wt) and mutant CT and LT were prepared. The C-terminal KDEL sequence in CT was replaced by seven unrelated amino acids: LEDERAS. In LT, a single point mutation substituting leucine for valine was made. Wt and mutant toxins displayed similar enzymatic activities as assessed by in vitro ADP-ribosylation (wt: 0.98±0.14 vs. 0.67±0.17 nmoles NAD/h, mean±sd, n=3). Neither mutation affected toxin binding to GM1 immobilized on plastic (apparent Kd ≈ 6 nM, assessed using a CT-fluorescein tracer). The action of recombinant toxins was assessed as a secretory response (Isc) elicited from monolayers of polarized human epithelial T84 cells using standard techniques. Mutations in KDEL of both CT and LT (20 nM) lengthened the lag phase and slowed the rate (25-30%) of toxin-induced Cl secretion ($n \geq 6$, $P<0.0001$). The magnitude of inhibition was greater at lower toxin concentrations. At T1/2, dose dependencies for both mutant toxins were increased ≥ 10-fold. Mutant CT displayed differentially greater temperature sensitivity. Incubations at 20° C completely inhibited signal transduction by KDEL-mutant (peak Isc 2.5±0.8 $\mu A/cm^2$, equal to baseline) but secretion in response to wt CT was clearly present (peak Isc: 10.5±0.6 $\mu A/cm^2$, mean± s.e., n = 7). In direct concordance with a slower rate of signal transduction, KDEL-mutants were trafficked to the basolateral membrane more slowly than wt CT (assessed by selective cell surface biotinylation as transcytosis of B-subunit). These data suggest that Golgi and ER are compartments through which CT/LT physiologically traffic, and thus anatomically identify a discrete trafficking step during the transcytotic events of CT-induced signal transduction.

ENDOTHELIAL CAVEOLAE HAVE THE MOLECULAR MACHINERY FOR DYNAMIC TRANSPORT BY BUDDING, DOCKING AND FUSION: VAMP, NSF, SNAP, ANNEXINS AND GTP-BINDING PROTEINS

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Transport by discrete vesicles is achieved by budding, docking and fusion of individual vesicular carriers with their target membranes. Our understanding of this process has improved because of recent discoveries describing the molecular machinery mediating vesicle formation, docking and fusion. The transport of a divergent group of vesicular carriers requires a general mechanism sensitive to N-ethylmaleimide (NEM). Many endothelia have an abundant population of noncoated plasmalemmal vesicles or caveolae which have been reported with considerable controversy to function in transport. Recently, we have developed a method to isolate endothelial caveolae to homogeneity from rat lung tissue. The endothelial caveolae are enriched in four known caveolar markers: caveolin, GM_1, Ca^{++}ATP-ase, and IP_3 receptor. Here, we report that purified caveolae possess key proteins known to mediate different aspects of vesicle budding, docking and fusion. Immunoblotting shows that docking and fusion proteins, i.e. VAMP, NSF and SNAP, are enriched in the endothelial caveolae. Moreover, the purified caveolae contain heterotrimeric and small G proteins, as well as annexins which are known to regulate membrane docking and fusion. Furthermore, treatment of endothelium in situ or in culture with NEM impaired caveolae-mediated transport. Hence, it appears that endothelial caveolae like other vesicle carriers appear to mediate endocytosis or transcytosis via a process of vesicle-membrane docking and fusion. (Work supported by NIH grants HL43278 and HL52766 and EI Award from AHA and Genentech.).

A FUNCTIONAL SUBDOMAIN OF THE CLATHRIN TRISKELION EXPRESSED IN BACTERIA

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Assembly of the clathrin triskelion creates the characteristic protein lattice surrounding coated pits and coated vesicles formed during receptor-mediated endocytosis. Each clathrin triskelion leg is composed of one heavy chain complexed to one light chain. The clathrin heavy chain contains all the structure features required to form the clathrin lattice, while the light chains are believed to perform regulatory functions. The clathrin triskelion is divided into three distinct domains. These include the hub comprising the vertex and the proximal segment of the leg, the distal segment of the leg and the compact N-terminal domain. Previous studies have indicated that the hub is formed by the C-terminal third of the clathrin heavy chain and includes sequences responsible for light chain binding and heavy chain trimerization, as well as sequences involved in triskelion assembly. We have cloned the bovine clathrin heavy chain gene, and the equivalent polypeptide constituting the hub has been expressed in bacteria. Recombinant hub appears to be trimeric upon sizing chromatography and binds light chain that is either co-expressed in bacteria or added *in vitro*. When exposed to low pH buffer, both hub and bovine brain clathrin show a similar acute increase of absorbance at 320nm which indicates their rapid assembly. Assembly of the hub fragment is independent of its association with clathrin light chain. Electron microscopy reveals that the assembled triskelion hub (with light chain) forms an open-ended lattice structure while intact clathrin forms closed cages. These results establish that interaction between the proximal segments of the triskelion drives cage assembly. Deletion analysis of the recombinant hub fragment has revealed sequences not only necessary but also sufficient for trimer formation. In addition, the essential clathrin light chain binding sites have been mapped to a compactly folded region of the proximal segment that is close to but independent of the trimerization sequence. Expression of the functional hub domain of the clathrin triskelion establishes a tractable system for further structural and functional studies of the molecular mechanism of receptor-mediated endocytosis.

MOLECULAR IDENTIFICATION OF A RAB-RELATED LOW-MOLECULAR-WEIGHT GTP-BINDING PROTEIN IN *SCHISTOSOMA MANSONI*

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Schistosomiasis is a parasitic disease initiated by the eggs deposited in host tissues by the blood fluke *Schistosoma spp.* Results of experiments in our laboratory with inhibitors of isoprenoid synthesis and with GTP binding assays indicate that prenylated low-molecular-weight GTP binding proteins (LMWGP's) may play an important role in the regulation of egg production in the schistosome. This role may be carried out *via* direct effect on female reproductive capacity and/or indirectly by affecting male viability, and thereby female fecundity.

A molecular biological approach was undertaken to isolate and to characterize one such LMWGP from *Schistosoma mansoni.* Degenerate oligonucleotide primers to two conserved GTP-binding regions of *ras*-related genes yielded a 200 bp PCR (Polymerase Chain Reaction) product from both genomic and complementary schistosome DNA. Screening of an adult *S. mansoni* cDNA library in lamda gt11 with this PCR product, and subsequent 5'RACE PCR (5' Rapid Amplification of cDNA Ends), yielded the full-length gene, termed *smrab*. Northern blot analysis hybridized *smrab* with a 1.0 kb band of mRNA; this mRNA appears to be abundantly expressed in male schistosomes, while only faintly in females. *Smrab* meets the criteria recently established for acceptance into the ras superfamily of GTP-binding proteins. Its highest amino acid sequence homology is with rab-related proteins of the electric eel (ora1 & 2) and of the slime mould (SAS1 & 2), which, in turn, are closely related to SEC4 of yeast. The function of SEC4 has been demonstrated in post-Golgi constituitive secretion. Consistent with its overall sequence similarity with rab-related proteins, *smrab* terminates in a CCXX motif, necessary for post-translational isoprenoid modification by geranylgeranyl protein transferase. Recombinant expression of *smrab* in the pET3a expression vector yielded insoluble protein which migrates as a single band on SDS-PAGE. N-terminal sequence information of the recombinant protein matched the predicted amino acid sequence of *smrab*. On the basis of the described observations and of what is understood of schistosome physiology, we suggest that the protein encoded by *smrab* serves an important function in maintenance of the male parasite's tegument, and hence indirectly in regulation of the fecundity of the worm pair.

RECEPTOR RECYCLING BETWEEN THE ENDOPLASMIC RETICULUM AND THE GOLGI IS ESSENTIAL FOR THE DELIVERY OF CERTAIN CYTOTOXIC PROTEINS INTO THE CYTOSOL OF TARGET CELLS

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Bacterial proteins such as diphtheria toxin (DT), *Pseudomonas* exotoxin A (PE) and *Escherichia coli* Shiga-like toxin-1 (SLT-1), and plant proteins such as ricin are potently toxic to mammalian cells. All of these toxins irreversibly inactivate target cell protein synthesis by catalytically modifying key components of the translational machinery. To do so, a catalytically-active polypeptide must enter the cytosol in order to reach their substrates. With the exception of DT, which enters the cytosol from acidified endosomes, the intracellular site of translocation of the other toxins is unknown.We have used a molecular approach to address the site of toxin translocation by investigating the effect of overexpressing mutant forms of Rab1a, ARF1 and Sar1, GTPases which are required for the assembly of biochemical complexes mediating anterograde and retrograde transport in the exocytic and endocytic pathways, on the susceptibility of HeLa cells to various toxins. The results provide evidence that ricin, PE and SLT-1, but not DT, require receptors which actively recycle between the endoplasmic reticulum and terminal Golgi compartments for cytotoxicity. These finding strongly support the contention that ricin, PE and SLT-1, in contrast to DT, must undergo retrograde transport through the Golgi stack to the endoplasmic reticulum before translocation to the cytosol occurs.

THE CHLOROPLAST PROTEIN IMPORT MACHINERY

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A large number of plastid localized proteins are posttranslationally imported as precursor proteins from the cytosol into the organelle. Recognition and translocation is accomplished by a subset of chloroplast envelope proteins. The outer envelope proteins OEP 86, OEP 75, OEP 70 (an heat shock cognate 70 homologue) and OEP 34 are clearly involved in the import event and can be isolated as one functionally active translocation unit. For two of these proteins cDNA clones have been obtained very recently in our laboratory, namely OEP 86 and OEP 34. OEP 86 seems to be a precursor protein receptor, which could be regulated by GTP binding and ATP dependent phophorylation-dephosphorylation. OEP 75 is traversing the membrane in multiple ß-sheets. OEP 34 is tightly associated with OEP 75, which could be part of the translocation pore. OEP 34 represents a new type of GTP-binding protein, which possesses endogenous GTPase activity. Multiple GTP binding and hydrolysis cycles as well as protein phosphorylation-dephosphorylation events might therefore regulate the interaction of a precursor protein with the translocation machinery of the outer envelope, making it very distinct from the mitochondrial outer membrane system. Further proteins of the inner envelope membrane namely IEP97 and IEP36 have been implied to function in the translocation event. We have cloned IEP 97 and investigated its orientation in the inner membrane in order to establish its role in translocation. IEP 97 shows significant sequence homologies to USO I, a component of the cytoskeleton in yeast. A new model for translocation into chloroplasts will be presented.

ENDOPLASMIC RETICULUM TO GOLGI ROUTING OF A CONSERVED DOMAIN OF MULTIDOMAIN PROTEOGLYCANS.

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Multidomain proteoglycans of the aggrecan family share a conserved C-terminal globular domain, G3. This domain appears to be implicated in ER to Golgi routing, based on the the following considerations: 1) a major subdomain of G3 is a common motif in secreted proteoglycans and other secreted or cell surface proteins; 2) G3 is absent in mutant avian aggrecan due to an upstream premature stop codon and; 3) the truncated mutant protein remains in the ER but is a substrate for glycosaminoglycan (GAG) chain addition; 4) G3 is usually cleaved from normal aggrecan after secretion, implying that it is expendable in the extracellular space. Thus, recognition of G3 within the ER may enable its routing into the Golgi network. We have tested this hypothesis by transfection of CHO cells with a construct containing a tandem arrangement of: *aggrecan signal peptide (SP)-5 GAG consensus sites (GAG5)-G3 domain-His6*. Transfection was initially optimized using a CMV promoter-driven vector containing a reporter gene, chloramphenicol acetyltransferase (CAT). Expression was >500 times the basal levels, per unit of protein in the assay. Subsequently, transfection under the same conditions with the *SP-GAG5-G3-His6* construct, instead of CAT, led to transcription (Northern analyses), translation of the transcript (immuno-fluorescence) and a secreted neoproteoglycan. The $^{35}SO_4$-labeled neoproteoglycan was isolated from spent culture media by chelation chromatography. It appears as a diffuse band on SDS/PAGE, ranging in size from 90-160 kDa; the GAG chains are mostly removed by chondroitinase ABC, with residual ones being cleaved by heparitinase. After partial digestion the neoproteoglycan yields several intermediate products, including a core protein with sulfate-labeled stubs at ca. 33 kDa. CHO cells transfected with an empty vector, as well as non-transfected cells, did not display the proteoglycan transcript or produce the translated, secreted neoproteoglycan. These results show that the G3 domain with tandem GAG consensus sites is sufficient for entry of the chimeric protein into the Golgi stacks, followed by glycosaminoglycan chain addition and secretion of the neoproteoglycan. Future studies will determine if G3 is necessary as well as sufficient for ER to Golgi routing of chimeric proteins.

MATURATION OF F-PILIN IS SEC-INDEPENDENT, TRAQ-DEPENDENT AND IS INHIBITED BY A TEMPERATURE SENSITIVE MUTATION AFFECTING LEADER PEPTIDASE.

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F pilus filaments extend from the surface of F^+ donor bacteria and initiate contacts with recipient cells. Pili are composed of F-pilin subunits that derive from a 121 amino acid precursor, propilin, product of the F plasmid transfer gene *traA*. Maturation of propilin proceeds in two steps. First, the leader peptide is removed yielding a 70 residue pilin peptide. Second, the 7-kDa pilin peptide is acetylated to yield mature F-pilin. These subunits are stable bitopic integral membrane proteins with an N_{out}-C_{out} conformation (Paiva et al. 1992, J. Biol. Chem. 267:26191-26197) that accumulate in the inner membrane. The maturation of propilin is dependent upon the function of the F *traQ* product since, in the absence of TraQ, propilin is rapidly degraded. In order to define the role of TraQ in propilin maturation, we examined the processing of TraA *in vitro* and *in vivo*. Propilin processing results in the conversion of the 13-kDa precursor into a 7-kDa pilin peptide product and a 5.5-kDa leader peptide product indicating that TraQ does not alter the size of the leader peptide and that only one polypeptide cleavage occurs during maturation. This cleavage event is dependent upon signal peptidase I since processing was inhibited in a $TraQ^+$, $LepB_{ts}$ host at 42° C. In contrast, propilin processing was unaffected in $secA_{ts}$ or $secY_{ts}$ hosts at 42° C when TraQ was present. Furthermore, neither sodium azide nor ethanol inhibited propilin processing. Attempts to stabilize the propilin polypeptide in a Lon^- Clp^- host led to a slight increase in the level of the 7-kDa peptide in the absence of TraQ. However, increased expression of host chaperone proteins did not alleviate the TraQ requirement. To test whether the unusual leader peptide is responsible for the TraQ-dependency of propilin maturation, we examined the processing of propilin missense and deletion mutants and of *traA*::*bla* gene fusion products. Our data indicates that TraQ-dependence reflects a feature of the mature protein portion of the precursor, rather than its leader peptide. Together, these findings suggest that TraQ permits efficient propilin insertion in the membrane in a *sec*-independent pathway and via an ethanol resistant mechanism. Interestingly, the transfer deficiency of an F *traQ* mutant could be suppressed by high levels of *traA* expression.

CALCIUM-DEPENDENT EXOCYTOSIS IN DENDRITES OF CULTURED HIPPOCAMPAL NEURONS: ROLE OF CaMKII.

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We wish to test the hypothesis that calcium/calmodulin-dependent protein kinase II (CaMKII) activation mediates insertion of glutamate receptors into postsynaptic membrane by vesicle exocytosis. Towards this goal, we have used quantitative time-lapse imaging of the fluorescent membrane probe FM 1-43 to monitor the exocytosis of vesicles in cultured hippocampal neurons at 2 to 10 days *in vitro* (DIV). Neurons exposed overnight to FM 1-43 accumulate dye in all neuronal processes. Postsynaptic localization of FM 1-43 was confirmed by indirect immunofluorescent staining with dendritic marker MAP-2. Release of the dye from dendritic sites was induced by the Ca^{++}-ionophore A23187 (2.5 μM). This release was observed at ages (DIV) when CaMKII and glutamate receptors (NMDAR1, GluR1 and GluR2/3) are normally expressed, but not before. We investigated the pattern of FM 1-43 release at earlier ages, following infection of the cultured neurons with a recombinant vaccinia virus expressing a full length (f) or truncated (t; constitutively active) CaMKII. Release of the dye from fCaMKII infected cells was evoked by A23187 at an age when NMDAR1 is expressed but CaMKII and GluRs are normally not expressed. Spontaneous exocytosis was detected in tCaMKII infected neurons at all ages, and not in fCaMKII-infected or non-infected neurons. These results suggest a role for CaMKII activation in exocytosis at postsynaptic sites.

PRESYNAPTIC COMPONENT OF LONG-TERM POTENTIATION VISUALIZED AT INDIVIDUAL HIPPOCAMPAL SYNAPSES

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Long term potentiation has been studied with electrophysiological techniques that do not separate presynaptic and postsynaptic contributions. Here, changes in exo-endocytic cycling were monitored at synapses between cultured rat hippocampal neurons by measuring the differential uptake of antibodies that recognized the intralumenal domain of the synaptic vesicle protein synaptotagmin. Vesicular cycling increased markedly during glutamate-induced long term pontentiation. The degree of potentiation was heterogeneous, appearing greater at synapses where the initial level of vesicular turnover was low. Thus, changes in presynaptic activity were visualized directly and the spatial distribution of potentiation could be determined at the level of single synaptic boutons.

UNIQUE COFACTOR REQUIREMENTS AND 3-DIMENSIONAL STRUCTURE OF A PROTEINASE ACTIVITY

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Human adenovirus serotype 2 virions contain a virus-coded proteinase activity whose function is to cleave 6 different virion proteins 2500 times to render a virus particle infectious. For maximal activity *in vitro,* the protein product of the L3 23-kDa gene and two cofactors are required- an 11 amino acid peptide (pVIc) that originates from the C-terminus of the precursor to virion protein VI and the viral DNA (Ad2 DNA). Proteinase activity in the virion is greatly diminished by DNase treatment but restored upon the addition of Ad2 DNA after inactivation of the DNase. The Ad2 DNA requirement is not sequence specific and actually is a requirement for a polymer of high negative charge density, a requirement fulfilled in the virion only by the viral DNA. The cofactors function by increasing the catalytic rate constant for substrate hydrolysis- pVIc by 355-fold, pVIc + Ad2 DNA by 6,072-fold. The dissociation constant for a recombinant form of the L3 23-kDa protein, recombinant endoproteinase (rEP), with pVIc is 13 nM; 23 nM for the binding of rEP-pVIc complexes to Ad2 DNA. The 3-dimensional structure of the rEP-pVIc complex has just been solved to a resolution of 2.6 Å and an R-factor of 0.186. At first glance, the active site contains a Cys-His ion pair. pVIc is connected to rEP by a disulfide bond with Cys-104. The 11 amino acids of pVIc form a beta-sheet at the periphery of the protein.

Our current model is that pVIc is required for proteinase activity to prevent premature activation of the proteinase before virion assembly and that the viral DNA serves as a guide wire along which the proteinase complex moves as it cleaves virion precursor proteins. Similar, unusual cofactors may be required for other enzymes involved in protein degradation.

A LYSOSOMAL TARGETING SIGNAL IN THE CYTOPLASMIC TAIL OF THE β CHAIN DIRECTS H-2M TO THE MIIC IN B LYMPHOBLASTOID CELLS. Michael S. Marks, Elly van Donselaar*, Lauren Woodruff, Paul A. Roche¶, Peter J. Peters* & Juan S. Bonifacino. Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development and ¶Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda MD; *Dept. of Cell Biology, University of Utrecht, The Netherlands.

Class II molecules of the major histocompatibility complex (MHC-II) function by binding peptides derived from antigens encountered within the endocytic pathway and presenting them on the cell surface to CD4+ T cells. In human B cells, class II molecules accumulate in a specialized endosomal compartment, the MIIC, in which they are thought to encounter and bind peptides. An additional molecule, HLA-DM (DM; H-2M in mice), has been localized to the MIIC and shown to be required for MHC-II peptide binding. Neither the relationship of the MIIC to the endosomal system nor the mechanisms by which DM functions or localizes to the MIIC are well understood. As a step toward addressing these questions, we analyzed DM localization in cells that do not express MHC-II. The human DM αβ heterodimer, independently of the invariant chain, was localized in transfected HeLa and NRK cells by immunofluorescence and immunoelectron microscopy to a late endosomal/lysosomal compartment. In order to identify a potential targeting determinant, we analyzed the localization of a chimeric protein, T-T-Mb, in which the cytoplasmic tail of the murine DMβ chain (Mb) was appended to the lumenal and transmembrane domains of a cell surface protein, Tac antigen. Like the DM heterodimer, T-T-Mb was localized to a lysosomal compartment in HeLa and NRK cells, as judged by immunofluorescence and immunoelectron microscopy. In this compartment, T-T-Mb was rapidly degraded; degradation was blocked by inhibitors of lysosomal proteolysis. The Mb cytoplasmic tail was also able to mediate internalization of anti-Tac antibody; internalized antibody was delivered to lysosomes. Deletion of the tyrosine-based motif, YTPL, from the Mb cytoplasmic tail resulted in cell surface expression of the T-T-Mb chimera and a loss of both degradation and internalization. Furthermore, alanine scanning mutagenesis showed that the Y and L residues were critical for these functions. Lastly, T-T-Mb was localized by immunoelectron microscopy to both lysosomes and the MIIC in a human B lymphoblastoid cell line. Our results suggest that a motif, YTPL, in the cytoplasmic tail of the β chain of DM is sufficient for lysosomal targeting in fibroblasts and for MIIC targeting in human B lymphoid cells. The presence of T-T-Mb in MHC-II-negative lysosomes in B lymphoid cells suggests that other determinants, perhaps through lumenal domain interactions, mediate segregation of the MIIC from classical lysosomes.

CELL-FREE RECONSTITUTION OF THE TRANSPORT OF VIRAL GLYCOPROTEINS FROM THE TGN TO THE BASOLATERAL PLASMA MEMBRANE OF MDCK CELLS.

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An *in vitro* system to study the transport of plasma membrane proteins from the TGN to the basolateral plasma membrane of polarized MDCK cells has been developed in which purified cell fractions are combined and transport between them is studied under controlled conditions. In this system, a donor Golgi fraction derived from VSV or Influenza virus-infected MDCK cells — in which ^{35}S-labelled viral glycoproteins were allowed to accumulate in the TGN during a low temperature block — is incubated with purified immobilized basolateral plasma membranes that have their cytoplasmic face exposed and are obtained by shearing-lysis of MDCK monolayers grown on cytodex beads. Approximately 15-30% of the labelled glycoprotein molecules are transferred from the Golgi fraction to the acceptor plasma membranes and are recovered with the sedimentable (1xg). beads. Transport is temperature, energy and cytosol dependent, and is abolished by alkylation of SH groups and by the presence of GTP-γ-S — which implicates GTP-binding proteins and the requirement for GTP hydrolysis in one or more stages of the transport process. Endo H-resistant glycoprotein molecules that had traversed the medial region of the Golgi apparatus are preferentially transported and their luminal domains become accessible to proteases, indicating that membrane fusion with the plasma membrane takes place in the *in vitro* system. Mild proteolysis of the donor or acceptor membranes abolishes transport, suggesting that protein molecules exposed on the surface of these membranes are involved in the formation and consumption of transport intermediates, possibly as addressing and docking proteins, respectively. Moreover, removal of peripheral proteins from the acceptor membranes by either high salt wash or a preincubation with cytosol and ATP greatly reduced their acceptor capacity, which could be restored by the addition of the protein extract.

Surprisingly, both VSV-G and influenza HA were transported with equal efficiencies to the basolateral acceptor membranes. However, low concentrations of a microtubular protein fraction preferentially inhibited the transport of HA, although this effect was not abolished by microtubule depolymerizing agents. This system shows great promise for elucidating the mechanisms that effect the proper sorting of plasma membrane proteins in the TGN and their subsequent targeting to the appropriate acceptor membrane.

UBIQUITIN-MEDIATED PROTEIN DEGRADATION IS ESSENTIAL FOR ACTIVATED TRANSCRIPTION OF PHOSPHOLIPID BIOSYNTHETIC GENES AND FOR ENDOCYTIC DEGRADATION OF THE YEAST *ITR1* INOSITOL PERMEASE IN THE VACUOLE

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Inositol supply in *Saccharomyces cerevisiae* is either though endogenous synthesis or uptake from the growth medium, and both processes are highly regulated. *INO1* encodes the key enzyme in the biosynthetic pathway and *ITR1* encodes the major inositol permease. Both inositol permease activity and Ino1p activity are derepressed when inositol is not present in the growth medium, and repressed to basal level when inositol is present. Regulation is through control of both transcription and protein stability. Transcriptional control of *ITR1* and *INO1* as well as a number of other phospholipid biosynthetic genes depends on the regulatory genes *INO2* and *INO4* for derepression of transcription, and on the *OPI1* gene for repression. Down-regulation of Itr1p is triggered by inositol and leads to its endocytic degradation in the vacuole.

We present evidence (1) that the ubiquitin conjugating enzymes Ubc4p or Ubc5p are required to quickly inactivate the Itr1 permease prior to the slower endocytic removal of the permease from the plasma membrane to the interior of the cell. (2) endocytic degradation of Itr1p does not occur if Ubc4p/Ubc5p-mediated ubiquitination is blocked or if ubiquitin recycling is blocked by*doa4* mutation. Endocytic removal of the permease from the cell surface is not affected in *ubc4, ubc5* mutants, and therefore down-regulation of activity is observed. We are currently investigating the role of ubiquitination in targeting the permease to the vacuole, and the role of the 26S proteosome in the endocytic degradation pathway.

We also show that (3) derepression of *ITR1* and *INO1* transcription depends on degradation of the negative regulator Opi1p, a process that requires a functional ubiquitin conjugating enzyme encoded by either *UBC4* or *UBC5*, and also the ubiquitin recycling enzyme encoded by *DOA4*. Thus, ubiquitin-mediated protein degradation regulates inositol supply and phospholipid synthesis in yeast at multiple levels.

SNAP-25 AND SYNAPTOTAGMIN INVOLVEMENT IN THE FINAL Ca^{2+}-DEPENDENT TRIGGERING OF NEUROTRANSMITTER EXOCYTOSIS

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In neurons, depolarization of the plasma membrane causes influx of Ca^{2+} ions, leading to the fusion of membranes of synaptic vesicles docked at the active zone and the plasma membrane, and release of neurotransmitter into the synaptic cleft. Recent studies have also identified an ATP hydrolysis-dependent step mediated by the ubiquitous N-ethylmaleimide-sensitive protein, NSF, as an important component in the mechanism of neurotransmission[1]. While studies with solubilized membrane proteins and recombinant proteins indicate that the assembly of NSF fusion protein into the initial vesicle docking complex followed by its ATPase activity leads to a disassembly of this complex, the mechanism which serves to ultimately trigger exocytosis has remained elusive. Using a cell-free, synaptosomal membrane preparation from mouse cortices, we show that Ca^{2+} activates this final triggering event resulting in secretion of the excitatory neurotransmitter, glutamate. Hydrolysis of ATP further potentiates this Ca^{2+}-dependent glutamate release but ATP addition by itself cannot stimulate any significant release. We also demonstrate directly that exocytosis signaled by Ca^{2+} in this membrane preparation involves SNAP-25, synaptosomal associated protein, 25 kD, a key constituent of the recently characterized membrane docking complex and also show that Ca^{2+} affects the association of synaptotagmin, a synaptic vesicle membrane protein proposed to serve as a calcium sensor and triggering device, with this docking complex. Our results of the role of ATP in glutamate release also show that at neuronal synapses, as suggested previously for PC12[2] and chromaffin cells[3], Ca^{2+}-dependent triggering acts after an ATP-dependent priming step, and that it alters the association of core proteins of the docking/fusion complex to immediately prompt membrane fusion for neurotransmitter release.

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CYTOPLASMIC CHAPERONES DETERMINE THE TARGETING PATHWAY OF PRECURSOR PROTEINS TO MITOCHONDRIA.

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Mitochondrial import stimulation factor (MSF) is an NEM-sensitive cytoplasmic chaperone which functions in precursor-targeting to mitochondria. Here we analyzed how hsp70 and MSF participate in mitochondrial protein import. We showed that mitochondrial precursor proteins can be imported into mitochondria by an NEM-sensitive, MSF and its receptor-dependent pathway, and NEM-insensitive, hsp70-dependent pathway. Hsp70-dependent precursor targeting occurred through a direct interaction of the precursors with the membrane receptor. The MSF-dependent import of the precursor proteins required extramitochondrial ATP for the MSF release from the receptor. On the other hand in the hsp70-dependent import, hsp70 was spontaneously dissociated from the hsp70-precursor complex upon its interaction with the outer mitochondrial membrane and therefore, cytoplasmic ATP was not required for precursor import into mitochondria. The relative importance of both pathways was determined by the affinity of MSF and hsp70 for mitochondrial precursors.

CHARACTERIZATION OF A DYNEIN RELATED GENE EXPRESSED SPECIFICALLY IN ADULT HUMAN TESTIS

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Dyneins are large, multisubunit ATPases that interact with microtubules to generate force. Dyneins are also present in a cytoplasm, where they are involved in the transport of particles and organelles along microtubules and in the transport of condensed chromosomes during the mitosis. Defects in axonemal dyneins in humans lead to various hereditary forms of sterility due to immotile sperm. Patients have also a long history of respiratory tract diseases - recurrent bronchitis and chronic sinusitis because their immotile cilia are unable to clear the mucus from lungs and sinuses. About half of the individuals with immotile cilia syndrome have a condition described as situs inversus, where the position of the heart, intestine, liver and other internal organs is reversed. The complex of abnormalities is known as Kartagener's syndrome.

Dyneins are large protein complexes with a mass between 1000-2000 kDa and have been characterized under the electron microscope. However, little is known about them at the molecular level. An important recent achievement has been the determination of the sequences of the ß-heavy chain from the axonemal dynein of two species of a sea urchin. We have characterized a human testis-specific transcript that shows a high degree identity and similarity to the 3-terminal part of the ß-heavy chain of the sea urchin dynein. To our knowledge our laboratory has been the first to obtain sequence information of a dynein intermediate chain in humans. We are currently initiating studies that will determine its structure and expression pattern. These studies combined with the analysis of patient material may give an insight into a role of intermediate chains in dynein complexes as well as the function of our testis-specific dynein gene in spermatogenesis and infertility.

REGULATION OF Fc RECEPTOR - MEDIATED ENDOCYTOSIS VIA CLATHRIN COATED PITS

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Cell surface receptors which bind the Fc domain of immunoglobulin molecules are capable of mediating endocytosis of bound ligands via clathrin coated pits. Coated pit - dependent internalization of soluble immune complexes by murine FcγRII-**B2** requires a di-leucine motif located within a conserved YXLL sequence at Y26 of the cytoplasmic tail domain. Y26 is not needed for endocytosis of the bound ligand, since mutating this residue to an alanine did not result in altered internalization efficiency in transfected lymphocytes. However, changing Y26 to a glycine residue abolished mFcγRII-**B2** mediated endocytosis in transfected lymphocytes. These observations suggest that the mFcγRII-**B2** cytoplasmic tail $L^{28}L^{29}$ residues interact with at least one component along the endocytic pathway, presumably a clathrin coated pit adaptor. In addition, a specific conformation of a cytplasmic tail $L^{28}L^{29}$ - containing sequence may be required for this interaction to occur. The murine FcγRII-**B1** isoform, which is identical to mFcγRII-**B2** except for a membrane proximal 47 amino acid insert in the cytoplasmic tail, is unable to endocytose soluble immune complexes. Although it has been previously established that the FcγRII-**B1** insert prevents coated pit localization, the mechanism is unknown. FcγRII-**B1** mediated inhibition of endocytosis via clathrin coated pits appears to involve the amino terminal half of this insert. One possible mechanism for this inhibition is that the FcγRII-**B1** insert may block the availability of, or disrupt the secondary structure of a downstream sequence that is required for commitment of receptor - ligand complexes to enter the endocytic pathway.

THE ASMA GENE PRODUCT IS INVOLVED IN THE ASSEMBLY OF *ESCHERICHIA COLI* OUTER MEMBRANE PROTEINS.

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The outer membrane proteins of *Escherichia coli* are first synthesized as high molecular weight precursors. The signal sequence present within these precursors directs them to a site in the inner membrane from where the proteins exit the cytoplasm. The signal sequence is cleaved during this process and mature monomers either remain in the periplasm (space between the two membranes) or are targeted to the outer membrane. For trimeric outer proteins, mature monomers either trimerize prior to or during the insertion in the outer membrane. To better comprehend this cellular process, we devised novel genetic schemes and isolated mutations within genes coding for outer membrane proteins that interfered with the assembly process. Suppressors of these assembly mutations were sought in order to identify other cellular components that may play a role in the assembly process. Suppressor mutations identified three distinct genetic loci on the chromosome. In this study we present our data on one such assembly suppressor locus (*asmA*). We have used OmpF and OmpC as our model trimeric outer membrane proteins.

Mutations in *asmA* suppressed assembly defects of a large number of mutant OmpF and OmpC proteins. These mutations even suppressed the assembly defects of wild type proteins in cells containing an incomplete lipopolysaccharide. This lack of allele specificity and the general suppressive nature of *asmA* mutations indicated that the suppression may not involve protein-protein interactions. A direct examination of *asmA* mutations revealed that they represent null alleles of a previously unidentified gene. Thus, it was the absence of AsmA and not the presence of an altered AsmA that provided the suppressive environment. Mutations in *asmA* did not alter the assembly of wild type outer membrane proteins. This suggests that the physiological role of AsmA is to prevent the assembly of mutant proteins. The amino-terminal end of AsmA contains a typical signal sequence. To facilitate in localizing AsmA, we constructed a MalE-AsmA chimeric protein and raised antibodies. These antibodies recognize MalE and AsmA only when the latter is over produced from a plasmid. This shows that the chromosomally-coded AsmA is present in very low amounts. Although *asmA* null mutations impose no assembly defects on the wild type OmpF protein, overproduction of AsmA does. This would be expected for a protein which under physiological conditions does not interfere with correctly folded proteins, but when overproduced, it may nonspecifically bind to wild type proteins and interfere with their proper assembly.

PROTEIN RETENTION IN THE GOLGI APPARATUS

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The resident proteins of the Golgi apparatus are able to stay in place despite the continuous flow of protein and lipid through the structure. The single transmembrane-domain (TMD) of the type II Golgi enzyme sialyltransferase (ST) specifies Golgi retention of the protein, and this now appears to be a general feature of Golgi glycosyltransferases. The TMDs of known Golgi enzymes are, on average, five residues shorter than those of the plasma membrane proteins. Addition or removal of residues showed that the efficiency of Golgi retention of both ST and a cell surface protein DPPIV is directly related to TMD length. Moreover when the type I plasma membrane protein CD8 was expressed with a synthetic TMD of 23 leucines, then as expected it accumulated on the cell surface, but when the polyleucine stretch was reduced to 17 leucines substantial accumulation in the Golgi apparatus was observed. In addition to the average length difference, Golgi TMDs contain 2.3 times the level of the bulky residue phenylalanine. If the four phenylalanines in the TMD of ST are changed to isoleucines then there is an increase in cell surface expression of the protein. All these observations have led us to propose that within the bilayer of a Golgi cisterna the mixed lipid populations separate into lipid microdomains with distinct compositions and hence thickness and degree of structural perturbability. The Golgi enzymes would selectively partition into one domain and so be prevented from entering transport vesicles comprised of the other domain. To test this model further, we have isolated mutants in the yeast *S. cerevisiae* in which TMD-mediated retention is defective. Cloning of the gene corresponding to one of these mutants has identified a new family of proteins, and intriguingly the protein encoded by this gene, Anp1p is located in the endoplasmic reticulum, although it affects the functioning of the Golgi apparatus.

MEMBRANE TARGETING OF PROTEINS INVOLVED IN PLANT STORAGE LIPID SYNTHESIS AND DEPOSITION.

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We are interested in understanding the synthesis of plant storage lipids and to that end are studying a number of different proteins involved in this process. Two proteins of particular interest are cytochrome b_5 and oleosin, both of which have unusual targeting pathways. Cytochrome b_5 is a small integral ER membrane protein involved in electron transport and plays a key role in the activity of fatty acid desaturases. In mammalian cells the targeting of this protein to the ER has been shown to be determined by the last 10 residues in the protein's C-terminal hydrophobic anchor. We have isolated a number of cDNAs encoding cytochrome b_5 from several plant species, and this region is not highly conserved. Therefore we wish to identify the key residues in plant cytochrome b_5 targeting and to confirm it does behave like a typical signal-anchor protein. We are using a number of different approaches to this problem including *in vitro* assays, expression in yeast and transgenic plants.

The second protein of interest is termed oleosin, which is the single major component of plant storage lipid bodies (or oil bodies). Oil bodies are small spherical organelles present in oil seeds that compartmentalize the storage lipid (usually in the form of TAG) away from other more hydrophilic subcellular environments. This organelle is very simple in structure, consisting of a central TAG core surrounded by a phospholipid monolayer into which the oleosin proteins are inserted and act as a shield between the cytosol and the TAG. However although the organization of the oil body is very simple, its biogenesis is less clear. Working from the hypothesis that it is an ER-derived organelle, since this is the site of TAG synthesis we have investigated the targeting of the oleosin protein, inferring that it may be targeted to the ER as a first step in oil body biogenesis. To that end we have shown that oleosin insertion in to the ER does occur, and that it is mediated by SRP. We have photocrosslinked oleosin nascent chains to SRP54 and are in the process of identifying the oleosin signal sequence recognized by SRP. Therefore we feel it likely that oil bodies are ER-derived in their origin.

AFFINITY-PURIFICATION AND IDENTIFICATION OF GrpE HOMOLOGS FROM MAMMALIAN MITOCHONDRIA

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DnaK, DnaJ, GrpE, GroEL and GroES proteins of *Escherichia coli* are heat shock proteins which function as molecular chaperones. Three mitochondrial chaperones with extensive sequence identity to their bacterial counterparts DnaK, GroEL and GroES have previously been identified from higher eukaryotes, but as yet DnaJ and GrpE homologues have only been identified in yeast. We used affinity chromatography on DnaK columns to identify a mitochondrial GrpE homologue from bovine, porcine and rat liver mitochondria. The 24 kDa GrpE homologue bound specifically to the DnaK column and was not eluted by 1 M KCl but eluted with 5 mM ATP. Sequence analysis of 85 residues revealed 42% positional identity to the essential mitochondrial GrpEp from *S. cerevisiae* and about 30% identity to the bacterial counterparts again illustrating the high degree of conservation chaperones exhibit from bacteria to mammals. The 24 kDa mitochondrial GrpE homologue should therefore be designated mt-GrpE. Based on the partially determined amino acid sequence of mt-GrpE, a 440 bp partial cDNA clone was obtained by RT-PCR of mRNA from rat hepatoma cells. The partial cDNA clone was then used as a probe to isolate the full length cDNA sequence of mt-GrpE from a cDNA library.

CLOSTRIDIAL NEUROTOXINS AND MEMBRANE TRAFFIC AT THE SYNAPTIC TERMINAL

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The clostridial neurotoxins (CNTs) block neurotransmitter release from nerve terminals. The toxins act intracellularly to cleave specific proteins believed to participate in the process of synaptic vesicle exocytosis. We have correlated synaptic blockade induced by either tetanus toxin (TeNT) or botulinum neurotoxin A (BoNT A) with the disappearance of their protein substrates, with alterations in the fine structure of the synaptic release site, with the activity-dependent uptake of the dye FM1-43, and with the stimulated uptake of horseradish peroxidase (HRP) into newly recycled synaptic vesicles.

Dissociated cell cultures of murine spinal cord are maintained for 3 wk before exposure to TeNT or BoNT A (10 ng/ml for 16-20 hr). Potassium-evoked release of both an inhibitory (glycine) and an excitatory (glutamate) neurotransmitter is blocked completely in toxin-treated cultures. Immunoreactivity for VAMP and for SNAP-25 is abolished in cultures exposed to TeNT or BoNT A, respectively. Electron microscopy shows that approximately twice the number of synaptic vesicles are "docked" (located within 10 nm of the membrane) at the active zones of toxin-blocked cultures as are seen in either spontaneously active or KCl-stimulated control cultures. Consistent with the block in synaptic activity, TeNT- (and BoNT B-, C-, or D-) treated cultures show no activity-dependent uptake of FM1-43. In contrast, cultures treated with BoNT A (up to 300 ng/ml) continue to show FM1-43 loading, indicating the persistence of synaptic vesicle endocytosis in the absence of exocytosis. These findings are confirmed by KCl stimulation in the presence of HRP. TeNT-blocked synapses show HRP-labeling of only occasional synaptic vesicles, whereas a large proportion of synaptic vesicles are labeled in BoNT A-blocked terminals. The labeled vesicles appear to form via conventional clathrin-coated intermediate structures.

These observations suggest that BoNT A may be used to uncouple the cycle of triggered exocytosis-endocytosis that occurs at the synaptic terminal, allowing discrete analysis of the endocytosis component. They further suggest that VAMP (or another as yet unidentified substrate of tetanus toxin) participates in the process of synaptic vesicle membrane retrieval.

NUCLEAR LOCALIZATION SIGNALS OF HUMAN PROTEASOMAL α-SUBUNITS ARE FUNCTIONAL IN VITRO

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Proteasomes are non-lysosomal, high molecular weight proteinases implicated in the degradation of both misfolded proteins and short-lived regulatory proteins. Proteasomes are located both in nuclei and cytoplasm of eukaryotic cells. Active transport through the nuclear pores mediated by nuclear localization signals (NLS) could be the mechanism of redistribution of proteasomes. A large number of proteasomal subunits from various organisms have been cloned and sequence analysis has shown that several subunits contain putative NLS.

We tested the functionality of three different putative NLS sequences from human α-type proteasomal subunits (Hsci, Hsc9, Hsc3, respectively KKVKKK, KKEK, KKQK) and the NLS-type sequence from the archaebacterium *Thermoplasma acidophilum* (KKVR) for their ability to direct non-nuclear proteins to the nucleus. Synthetic peptides containing these sequences were generated and conjugated to allophycocyanin (APC) or BSA-FITC as large fluorescing non-nuclear reporter molecules. The conjugates were introduced into digitonin-permeabilized HeLa and 3T3 cells. Nuclear import was analyzed by fluorescence microscopy.

All three putative NLS sequences from human proteasomal subunits were able to direct the reporter molecules to the nucleus in both cell types. Substitution of the first lysine residue of the NLS motifs with a threonine residue (TKVKKK, TKEK, TKQK) inhibited nuclear import. Interestingly, also the putative NLS sequence found in *Thermoplasma acidophilum* was functional as a nuclear targetting sequence.

These results indicate that all three NLS sequences found in human proteasomal α-subunits can function as a nuclear targeting sequence in vitro and could be functional in vivo to direct proteasomes to the nucleus. This would indicate that per 20S complex several NLS are functional. This redundancy could increase the efficiency of transport. Alternatively, interaction of the 20S complex with regulatory complexes could result in masking of one or more NLS sequences.

CHARACTERIZATION OF A RAN BINDING NUCLEOPORIN IN YEAST

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Ran has been shown to be an essential factor for nuclear import in both higher eucaryotes and yeast. To understand the molecular mechanisms of yeast Ran (GSP1) function at the nuclear periphery we tried to identify GSP1 binding proteins at the nuclear pore complex (NPC) in yeast. Recombinant GSP1 was therefore purified, labelled with ^{32}P-GTP and used for ligand blotting experiments on yeast nuclear envelope preparations immobilized on nitrocellulose. Several bands specifically labelled with GTP-GSP1, including one at 36kD. Data available from the yeast genome sequencing project showed that a novel protein with a calculated molecular weight of 36kD shares significant sequence homology with Ranbp1 in its putative Ran binding motif. In addition it contains FXFG repeat motifs that are characteristic for nucleoporins. We therefore termed this protein Nup36. The gene coding for Nup36 was cloned and recombinant Nup36p was shown to bind GTP-Ran. Moreover, Nup36p was shown to bind purified yeast karyopherin, an α/β heterodimer that has been shown to be required for recognition of import substrate and docking at the NPC. We are currently investigating the molecular interaction of the different nuclear import factors binding to Nup36p.

IDENTIFICATION OF A NEW MUTANT DEFECTIVE IN ER TO GOLGI TRANSPORT. Aaron Neiman and Rolf Sternglanz, Dept of Biochemistry and Cell Biology, SUNY Stony Brook, Stony Brook NY 11794

We have used the lipophilic dye DiOC6 to screen through a bank of temperature-sensitive yeast strains for mutants with abnormal nuclear envelope/ER morphologies. In attempting to clone one of the mutants identified in this screen, *nin4*, we isolated two genes, *SAR1* and a novel gene, as high copy suppressors of the *nin4* temperature-sensitive phenotype. *SAR1* encodes a GTPase required for vesicle budding from the ER[1].

The isolation of *SAR1* as a suppressor led us to examine secretion in the *nin4* mutant. *nin4* mutants accumulate the ER form of carboxypeptidase Y at the non-permissive temperature, indicating a block in ER to Golgi transport. Complementation tests indicate that *nin4* is not an allele of *sec12, 13, 16, 17, 18, 19, 20, 22, 24, 31, or sar1*. Additionally, *nin4* displays unlinked non-complementation with *sec23*, which encodes a coatomer complex II subunit[2]. The genetic interactions with *SAR1* and *SEC23* suggest a role for *NIN4* in vesicle budding from the ER. Cloning of *NIN4* and further investigation of the second high copy suppressor of *nin4^{ts}* are currently underway.

[1]Nakano, A. and Muramatsu, M. (1989) J.Cell Biol. 109:2677-2691

[2]Barlowe, C. et al. (1994) Cell 77:895-907.

β-NAP, A CEREBELLAR DEGENERATION ANTIGEN, IS A CANDIDATE SYNAPTIC VESICLE COAT PROTEIN. Lori S. Newman, Matthew O. McKeever, Hirotaka J. Okano and Robert B. Darnell, Laboratory of Molecular Neuro-Oncology, The Rockefeller University, New York, N.Y. 10021.

We have identified a target antigen in autoimmune cerebellar degeneration, β-NAP, that is closely related to the β-adaptin and β-COP coat proteins. β-NAP is expressed exclusively in the nervous system; in developing mice it is specifically expressed in post-mitotic neurons from E12 through adulthood. β-NAP is a non-clathrin associated phosphoprotein present as both a cytoplasmic pool and a membrane bound fraction. Within neurons, β-NAP is found within the soma and nerve terminal. Our data suggest a model in which β-NAP mediates synaptic vesicle coating and transport to the axon terminus, and may represent a general class of coat proteins that mediates transport to the apical surface of polarized cells.

CLATHRIN HEAVY CHAIN IS REQUIRED FOR CELL-TYPE DIFFERENTIATION.

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Clathrin heavy chain (CHC) is a major component of clathrin-coated vesicles that line the plasma membrane of eukaryotic cells and ferry specific receptors and soluble nutrients to internal compartments within the cell. We targeted the clathrin heavy chain gene in *Dictyostelium* cells to generate a stable clathrin heavy chain-deficient (CHC^-) cell line. *Dictyostelium* cells exhibit a simple developmental program and the CHC^- mutants provide us the opportunity to test the role of clathrin in development. When plated on starvation plates, wild-type *Dictyostelium* cells use extracellular cAMP as a signal to coordinate a mass migration of 10^5 cells into a mound. Within 24 hours, this mass differentiates into a multicellular fruiting body consisting of two kinds of specialized cells: stalk and spore cells. When induced to develop, CHC^- cells express lower numbers (33% of wild-type) of cAMP-binding sites at their cell surface. Despite this deficiency, mutant CHC^- cells can chemotax, albeit inefficiently, into a mound of cells. Thus clathrin is not required for chemotaxis. However, in contrast with the differentiated fruiting body constructed by wild-type cells, clathrin-minus mutants construct a poorly formed elongated structure that is poorly differentiated. Examination of the elongated structures reveals highly vacuolated cells with a morphology characteristic of differentiated stalk cells, however viable spore cells are never found. Staining with calcoflour demonstrates that the clathrin-minus cells secreted extracellular cellulose, a major product of fully differentiated stalk and spore cells. In order to test whether the block of CHC- cells to fully differentiate into spore cells was cell-autonomous, we tested the ability of CHC^- differentiate if provided with a wild-type extracellular environment. We mixed clathrin-minus mutants with wild-type cells and examined the ability of clathrin-minus cells to differentiate into viable spores. Even when provided with an environment of 90% wild-type cells and 10% mutants, CHC^- mutants were unable to sporulate. These results suggest that clathrin heavy chain is required for terminal differentiation into spore cells, but is not required for differentiation into stalk cells.

GEOMETRICAL COINCIDENCE BETWEEN TWO FILAMENTS OF STRIATED MUSCLE

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The coincidence of the period between actin and myosin filaments of striated muscles has been identified. Assuming that the interaction sites of the myosin heads conform their spacing to the period during contraction, the smallest helix unit of the myosin sites is the same length as that of the one period helix of the actin filament. The coincidence is observed both in rabbit skeletal muscle and in crab striated muscle. The geometry of the coincidence suggests a sliding unit and the rotation of the actin and myosin filaments in the transversal direction of the filament-axis at free interaction. The *in-vivo* interaction specified here predicts the sliding unit per crossbridge that correlates with the actin size. Furthermore, we calculated and discuss the twisted degrees of actin filament, the sliding distance per ATP hydration, and the unitary velocity produced by a single crossbridge.

SUBCELLULAR LOCALIZATION OF B-50/GAP-43 TRAVELLING FROM THE NEURONAL CELL BODY TO THE GROWTH CONE

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Neurons convey building materials by axonal transport to maintain and extend axons and dendrites. In the nervous system, the growth-associated protein B-50/GAP-43 is highly expressed during neuritogenesis and axonal regeneration. There is much evidence which indicates that this protein is involved in neurite outgrowth, neurotransmisssion and neuroplastic events (e.g. LTP). B-50 is synthesized in the cell body, membrane bound and moved out into processes by fast axonal transport to the plasma membrane, growth cones and nerve terminals, where it is involved in signal transduction.

By means of immunocytochemistry and electron microscopy we have examined the ultrastructural distribution of B-50 in various subcellular compartments of hippocampal cells grown in vitro. We demonstrate by pre- and post-embedding procedures, using affinity-purified polyclonal B-50 antibodies and secondary antibodies coated on gold probes, that B-50 can be detected on the membrane of electron lucent vesicles in the trans region of the Golgi, adjacent to microtubules in the cell body, in neurites and growth cones. The average size of the B-50 decorated vesicles was 100 nm. The plasma membrane of the cell body, but, even more, that of the neurites and growth cones was richly immunolabeled for B-50, indicating that this cellular element may be a site of destination.

In an ultrastructural study of the regenerating rat sciatic nerve, in which fast axonal transport was interrupted by a nerve ligature, we found that numerous organelles and vesicles of various sizes accumulated. B-50 was detected on vesicles of 50 nm diameter present proximally to the ligature. In part, these vesicles were double-immunolabeled by an antibody to synaptophysin, a protein which is finally targeted to synaptic vesicles. In contrast, the immunolabel to the CGRP neuropeptide was found to not be colocalized with B-50, but present, as expected, on large dense core vesicles.

Our ultrastructural results indicate that B-50 travels in neurons, in part, associated with transport vesicles from the cell body to the growth cone. The vesicles decorated by B-50 may be accumulated by inhibition of fast axonal transport. In sciatic nerve, B-50 and synaptophysin share transiently a common vesicle carrier. The size of the vesicles appears to differ in various types of neurons.

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MOLECULAR BIOLOGICAL STUDIES ON AUTOPHAGY IN YEAST, *SACCHAROMYCES CEREVISIAE*

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Yeast, *S. cerevisiae*, induces nonselective degradation of cytosolic components in the vacuole under various starvation conditions. This autophagic process is homologous to that in mammalian cells (1, 2). Signal transduction pathway of nutrient starvation for the induction of autophagy is completely unknown. Recently we obtained fifteen mutants defective in the autophagy (*apg1-apg15*) by a method based on the morphological criteria (3). They do not accumulate autophagic bodies, single-membrane structures delivering the cytosolic components to the vacuole, in the presense of protease inhibitor PMSF. Consequently in these mutants protein degradation during starvation is severely reduced. All *apg* mutants can not retain their viability under nitrogen starvation condition. But they undergo normal vegitative growth in a growth medium. Electron microscopic analysis of every *apg* mutant showed no accumulation of intermediate membrane structures, such as autophagosomes in the cytosol. Up to now ten *APG* genes were cloned by complementation of the loss of viability of *apg*, and nucleotide sequences of most of them had been determined. Interestingly all *APG analyzed* appeared to be unknown genes and showed no homology to the known genes. Among them *APG1* funtions as a protein kinase essential for induction of autophagy. Genetic and molecular biological analyses of these *APG* genes would provide new insights for the novel signal transduction for the autophagy in yeast.

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CafA & MreB, CYTOSKELETAL PROTEIN CANDIDATES OF *E. COLI*

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Cell growth and division are among the most fundamental biological phenomena and their processes are well organized topologically, temporally, and quantitatively. In eukaryotic cell, several cytoskeletal proteins have been found to function as major growth and division regulatory machinery. By contrast, the existance of prokaryotic cytoskeleton is still doubtful. Among cytoskeleton candidate proteins, CafA and MreB still lacks complete evidence. However, unusual phenotype of *cafA* overproducer and *mreB* mutant, and the amino acid sequence similarity of CafA and MreB to eukaryotic cytoskeletal proteins support the possible role of these proteins as cytoskeletal component of cell.

Overexpression of the *cafA* gene on the plasmid caused [1]inhibition of cell division and [2]formation of cytoplasmic axial filament bundles. The CafA filament bundles are strictly oriented longitudinally, run throughout the entire cell at the center of the cell with its end specifically attached to the cell pole(*ref. 1). Structure formation and degradation cycle was observed, and purified CafA protein polymerize to form large sheet.

In *mreB* mutant, processes of cell growth and division is disordered with respect to topology of the cell axis and quantity of the cell volume. Normally, *E. coli* cell is in rod shape, grows uniformly along the cell axis, and divide at the middle of the elongated cell to form unit sized daughter cell. In *mreB* mutant, cell is in varied shape, grow in diverse directions, and divide to form daughter cell in different shape and in different size. Chromosome distribution in the mutant cell is also quite strange.

CafA and MreB are very closely encoded on *E. coli* chromosome. Deduced amino acid sequences shows that CafA is similar to kinesin/ myosin and MreB is similar to actin, indicating strong candidacies of CafA and MreB for prokaryotic cytoskeletal element functioning as cell proliferation machinary, cell polarity determinant, or chromosome segregating apparatus.

*ref. 1: Okada et.al. J. Bact. **176**:917-922
CafA: Cytoplasmic axial filament A, MreB: Murein-e-B

SRC HOMOLOGY 3 (SH3) DOMAIN BINDING STUDIES OF RAT DYNAMIN ISOFORMS P.M. Okamoto, J. Taylor*, C. Seidel-Dugan* and R.B. Vallee Worcester Found. for Exp. Biol., Shrewsbury, MA, 01545; *ARIAD Pharmaceuticals, Cambridge, MA 02139

Dynamin is a 100 kDa GTPase involved in receptor-mediated endocytosis. A number of SH3 domains have been shown to bind to the proline-rich C-terminus of dynamin, consistent with a role for the protein in receptor down-regulation. Multiple isoforms of rat dynamin have been identified that are a result of alternate splicing from three different genes. These isoforms are closely related in sequence with the proline-rich C-terminal domain being the least conserved. To determine whether functional differences exist among the various isoforms, we raised polyclonal antibodies against unique peptide sequences in two isoforms of dynamin-1, and in one isoform of dynamin-2 and dynamin-3. Western analysis of the isoforms showed different levels of expression within a variety of rat tissue extracts, and no cross-reactivity was observed between the antibodies. Binding studies of dynamin in rat brain and testis cytosol with SH3-GST fusion proteins revealed that all four isoforms bound to SH3 domains, though the binding affinities appeared to differ. These results suggest that the isoforms may differentially interact with different receptor pathways in vivo. In addition, binding sites for various SH3 domains were mapped within the dynamin-1 C-terminus using expressed epitope-tagged dynamin-1 deletion mutants. These results further suggest that there are distinct binding sites within dynamin for different SH3 domains. Supported by NIH grant GM26701.

GLYCOSYLATION SPECIFIC CHAPERONE BINDING IN THE LUMEN OF THE ER

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Calnexin is an integral membrane protein that acts as a chaperone during glycoprotein folding in the endoplasmic reticulum. Cross-linking studies were carried out with the aim of investigating the interactions of calnexin with glycoproteins *in vitro*. A truncated version of an integral membrane protein, the glucose transporter (GT_{155}) was synthesised in a rabbit reticulocyte translation system in the presence of canine pancreatic microsomes. Following immunoprecipitation with an anti-calnexin antiserum an apparently cross-linker-independent association was seen between GT_{155} and calnexin. In addition the anti-calnexin antiserum immunoprecipitated a UV-dependent cross-linking product between GT_{155} and a protein of approximately 60kDa initially designated CAP60 (calnexin associated protein of 60kDa). This GT_{155}-CAP60 cross-link occurred via UV-activation of a reticulocyte lysate specific factor.

Both the GT_{155}-calnexin and the GT_{155}-CAP60 associations were dependent on the presence of the correctly modified oligosaccharide group on the GT_{155}, a characteristic of calnexin interactions. A GT_{155} mutant that was not glycosylated (aglycoGT_{155}) did not associate with calnexin or CAP60.

CAP60 was identified as calreticulin (a lumenal ER protein) by immunopreciptation of the cross-linked product GT_{155}-CAP60 with anti-calreticulin antiserum. Similar results were observed using a glycosylated mutant of preprolactin (a secretory protein that is normally non-glycosylated and which has no interaction with calnexin) indicating that the results with GT_{155} are not unique. Our data implies the existence of a putative glycoprotein-specific chaperone complex involving calnexin and calreticulin and interacting with newly synthesised proteins in the lumen of the ER.

A Mutation in the Sucrase Subunit causes an Accumulation of Sucrase-Isomaltase in the Golgi Apparatus.

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In the case of congenital Sucrase-Isomaltase deficiency (CSID) we describe here, SI accumulates as a mannose-rich precursor on one side of the Golgi apparatus and is in contrast to control brush border proteins not transported to the brush border membrane. We isolated the SI cDNA out of a jejunal biopsy specimen of this patient by reverse-transcriptase-PCR technology and identified a mutation in a region of the sucrase subunit. This region is highly conserved between sucrase and isomaltase from different species and several other structurally and functionally related proteins. Normal and mutant hSI cDNA were analyzed by transfection of COS-1 cells. Normal hSI appeared as an enzymatically active complex-glycosylated precursor on the plasma membrane of COS-1 cells similar to the wildtype SI in intestinal cells. By contrast, the CSID mutant persisted as a high-mannose precursor comparable to its counterpart in the biopsy specimen of the patient. No enzym activity could be identified. The introduction of a comparable mutation in the structurally related lysosomal acid α-glucosidase also lead to an accumulation of mutant proteins in the Golgi apparatus in stead of transport to the lysosomes.

We show here that a mutation in a highly conserved region is responsible for the transport incompetence of sucrase-isomaltase in a patient with this type of CSID. The transferability of the Golgi block to lysosomal α-glucosidase provides us with two candidate proteins for studying intra-Golgi transport mechanisms or for the identification of possible Golgi retention motifs.

ENDOCYTOSIS IN *DICTYOSTELIUM DISCOIDEUM*: THE ROLE OF ACIDOSOMES IN RECYCLING OF THE INTERNALIZED RECEPTORS

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In *D. discoideum*, buoyant membrane fraction called 'acidosomes' contained ~90% of the vacuolar proton pump ($V\text{-}H^+$-ATPase) activity. Further examination of the putative function(s) of acidosomes suggests that it is the site where internalized receptors for cyclic AMP are localized. In addition, the acidosomes also contain an abundance of Rab4 protein (Bush et al. 1994), a marker for an early endosome, suggesting that the acidosome is an equivalent of an early endosome in higher eukaryotes. Here, we describe and discuss the process of endocytosis in this organism and evaluate the possible function(s) of acidosomes. When cells were fed colloidal iron-dextran particles, and run through an electromagnet column in the presence of 1.5 mM $MgCl_2$, a good fraction (50%) of acidosomes were retained on the column along with the lysosomes. The acidosomes contained sparse colloidal iron. The iron particles per acidosome were much fewer than that observed in the lysosomes. It was observed that the multivesicular bodies were associated with the acidosomes as if they were budding out of the acidosome reticulum. The acidosomes exist as a tubulo-vesicular reticulum *in vivo*. *In vitro*, they maintain such structure only in the presence of Mg^{2+}. In the absence of Mg^{2+}, the acidosomes fragment into smaller round vesicles. Collectively, these findings suggest that the structure earlier defined biochemically and morphologically as acidosomes represents an early and/or recycling endosomes in this protist. It is inferred that an incoming cargo and the internalized receptors enter preexisting compartment, characterized earlier as the acidosomes. The receptors may then recycle back to the plasma membranes and the pinocytic cargo follow the subsequent route through the late endosomes and lysosomes by a process of maturation.

TOWARDS UNDERSTANDING NUCLEAR PORE COMPLEX STRUCTURE AND FUNCTION AT MOLECULAR DETAIL

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Bidirectional molecular trafficking between the nucleus and the cytoplasm of eukaryotic cells occurs through the nuclear pore complexes (NPCs), large (~120 megadalton) macromolecular assemblies embedded in the double-membraned nuclear envelope (NE). Extensive structural studies from our group and others (reviewed by Panté and Aebi, 1993; 1994a) have revealed that the NPC consists of a 52-MDa basic framework - made of eight multi-domain spokes embracing a central pore which is 'plugged' with a central channel complex - sandwiched between a nuclear and a cytoplasmic ring to both of which distinct filamentous assemblies are attached: the cytoplasmic ring is decorated with eight short fibrils, whereas the nuclear ring is topped with a basket-like assembly made of eight thin filaments joined distally by a terminal ring. In an effort to establish the chemical composition of the various NPC components an increasing number of NPC proteins has now been identified, caracterized and cloned and sequenced (reviewed by Panté and Aebi, 1994b). However they represent only about 15% of the entire NPC mass, thus the chemical composition of the NPC is far from being complete. Moreover, there remain a number of questions concerning the conformation, chemical composition and functional role(s) of the different components of the NPC. We have been combining high resolution conventional EM with immuno-labeling protocols to determine the protein chemical composition of distinct NPC components. In doing so, we have now identified epitopes of five different NPC proteins (i.e., p62, NUP153, p250/NUP214/CAN, p265/Tpr, RanBP2) within the 3-D architecture of the NPC. With the exception of p62, which appears to be associated with a component of the central channel complex, these proteins are constituents of the peripheral components of the NPC: NUP153 is a constituent of the nuclear baskets with epitopes at its terminal ring, whereas p250/NUP214/CAN, p265/Tpr and RanBP2 are constituents of the cytoplasmic fibrils. To determine the functional role of distinct NPC components, we are (i) exploring the effect of conditions that mediated or interfere with nucleocytoplasmic transport on the structure of the NPC, and (ii) entertaining the hypothesis that the cytoplasmic fibrils and nuclear baskets are the initial docking sites for ligands involved in nuclear import and export, respectively.

Antibodies for this study have been provided by Drs. B. Burke (University of Calgary, Canada), L. Gerace (Scripps Research Institute, La Jolla), and T. Nishimoto (Kyushu University, Japan).

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3-D STRUCTURE OF THE TRANS-GOLGI NETWORK. K. E. Howell*, M.S. Ladinsky, J.R. Kremer, J.R. McIntosh. University of Colorado, C&S Biology, School of Medicine, Denver, CO* and MCDB, Boulder, CO

Earlier studies by Rambourg and colleagues described the Golgi ribbon and the 3-D structure of the three cisternal subcompartments: cis, medial and trans (Rambourg et al., Am. J. Anat. 154: 455-476, 1979). However, their studies did not clearly define the TGN because its 3-D structure is far less regular than that of the cisternae and varies significantly throughout the Golgi ribbon. Our high voltage electron microscopic and computer axial tomographic data on the TGN in NRK cells confirms that the TGN is abundant in some regions and absent in other regions of the Golgi ribbon. We have made 8 reconstructions from regions of the Golgi ribbon with a pronounced TGN which together provide a consistent model (Ladinsky et al., J. Cell Biol. 127: 29-38, 1994).

Our data have profound implications on the mechanisms of sorting and vesicle formation in the TGN. (a) Regions of the Golgi ribbon with pronounced TGN contain multiple cisternae that can be defined as trans. All such trans-cisternae "peel off" from the well-aligned cis-medial cisternae and are continuous with tubules that form the TGN. (b) The TGN is predominantly tubular; most vesicular profiles visualized in a single slice from a TGN tomogram were identified by 3-D reconstruction to be buds emerging from TGN tubules. There is little, if any, anastomosis between TGN tubules. (c) There are two morphologically distinct coat structures on vesicles emanating from TGN tubules: clathrin and a novel, lace-like structure. Individual TGN tubules produce vesicles with only one coat morphology. There are two major populations of vesicles that bud from the TGN in constitutively secreting, nonpolarized cells such as the NRK. One, which is clathrin coated, carries newly synthesized lysosomal proteins via the mannose-6-phosphate receptor to the endocytic-lysosomal pathway. The second carries newly synthesized molecules to the plasma membrane; a distinct coat-structure has not been identified on these vesicles. We and others (Pepperkok et al., Cell. 74: 71-82, 1993) have shown that neither clathrin nor coatomer are required for budding of exocytic vesicles from the TGN. Our hypothesis is that the vesicles bearing the lace-like coat are the exocytic vesicles. (d) Vesicle formation appears to occur synchronously along the length of a tubule. Together the data suggest the following hypotheses: (1) the process of sorting and budding are spatially separated, (2) distinct factors are required for the sorting, budding and scission processes and (3) that the TGN tubules are consumed during the budding process and are subsequently reformed.

IN VITRO GENERATION OF POST GOLGI VESICLES CONTAINING VIRAL GLYCOPROTEINS AND THEIR TRANSPORT TO ISOLATED PLASMA MEMBRANES.

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We have undertaken a comprehensive study of the sorting and segregation into post Golgi vesicles of viral glycoproteins (VSV-G and Influenza HA) destined to the opposite cell surface domains of polarized MDCK cells. There are three different components to this work. The first involves an *in vitro* system that utilizes Golgi fractions from pulse-labeled virus-infected cells in which the viral glycoproteins had been accumulated in the TGN. In this system, vesicle generation, monitored by release of the labeled glycoproteins from the sedimentable Golgi, is temperature and ATP-dependent and requires a supply of cytosolic proteins, including an NEM-sensitive factor distinct from NSF, as well as the activity of a protein kinase C-like enzyme. During the incubation of a Golgi fraction from doubly infected cells, the two viral glycoproteins were segregated into different post Golgi vesicle populations. Although, when supported by cytosol, vesicle release was totally ATP-dependent, it was possible to obtain by chromatographic procedures a cytosolic protein subfraction containing a high molecular weight (800-1000 kDa) protein complex that was depleted of known coat components but supported the release of the glycoproteins in the absence of ATP.

We have also reconstituted *in vitro* the transport of glycoproteins from an isolated Golgi fraction or post Golgi vesicles to dextran bead-immobilized basolateral plasma membranes. Because in this system vesicles that have docked but not yet fused with the acceptor membranes could be removed from them by high salt treatment, it could be determined that docking still takes place at 20°C, whereas fusion requires incubation at 37°C. Mild proteolysis or salt extraction of the acceptor membranes abolished the transport, indicating that peripheral proteins on the cytoplasmic face of the membranes play a role in the docking or fusion of the transporting elements. Isolated post Golgi vesicles obtained from the cytosol and ATP-dependent system did not require additional ATP to deliver their content to the plasma membrane.

Two rab proteins, rab8 and rab11, have been implicated in transport from the TGN to the basolateral and apical membranes of polarized cells, respectively. To understand their role in sorting or transport we have sought to identify other proteins (rabIPs) that specifically interact with these rabs and may serve as effectors or regulators of their function. We have identified and cloned a rab8 interacting protein that recognizes the GTP containing, but not the GDP form of rab8, and has ser/thr protein kinase activity. We have also purified a rab11 interacting protein that not only requires a GTP-charged rab11 but only binds to it if the latter has an intact effector domain. We are currently engaged in studies directed towards elucidating the role of the rabs and their interacting proteins in sorting and/or protein transport.

BUDDING OF AN ENVELOPED VIRUS IN THE GOLGI COMPLEX: ROLE OF THE SPIKE PROTEINS.

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Enveloped viruses aquire a lipoprotein coat by budding through one of the cellular membranes. The lipids are derived from the cellular membrane, while host proteins are excluded and replaced by the viral membrane proteins. Many viruses bud at the plasma membrane, whereas others mature at internal membranes, such as the ER, the 'intermediate compartment', the Golgi complex (GC), or the inner nuclear membrane. What determines the site of budding is not fully understood, although it is thought that the accumulation of spike proteins in the budding compartment (BC) plays a key role. Viral membrane proteins usually form oligomers in the ER and are then targetted to the BC, where they become retained. Thus, these spike complexes must contain retention signals for localization to the BC. Are these signals of the same kind as those used by resident cellular proteins?

It is well documented that members of the *Bunyaviridae* family acquire their envelope in the GC. We are using one particular bunyavirus, Uukuniemi (UUK) virus, as a model to study the mechanism underlaying budding into the cisternae of the GC. The spike complex of bunyaviruses consists of two glycoproteins, G1 and G2, formed from a precursor by cotranslational cleavage in the ER. G1 folds rapidly and G2 slowly, and only properly folded monomers appear to be able to enter into heterodimeric complexes. G1-G2 heterodimers are then slowly transported to the GC, where they become arrested. By expressing G1 and G2 separately, we have found that G1 contains a signal for Golgi localization. It is likely that G2 becomes retained in the GC through its association with G1. To map the retention signal, various chimeric proteins have been constructed between different domains of G1, VSV G, lysozyme, or CD4. These results indicate that neither the ectodomain nor the transmembrane domain of G1 are essential for Golgi localization. Instead, the cytoplasmic tail of G1 is both necessary and sufficient for Golgi retention. Although not sufficient on its own, the transmembrane domain (19 aa) seems to enhance Golgi-retention. Within the tail (98 aa), the retention signal has been mapped to the membrane-proximal domain. The fine-mapping of this region and the importance of the length of the transmembrane domain are currently being analyzed.

Future work will focus on the mechanism by which the G1-tail is able to retain G1/G2 in the Golgi complex.

A NOVEL Ca^{2+}-REGULATED ACTIN- AND TROPOMYOSIN-BINDING PROTEIN (CAPS) IS REQUIRED FOR A LATE STEP IN REGULATED DENSE CORE GRANULE EXOCYTOSIS.

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Ca^{2+}-regulated, botulinum neurotoxin-sensitive neurotransmitter secretion via large dense core vesicle (LDCV) exocytosis is reconstituted in semi-intact PC12 cells by 10 μM Ca^{2+}, 2 mM MgATP and cytosolic proteins. Exocytosis proceeds through sequential ATP-dependent priming and Ca^{2+}-dependent triggering steps that require distinct cytosolic proteins. Ca^{2+}-dependent secretion in ATP-primed semi-intact cells requires a neuroendocrine-specific 145 kD cytosolic protein termed CAPS (for Ca^{2+}-dependent activator protein for secretion), which is a necessary and sufficient soluble factor for Ca^{2+}-triggered LDCV fusion. To clarify the role of CAPS, a rat brain CAPS cDNA was isolated, which contained an ORF coding for a novel 146.3 kD protein. The cDNA corresponded to a 5.6 kb mRNA expressed exclusively in neuroendocrine secretory tissues (brain, pituitary, endocrine pancreas and adrenal). Since aminoterminal CAPS protein domains of ~30 and ~100 amino acids exhibited significant similarity to the actin-binding proteins troponin T (80% similarity) and tropomyosin (71% similarity), respectively, F-actin cosedimentation studies were conducted that demonstrated CAPS to be an F-actin binding protein that exhibits an apparent K_D of ~0.3 μM for actin and a binding stoichiometry of 1 mol CAPS per 7 mol actin. Additional *in vitro* binding studies revealed an interaction between CAPS and tropomyosin that is Ca^{2+}-stimulated. CAPS fusion protein fragments containing the aminoterminal 30 amino acid troponin T-like domain interacted with tropomyosin. Peptides containing sequences within the aminoterminal troponin T- and tropomyosin-like domains inhibited Ca^{2+}-activated neurotransmitter secretion from semi-intact PC12 cells. We conclude that a late ATP-independent step in Ca^{2+}-triggered LDCV fusion involves cytoskeletal protein interactions that require CAPS. CAPS may function by exerting Ca^{2+}-dependent regulation on tropomyosin.

BIOGENESIS OF SYNAPTIC VESICLES.

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The synaptic vesicle has a unique size and has unique membrane components. These properties simplify the task of identifying what causes some membrane proteins to be targeted to synaptic vesicles and others to be excluded. We have shown that markers of recycling endosomal, transcytotic, and surface modifying vesicles are excluded from synaptic vesicles. To determine what is targeted to them we have performed extensive mutagenesis on one synaptic vesicle protein, synaptobrevin or VAMP. Mutations that affect subcellular distribution fall into the domain of VAMP conserved in the non-neural homologues that are also involved in plasma membrane targeting. Mutations can be subdivided into those that allow endocytosis but no synaptic vesicle targeting, those that give cell surface retention and those that enhance dramatically synaptic vesicle targeting. The latter have been used to reconstitute synaptic vesicle biogenesis from extracts of PC12 cells. Brain cytosol is essential for vesicle biogenesis, as is GTP hydrolysis.

In a separate series of experiments we have evidence that the mammalian protein galectin is secreted from yeast by a novel, non-classical mechanism. Secretion is not reduced by inactivating genes in the classical export pathway, such as sec18, or the A-factor translocation mechanism (ste6).

THE LAST SECONDS IN THE LIFE OF A SECRETORY VESICLE. J.M.Coorssen, T.D. Parsons, T. Horstmann, W. Almers (Dept. Molecular Cell Research, Max Planck Inst. f. Medizinische Forschung, Heidelberg, Germany) and P. Thomas (Dept. Human Physiology, Univ. of California, Davis, CA).

In neurons and neuroendocrine cells, exocytosis is triggered by an increase in cytosolic [Ca]. We have investigated the last stations on the secretory pathway in single pituitary and chromaffin cells.

(A) While sustained exocytosis in these cells requires Mg-ATP, each cell keeps a store of vesicles (about 3000 in a melanotroph, 710 in a chromaffin cell) that can be triggered to exocytosis also when [Mg] < 100 nM and ATP is replaced by nonhydrolyzable analogs. Evidently the Ca-triggered fusion of such "primed" vesicles with the plasma membrane requires no ATP-hydrolysis. In this, they differ from transport vesicles fusing with the Golgi stack.

(B) Exocytosis was investigated in the absence of Mg-ATP. Following a step increase in [Ca], there is a brief burst of exocytosis releasing 250 vesicles in a melanotroph (100 in a chromaffin cell). This exocytic burst is followed by slower components of exocytosis. Kinetic analysis indicates at least four distinct reactions in the exocytic cascade. In melanotrophs they are, in sequence: (a) a steeply temperature sensitive step (requiring tens of seconds) followed by (b) a step that is blocked by mild acidification (cytosolic pH 6.2, lasts 1 s), (3), the binding of 3 Ca ions to their regulatory site (10 ms) and (4) exocytosis (25 ms; all times at 24 °C). Clearly, the last ATP-requiring step in the exocytic cascade is followed by at least four reaction steps. Only a small subset of primed vesicles (those participating in the exocytic burst) are completely ready for exocytosis in response to Ca.

(C) Analysis of vesicle locations in quickly frozen, unfixed cells shows 2100 dense-core secretory vesicles in melanotroph (720 in chromaffin cells) docked beneath the plasma membrane of each cell. Evidently docked vesicles, and those capable of exocytosis without Mg-ATP, are similar in number. From the size of the exocytic burst, only 10-15% of the docked vesicles are readily releasable. Clearly not all are equal. We suggest that docked vesicles must mature before they are ready for exocytosis.

(D) After Ca-triggered exocytosis, the added membrane is retrieved in about 4 s. This rapid mechanism may be arrested at a late step and requires Ca (or a consequence of raised [Ca]) to go to completion. Unlike clathrin-mediated retrieval, it is unaffected by cytosolic pH 6.2, does not require cytosolic K, and some of the vesicles thus retrieved have a diameter $> 1\ \mu m$. We suggest that it is the physiologic retrieval route, and that only membrane escaping this mechanism is retrieved in clathrin-coated vesicles.

ESSENTIAL ROLE FOR PROTEIN AND PI KINASES IN THE YEAST SECRETORY PROTEIN TRAFFIC

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Yeast *vps* (vacuolar protein sorting) mutants missort and secrete vacuolar/lysosomal hydrolases. *vps*15 and *vps*34 mutants appear to form a unique subset among the *vps* mutant collection. They share a common set of growth, morphology and protein sorting defects. The *VPS*15 gene encodes a membrane-associated protein kinase that is myristoylated at its N terminus. The *VPS*34 gene encodes a phosphatidylinositol 3-kinase (PI 3-kinase) that is homologous to the catalytic subunit (p110) of mammalian PI 3-kinase. Changes in residues of Vps15p as well as Vps34p that are conserved among protein kinases and lipid kinases, respectively, inactivate each kinase. The mutant cells are *ts* for growth and exhibit extreme defects in vacuolar protein sorting. The vacuolar hydrolase carboxypeptidase Y (CPY) is secreted by these mutant cells as the Golgi-modified precursor form. A direct association between the Vps15 and Vps34 proteins has been demonstrated using both native immunoprecipitation and chemical cross-linking experiments. This protein-protein interaction and the activation of the Vps34 PI 3-kinase requires an active Vps15 protein kinase. Vps15 protein kinase activity may itself be regulated by direct interaction with transmembrane receptors, such as the CPY-specific sorting receptor coded for by the *VPS*10 gene. In the late Golgi, ligand (CPY) binding to the sorting receptor (Vps10p) appears to result in the sequential activation of the Vps15 and Vps34 kinases. The Vps10, Vps15 and Vps34 proteins effectively act as components of a novel signal transduction complex which converts the signal received by the Vps10 sorting receptor into a second messenger (PI 3-phosphate) that then triggers the action of as yet unknown effector proteins (e.g., vesicle coat proteins) which are required for initiating vesicle-mediated transport of vacuolar hydrolases.

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PROTEIN TARGETING TO THE YEAST ENDOPLASMIC RETICULUM MEMBRANE.

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Protein translocation across the membrane of the endoplasmic reticulum (ER) in the yeast *Saccharomyces cerevisiae* can occur by two partially redundant pathways. One pathway targets proteins co-translationally to the ER membrane and is mediated by the signal recognition particle (SRP) and its receptor, while the other pathway targets proteins post-translationally and is SRP and SRP receptor-independent. We have isolated novel mutant alleles of the ER membrane proteins Sec62p and Sec63p that specifically block *in vivo* and *in vitro* the SRP-independent post-translational translocation pathway. Mutant cells are viable, as are cells that lack SRP or SRP receptor; the combination of mutations from both pathways, however, leads to cell death. Targeting of different proteins into either one of the two pathways is determined by their signal sequences. We entertain a model where a common translocation apparatus, probably composed of the Sec61p complex, receives input from both targeting pathways, in one case via SRP and SRP receptor and in the other via the Sec62,63p complex. Association with such "accessory functions" would allow the translocation apparatus to receive translocation substrates either co- or post-translationally.

During these studies, we observed that yeast cells in which the genes encoding either one of the two subunits of the SRP receptor are deleted are not complemented when the corresponding wildtype genes are reintroduced into these cells. Wildtype levels of SRP receptor are made from the reintroduced genes, and subunits form a complex with one another, but remain inactive. This unusual phenotype, which we call lack of SRP receptor function, [srf$^-$], behaves as a stable, recessive, non-Mendelian trait. One exciting interpretation of these results is that the SRP receptor requires itself for its biogenesis, and that in cells in which no active, parental SRP receptor is present, no new active SRP receptor is formed. We propose that this mechanism helps to maintain the spatial and temporal continuity of the ER by assuring that new ER membrane will be formed only from existing ER membrane.

COMPONENTS AND MECHANISMS INVOLVED IN PROTEIN TRANSLOCATION ACROSS THE ENDOPLASMIC RETICULUM MEMBRANE

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In all eukaryotic cells, protein translocation across the endoplasmic reticulum (ER) membrane can occur co- or posttranslationally. The cotranslational mode, prevalent in mammals, is initiated by the targeting of a ribosome- bound nascent polypeptide chain to the ER membrane, mediated by the signal recognition particle (SRP) and its membrane receptor. The nascent chain is subsequently transported across the membrane, through a protein- conducting channel, most likely formed from the heterotrimeric Sec61p- complex. During this translocation process, the Sec61p- complex is tightly associated with the membrane- bound ribosome. The heterotrimeric Sec61p- complex, together with the SRP- receptor, is sufficient for the translocation of some polypeptides into reconstituted proteoliposomes, but most polypeptides also require the translocating chain-associating membrane (TRAM) protein (1).

We have now reproduced the posttranslational mode of protein translocation in yeast using reconstituted proteoliposomes which contain a purified complex of seven yeast proteins (2). This Sec-complex includes a heterotrimeric Sec61p- complex, homologous to that in mammals, as well as all other membrane proteins found in genetic screens for translocation components. Efficient posttranslational translocation also requires the addition of lumenal Kar2p (BiP) and ATP. The trimeric Sec61p- complex also exists as a separate entity in yeast that, in contrast to the large Sec-complex, is associated with membrane- bound ribosomes. We therefore hypothesize that distinct membrane protein complexes function in co- and posttranslational translocation pathways.

In mammals, a class of membrane proteins, called "tail anchored", is also inserted into the membrane posttranslationally. One example of this class, synaptobrevin, is shown to be inserted into the ER membrane posttranslationally in a process that requires ER protein(s) different from the translocation components needed for proteins with signal sequences, thus suggesting a novel mechanism of insertion (3).

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FUNCTION AND REGULATION OF BIP DURING PROTEIN FOLDING AND ASSEMBLY IN THE ENDOPLASMIC RETICULUM

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BiP, the endoplasmic reticulum (ER)-located member of the hsp70 family of molecular chaperones, is required both for translocation of nascent proteins across the ER membrane and for their subsequent folding and assembly in the ER lumen. Peptides that bind to BiP display extensive sequence diversity, consistent with the observed "promiscuity" of BiP's interaction with nascent secretory proteins, and usually exhibit marked hydrophobicity, consistent with the likelihood that BiP interacts with sequences normally located in the interior of a fully folded protein. Analysis of a large number of BiP-binding peptides revealed a heptameric binding motif containing bulky aromatic or hydrophobic residues in an alternating pattern compatible with peptides being bound to the chaperone in an extended conformation. Comparison of the peptide binding specificity of BiP with that of DnaK and hsc70 indicates that the specificity divergence is determined by the differing extents to which their peptide binding pockets tolerate polar and charged residues. We have developed a computer program that predicts BiP-binding sites in natural proteins. Analyses of the positions of BiP-binding sites in proteins of known structure indicate that in the native protein, these sites are located either in the interior of a folded domain or at the interface between subunits.

Transcription of the BiP gene is increased many fold when mutant or unfolded proteins accumulate within the ER. This unfolded protein response appears to be initiated by the decrease in the concentration of free BiP that occurs when complexes are formed between BiP and unfolded proteins. Thus a novel intracellular sensing system must exist that monitors events in the lumen of the ER and transduces signals across the ER membrane and to the nucleus. A genetic screen designed to isolate yeast mutants that are unable to activate the BiP (*KAR2*) gene when unfolded proteins are present in the ER identified a gene (*ERN1*) encoding a 1115 amino acid integral membrane protein with a glycosylated N-terminal domain in the lumen of the ER and a cytosolic C-terminal domain that contains a region homologous to the catalytic domains of serine/threonine-specific protein kinases. Additional genes encoding putative components of the signalling pathway are currently being characterized.

PATHWAYS OF CHAPERONE-MEDIATED PROTEIN FOLDING,
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Protein folding in the cell depends on helper proteins, so-called molecular chaperones and folding catalysts. Of specific interest are the functions of members of the hsp70 and hsp60 (chaperonin) families of molecular chaperones in *de novo* protein folding. Hsp70 and hsp60 can act sequentially in this process, determined by their differential specificity for structural elements exposed by a polypeptide chain at different stages of the folding pathway. Hsp70 recognizes extended polypeptide segments emerging from ribosomes. The fully synthesized polypeptides, adopting the conformation of partially folded intermediates, may subsequently be transferred to the central cavity of the chaperonin double-toroid for folding to the native state. In the eukaryotic cytosol, polypeptide transfer from hsp70 to the chaperonin TRiC can occur co-translationally, probably reflecting a coordinated process of translation and domain-wise folding. The function of hsp70 in this process is regulated by DnaJ homologs, such as hsp40, and by further components that interact with the ATPase domain of hsp70. The yeast two-hybrid system has been employed to identify such proteins.

The mechanism by which chaperonin ring complexes mediate protein folding can be studied *in vitro*, using GroEL and GroES of*E. coli*: GroEL is known to be dependent on the single-ring co-factor GroES. Under most conditions, GroES binds to one end of the GroEL cylinder, forming an asymmetrical GroEL:GroES complex. Stimulated by the recent observation of symmetrical GroES:GroEL:GroES complexes, we have reinvestigated the stoichiometry of GroEL and GroES in the functional chaperonin complex. Our kinetic analysis indicated that formation of symmetrical GroES:GroEL:GroES particles is not required for the normal function of the chaperonin reaction cycle. Moreover, biochemical binding assays and structural analysis by electron microscopy (collaboration with A. Engel, Basel, and W. Baumeister, Munich) revealed that the symmetrical complex only assembles at high concentrations of Mg^{2+} and at elevated pH. We conclude that, in agreement with previous observations, the asymmetrical GroEL:GroES complex is sufficient to mediate the process of chaperonin-mediated protein folding.

STRUCTURE AND FUNCTIONAL ANALYSIS OF THE CHAPERONIN GROEL. A.L. Horwich, W.A. Fenton, J.S. Weissman, D.C. Boisvert, K. Braig, P.B. Sigler. Depts. of Genetics and Molecular Biophysics and Biochemistry and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven CT.

Chaperonins are ring-shaped oligomeric protein assemblies that provide essential assistance in vivo in folding many newly-translated and translocated proteins. Recent structural and functional studies of the bacterial chaperonin GroEL suggest that it binds non-native substrate proteins in a central channel ~45 Å in diameter via flexible hydrophobic surface of its apical domains. ATP binding/hydrolysis in the equatorial domains promotes allosteric changes in apical conformation that direct release of substrate proteins in non-native forms that are kinetically partitioned in the bulk solution between folding to native form or rebinding to other chaperonin molecules. For many proteins productive release also requires the action of the cochaperone GroES, that binds to the apical domains through residues that include those involved in peptide binding. We are attempting to further understand the cycle of peptide binding and release through: structural studies of binary complexes; biochemical studies addressing the physical arrangement of GroES and polypeptide; and functional studies of single ring versions of chaperonins.

THE FUNCTIONALLY DISTINCT CYTOSOLIC HSP70s OF YEAST, THE *SSA* and *SSB* PROTEINS.

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SSA and *SSB* proteins represent two classes of abundant cytocolic hsp70s of the yeast *Saccharomyces cerevisiae*. The essential *SSA* subfamily is encoded by 4 genes, *SSA1-4*. The *SSA* proteins have been shown to be involved in protein translocation into organelles and the regulation of the heat shock response. The *SSB* class is encoded by the *SSB1* and *SSB2* genes. Disruption of both *SSB1* and *SSB2* results in sensitivity to certain protein synthesis inhibitors and slower than wild-type growth rates at all temperatures but particularly at lower temperatures. These phenotypes and the association of the majority of *SSB* protein with translating ribosomes suggest a role of these proteins in the process of translation. The *SSA* and *SSB* families are functionally distinct: Overexpression of an *SSA* protein fails to rescue the phenotype of an *SSB* protein, and overexpression of an *SSB* protein fails to rescue an *SSA*-deficient strain.

To determine regions critical for the specific functions carried out by the *SSA* and *SSB* proteins we constructed chimeric *SSA/SSB* genes. Using PCR restriction endonuclease cleavage sites were created at identical sites in the *SSA1* and *SSB1* genes separating the two lobes of the ATPase domain and the peptide-binding domain. None of the chimeras were able to support the growth of an *ssa1 ssa2 ssa4* strain. However, rescue of the phenotypes of cold-sensitivity and hygromycin- sensitivity of *ssb1 ssb2* was observed with some chimeras. For example, a chimera containing the N-terminal portion of the ATPase domain of *SSA1* protein, but the remainder of *SSB1* protein was able to completely rescue the sensitivity to hygromycin and partially rescue the cold-sensitivity of *ssb1 ssb2* cells. A chimera containing only the peptide binding domain of *SSB* protein was able to rescue the hygromycin sensitivity, but not the cold-sensitivity. This initial analysis of chimeras indicates that certain regions of the *SSB* proteins are required for specific functions. Analysis of additional chimeras and the biochemical properties of *SSA* and *SSB* proteins which is in progress should provide information as to the differences amongst related members of the hsp70 family.

HSP100: UNIQUE FUNCTIONS IN STRESS TOLERANCE AND PRION MAINTENANCE

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When cells of all types are exposed to stress – mildly elevated temperatures, ethanol, anoxia, heavy metal ions, etc. – they respond with the vigorous induction of a small number of proteins known as the heat-shock proteins (HSPs). In the yeast *Saccharomyces cerevisiae* one of these proteins, Hsp104, plays a particularly important role in helping cells survive extremes. It is not required for growth at any temperature but is vital for survival during short exposures to very high temperatures or to high concentrations of ethanol. Cells carrying mutations in *HSP104* can be rescued at high temperatures by a related gene isolated from the vascular plant, *Arabadopsis thaliana* , demonstrating the conservation of Hsp100 functions in evolution. Three different lines of analysis (electron microscopy of heat-shocked cells, analysis or a temperature sensitive reporter enzyme, and reconstitution of temperature-sensitive RNA splicing) indicate that Hsp100 proteins have a novel function, rescuing proteins that have been damaged and driven into aggregates by high temperatures. Most recently, we have found that hsp104 plays a crucial role in the propagation of prion-like phenotypes in yeast. In particular, the levels of Hsp104 in the cell determine the phenotypic state of the translational fidelity factor known as [psi]. We are currently attempting to define the mechanism by which Hsp104 acts in molecular terms.

THE N-END RULE

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The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue [1-3]. Similar but distinct N-end rules operate in all organisms examined, from bacteria to mammals. A short-lived protein targeted by the N-end rule pathway bears a specific degradation signal, the N-degron. In eukaryotes, the N-degron comprises two determinants: a destabilizing N-terminal residue and a specific lysine (or lysines) of a substrate. The lysine residue is the site of attachment of a multiubiquitin chain. I will discuss recent advances in the understanding of mechanics and functions of the N-end rule.

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INVOLVEMENT OF MOLECULAR CHAPERONES IN THE SELECTIVE DEGRADATION OF ABNORMAL PROTEINS

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In addition to functioning in protein folding and translocation, molecular chaperones are essential for the rapid degradation of certain abnormal proteins. We propose that the chaperones attempt to refold the aberrant polypeptide, and if unsuccessful, they can target it for rapid degradation. In E. coli, members of the hsp70 and hsp60 families function in concert with the ATP-dependent proteases La (lon) and Clp (Ti) in the selective elimination of abnormal polypeptides. For example, the short-lived mutant protein, phoA6l, requires dnaK, dnaJ, and grpE for its hydrolysis by La, and phoA61 is found in large complexes containing these hsps and La prior to degradation. By contrast, the degradation of the fusion-protein, CRAG, by clpP requires the formation of complexes of CRAG with GroEL and a 50K polypeptide identified as Trigger Factor, which seems to be rate-limiting for proteolysis. The GroES-dependent release of CRAG in an unfolded state from complex with GroEL and Trigger Factor appears to allow proteolytic attack.

Chaperones are also necessary for the degradation of certain abnormal proteins by the ubiquitin-proteasome pathway in the yeast cytosol. Mutants lacking certain hsp70s (SSA1, 2) and TS-mutants of the dnaJ homologs, Ydj and Sis, reduce the degradation of analog-containing proteins and short-lived normal proteins without affecting degradation of long-lived proteins. Ydj and Sis are necessary for ubiquitin-dependent degradation of certain fusion proteins, e.g. Ub-Pro-β-gal, but not for the hydrolysis of the N-end rule substrates, Arg-β-gal. Ydj is necessary for the ubiquitin conjugation to Ub-Pro-β-gal, with which it associates, while Sis promotes the hydrolysis of the ubiquitinated proteins by the 26S proteasome.

PHYSIOLOGICAL ROLES AND REGULATION OF THE UBIQUITIN-MEDIATED PROTEOLYTIC SYSTEM

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Degradation of a protein via the ubiquitin system involves two steps. Initially, multiple molecules of ubiquitin are covalently linked to the substrate. The targeted protein is then degraded by a specific 26S proteasome complex, and free and reutilizable ubiquitin is released. Ubiquitin-mediated degradation of cellular proteins plays important roles in a variety of cellular processes. Among these are programmed degradation of cyclins, degradation of oncoproteins, tumor suppressors and transcriptional activators, regulation of cell surface receptors and the secretory pathway, involvement in long-term memory, presentation of antigens via Class I MHC molecules, involvement in programmed cell death, and removal of abnormal and mutated proteins. Central to all these complex processes is recognition of the different protein substrates by the conjugating and proteolytic machineries. While a few proteins are targeted via their N-terminal residues, most cellular proteins are recognized by different signals that reside, most probably, downstream to the N-terminus. Furthermore, in addition to primary targeting signals, many protein substrates are recognized only following binding to a "trans" recognition protein or after post-translational modifications. Few examples demonstrate the complexity of the recognition process: (1) c-Fos is targeted for conjugation only following formation of a complex with phosphorylated c-Jun. (2) NF-κB is activated following limited processing and removal of its 65 kDa C-terminal region. The nmechanism that spares the active N-terminal domain is not known. (3) IκBα is targeted only in response to an environmental stimulus that elicits signal transduction-induced phosphorylation. (4) The degradation of a subset of proteolytic substrates depends on the presence of hsc70. The molecular chaperone probably presents a denatured substrate to the conjugating machinery following a failure to refold it to its native form.

MECHANISMS AND FUNCTIONS OF UBIQUITIN-DEPENDENT PROTEIN DEGRADATION IN THE YEAST *SACCHAROMYCES CEREVISIAE*.

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Selective protein degradation is an important component of many cellular regulatory mechanisms. Proteins that are short-lived *in vivo* include a variety of proto-oncogene products, many transcription factors, and cell cycle regulators such as cyclins. We have found that the yeast MATα2 transcriptional repressor is rapidly degraded *in vivo*, and that prior to its degradation, α2 is covalently linked to oligomers of the highly conserved polypeptide ubiquitin. Attachment to ubiquitin targets proteins for intracellular degradation by a complex protease known as the 26S proteasome. Using a MATα2 derivative as substrate, we have identified a ubiquitin-dependent degradation pathway in *S. cerevisiae*, the DOA (*d*egradation *o*f *a*lpha2) pathway. Among the gene products of the DOA pathway important for α2 turnover that have now been identified by this molecular genetic analysis are a ubiquitin-conjugating enzyme (Doa2/Ubc6), two subunits of the 20S proteasome (Doa3 and Doa5), which forms the catalytic center of the 26S protease, and a ubiquitin isopeptidase (Doa4), i.e., an enzyme that removes ubiquitin from proteins. Detailed genetic and biochemical analyses of these proteins have provided important mechanistic insights into the ubiquitin system. Interestingly, the Doa4 isopeptidase is closely related to a number of previously enigmatic proteins involved in mammalian growth control. We have demonstrated that one of these, the human *tre*-2 proto-oncogene product, is also a ubiquitin isopeptidase. This is the first component of the ubiquitin system whose alteration has been shown to lead to tumorigenic growth.

STRUCTURE AND FUNCTION OF 26S- AND 20S PROTEASOMES

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Proteasomes are high molecular weight proteinases implicated in the degradation of misfolded proteins and of short-lived regulatory protein via the ubiquitin pathway. The 20S proteasome is an ATP-independent proteinase degrading only unfolded proteins; the peptides generated fall into a narrow size range (4mers to 10mers) suggesting that a molecular ruler exists. The 20S forms the catalytic core of the ATP-dependent 26S proteasome in which several regulatory subunits associate with its termini. The 20S core complex is made of four seven-membered rings stacked together to form a barrel-shaped complex. In the proteasome from *Thermoplasma acidophilum* which has taken a pivotal role in elucidating proteasome structure and function, the α-subunits form the two outer and the ß-subunits the inner rings. Eukaryotic proteasomes are formed by 14 different subunits which are related to either the α- or ß-subunits of the *Thermoplasma acidophilum* proteasome. The active site is formed by the N-terminal Thr of the ß-subunit and located in the innermost compartment of the 20S barrel. Access to this proteolytic compartment is controlled by a narrow polypeptide channel allowing only unfolded proteins to enter. The autocatalytic activation of the ß-subunit which requires the removal of a pro-sequence is tightly linked to the assembly of the complex, thus preventing that free proteolytically active ß-subunits exist in the cytoplasm. It is proposed that the ATPases which are components of the 26S complex have a role in unfolding substrate proteins (acting as reverse chaperones), and in threading them through the polypetide channel.

THE CRYSTAL STRUCTURE OF THE 20S PROTEASOME FROM *THERMOPLASMA ACIDOPHILUM* REVEALES ITS CATALYTIC MECHANISM

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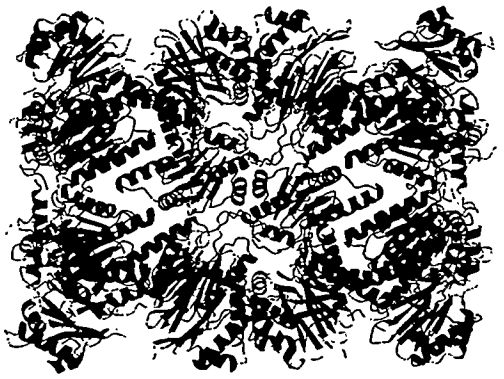
The three-dimensional structure of the proteasome from the archaebacterium *Thermoplasma acidophilum* has been elucidated by X-ray crystallographic analysis using isomorphous replacement and cyclic averaging. The atomic model was built and refined to a crystallographic R factor of 22.1% at 3.4Å resolution. The 673 kDa protease complex consists of 14 copies of two different subunits, α and β, that form a barrel-shaped structure of four stacked rings. The two inner rings consist of seven β subunits each, the two outer rings of seven α subunits each. A narrow channel controls access to the three inner compartments. The structure of the α and β subunits is similar, consisting of a core of two antiparallel β sheets flanked by α helices on both sides. The binding of a peptide aldehyde inhibitor marks the active site in the central cavity at the N-termini of the β subunits and suggests a novel proteolytic mechanism. The hydroxyl group of the β subunit N-terminal threonine acts as nucleophile and is probably activated by its own amino-group.



SELECTIVE AND ENERGY-DEPENDENT DEGRADATION BY THE LON AND CLP PROTEASES

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Most cytoplasmic proteolysis in both prokaryotes and eukaryotes is carried out by energy-dependent proteases. We have been studying the *E. coli* Lon and Clp proteases, which are representative of major classes of ATP-dependent proteases found in prokaryotes and in eukaryotic organelles. Lon is a homotetramer, whereas ClpAP contains two seven-membered rings of ClpP, encoding the serine-protease active site and six subunits of the ClpA ATPase. ClpAP is similar in structural organization to the more complex 26S eukaryotic protease. Replacing ClpA with a different ATPase subunit, ClpX, alters the substrate selectivity of ClpP, demonstrating that the substrate selectivity of ClpP is governed by the ATPase subunit.

For ClpAP, ATP is necessary both at steps at which no ATP hydrolysis is required (assembly of Clp protease and substrate recognition) and at which hydrolysis is necessary (processive degradation of proteins or release of proteins). In the presence of nucleotide, ClpA can interact with protein substrates, and, upon ATP hydrolysis in the presence of ClpP, rapid degradation occurs. In the absence of ClpP, substrates can be released from ClpA upon ATP hydrolysis. For the Pl replication protein RepA, binding and release of RepA by ClpA alone results in monomerization and activation of RepA for DNA binding, a reaction also catalyzed by the DnaJ/DnaK/GrpE chaperone system.

The specificity of substrate recognition by these classes of proteases may be complex, and is not dictated simply by cleavage specificity. Peptide bond cleavage occurs at multiple sites, with a slight preference for cutting C-terminally to hydrophobic residues. The ability of energy-dependent proteases to degrade abnormal and naturally unstable proteins probably reflects the ability of the ATPase domains to recognize and further unmask appropriate hydrophobic regions normally inaccessible in properly folded proteins.

THE ROLE OF DYNAMIN IN CLATHRIN COATED VESICLE FORMATION

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Dynamin, a 100 kD protein, was originally isolated as a microtubule-dependent GTPase (1), and was later identified as the mammalian homologue of the Drosophila *shibire* gene product (2,3). Temperature-sensitive mutations in *shibire* cause a pleiotropic block in endocytosis, a process not known to involve microtubules. To resolve this parodox, we have explored the role of dynamin both *in vivo* and *in vitro*. Using a tetracycline-regulatable promoter, we have generated stable HeLa cell lines expressing either wild-type dynamin, a mutant (G44A) defective in GTP binding and hydrolysis or a human dyn^{ts} mutant (G273D) homologous to the $shibire^{ts-1}$ allele. Phenotypic analysis of these cells established that dynamin is specifically and directly required in receptor-mediated endocytosis for the formation of constricted coated pits and for coated vesicle budding. No other membrane trafficking events were affected, including coated vesicle-mediated trafficking of Cathepsin D from the TGN to the lysosome. Consistent with this specificity of function, dynamin was exclusively localized to clathrin-coated pits on the plasma membrane, although there exists a cytosolic pool. Dynamin has been purified in high yield from baculovirus-infected Sf9 cells. We find that dynamin is a tetramer in solution at high salt, but that it spontaneously self-assembles into rings and stacks of rings upon dilution or dialysis into low salt buffers. The dimensions of these dynamin rings (20-25 nm internal diameter, 40-50 nm external diameter with 10 nm wide rims) are identical to electron dense 'collars' seen surrounding the necks of membrane invaginations that accumulate on the presynaptic membranes of *shibire* flies at the non-permissive temperature (4). We propose that dynamin self-assembles into rings at the necks of invaginated coated pits to form 'constricted coated pits' and that a concerted conformation change driven by GTP-hydrolysis drives coated vesicle budding by tightening the rings and severing the necks.

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MOLECULAR MECHANISMS IN SYNAPTIC VESICLE ENDOCYTOSIS

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A large body of information has recently been accumulated on the mechanisms involved in the docking and fusion of synaptic vesicles with the plasmalemma. In contrast, the mechanisms by which these vesicles are reformed by endocytosis and recycling remain largely unclear. Many vesicular transport steps require GTP hydrolysis and non-hydrolyzable analogs of GTP have been used in several studies to identify transport intermediates. We speculated that a similar approach may provide clues on mechanisms of synaptic vesicle endocytosis and recycling. We have found that incubation of lysed nerve terminals with cytosol, ATP and GTPγS results in the formation of tubular membrane invaginations of the plasmalemma which are surrounded by regularly spaced rings stacked onto each other to form a tight spiral. These tubules often terminate in a clathrin coated bud. Using immunogold electron microscopy we have demonstrated that dynamin is a major component of these rings. Dynamin is a GTPase which has been implicated in synaptic vesicle endocytosis, and more generally in clathrin-mediated endocytosis, by genetic studies in *Drosophila* and transfection studies in mammalian cells. Our results indicate that dynamin participates selectively in the fission reaction of endocytosis by forming a collar at the neck of invaginated coated vesicles. They suggest that GTP hydrolysis produce a conformational change (twist?, constriction?) of the dynamin ring which leads to constriction of the vesicle neck. Inhibition of this conformational change by GTPγS may stabilize the rings, lead to ring stacking and elongation of the vesicle neck to form a tubular invagination. It is likely that the function of dynamin, like that of clathrin, may be regulated by accessory proteins. We have identified two proteins which may participate with dynamin in the endocytotic process: p145 and amphiphysin. p145 is a brain protein which we identified by its property shared with dynamin to bind SH3 domains (dynamin and p145 are the two major brain SH3 binding proteins). Its distribution in brain tissue is very similar to the distribution of dynamin. Furthermore, it undergoes dephosphorylation in parallel with dynamin after nerve terminal depolarization. Amphiphysin is another brain protein closely colocalized with dynamin and p145. It contains an SH3 domain which binds the proline-rich tail of dynamin as well as p145.

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COMPARTMENTALIZED CELL SIGNALING IN CAVEOLAE

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Caveola undergo an internalization cyclic that begins with the conversion of a flat segment of membrane into a vesicle and ends when that vesicle returns to the cell surface. During this cycle, resident molecules are sequestered in the interior of the vesicle where they perform special functions. Previously we showed that the internalization step is interrupted by activators of PKC such as phorbol-12,13-dibutyrate (PDBu). Recently we found that caveolae contain a resident population of PKCα molecules that phosphorylate a 90-kDa protein during invagination. In unstimulated cells, virtually all of the detectable PKCα in plasma membranes is in caveolae. Treatment of the cells with PDBu or agents that raise the concentration of diacylglycerol (DAG) in the cell displaced PKCα from caveolae, which blocked the phosphorylation of the 90-kDa protein and membrane invagination. Caveolae also contain a protein phosphatase that dephosphorylated the 90-kDa protein once PKCα was gone or inactive. PKCα was also dissociated from caveolae and invagination was inhibited after cells were incubated in the presence of histamine. This effect was blocked by pyrilamine but not cimetidine, suggesting that histamine was acting through H_1 receptors. Therefore, the caveolae internalization cycle can be hormonally regulated by displacing PKCα from the membrane. Another molecule we found to be highly enriched in caveolae is sphingomyelin. We have now found that interleukin 1β (IL-1β) stimulation of normal human fibroblasts initiates a lipid messenger cascade in caveolae. Hormone binding first stimulated the formation of diacylglycerol (DAG) in caveolae. This was immediately followed by the conversion of a resident population of sphingomyelin to ceramide. The ceramide produced in caveolae, in turn, blocked PDGF-stimulated DNA synthesis. IL-1β stimulated DAG production at other locations within the same cell, but this DAG was not coupled to ceramide production. These results suggest that caveolae can compartmentalize ceramide production for delivery to specific sites within the cell. The identification of two different signaling pathways in caveolae is strong evidence that this organelle has a major role to play in coordinating both intercellular and intracellular communication.

THE ARF6 GTPase: MODULATING THE STRUCTURE OF THE PLASMA MEMBRANE THROUGH REARRANGEMENTS OF CORTICAL ACTIN.

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The ADP-ribosylation factor (ARF) family of GTP binding proteins has been implicated in the regulation of membrane traffic in the secretory pathway. Although the ARF1 protein has been shown to be required for the binding of coatomer complex onto Golgi membranes and thus may regulate the structure and function of the Golgi complex, the functions of the other ARF family members have not been defined. We have shown by transient transfection with epitope-tagged ARF6 proteins that ARF6 is associated with both endosomes and plasma membrane and that the overexpression of the tagged, wild type protein does not radically alter membrane structure. In contrast, the transfection of a mutant ARF6 (T27N) predicted to bind GTP poorly, results in accumulation of ARF6-labeled, endocytic structures coated with a novel coat. Conversely, cells transfected with the GTPase deficient ARF6 mutant (Q67L) show a depletion of endosomal structures and an elaboration of the plasma membrane where all of the ARF6 is now localized. Thus, the ARF6 protein appears to cycle between the endosomal (GDP-ARF6) and plasma membranes (GTP-ARF6) according to its nucleotide state.

To identify the effector function of ARF6, we have attempted to shift the GTP status of the wild type protein expressed in Hela cells to mimic the GTP-active state of the Q67L mutant. Here we report that the G protein activator aluminum fluoride (AlF) indirectly activates ARF6 and results in a redistribution of ARF6 to peripheral sites along the edge of the cell. Concomitant with the movement of ARF6, cortical actin and the actin binding protein, gelsolin, also redistribute to these peripheral sites and by 10 min of AlF treatment these sites develop into structures resembling pseudopodia. By 2 h the morphology of these AlF-treated cells resembles cells transfected with the GTPase defective ARF6 mutant (Q67L). The actin rearrangement and pseudopodia formation is only observed in cells transfected with the wild type, myristoylated ARF6 protein. The relationship between ARF6-induced actin rearrangements and membrane traffic, and the possible interactions between ARF6 and the two other GTPases implicated in actin cytoskeletal organization, Rac and Rho, will be discussed.

FORMATION OF FUNCTIONAL ENDOSOMAL TRANSPORT VESICLES IN VITRO DEPENDS ON ENDOSOMAL ßCOP

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We have previously shown that membrane transport from early to late endosomes in vivo and in vitro is mediated by transport vesicles, which we termed Endosomal Carrier Vesicles (ECVs). We also found that ECV formation from early endosomes in vivo was blocked after inhibition of the vacuolar ATPase, whereas internalization, recycling and endosome fusion properties in vitro were unaffected. We have now reconstituted ECV formation from early endosomes in vitro. The process is cytosol-, temperature- and ATP-dependent. ECVs formed in vitro are functionnal. They exclude early endosomal markers, and have acquired the capacity to undergo fusion with late endosomes in vitro. This fusion process is specific, since the donor early endosomes do not become fusogenic with late endosomes in the assay (but retain their homotypic fusion properties).

We have investigated whether coat proteins are implicated in ECV formation. We find that the process is blocked by antibodies against ßcop, a coat protein present on biosynthetic transport vesicles. Direct evidence for the presence of ßcop, or a closely related homologue, on endosomes was obtained by electron microsocopy. In addition, early endosomes containing internalized HRP, rab5 and the transferrin receptor can be immunoisolated using anti-ßcop antibodies. We find that inhibition of the vacuolar ATPase blocks ECV formation in vitro, as in vivo, and concomitantly decreases the amounts of ßcop associated to early endosomal membranes. In contrast, GTPγS and brefeldinA, two drugs known to affect biosynthetic ßcop, have no effect on endosomal ßcop. Our data show that endosomal ßcop is implicated in ECV formation from early endosomes, and that the biochemical properties of endosomal and biosynthetic forms of ßcop are distinct. Our data also suggest that coat formation is controlled by the acidic properties of early endosomes, presumably via a transmembrane sensor.

FUNCTIONAL MECHANISM OF THE SMALL GTP-ASE RAB5

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The small GTPase Rab5 regulates transport in the early endocytic pathway. This protein is associated with the plasma membrane, clathrin-coated vesicles and early endosomes, is necessary for the homotypic fusion of early endosomes *in vitro* and regulates the rate of endocytosis *in vivo* . To study its functional mechanism we have searched for regulatory factors and effctors using both biochemical and genetic approaches. We have investigated whether Rab5, which regulates the clathrin coated vesicle-mediated endocytic pathway, can directly associate with the membrane of clathrin coated vesicles (CCV) purified from bovine brain. Having reconstituted the cytosolic form of Rab5 complexed to Rab GDP Dissociation Inhibitor (GDI), we found that RabGDI can specifically deliver Rab5 to these vesicles but not Rab7, which is localized to late endosomes. Furthermore, CCV contain a heat- and trypsin-sensitive activity which stimulates the dissociation of GDP from Rab5 but not from Rab7. The activity was found to be associated with the CCV membrane but not with the components of the coat. Maximal GDP-dissociation stimulation required the presence of RabGDI, suggesting that the factor(s) responsible for the membrane association and GDP/GTP exchange of Rab5 recognize the protein complexed to RabGDI. These data indicate that the membrane receptors of Rab5 are not restricted to the donor compartment but cycle between plasma membrane and early endosomes. Since CCV are competent for acquiring Rab5 and for converting the molecule into the GTP-bound active form, we have used this membrane preparation to purify the factors responsible for these activities.

In addition, we made use of the yeast two-hybrid system to search for downstream targets of the GTP-bound active form of Rab5. For this purpose the screening was carried out with the activated mutant Rab5 Q79L. Yeast cells transformed with a human cDNA library were screened for the expression of both the auxotrophic reporter marker *His3* and the independent reporter β-Galactosidase. A clone was isolated which interacts strongly with Rab5 Q79L, weakly with wt Rab5 but interacts neither with Rab5 S34N nor with three other small GTPases, Rab4, Rab6 and Ras. The protein encoded by this cDNA clone binds specifically Rab5:GTP and is recruited onto endosomes expressing wt Rab5, Rab5 Q79L but not Rab5 S34N. Preliminary studies suggest that this protein acts as an effector specifically interacting with the GTP-bound active form of Rab5.

SELECTIVE MEMBRANE RECRUITMENT OF RAB GTPASES

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Rab GTPases represent a family of over 30 proteins that are localized to the surfaces of distinct membrane-bound organelles and function in transport vesicle docking and/or fusion. We showed previously that purified, prenylated rab9, bound to GDI-α, is recruited selectively onto a membrane fraction enriched in late endosomes; this process is accompanied by nucleotide exchange. We have used this system to address whether each rab uses a distinct machinery to associate with its cognate organelle. Purified, prenylated rab1B, rab7 and rab9 proteins were each reconstituted as stoichiometric complexes with purified GDI-α, and rab recruitment onto endosome- or ER-enriched membrane fractions was quantified. The two late endosomal proteins, rab9 and rab7, were each recruited onto endosome membranes with apparent Km values of 4-6 nM. However, while myc-tagged rab9, complexed with GDI-α, inhibited wild type rab9 recruitment with an apparent K_i of ~9nM, rab7 complexes inhibited this process much less effectively (K_i ~ 112nM). Similarly, complexes of the ER-localized rab1B protein were even less potent than rab7 complexes (K_i ~ 405 nM). Rab9 complexes inhibited rab7 recruitment with the same low efficacy as rab7 complexes inhibited rab9 recruitment. These experiments lend strong support to the notion that each rab utilizes a distinct machinery for recruitment onto a given organelle. Since rab7 and rab9 are both localized at least in large part, to late endosomes, this suggests that a single organelle may bear multiple rab recruitment machines. Experiments are in progress to identify the membrane-localized proteins responsible for rab-specific recruitment onto distinct organelles.

ANTIGEN PRESENTATION: ASSEMBLY AND TRAFFICKING OF MHC PRODUCTS.

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Antigen processing and presentation are the processes by which the mammalian cell generates complexes between the products of the Major Histocompatibility Complex (MHC) and short peptides derived from antigenic proteins. There are two classes of MHC product, each of which samples distinct intracellular compartments as the source of peptides, and each of which serves a unique category of T lymphocyte.

The generation of peptide-MHC complexes is intimately linked to assembly of these multimeric membrane glycoproteins, and their movement through the cell. MHC Class I products present peptides derived from predominantly cytosolic proteins to CD8 T cells. Peptides are generated in the cytosol, and are translocated into the ER in an ATP-dependent fashion by a dedicated peptide transporter. MHC Class II molecules combine with peptides generated in the endocytic pathway and present them to CD4 T cells. The delivery of Class II molecules to what appear to be unique endocytic structures requires the action of the invariant chain (Ii), a type-II transmembrane protein. Removal of Ii is a prerequisite for proper binding of peptides, a process in which the unusual class II product HLA-DM appears to be involved. The assembly and trafficking of MHC products will be described from the perspective of membrane glycoprotein synthesis in general, and as a process with aspects unique to the function of MHC products in immunity.

Not surprisingly, in view of the role of MHC products in warding off infections with pathogens, several viruses have evolved strategies to elude these mechanisms of antigen presentation. A molecular understanding of how viruses achieve this is relevant not only for pathology but also for understanding the events that take place in the course of normal assembly and intracellular transport of MHC products.

FUNCTIONS OF THE TRANSPORTERS ASSOCIATED WITH ANTIGEN PROCESSING (TAP)

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Peptides which associate with newly synthesized Major Histocompatibility Complex (MHC) class I molecules are translocated in an ATP-dependent fashion into the endoplasmic reticulum (ER) by the Transporter associated with Antigen Processing (TAP), which is a member of the ATP-binding cassette family of membrane transporters. TAP molecules consist of a non-covalently associated dimer of two MHC-encoded subunits, TAP.1 and TAP.2, each of which contains multiple transmembrane domains and a cytoplasmic ATP-binding domain. Using photoactivatable peptide derivatives, we have defined a TAP binding site which contains elements of both TAP.1 and TAP.2. Heterodimers of MHC class I heavy chains and β2 microglobulin physically associate with the TAP.1/TAP.2 complex prior to their association with peptides. The class I binding site is on the TAP.1 subunit. The association of MHC class I molecules with TAP induces a 10-fold increase in the affinity of peptides for the TAP binding site, which results in a more rapid rate of peptide translocation into the ER. These results argue for a highly coordinated mechanism of peptide loading into MHC class I molecules.

GENETIC STUDIES ON PROTEIN SECRETION IN *E. COLI*

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Analysis of the SecE protein: The SecE/SecY membrane protein complex of *E. coli* is essential for protein translocation. The 13.6 kD SecE has three membrane spanning segments (MSS). Deletion, replacement mutation and gene fusion analysis shows that the only essential components of SecE are a 17 amino acid stretch in the second cytoplasmic domain and the third MSS (MSS3) which follows it. The important feature of MSS3 is its ability to act as a membrane anchor for the 17 amino acid stretch as it can be replaced by another unrelated MSS. In contrast, replacement or deletion of the 17 amino acid stretch yields inviable *secE* mutants. A consensus sequence was found among a number of prokaryotic SecE's comprising 7 amino acids in the second cytoplasmic domain. This region may from an amphipathic helix. We have used oligonucleotide mutagenesis to generate single and multiple mutations of the conserved amino acids, allowing us to identify the most important residues.

The SecE/SecY complex stabilizes SecY and results in co-immunoprecipitability of the two proteins. Both deletion of the consensus sequence and replacement of MSS3 result in a weakening or elimination of this complex. Our results suggest multiple contacts between SecE and SecY, with the interactions of the 17 amino acid sequence being the most important.

Export of signal sequence-less proteins: When the signal sequence of alkaline phosphatase (AP) is deleted, only 1% of AP is exported across the membrane. However, certain mutations in the *secY* gene (*prlA*) mutations allow export of 25% of this protein. Two models for signal sequence-less AP export are: 1)recognition by the altered SecY of an internal "cryptic" signal sequence or 2)recognition of the unfolded state of AP. To test models we isolated mutants eliminating the ability of the cell to export this AP. The mutations all cause an increase in the net positive charge at the amino-terminus of AP. These findings will be discussed in terms of models for the interaction of exported proteins with the export apparatus.

E.COLI PREPROTEIN TRANSLOCASE: TOWARDS A MECHANISM OF ITS FUNCTION

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The combination of powerful genetic analyses and *in vitro* reconstitution using purified components, has led to significant progress in our understanding of procaryotic preprotein translocation. Translocation of preproteins across the cytoplasmic membrane of *E.coli* is catalyzed by preprotein translocase. Translocase is a complex membrane protein which requires acidic phospholipids for its function. It is composed of: an integral membrane domain of three polypeptides (SecY, SecE and SecG) which is conserved from *E.coli* to mammals and the SecA subunit which is peripherally associated with SecYEG and posesses preprotein-stimulated ATPase activity.

Preproteins synthesized in the cytosol reach the membrane in a non-native state, complexed with chaperones such as SecB. SecA acts as a receptor for preprotein/SecB complexes. The translocation reaction consumes energy from both of the cell's energy stores: chemical energy in the form of ATP and electrochemical energy from the transmembrane proton gradient. These energy forms are used at distinct stages of the reaction.

Recent observations have suggested how ATP energy may be harnessed to produce translocation work. At least part of the energy of ATP is used to drive profound conformational changes in the SecA subunit. As a result a substantial 30kDa domain of SecA penetrates deeper into and partially across the membrane. The binding energy of ATP is sufficient to drive domains of 20-30 aminoacyl residues of the preprotein into the membrane. We postulate that SecA transports into the membrane short segments of bound preprotein, during its own membrane insertion. The preprotein crosses the membrane through a conduit composed of at least SecY and SecA and is apparently shielded from the phospholipid bilayer. ATP hydrolysis allows the release of the preprotein from SecA and the retromovement of SecA. At this stage SecA can bind to the succeeding domain of the preprotein and engage in one more insertion cycle or exchange with the cytoplasmic SecA pool.

Further dissection of the system will require the use of translocase proteoliposomes. To this end the SecY, SecE and SecG polypeptides have been overexpressed. Significantly increased amounts of functional translocase have been obtained in inverted inner membrane vesicles and in reconsituted proteoliposomes.This paves the way for future structural and mechanistic characterization of translocase.

INTRACELLULAR TRAFFICKING AND SUBSTRATE SPECIFICITY OF STE6, THE YEAST a-FACTOR TRANSPORTER.

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The *Saccharomyces cerevisiae* mating pheromone a-factor is an isoprenylated, carboxylmethylated peptide signaling molecule that uses a "non-classical" mechanism for secretion. The export of a-factor is mediated by STE6, a membrane protein comprised of two homologous halves, each of which contains six membrane spanning segments and a nucleotide binding fold (NBF) domain. STE6 is a member of the ATP binding cassette (ABC) transporter superfamily, which also incudes the multidrug resistance protein, MDR, and the cystic fibrosis transmembrane conductance regulator, CFTR. We are using STE6 to study general questions concerning the intracellular trafficking and topology of a complex membrane protein. By examination of the metabolic stability and intracellular localization of STE6, we have shown that STE6 is metabolically unstable in a wild-type strain and is significantly stabilized in a *pep4* mutant, suggesting that STE6 degradation occurs in the vacuole. STE6 stability is also increased at non-permissive temperature in mutants defective in the secretory pathway (*sec1*, *sec6*) and in endocytosis (*end3*, *end4*). These results, together with localization studies by immunofluorescence, provide evidence that STE6 transits through the secretory pathway to the plasma membrane and is subject to rapid, constitutive endocytosis. STE6 is also stabilized in a *doa4* mutant, suggesting that the ubiquitin pathway plays a key role in STE6 trafficking and raising the question of whether ubiquitination is necessary for transit of STE6 to the vacuole or for its turnover there.

We are currently screening through a collection of *ste6* loss-of-function mutants to identify mutant forms of STE6 which exhibit abnormal localization and/or stability. We have isolated two alleles which result in ER retention of STE6, reminiscent of the ER retention phenotype observed for certain alleles of CFTR. Interestingly, unlike ER-retained forms of CFTR which are degraded, our mutant forms of STE6 are hyperstable, presumably because they are not degraded by proteases that recognize and eliminate misfolded proteins and because they never reach the vacuole. Both of these *ste6* mutations map to the C-terminal soluble NBF domain of STE6 and not to the membrane spans. We have also identified a third mutant allele of STE6 which exhibits a hyper-rapid degradation phenotype. Preliminary experiments suggest that the degradation of this latter mutant version of STE6 is *PEP4*-independent and thus is likely to involve a non-vacuolar pathway. Ultimately, the mutants described here will aid in dissecting the intracellular life cycle of STE6 and in identifying "ER quality control" components and other machinery required for the folding and integration of STE6 in the membrane.

We have also asked which features of a-factor are recognition determinants for STE6. Analysis of a large collection of a-factor substitution mutations has revealed that most changes in a-factor have little impact on export. This low apparent specificity in a-factor recognition by STE6 is reminiscent of the broad range of drug substrates transported by MDR. In contrast to the minimal contribution of particular amino acids to export, in a *ste14* mutant strain (which lacks a functional carboxlmethyltransferase) export of a-factor is completely blocked. This finding suggests that the carboxylmethyl moiety of a-factor may be a critical recognition determinant for the STE6 transporter.

PROGRAMMING OF CELL POLARITY IN BUDDING YEAST BY ENDOGENOUS AND EXOGENOUS SIGNALS

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The yeast cytoskeleton is highly polarized towards the nascent bud site. The position of this site is genetically programmed by the mating type locus: a and α cells exhibit an axial budding pattern); a/α exhibit a bipolar pattern. We have identified five genes (*BUD1 - BUD5*) necessary for proper selection of the bud site (Chant & Herskowitz, 1991; Chant et al., 1991) and have proposed that they are part of a morphogenetic hierarchy: they organize proteins necessary for cell polarity such as CDC24p, CDC42p, and BEM1p, which, in turn, organize the cytoskeleton.

Bud3p and Bud4p appear to be involved in identifying the axial budding site: *bud3* or *bud4* mutants exhibit bipolar budding. These proteins are located at the mother-bud neck and are associated with the neck fibers located there (Chant et al., 1995; Sanders, unpublished). Bud3p/Bud4p appears to participate in a cycle of protein localization: the neck fibers determine the position Bud3p/Bud4p, which then determines the position of the neck fibers for the next cell division, etc.

BUD1p, BUD2p, and BUD5p are functionally related: mutants lacking any of these products exhibit random budding. BUD1p is a GTPase; BUD2p and BUD5p are GAP and GNEP for BUD1p, respectively (Park et al., 1993; Zheng et al., 1995). We have proposed that BUD1p associates with CDC24p or other members of this group and brings it to the nascent budding site. Using fusion proteins produced in *E.coli*, we have now shown (Park, unpublished) that Bud1p-GTP associates with CDC24p, CDC42p, and BEM1p *in vitro*: Bud1p may thus facilitate nucleotide exchange for the GTPase, CDC42p, by bringing it together with its exchange protein, CDC24p.

Yeast cells reorient their cytoskeleton in response to extracellular signals produced by a mating partner. We have recently found that the FAR1 protein is necessary for this reorientation (Valtz et al., submitted). FAR1p may thus be the link (or control the link) between the cell surface and the cytoskeleton.

ESTABLISHMENT OF CELL POLARITY IN YEAST

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Bud formation in yeast occurs by polarized growth of the cell surface, which involves a polarized movement of secretory vesicles carrying new cell-surface material to the bud site and into the growing bud. This movement seems to be mediated primarily by the actin/myosin system, but other cytoskeletal elements such as the cytoplasmic microtubules and the septin-containing neck filaments also polarize before bud emergence and may play roles in modulating cell-surface growth or in the segregation of organelles along the mother-bud axis. Central questions about the establishment of cell polarity in yeast concern how the axes of polarization (bud sites) are chosen and how this choice is then communicated to the cytoskeleton. Recent work suggests that bud-site selection and polarity establishment involve a morphogenetic hierarchy of several levels.

Bud sites are selected in one of two patterns, axial and bipolar, depending on cell type. Detailed analysis of the axial pattern has shown that the new bud forms directly adjacent to the immediately preceding division site. This and other evidence suggests that the axial pattern depends on a transient signal that is deposited at the division site in one cell cycle and directs the selection of the bud site in the next cell cycle. Immunolocalization of the *BUD3* gene product (identified originally by mutations that specifically disrupt the axial pattern) shows that it behaves just as predicted for a component of the transient signal: Bud3p assembles on the neck filaments during each cell cycle and remains after division as a ring marking the division site until the next bud site begins to organize. In contrast, detailed analysis of the bipolar pattern suggests that it depends on persistent signals that are found in a ring at the division site and at the distal tip of a daughter cell as well as in a ring marking each previously used bud site on a mother cell. New bud sites can assemble at any of the marked sites. Analysis of mutants defective specifically in the bipolar pattern has identified several genes whose products may be components of these signals or involved in positioning or reading them.

The axial- and bipolar-specific spatial signals appear to be read by a set of "general site-selection functions" that are necessary for either budding pattern; these include a GTPase (Rsr1p) and the GAP (Bud2p) and GEF (Bud5p) that regulate it. Rsr1p-GTP appears to be able to interact with Cdc24p, the paradigmatic member of the set of "polarity-establishment functions", necessary for the establishment of cytoskeletal and growth polarity. At least one function of Cdc24p is the activation of Cdc42p, a GTPase also necessary for polarity establishment. A major question is now how Cdc42p-GTP communicates positional information to the cytoskeleton. Recent work has identified several proteins (including protein kinases and putative Cdc42p-GAPs) that may be involved in this communication.

MECHANISMS OF MOLECULAR SORTING IN POLARIZED AND NON-POLARIZED CELLS

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During intracellular transport, membrane proteins are selectively targeted to, or retained within, their characteristic compartments. This feature is perhaps most dramatically illustrated in the case of polarized epithelial cells, where plasma membrane components, whether on the endocytic or secretory pathways, must be directed to either of two cell surface domains. We have found that these events are governed by a surprisingly simple logic. Membrane proteins destined for the basolateral surface of MDCK cells contain a cytoplasmic domain signal that specifies basolateral sorting which co-exists with, but is dominant to, a second lumenal domain signal for apical sorting. Thus, if the basolateral signal is absent or inactivated, apical transport results. Both signals are widely distributed suggesting that they may function in all polarized and perhaps non-polarized cells and that the mechanisms of sorting are widely conserved. Moreover, the same logic applies to both endosomes and the TGN and almost certainly mus involve recognition of basolateral signals by cytoplasmic coat proteins. To identify the elements that decode these signals, we have focussed on molecular sorting in endosomes. We have reconstituted the processes of vesicle budding and receptor sorting in endosomes isolated from MDCK cells and found that the in vitro reactions have the same requirements as does sorting in vivo. These include critical roles for ARF-dependent coat proteins and heterotrimeric G proteins. Indeed, endosomes may generate two different types of coated vesicles, at least one of which contains specific ARF proteins (not ARF6) and components related or identical to subunits of the COPI coatomer complex. These components are also important for function in intact cells. Thus, sorting and transport in endosomes involves the regulated assembly of specific cytoplasmic coats.

BIOGENESIS OF A POLARIZED CELL SURFACE IN EPITHELIAL CELLS

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Vesicular carriers deliver their cargo from the TGN to the apical and basolateral plasma membranes in polarized epithelial cells. In our attempts to identify the molecular machinery involved in protein sorting, we have described a high molecular weight, CHAPS insoluble complex of exocytic TGN-derived carrier vesicles specifically including influenza virus HA but excluding VSV G protein. Using detergent insolubility as one means of purification we have identified three integral membrane proteins (VIP21-caveolin, VIP36, and VIP17) present in the CHAPS complex. These proteins are complexed together in the apical vesicles but recent results show that they are in an uncomplexed state in the basolateral vesicle. To study the involvement of NSF, SNAP, SNAREs and Rab proteins in polarized membrane transport of epithelial cells, we have used an in vitro system based on streptolysin O-permeabilized MDCK cells. In MDCK cells, transport from the trans-Golgi network to the basolateral plasma membrane is inhibited by anti-NSF antibodies and stimulated by a-SNAP. In contrast, transport from the TGN to the apical cell surface is not affected by anti-NSF antibodies or a-SNAP. Furthermore, apical transport is insensitive to Rab-GDI and tetanus and botulinum neurotoxins which inhibit basolateral transport. These results provide clear evidence that the Rab/NSF/SNAP/SNARE-mechanism operates in basolateral transport. Our present evidence is against involvement of this mechanism in apical transport. Instead we have evidence suggesting the involvement of a mechanism involving annexins, VIPs and glycolipids as a new mode of vesicular transport.

ESTABLISHMENT OF EPITHELIAL CELL SURFACE POLARITY: REGULATION OF PROTEIN SORTING AND RETENTION PATHWAYS

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Cadherin-mediated cell-cell adhesion is required for the establishment and maintenance of epithelial cell surface polarity. Induction of cell-cell adhesion by E-cadherin results in the re-organization of Na/K-ATPase distribution to the lateral membrane domain in MDCK cells and in a cell line derived from rat retinal pigmented epithelium (RPE-J+EC). Direct sorting of Na/K-ATPase from the Golgi complex to the lateral membrane domain is not required to either establish or maintain cell surface polarity: Na/K-ATPase delivered to the lateral membrane is retained through an interaction with the membrane-cytoskeleton; Na/K-ATPase delivered to the apical membrane is rapidly internalized and degraded. Assembly of the membrane-cytoskeleton at sites of cell-cell adhesion appears to be induced through direct protein interactions with the cadherin/catenin complex. Although Na/K-ATPase is not sorted directly to the lateral membrane in one clone of MDCK cells (II/J) and RPE-J+EC cells, another clone of MDCK cells (II/G) sorts ~80% of newly-synthesized Na/K-ATPase to the lateral membrane. Differences in Na/K-ATPase sorting to the basal-lateral membrane correlate inversely with sorting of a GPI-anchored protein (GP-2) and a glycosphingolipid (glucosylceramide; GluCer); GP-2 and GluCer are preferentially delivered to the apical in II/G cells, but to both apical and basal-lateral membranes in II/J cells. Inhibition of sphingolipid synthesis in II/G cells with the fungal metabolite fumonisin B1 (FB1) results in delivery of GP-2 and Na/K-ATPase to both apical and basal-lateral membranes, similar to II/J cells; addition of exogenous ceramide, to circumvent the FB1 block, restores GP-2 and Na/K-ATPase sorting to the apical and basal-lateral membranes, respectively. Note that sorting of E-cadherin is direct to the basal-lateral membrane of both II/J and II/G cells and is unaffected by FB1. These results show that the generation of complete cell surface polarity of Na/K-ATPase involves a hierarchy of sorting mechanisms in the Golgi complex and plasma membrane, and that Na/K-ATPase sorting in the Golgi complex may be regulated by exclusion from an apical pathway(s). These results also provide new insight into the regulation of sorting pathways for other apical and basal-lateral membrane proteins.

REGULATION OF PROTEIN TRAFFIC IN POLARIZED EPITHELIAL CELLS

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Polarized epithelial cells contain apical and basolateral surfaces with different compositions. As a model system to study the regulation of protein traffic to these two surfaces, we are studying the polymeric immunoglobulin receptor. This receptor is sent from the TGN to the basolateral surface and is then endocytosed and transcytosed to the apical surface. Transcytosis is regulated by multiple mechanisms, including phosphorylation of Ser664 on the receptor, Gs, cAMP, Ca^{++}, protein kinases A and C, and binding of the ligand, IgA . Ligand binding to the receptor causes rapid tyrosine phosphorylation of several proteins, including PI-PLCγ1. Subsequently, PKC is activated and IP3 is produced, leading to an increase in intracellular free Ca^{++}. Both of these signals stimulate transcytosis. Transcytosis can be divided into three steps: 1) Endocytosis and delivery to basolateral early endosomes. 2) Delivery to an apical recycling compartment, which receives membrane-bound material from both surfaces and sorts them to the correct surface. 3) Exocytosis at the apical surface. Step 1 is regulated by phosphorylation of receptor Ser726. Step 2 is regulated by phosphorylation of receptor Ser664. Step 3 is regulated by phosphorylation of Ser664, ligand binding, protein kinases A and C, cAMP, and Ca^{++}. Step 3 has been reconstituted in permeabilized cells. This step requires NSF and a SNAP-25 homologue.

MORPHOGENESIS AND SIGNALLING MEDIATED BY ACTIN BINDING PROTEINS IN POLARIZED EPITHELIAL CELLS

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Epithelial cells display distinct morphological features that are acquired during cell differentiation. This includes the biogenesis of plasma membrane domains and assembly of specialized structures such as junctional complexes and microvilli. An assymetric organization of the overall architecture leads to the functional polarized state of this cell type. We have focused our studies on components of the apical domain and tight junctions. Towards this goal, we have investigated the role of several actin binding proteins associated with the cortical actin microfilaments network of intestinal cells during epithelial morphogenesis and intracellular signalisation of epithelial cells.

First, the functions of villin and fimbrin/plastin isoforms, two different actin binding proteins found in microvillus microfilaments have been assayed. When overexpressed in cultured cells, these proteins induce actin microfilaments reorganization. As a result, the shape of cells and the plasma membrane undergo a drastic remodelling. Conversely knock out expression of villin by overexpression of villin antisense RNA impairs the assembly of microvilli in cultured intestinal cells.

Second, variants of the procaryotic actin nucleator ActA (produced by *listeria monocytogenes*) have been designed and overexpressed in animal cells. One interesting construct to probe ActA activities in transfected cells interacts with the inner leaflet of the plasma membrane. For this purpose the carboxy terminal sequence of ras (containing a CAAX box sequence) was fused to the carboxy terminal end of ActA. This variant leads to complete redistribution of F actin to the cell cortex and consequently to spectacular cell-shape changes.

Third, recent genetic studies subject that a new picture of the functions of junctional complexes has emerged. These studies demonstrated that mutations in several peripheral membrane proteins which normally localized to adherens or tight junctions give rise to tumor supressors gene products.

We are interested in how ZO1 (a marker of tight junctions) and Rab13 (a small GTP binding protein closely associated with tight junctions) contribute to tumor suppression or junction function. As a first attempt the subcellular distribution of overexpressed wild type proteins or dominant negative variants have been investigated.

Work in progress aims to analyze the cross talk between proteins which contribute to cellular morphogenesis and cellular signalling in polarized cells.

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Overexpression of Wild Type and Mutant ARF1 and ARF6: Distinct Perturbations of Non-overlapping Intracellular Targets

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The ARF family of GTP binding proteins are believed to function as regulators of membrane traffic in the secretory pathway. While the ARF1 protein has been shown in vitro to mediate the membrane interaction of the cytosolic coat proteins coatomer (COP1) and γ-adaptin with the Golgi complex, the functions of the other ARF proteins have not been defined.

Here we show by transient transfection with epitope tagged ARFs that whereas ARF1 is localized to the Golgi complex and can be shown to predictably perturb the assembly of COP1 and γ-adaptin with Golgi membranes in cells, ARF6 is localized to the endosomal/plasma membrane system and has no effect on these Golgi-associated coat proteins. By immunoelectron microscopy, the wild type ARF6 protein is observed along the plasma membrane and associated with tubular endosomes, and overexpression of ARF6 does not alter the morphology of the peripheral membrane system. In contrast, over-expression of ARF6 mutants predicted either to hydrolyze or bind GTP poorly shifts the distribution of ARF6 and affects the structure of the endocytic pathway. The GTP hydrolysis-defective mutant is localized to the plasma membrane and its overexpression results in a profound induction of extensive plasma membrane invaginations and a depletion of endosomes. Conversely, the GTP binding defective ARF6 mutant is present exclusively in endosomal structures and and its overexpression results in a massive accumulation of non-clathrin coated endocytic structures.

THE THYROTROPIN-RELEASING HORMONE RECEPTOR IS INTERNALIZED IN CLATHRIN COATED VESICLES DISSOCIATED FROM Gq.

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It has been reported that after ligand binding, the thyrotropin-releasing hormone receptor (TRHR) becomes internalized associated with Gq, as part of a TRH-TRHR-Gq tertiary complex (Mol. Endocrinol. 7: 1105, 1993). We have tested this hypothesis further by examining directly the intracelllular redistribution of the TRHR and Gqα, after agonist binding. The 9-amino acid influenza hemaglutinin epitope (HA) was fused to the extracellular N-terminus of the TRHR, and the tagged TRHR (HA-TRHR) was transiently expressed in both TRHR minus (GH_12C_1) and TRHR positive (GH_4C_1) rat pituitary cells. The HA-TRHR coupled to Gq because agonist in transfected GH_12C_1 cells induced an increase in $[Ca^{2+}]_i$. The cellular localization of the HA-TRHR and Gqα was studied by confocal immunofluorescence microscopy and by biochemical isolation of endocytic compartments. Using confocal microscopy, both the HA-TRHR and Gqα, were localized at the plasma membrane of untreated GH_4C_1 cells, and cells treated with agonist at 4°C. After incubation with MeTRH at 37°C for 60 min, the HA-TRHR was translocated to intracellular vesicles; however, Gqα remained associated with the plasma membrane. The TRH-TRHR complex was internalized via the clathrin coated pit pathway. Clathrin coated vesicles (CCVs) from GH_4C_1 cells, which had internalized [^{3}H]MeTRH for different periods of time contained 3- to 8-fold higher levels of radioactivity than CCVs from cells incubated with [^{3}H]MeTRH at 4°C. Although the receptor-ligand complex was internalized in CCVs, Gqα was not translocated into these endocytic vesicles. CCVs with internalized [^{3}H]MeTRH were not enriched in Gqα compared to vesicles lacking sequestered agonist. These new results demonstrate that after agonist-induced TRHR-Gq coupling, the TRH-TRHR complex dissociates from Gqα at the plasma membrane. The receptor-ligand complex alone becomes internalized via the clathrin coated pit pathway, while Gqα remains at the plasma membrane.

SECRETION OF A LUMINAL INVARIANT CHAIN FRAGMENT BY HUMAN MELANOMA CELLS

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Major Histocompatibility Complex (MHC) Class II molecules consist of a dimer of an alpha chain and a beta chain that function in the presentation of antigenic peptides to CD4+ T cells at the plasma membrane of antigen presenting cells. Directly after biosynthesis, the MHC class II molecules are associated with an invariant chain (Ii). Ii is a type II transmembrane molecule which function: (i) to prevent MHC class II molecules from association with peptides in the endoplasmic reticulum (ER); (ii) to assist folding of class II molecules and their export from the (ER); and (iii) to target the class II complex to the MHC class II compartment where peptides are subsequently bound. Targeting information for the MHC class II compartment resides within the Ii cytoplasmic and transmembrane domains.

In a number of cell types, in addition to full length Ii, an additional Ii related product of a molecular weight of 25 kD is present (IiP25). The biosynthesis and intracellular transport of IiP25 was investigated in a human melanoma celline. Using luminal and cytoplasmic domain specific antibodies IiP25 was shown to contain the intact luminal domain, but to lack the Ii cytoplasmic tail and transmembrane region. Newly synthesized IiP25 was associated with MHC class II molecules and transported through the secretory pathway. Within two hours after biosynthesis after biosynthesis IiP25 was found to be present in the culture medium. As IiP25 represents the domain of Ii that has been implicated in blocking antigenic peptide binding to MHC class II molecules, we asked the question whether IiP25 could prevent antigen presentation. Preliminary results indicate that IiP25, produced in insect cells has an inhibitory effect on peptide presentation to T cell hybroma. Inhibition of antigen presentation by IiP25 may be one of the mechanisms that are used by tumor cells to evade immune recognition.

AGENTS THAT ENHANCE RICIN CYTOTOXICITY AFFECT THE FUSION OF EARLY ENDOCYTIC COMPARTMENTS (EECs). D.D. Pless and R.B. Wellner, USAMRIID, Frederick, MD 21702-5011

Agents that increase EEC pH enhance ricin cytotoxicity. We studied the effects of these agents on the fusion of ricin-containing EECs, an early event in the cellular uptake of ricin. K562 cells were incubated for 5 min at 37° with biotinylated ricin (B-RIC) or for 60 min at 18° with avidin-β-galactosidase (AV-βGAL), and the fusion of EECs containing these markers was studied in vitro using an ELISA procedure that detects the fusion product βGAL-AV-B-RIC [Braell (1992) Meth. Enz. 219:12]. EEC fusions were studied in the presence of the following agents: the ionophores monensin (MON) and nigericin (NIGE), the weak bases NH_4Cl and chloroquine (CQ), and the vacuolar H^+-ATPase inhibitor bafilomycin A1 (BAF). The effects of these agents on fusion were tested using (i) EECs in PNS fractions, and (ii) EECs partially purified from PNS on a sucrose step gradient used to obtain early endosomes from BHK cells [Clague et al. (1994), J. Biol. Chem. 269:21]. In PNS fractions, CQ, MON, NIGE, but not BAF, stimulated the rate of EEC fusion. BAF blocked the enhanced fusion rates produced by CQ, MON, and NIGE. These results suggest that in PNS, CQ, MON, and NIGE increase fusions by altering intravesicular ionic composition in a manner not solely accounted for by a change in intravesicular pH (lack of effect by BAF alone). EECs obtained from sucrose gradients had different fusion properties from those of PNS fractions: fusion activity/mg protein was markedly increased; BAF substantially reduced the fusion rate; CQ, after a lag period, also inhibited EEC fusion. Thus, although we did not detect an effect of pH on EEC fusions in PNS, a fraction of EEC isolated from PNS on a sucrose density gradient exhibited pH sensitive fusion rates as judged by the effects of BAF and CQ.

HOW DOES *ESCHERICHIA COLI* SENSE AND RESPOND TO POSTTRANSLOCATIONAL PROTEIN FOLDING DEFECTS?

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Posttranslocational protein folding in the periplasm of E. coli is a catalyzed process. This process can be disrupted either by mutations which reduce the efficeincy of this process or by overproduction of outer membrane proteins, which may saturate the system. Cells respond to such perturbations by activation of a stress-responsive alternative sigma factor, σE , which subsequently produces the periplasmic protease DegP. The mechanisms by which cells sense defects in the folding of extracytoplasmic proteins, transmit these signals across the inner membrane, and mount a response are largely unknown. Recently, the Cpx proteins have been implicated in playing a role in sensing and responding to the overproduction of the outer membrane protein NlpE. The Cpx proteins are members of the two-component signal transduction family. CpxA is an inner membrane protein homologous to other "sensors" while CpxR is the cognate response regulator. CpxR is thought to act in concert with σE to activate DegP transcription. DegP induction by overproduction of many other outer membrane proteins (OmpC and OmpF, for example) was independent of CpxA and CpxR, suggesting that the Cpx proteins play a limited or specific role. We have found that overproduction of the periplasmic protein Alkaline Phosphatase (AP) from a controllable promoter induces DegP six fold. Induction of DegP is dependent upon the *cpx* genes. While high level synthesis of AP also mildly inhibits the export of other cell envelope proteins such as OmpA or MBP, comparable export defects due to mutations in the *secE*, *secD*, or *secY* genes do not strongly induce DegP. In contrast to weak *sec* mutations, very strong *sec* mutations, such as a *secDF::kan* null mutation, induce DegP. Since strains containing these severe mutations are barely viable, induction of DegP due to secondary consequences is very likely. Our results suggest that the Cpx proteins play an important role in monitoring posttranslocational folding and assembly of cell envelope proteins.

IN VIVO ANALYSES OF INTERACTIONS BETWEEN SECE AND SECY, CORE COMPONENTS OF THE *ESCHERICHIA COLI* PROTEIN TRANSLOCATION MACHINERY

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SecE and SecY are integral cytoplasmic membrane components of the *E. coli* secretion machinery. While several lines of evidence suggest that protein secretion requires the interaction of these two components, the role of both proteins is largely unknown. We are working to understand the functional significance of the SecE-SecY interaction as well as to identify the regions of both proteins required for interaction. We have coupled mutagenic analyses of SecE with two assays to assess the extent to which SecE and SecY can interact *in vivo*: 1. the ability of SecE and SecY to coimmunoprecipitate using antibodies directed at either protein and 2. the extent to which stable overproduction of the labile protein SecY can be sustained by the simultaneous overproduction of SecE.

Several mutant forms of SecE were analyzed for their ability to interact with SecY. A SecE construct altered in the essential second cytoplasmic domain (non-functional), as well as a construct with a heterologous third membrane-spanning segment (partially functional), failed to coimmunoprecipitate SecY. Furthermore neither of the SecE constructs was able to stabilize SecY as efficiently as the wildtype SecE when cooverexpressed. We have isolated additional non- and partially functional SecE missense mutants, altered at positions in the molecule that are conserved across species. Studies are currently underway to analyze the effect of these mutations in SecE on its ability to interact with SecY. Our results suggest that SecE and SecY have more then one site of interaction and that the stable interaction is probably required for efficient protein secretion.

RETENTION OF UNASSEMBLED LIGHT CHAINS IN CELLS INVOLVES FORMATION OF COVALENT COMPLEXES THROUGH CYS214

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Exposed thiol groups can act as intracellular retention / degradation signals for proteins of the secretory type. Yet unassembled Ig-light chains can be secreted despite the presence of a conserved and unpaired cysteine in the C-terminal region (Cys214). The rate of light chain secretion is increased on assembly with heavy chains, which involves the formation of a disulfide bond through Cys214, or by substituting the cysteine for a serine (Ser214). Reducing agents like 2-mercaptoethanol at concentrations that do not affect other processes, also have a similar effect. Replacing the conserved acidic residue (Asp 213) with either a Gly or a Lys increases the retention and degradation of the light chains, suggesting that the unassembled light chains escape Cys214 mediated retention due to the presence of this acidic residue.

Western blot analyses of microsomal lysates on non-reducing gels show some of the intracellular light chains to be covalently bound to proteins. These complexes are also present in the transfectants expressing the λGly213 and λLys213 light chains that are poorly secreted. These complexes involve Cys214 as they are absent in the λSer214 transfectant. They are very abundant in the ER, fewer in the Golgi and play a role in the retention of the light chains.

An additional feature is that the monomeric light chains in the ER can be labeled by radioactive N-ethylmaleimide. Light chains from the Golgi or the supernatant are however not labeled, suggesting that Cys214 gets masked during intracellular transport. We conclude that the thiol-mediated retention involves the formation of weak, reversible disulfide bonds with proteins of the ER. The presence of the acidic residue (Asp213) next to Cys214 allows the masking of the thiol group and thereby its transport to the Golgi. Studies are in progress to understand the nature of the masking group.

IDENTIFICATION OF RAB8 AND RAB11 BINDING PROTEINS THAT MAY HAVE A REGULATORY ROLE IN VESICULAR TRANSPORT.

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Small GTPases of the rab family are regarded as key regulators of vesicular transport in eukaryotic cells, but their precise function is still obscure. We have focused on rab8 and rab11, which have been localized to the trans-Golgi network (TGN) and post-Golgi transport vesicles and, therefore, may play a role in membrane traffic from the Golgi to the cell surface. Moreover, in MDCK cells, rab8 seems to participate selectively in transport to the basolateral, but not to the apical plasma-membrane domain (Huber et al., *J. Cell Biol.* **123**:35-45, 1993). On the other hand, rab11 has been shown to be present in the apical membranes and in apical subplasmalemmal vesicles of certain epithelial cells (Goldenring et al., *Am. J. Physiol.* **267**:G187-G194, 1994; Cameron et al., *Mol. Biol. Cell* **5**:447a, 1994), suggesting that it plays a role in regulating vesicular traffic to the apical membrane.

To understand how rab proteins participate in vesicular traffic, it is necessary to identify other proteins with which they must interact. Using a filter-overlay assay, we identified a 130Kd cytosolic protein (rab11ip) that specifically binds rab11 only in its GTP-bound form. A point mutation in the effector domain of rab11 abolishes this binding, suggesting that rab11ip might be an effector of rab11. Several rab11ip tryptic peptides obtained from rab11ip purified from bovine brain cytosol indicated that it is a novel protein. Moreover, the sequence of a cDNA for rab11ip obtained by PCR using degenerate oligonucleotide primers showed 48% amino-acid sequence similarity to β'-COP, suggesting that rab11ip is also a coat component, probably of post Golgi vesicles. It, therefore, seems likely that the cyclic function of rab11, and probably of other rabs, involves an interaction of the active form of the rab with coat components, as would be the case if the GTP-binding protein promotes coat assembly.

We have also identified, using the yeast two-hybrid system, a rab8-interacting protein (rab8ip) that shows >90% amino-acid sequence identity to a recently identified human Ser/Thr protein kinase. Purified recombinant Rab8ip was able to phosphorylate casein and myelin basic protein, and also underwent autophosphorylation. Binding of rab8ip to rab8 is specific, since in the two-hybrid system rab8ip did not interact with rab11 nor with rab8 mutants that fail to achieve the GTP "active" conformation. Rab8ip is predominantly a cytosolic protein, but the Golgi fraction is highly enriched in it, as compared to total cellular membranes,. We are currently examining the possibility that the participation of rab8 in vesicular transport is modulated through the kinase activity of rab8ip and that an interaction with protein kinases is a general property of rab proteins important for their function.

INVOLVEMENT OF DILEUCINE MOTIFS IN INTERNALIZATION AND DEGRADATION OF INSULIN RECEPTORS

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Dileucine-containing motifs have been shown to be involved in trans Golgi sorting, lysosomal targeting and internalization. The insulin receptor (IR) has four dileucine pairs in its cytoplasmic domain. Previously, we have shown that the dileucine motif (EKITLL, residues 982-987) found in the juxtamembrane region of the IR is involved in receptor internalization (Renfrew Haft et al., *J. Biol. Chem.* **267**, 26286-26294). Mutation of the two leucines in this juxtamembrane sequence to alanines (EKITAA) resulted in a 3 to 5-fold attenuation of insulin uptake. Interestingly, mutation of the same motif to EKITMS, the sequence found in the homologous position in the Type I insulin-like growth factor receptor, had no effect on insulin uptake. These findings suggest that EKITMS in the context of the rest of the IR cytoplasmic domain may be recognized by the endocytic sorting machinery as readily as the native dileucine-containing sequence. Alternatively, secondary internalization signals (perhaps the other dileucine motifs) might act collectively with EKITMS to yield efficient internalization of the mutant IR. To address these points, we constructed chimeric receptor molecules containing the human Tac antigen fused to each isolated motif. A chimera containing the wild-type motif, Tac-EKITLL, was found predominantly in lysosomes by immunofluorescence microscopy, whereas the mutant chimera Tac-EKITAA localized to the plasma membrane. In contrast, Tac-EKITMS was found in endosomes and at the plasma membrane, with only small amounts of chimera detectable in lysosomes. These findings suggest that EKITMS can direct internalization as an isolated motif. However, it is not the functional equivalent of EKITLL, as it fails to direct efficient targeting to lysosomes. These results also support the idea that more than one cellular component may recognize dileucine motifs and help to determine the fate of proteins in the peripheral membrane system.

A NEW SEC1-RELATED PROTEIN CLONED FROM MDCK CELLS IS EXPRESSED UBIQUITOUSLY IN MAMMALIAN TISSUES

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Protein secretion in eukaryotic cells is controlled by a machinery conserved in evolution from yeast to mammals. Since there are a number of distinct vesicle transport steps in a single cell and different cell types perform different transport functions, the machinery involved comprises families of related proteins. The Sec1 protein is essential for yeast cell secretory function and viability. In mammalian neurons a homologous protein, n-Sec1, is associated with syntaxin 1A and has been suggested to regulate complex formation between SNAREs present on synaptic vesicles and on the presynaptic plasma membrane.

We made use of sequence conservation between the yeast and the neuronal Sec1 proteins to isolate an MDCK epithelial cell cDNA encoding a novel Sec1-related protein. The predicted protein shows 63.5% amino acid identity with n-Sec1, 56.7% with the *D. melanogaster* Rop protein, 54.6% with the *C. elegans unc-18* gene product, and 28.9% with the *S. cerevisiae* Sec1p. Northern blot analysis demonstrates that the MDCK Sec1 messages (2.0 and 5.1 kb) are expressed ubiquitously in canine tissues. By immunofluorescence microscopy using an affinity purified antibody, the novel protein localizes in MDCK cells to the perinuclear Golgi region and to more peripheral vacuolar-appearing and punctate elements as well as to patches of the plasma membrane. During an incubation at 20°C, the labeling concentrates in the Golgi region. These results indicate that the protein has a function in a TGN/post-Golgi membrane transport event.

THE APICAL MICROVILLI OF THE RETINAL PIGMENT EPITHELIUM CONTAIN THE NA,K-ATPASE AND 5A11 PROTEIN, BUT NOT SPECTRIN

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These studies examine the relationship of the cytoskeletal protein, spectrin, to the distribution of the plasma membrane proteins, Na,K-ATPase and 5A11 antigen (Fadool and Linser, *Roux's Arch Dev Biol* 203:328-339, 1994). Indirect immunofluorescence was used to determine the distribution of these proteins. Semi-thin (0.5 μm) frozen sections were prepared from the eyecups of chick embryos that were isolated from embryonic day 6 (E6) to E18. The sections were stained using a polyclonal antibody to spectrin and monoclonal antibodies to either the Na,K-ATPase or 5A11 antigen. The association of the membrane proteins with the cytoskeleton was examined by extraction with Triton X-100. The isoforms of β-spectrin were revealed by co-immunoprecipitation with antibodies to α-spectrin and SDS polyacrylamide gel electrophoresis. Initially, each of these proteins was distributed to the apical and lateral membranes. The Na,K-ATPase was the first to polarize by shifting its distribution to just the apical membrane. Later, beginning on E12, patches that contained spectrin and 5A11 appeared on the basal membrane. By E16, a pool of spectrin and 5A11 was revealed as a continuous stain along the basal membrane. Spectrin also occupied a position beneath the microvilli, but the Na,K-ATPase and 5A11 localized to the microvilli themselves. Although the bulk of the Na,K-ATPase and spectrin failed to colocalize, a small fraction of the Na,K-ATPase did appear to associate with some cytoskeletal element. Under conditions that preserved the cortical cytoskeleton, approximately 40% of the Na,K-ATPase and 25% of 5A11 were not extracted by Triton X-100. Unlike intestinal epithelia which have different isoforms of spectrin that underlie the apical and lateral membranes, only one isoform of spectrin was expressed during development. These data suggest that RPE, like other epithelia, exclude spectrin from the microvilli. Further, the RPE has a mechanism to sequester the Na,K-ATPase away from the bulk of the spectrin. By contrast, 5A11 localizes to microvilli and regions enriched in spectrin.

ABSENCE OF FUSION READY GRANULES IN MAST CELLS AND CHROMAFFIN CELLS.

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We measured the exocytotic response induced by a rapid step increase in the concentration of Ca^{2+} or GTPγS (achieved by flash photolysis of caged compounds) in mast cells and chromaffin cells. We measured secretion by following both cell membrane capacitance and amperometry. When Ca^{2+} was used to trigger secretion, we observed an immediate increase in capacitance, however, the first amperometric spike was observed after a long delay. The time integral of the amperometry over the secretory response was sigmoidal and lagged the capacitance trace by 4.02±0.70 s in mast cells and 0.52±0.12 s in chromaffin cells. In contrast, in response to a GTPγS stimulus, we found that the integral of the amperometry followed the same sigmoidal time course as the capacitance. These results suggest that the capacitance increase, when triggered by a large Ca^{2+} step, reports events that do not result in the exocytotic release of oxidizable substances. Capacitance monitors the combined effects of, i) the fusion of different types of vesicles; ii) endocytosis; iii) changes in the electrical equivalent circuit of the cell, whereas, amperometry follows only the release of oxidizable secretory products. Thus the integral of the amperometric recording is a true reflection of the time course of an exocytotic response. The lag observed in the amperometric signal in response to the first step increase in Ca^{2+} or GTPγS suggests the absence of fusion ready granules. We propose that this long lag phase represents a priming step required before fusion of such granules can occur.

THE TRANSMEMBRANE AND CYTOPLASMIC DOMAINS OF THE CD-MPR PREVENT SEQUESTRATION OF THE RECEPTOR INTO LYSOSOMES

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The cation-dependent mannose 6-phosphate receptor (CD-MPR) is a type I transmembrane protein that cycles between the trans-Golgi network (TGN), endosomes and the plasma membrane. To identify signals of CD-MPR that mediate sorting of the receptor out of the endosomal system back to the Golgi, mutant forms were stably expressed in mouse L cells and the intracellular distribution determined by time-course experiments, immunofluorescence microscopy and Percoll density gradients. When 40 residues were deleted from the 67aa cytoplasmic tail of CD-MPR, the half-life of the receptor was drastically decreased and a significant portion of the mutant accumulated in dense lysosomes. A considerable fraction of two chimeric proteins in which either the cytoplasmic tail or the transmembrane domaine (TMD) of CD-MPR were replaced by the corresponding domains of the lysosomal membrane protein Lamp1 was also recovered in lysosomes. Simultaneous substitution of both domains created a chimeric molecule that had a very short half-life and was distributed like Lamp1. In a reciprocal analysis, lysosomal delivery was efficiently prevented when the cytoplasmic tail and TMD of Lamp1 were substituted by CD-MPR sequence. While the TMD of CD-MPR alone did not prevent lysosomal localization, the cytoplasmic tail of CD-MPR had a partial effect. Taken together, these results show that both the TMD and the cytoplasmic tail of CD-MPR contain information that regulate recycling of the receptor from endosomes to the TGN.

Mutational analysis of the 11aa cytoplasmic tail of Lamp1 failed to identify a specific signal for delivery from endosomes to lysosomes. We could, however, not exclude that the Golgi sorting and endocytosis motif (YXXI) of Lamp1 is used in late endosomes as a lysosomal targeting signal.

MOLECULAR CHARACTERIZATION OF RCBP, A NOVEL RCC1 RELATED PROTEIN THAT BINDS TO CLATHRIN AND HSP70.

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We have recently identified a human oncogene that contains sequences related to the cell cycle control gene RCC-1. These sequences are derived from a novel locus that encodes a single high molecular weight (≈15 kb) mRNA ubiquitously expressed in all tested human cell lines and mouse tissues. Isolation of overlapping cDNA clones from a human fetal brain library has revealed a transcript capable of directing the synthesis of a 4,862 amino acid long polypeptide. Analysis of its predicted sequence unveiled several structural domains including two RCC1-like domains, three putative SH3 binding sites, seven β repeats, four leucine rich regions and seven very polar domains with predominantly acidic residues. Using specific antisera, we have identified this protein as a non-glycosylated polypeptide of ≈ 500 kDa with a half-life of 18 h. This protein interacts with the clathrin heavy chain, an association mediated at least by the carboxy-terminal RCC-1 like domain. Therefore, we have designated this protein as RCBP for RCC1-related clathrin binding protein. RCBP also forms ATP-dependent ternary complexes with clathrin and the heat shock 70 kDa protein (Hsp70). Point mutations in the carboxy-terminal RCC1 domain of RCBP completely abolish its clathrin-binding activity. Subcellular fractionation experiments show that RCBP is located in the cytosol as well as in association with intracellular membranes. However, only the cytosolic form is complexed with clathrin and Hsp70. The possible role of RCBP in cellular trafficking pathways is currently being investigated.

IDENTIFICATION OF A SORTING SIGNAL IN THE H,K-ATPase β SUBUNIT

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The H,K-ATPase and Na,K-ATPase are closely related members of the E_1-E_2 family of ATPases. Both proteins are comprised of an α and a β subunit. The α subunits manifest approximately 65% identity, the β subunits manifest approximately 35% identity. While the presence of a β subunit is required for exit of the functional protein complex from the ER, all of the known catalytic activity has been ascribed to the α subunits. In addition, the NH_2 terminal half of the α subunits has been implicated in the differential sorting of the Na,K-ATPase and the H,K-ATPase to the basolateral or the apical domains respectively in the polarized renal proximal tubule epithelial cell line, LLC-PK_1 (Gottardi and Caplan. *J. Cell Biol.* 121:283-293). Recent results indicate, however, that some proteins (Matter, Hunziker and Mellman. *Cell.* 71:741-753) or protein complexes (Fuhrer, Geffen, Huggel and Speiss. *J. Biol. Chem.* 269:3277-3282) maintain multiple sorting determinants. The presence of a tyrosine based endocytosis signal had been previously observed in the cytoplasmic tail of th H,K-ATPase β subunit. In order to determine whether the β subunits contained any sorting information, constructs exchanging the endocytosis signal region of the H,K-ATPase and the analogus region of the Na,K-ATPase β subunits where created. The inactivation of the tyrosine based endocytosis signal of the H,K-ATPase β subunit either through substitution or point mutation results in the relocalization of this protein from the basolateral to the apical domain in the polarized renal distal tubule epithelial cell line, MDCK. Interestingly, the H,K-ATPase β subunit is sorted to the apical domain of LLC-PK_1 cells whether or not the tyrosine based endocytosis signal is intact. These data demonstrate the presence of multiple sorting determinants in the H,K-ATPase. Furthermore, it appears that two similar renal cell lines appear to differ in their ability to interpret a tyrosine based sorting signal.

ROLE OF ARF1 AND SAR1 IN VESICLE BUDDING FROM THE ENDOPLASMIC RETICULUM

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We have developed an in vitro ER to Golgi transport assay using a membrane homogenate derived from NRK cells infected with the ts045 strain of vesicular stomatitis virus (VSV). The reaction is initiated by incubation of the membrane fraction in the presence of cytosol and an ATP-regenerating system. Transport is monitored by following the appearance of Endo H resistant (Endo Hr) forms of VSV G protein (VSV-G). We are also able to follow the release of ER-derived vesicular intermediates containing Endo H-sensitive VSV-G during the incubation by employing a differential centrifugation procedure. We are using this new assay to dissect the role of the small GTP-binding proteins Sar1 and Arf1 in ER to Golgi transport. Mutants of these proteins which are restricted to either the GDP- or GTP-bound conformations potently inhibit the transport reaction. The GDP-bound form of Sar1 blocks the appearance of VSV-G in the vesicle fraction. In contrast, GTP-bound mutants of Arf1 or Sar1 cause VSV-G containing vesicles to accumulate, indicating a block in vesicle fusion. Our results indicate a role for both COPI and COPII non-clathrin coat components in ER to Golgi transport in mammalian cells.

A 340kDa PROTEIN WHICH BINDS TO RAN AND ITS GEF, RCC1: STUDIES IN *XENOPUS* EGG EXTRACTS

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The RCC1 protein is required for many nuclear functions including nuclear transport, RNA metabolism, DNA replication and cell cycle control. RCC1 is a chromatin-associating protein that acts enzymatically as a guanine nucleotide exchange factor (GEF) for a small, *ras*-related, nuclear GTPase called Ran. Ran is required for similar spectrum of nuclear activities and has an essential role in nuclear transport. In order to understand how RCC1 and Ran function in nuclear functions and cell cycle control, we are looking for proteins that associate with RCC1 and Ran. We wish to identify and characterize those proteins in order to determine whether they might regulate RCC1/Ran activity, or might be downstream targets of Ran's activity. Using GST-RCC1 and GST-Ran fusion proteins as affinity-reagents, we purified RCC1/Ran-binding proteins from *Xenopus laevis* egg extracts. We found at least four polypeptides in the GST-RCC1-affinity-purified fraction and at least eight polypeptides in the Ran-affinity-purified fraction. Among those polypeptides, three polypeptides were commonly detected in both the RCC1- and the Ran-affinity-purified fractions. We identified two of them as hsp/hsc70 and RanBP1, respectively. The third protein had a molecular weight 340kDa on SDS-PAGE. p340 was particularly intriguing, since it associated with Ran in an RCC1-independent fashion, and might also associate with RCC1 in a Ran-independent manner. p340 was therefore an excellent candidate for a molecule that modulate the activity of the RCC1/Ran system. Although the peptide sequences obtained from p340 protein did not indicate any significant homology to sequences in data base, we found several nucleoporin XGXGF repeat motifs. Immunofluorescence data using anti-p340 antibodies showed punctate staining on nuclear surface of the interphase cell, suggesting that p340 might associate with the nuclear pore structure. In order to determine detailed localization of this protein, we are doing immunoelectron microscopic analysis. We are also in the process of obtaining cDNA clone and of checking the effect of anti-p340 antibodies on *in vitro* nuclear assembly and nuclear transport using *Xenopus* egg system. In addition to the p340, we will discuss other RCC1/Ran-binding proteins found in our affinity-purified fractions and data regarding our preliminary characterization of the interactions between these proteins.

REINITIATION OF PROTEIN TRANSLOCATION ON ER MEMBRANE

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Membrane proteins on the ER derived organelles are integrated into the ER membrane cotranslationally and then sorted to their final destinations. For the topogenesis of multispanning integral membrane proteins, the internal signal sequence for reinitiation of protein translocation (termed here as "reinitiation sequence" or "R") is essential in addition to the signal sequence (S) and the stop transfer sequence (St). We systematically and quantitatively investigated the structural requirements of R, using the model proteins, which possess the three topogenic sequences in the S-St-R context, and *in vitro* expression system. Systematically designed sequences were introduced as R and the topogenic activities of the sequences were examined.

(1) The model proteins showed the expected topologies in the membrane, depending on the sequence inserted as R. (2) The structural requirements of S and R overlapped to some extents but substantial differences could be demonstrated: some sequences showing type-I (N_{out}/C_{cyto}) signal-anchor function showed R function (N_{cyto}/C_{out}) in the above context. Some sequences with SRP-dependent S function could not function as R. (3) A certain SRP-dependent signal sequence could function as R and the reinitiation efficiency was not affected by SRP concentration when the cytoplasmic loop between St and R of the model protein was relatively short (58 aa). However, in the case of other constructs with longer cytoplasmic loop (100 aa), SRP did increase the overall reinitiation efficiency and, importantly, also affected an accessibility of the processing site after R toward the signal peptidase.

It was suggested that a unique mechanism must be operative in the reinitiation process and that SRP can mediate the translocation reinitiation when the cytoplasmic loop is long.

CHARACTERIZATION OF THE *SAR1* GENE FROM THE FILAMENTOUS FUNGUS *TRICHODERMA REESEI*

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Filamantous fungi have a long history in production of extracellular enzymes and are well known for their high capacity in protein secretion. The largest reported amount of secreted protein from the cellulolytic fungus *Trichoderma reesei* is about 40 g/l. Despite the above, the protein secretion machineries of filamentous fungi are poorly known. As one of the first studies in this field, we report here the characterization of the *sar1* gene from *Trichoderma reesei.*

The *Saccharomyces cerevisiae SAR1* gene product is a small GTP-binding protein involved in the ER to Golgi step of protein secretion. The Sar1p is thought to be regulating the budding of vesicles from the endoplasmic reticulum. The corresponding gene, *sarA*, has been isolated from the fungus *Aspergillus niger* (1). Using this gene as a probe, we isolated the cDNA and chromosomal copies of *sar1* gene of *Trichoderma reesei.* The *Trichoderma* sequence shows 72 % conservation with yeast *SAR1* and 86 % conservation with *Aspergillus sarA* at the amino acid level. The regions responsible for GTP binding are well conserved. The exon-intron structure of *Trichoderma sar1* shows some divergence when compared to that of *Aspergillus sarA.* Complementation experiments with yeast *sar1* mutants will be discussed.

(1)Punt, P.J., Veldhuisen, G. and van den Hondel, C.A.M.J.J., 1994. Antonie van Leeuwenhoek 65, 211-216

BIOSYNTHETIC BIOTINYLATION OF RAB5: A NOVEL MEANS OF CAPTURING RAB PROTEIN ACCESSORY FACTORS. Jack C. Sanford and Marianne Wessling-Resnick, Harvard School of Public Health.

Rab5 is a 25 kD GTP-binding protein involved in endocytosis. Cell-free translation of Rab5 with reticulocyte lysate accommodates efficient in vitro geranylgeranylation which is necessary for its function. When biotin-lysine charged tRNA is added to the cell-free system, the peptide biosynthetically incorporates biotin and can be captured on streptavidin-linked agarose and detected on Western blots using streptavidin-linked alkaline phosphatase. Biotinylated Rab5 is efficiently prenylated, indicating that the protein folds properly and binds GDP [Sanford *et al.* (1993) J. Biol. Chem. *268*: 23773]. Sucrose density gradient experiments indicate that biotin-Rab5 sediments with an apparent Mr ~ 70 kD, indicating a complex with accessory factors. Association with GDI-2 is demonstrated by its co-isolation with biotin-Rab5 from gradient fractions using streptavidin-linked agarose. GDI-2 is the only isotype of guanine nucleotide dissociation inhibitor present in the reticulocyte lysate, and our results suggest that it may provide a "rab escort" function during the post-translational modification reaction. Non-prenylated biotin-Rab5 does not co-sediment with GDI-2, confirming that post-translational modification is necessary for complex formation. Using this approach, we plan to utilize biosynthetically biotinylated Rab5 to isolate novel Rab-associated factors.

CLATHRIN COATED PIT FORMATION AND RECEPTOR ACTIVATION: A MODEL SYSTEM USING IMMOBILIZED IgE RECEPTOR-ANTIGEN COMPLEXES. Francesca Santini, and James H. Keen. Dept. Pharmacology - Jefferson Cancer Institute - Thomas Jefferson University - Philadelphia, PA 19107

IgE-bound FcεRI receptors are internalized via clathrin (Cl) coated pits only when cross-linked by the binding of multivalent antigens (Ag). We explored the relationship between activation of receptors and their association with Cl coated pits by halting the process using an immobilized Ag. When FITC-IgE-primed RBL-2H3 were exposed to immobilized Ag, the FcεRIs, initially uniformly distributed over the cell surface, relocalized and aggregated on the Ag-exposed membrane. The process was specific for Ag, and temperature- and time-dependent. This stimulation initiated a cascade of cellular responses characteristic of FcεRI signaling. Morphologically, cells spread and their membranes ruffled following cytoskeletal rearrangements including F-actin mobilization and formation of actin plaques. Incubation on immobilized Ag in the presence of Ca^{2+} also resulted in cell exocytosis which indicated that Ca^{2+} mobilization had occurred. Despite these responses, the distribution of Cl and AP2, as monitored by immunoblot and immunofluorescence using confocal microscopy analysis, did not undergo any changes. There was no decrease in the density of Cl coated pit in regions of the cell membrane not in contact with the Ag, and there was no apparent increase in Cl coated pits underneath the stimulated/engaged Fcε receptors as we would have expected if the receptors were inducing formation of new pits by active recruitment. These results suggest that activated receptors must encounter pre-existing Cl coated pits for endocytosis to occur.

MEMBRANE PROTEIN RETRIEVAL FROM THE GOLGI APPARATUS TO THE ENDOPLASMIC RETICULUM (ER): CHARACTERIZATION OF THE *RER1* GENE PRODUCT AS A COMPONENT INVOLVED IN ER LOCALIZATION OF Sec12p

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Yeast Sec12p, a type II transmembrane glycoprotein, is required for formation of transport vesicles from the endoplasmic reticulum (ER). Biochemical and morphological analyses have suggested that Sec12p is localized in the ER by two mechanisms: static retention in the ER and dynamic retrieval from the early region of the Golgi apparatus. The *rer1* mutant we isolated in a previous study mislocalizes the authentic Sec12p to the later compartments of the Golgi. To understand the role of *RER1* on Sec12p localization, we cloned the gene and determined its reading frame. *RER1* encodes a hydrophobic protein of 188 amino acid residues containing four putative membrane spanning domains. The *rer1* null mutant is viable. Even in the *rer1* disrupted cells, immunofluorescence of Sec12p stains the ER, implying that the retention system is still operating in the mutant. To determine the subcellular localization of Rer1p, an epitope derived from the influenza hemagglutinin (HA) was added to the C-terminus of Rer1p and the cells expressing this tagged but functional protein were observed by immuno-fluorescence microscopy. The anti-HA monoclonal antibody stains the cells in a punctate pattern which is typical for Golgi proteins and clearly distinct from the ER staining. This punctate staining was in fact exaggerated in the *sec7* mutant which accumulates the Golgi membranes at the restrictive temperature. Furthermore, double staining of Rer1p and Ypt1p, a GTPase that is known to reside in the Golgi apparatus, showed their good colocalization. From these, we suggest that Rer1p functions in the Golgi membrane to return Sec12p that has escaped from the static retention system of the ER.

A COMPLEX IN THE MITOCHONDRIAL INNER MEMBRANE WHICH FACILITATES PREPROTEIN TRANSLOCATION

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Protein translocation into mitochondria requires mitochondrial Hsp70. This molecular chaperone of the mitochondrial matrix is recruited to the inner membrane by MIM44. MIM44 is associated with a complex (MIM complex) which mediates preprotein translocation across the inner membrane.

Formation of the mt-Hsp70/MIM44 complex is regulated by ATP. MIM44 and mt-Hsp70 interact in a sequential manner with incoming segments of unfolded preproteins and thereby facilitate stepwise vectorial translocation of proteins across the mitochondrial membranes. The mt-Hsp70/MIM44 system appears to act as a molecular ratchet which is energetically driven by the hydrolysis of ATP.

We propose a structure of the inner membrane import machinery in which the MIM complex (i) constitutes a proteinaceous channel that accepts preproteins from the outer membrane import machinery, (ii) facilitates their reversible transmembrane movement, and (iii) mediates unidirectional transport by linkage to the ATP-dependent mt-Hsp70/MIM44 system.

Schneider, H.C., Berthold, J., Bauer, M.F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994). Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature 371*, 768-774.

DIFFERENTIAL PLASMALEMMAL DISTRIBUTION OF LIPID-ANCHORED MOLECULES: CAVEOLAE ARE RICH IN GM_1 AND YES BUT NOT GPI-ANCHORED PROTEINS

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The functions of and interrelationships between caveolae, detergent-resistant microdomains, and lipid-anchored molecules including GPI-anchored proteins, src-like nonreceptor tyrosine kinases (NRTK) and the ganglioside GM_1 remain undefined. To explore their normal physiological relationship at the cell surface under conditions that avoid the potential influences of antibody or ligand sequestration, cell culture, and contamination from intracellular compartments, we specifically purified and then subfractionated the luminal plasma membrane of rat lung endothelium into unique microdomains. Here, we show that caveolae and microdomains of GPI-anchored proteins can exist as distinct regions of the cell surface. In situ coating of the endothelial cell surface with colloidal silica particles allowed separate isolation of detergent-resistant plasmalemmal microdomains rich in GPI-linked proteins (but devoid of caveolin) from pure plasmalemmal caveolae enriched in caveolin, GM_1 and the NRTK Yes but not GPI-anchored proteins (5'-nucleotidase, carbonic anhydrase, and urokinase receptor). Immuno-electron microscopy showed that these two microdomains can associate as a unit in the membrane with an annular region rich in GPI-linked proteins attached to the neck of the caveola. This functional unit has been named the **caveolar processing center** (CPC). Specific antibodies and ligands can induce clustering of crosslinked cell surface molecules into the GPI-anchored protein microdomains and then the caveolae. This movement into the caveolae may play a role in signaling and cell activation through direct association with the NRTK. Moreover, molecular mapping of the CPC further reveals the presence of specific molecular machinery necessary for regulated ligand processing by integrating signal transduction with transport via endocytosis, transcytosis and/or potocytosis (see other abstract). (Work supported by NIH grants HL43278 and HL52766 and EI Award from AHA and Genentech.).

THE MUTATION E171A OF THE MOLECULAR CHAPERONE DNAK IS DEFICIENT IN ATP HYDROLYSIS BUT STILL COUPLES THE BINDING OF ATP TO PEPTIDE RELEASE.

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Molecular chaperones have the interesting property of interacting with other proteins in their non-native conformations and of mediating their folding to the native state. We are investigating one representative of the hsp70 family the chaperone DnaK, in order to understand how chaperones work, specifically how the hydrolysis of ATP is coupled to the chaperone function.

A point mutation at position 171 E→A, DnaK(E171A), was created based on computer modeling studies with the known structure of the ATPase domain of bovine hsc70 (Buchberger et al., 1994; Flaherty et al., 1990). It was suggested that this mutation could create a decoupled system since glutamate 171 is located in the so called hinge region that is supposed to mediate events at the nucleotide binding site to the peptide binding site and also complexes the catalytic Mg^{2+} ion.

Initial analysis of the ATPase activity by steady state kinetic measurements suggested that the ATPase activity of E171A was intact but unstimulated upon addition of peptide. Furthermore, addition of ATP to a E171A.peptide complex did not cause release of the bound peptide. It was therefore concluded that the ATPase activity of E171A is decoupled from the chaperone function (Buchberger et al., 1994).

We found that E171A has nucleotide binding properties that are comparable to DnaK(Wt) with a dissociation constant for ADP of 0.5 μM and a rate constant for dissociation of the fluorescently labeled nucleotide analog (MABA-ADP) of 80 x 10^{-3} s^{-1} for DnaK(E171A) and 0.1 μM. and 20 x 10^{-3} s^{-1} for the wild type.

Peptides are bound with virtually identical affinity to the ADP.P_i.protein complexes with K_d = 1 μM and similar association rate constants of 0.0026 s^{-1} μM^{-1} for DnaK(Wt) and 0.004 s^{-1} μM^{-1} for DnaK(E171A).

However, single turnover experiments showed that the rate constant for ATP hydrolysis of DnaK(E171A) is 1.6 x 10^{-6} s^{-1} and therefore some 1000 times slower than that of the wild type (1.5 x 10^{-3} s^{-1}). The dissociation constant of magnesium from its complex with DnaK.MABA-ADP is 10 μM for DnaK(Wt) and 300 μM for DnaK(E171) which indicates that the deficiency in ATP hydrolysis of DnaK(E171A) is not caused by the inability to saturate the enzyme with Mg^{2+} but may be caused by an inappropriate positioning of the catalytic metal ion.

Addition of stochiometric amounts of ATP to a DnaK(E171A).peptide complex immediately releases the peptide. with k_{off} = 7.5 s^{-1} compared to 3.9 s^{-1} with DnaK(Wt). We conclude that for DnaK(E171A) a coupling of ATPase activity to the chaperone function cannot exist because of the lack in ATPase activity, but that the energy available upon binding of ATP is still transferred to the peptide binding site and causes peptide release.

PROTEIN PRENYLATION, RAB GTPases, VESICULAR TRANSPORT AND RETINAL DEGENERATION: UNDERSTANDING THE PATHOGENESIS OF CHOROIDEREMIA.

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Choroideremia (CHM), an X-linked form of retinal degeneration, results from deletions in the Rab Escort Protein-1 (REP-1) gene. REP-1 is essential for the covalent attachment of geranylgeranyl (GG) groups to Rab proteins. Geranylgeranylation of Rab GTPases is essential to their membrane targeting, where they function as molecular switches, regulating intracellular vesicular transport. Two forms of REP, REP-1 and REP-2, have been identified, and REP-2 likely substitutes for the absent function of REP-1 in the prenylation of Rab proteins in most tissues of CHM patients, except the retina. In the retina of CHM patients, the existence of Rabs that depend exclusively on REP-1 for prenylation might explain why REP-2 is only partially effective in compensating for the loss of REP-1 function.

In the current study, cytosolic extracts from CHM and normal lymphoblasts were obtained and subjected to *in vitro* prenylation using [^{3}H] GG pyrophosphate. Upon prenylation, endogenous Rabs become covalently labeled with [^{3}H] GG and can be detected by autoradiography. While normal cells contain no unprenylated Rabs, all CHM cell lines studied reveal the presence of a 27 kDa protein, out of all possible Rabs, that selectively accumulates unprenylated in the cytosol. Furthermore, it shows a strong preference *in vitro* for prenylation with REP-1 as compared with REP-2. The 27 kDa meets the criteria for a protein that requires REP-1 selectively for prenylation both *in vivo* and *in vitro*. Its identification is under way and the future study of its function in the retina may reveal the molecular defect underlying the retinal degeneration in CHM.

A PLANT TRANSFER RNA NUCLEOTIDYLTRANSFERASE CONTAINS POTENTIAL NUCLEAR AND MITOCHONDRIAL TARGETING SIGNALS

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The enzyme ATP (CTP):tRNA-specific tRNA nucleotidyltransferase (CCA enzyme) catalyzes the addition of CMP and AMP to the 3' end of tRNA. This enzyme is required for the synthesis of functional tRNAs in eukaryotes because eukaryotic nuclear, mitochondrial and chloroplast tRNA genes do not encode this essential 3'-CCA sequence. A role in the repair of tRNAs in the cytosol has also been ascribed to this enzyme. In yeast a single gene provides the CCA enzyme for tRNA biosynthesis in both the nucleus and mitochondrion and for repair of damaged tRNAs in the cytosol (Wolfe *et al.*, 1994, J. Biol. Chem. 269:11361-13366). We are interested in determining if this might also be the case in plants where this enzyme is required for tRNA biosynthesis not only in the nucleus and the mitochondrion but also in the chloroplast.

A lupin CCA enzyme has been purified and its corresponding cDNA isolated. Analysis of the predicted amino acid sequence revealed a potential nuclear targeting signal characteristic of those found in other plant proteins (Raikhel, 1992, Plant Physiol. 100:1627-1632). Like the yeast protein, the lupin enzyme contains additional amino-terminal sequences not present in the *E. coli* enzyme which could encode a mitochondrial targeting signal. Antibodies to the lupin enzyme will be raised to determine its distribution in plant cells. As a more rapid way to assess the role of potential targeting signals we are studying the targeting of the lupin enzyme in a yeast strain bearing a temperature-sensitive mutation that affects both the nuclear and mitochondrial activities of this enzyme. Results of these experiments to determine whether the lupin enzyme can complement the nuclear and/or mitochondrial defects in yeast will be presented.

A CLATHRIN BINDING SITE IN THE HINGE OF THE β2 CHAIN OF THE MAMMALIAN AP COMPLEXES

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The assembly of cytosolic clathrin into coated pits and coated vesicles appears to be driven by the clathrin-associated protein (AP) complexes. One of the large chains of the AP complexes, the β chain, is sufficient to drive coat assembly *in vitro*. This chain consists of two domains, the amino-terminal trunk and the carboxyl-terminal ear, linked by a "hinge". We have found that presence of the hinge in recombinant β2-trunk or in recombinant β2-ear fragments is essential in order to drive the in *vitro* assembly of clathrin into coats. A binding assay in which the β-hinge-ear construct is displayed on the surface of agarose beads was used to map the clathrin-binding site to a 50-residue region in the center of the hinge. This sequence turns out to be conserved in all known β sequences from multicellular organisms. It is proposed that recruitment of cytosolic clathrin to a forming coated pit involves simultaneous contacts between the legs of single clathrin trimers and the β-hinge of two or even three membrane-bound AP complexes. Lateral association of these AP complexes can bring several bound clathrins into proximity, allowing formation of a lattice through incorporation of additional clathrin trimers from the cytosol.

KINETIC EFFECTS IN CLATHRIN SELF-ASSEMBLY: A THEORETICAL MODEL

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A theoretical model of clathrin cage and clathrin coated pit formation is proposed. The thermodynamics of assembly is considered for a general class of assumed clathrin-clathrin interactions starting with the simplest experimentally relevant case of in vitro formation of clathrin cages. A phase diagram in the temperature and clathrin concentration (T,c) variables is constructed. The phase diagram has a region of stability of clathrin trimers, the region of stability of clathrin cages and the random lattice ("monster" aggregate) region. It is argued that within the domain of cage stability, the cage size distribution is determined by the assembly kinetics rather than by equilibrium energetics such as the preferred curvature of the triskelion. The distribution is determined by the competition of the rates of addition of hexagonal and pentagonal faces to the growing cluster. The ratio of the two depends on the rigidity of the structure and the rate of triskelion addition to the structure which is proportional to the triskelion concentration leading to the prediction that the average size of the cage should increase with c. The cage distribution (as a function size and isomer type) is constructed by a Monte Carlo simulation of the assembly model. Similar physical considerations are applied to the cases of clathrin and AP-class protein coassembly into cages and into coated pits. The proposed mechanism of coated vesicle formation is of a "thermal ratchet" type. The model makes a number of qualitative predictions which can be studied experimentally.

PROCESSING AND TRAFFICKING OF THE PRION PROTEIN. S.-L. Shyng and D. A. Harris. Washington University School of Medicine, St. Louis, MO 63110, USA.

The cellular prion protein (PrP^c) is a glycolipid-anchored cell surface protein of unknown function. A posttranslationally modified isoform of PrP^c, designated PrP^{sc}, is involved in the pathogenesis of prion diseases, a group of transmissible neurodegenerative disorders including scrapie in animals, and kuru and Creutzfeldt-Jakob disease in man. To understand the cellular mechanism underlying prion diseases, and to gain insight into the function of PrP^c, we have conducted a series of studies to delineate the cellular trafficking of PrP^c. Using mouse N2a neuroblastoma cells stably transfected with a cDNA encoding mouse PrP or chPrP, a chicken homologue of mammalian PrP^c, we have shown that PrP^c constitutively cycles between the cell surface and an endocytic compartment, with a transit time of ~60 min. During each cycle, some of the PrP molecules are proteolytically cleaved within a highly conserved domain in the N-terminal half of the protein, and this cleavage occurrs in a compartment sensitive to lysosomotropic amines and lysosomal protease inhibitors (Harris et al., *Biochem.* 32:1009[1993]; Shyng et al., *J. Biol. Chem.* 268:15922[1993]). We demonstrated by immunogold labeling and biochemical analyses that PrP^c is endocytosed via clathrin-coated pits. However, PrP^c is a glycolipid-anchored protein that lacks a cytoplasmic domain, and so is not capable of interacting directly with the intracellular components of clathrin-coated pits. We therefore proposed that PrP^c associates with the extracellular domain of a transmembrane protein that contains a coated-pit internalization signal (Shyng et al., *J. Cell Biol.* 125:1239[1994]). Deletion analyses showed that the N-terminal half of PrP^c is essential for its internalization, and for its localization in clathrin-coated pits (Shyng et al., *J. Biol. Chem.*, in press [1995]). The detailed cellular pathway of PrP^c depicted here suggests that the protein might function as a cell surface receptor, and that conversion of PrP^c to PrP^{sc} is likely to occur along an endocytic recycling pathway.

To search for cell surface proteins that mediate the internalization of PrP^c, we recently performed binding assays in N2a cells using a bacterially expressed maltose binding protein (MBP)-chPrP fusion protein. We found that the fusion protein binds to N2a cells in a specific and saturable manner, with an estimated K_d of ~0.5mM and a binding capacity of $\sim 10^6$ sites per cell. The MBP alone or a fusion protein of MBP and a mutant chPrP where the 91 amino acids at the N-terminus have been deleted showed no significant binding. The binding of wild-type chPrP fusion protein in N2a cells is diminished by trypsin treatment as well as by salt wash and heparinase treatment. The binding can be competed by exogenous glycosaminoglycans (GAGs), and is greatly reduced in CHO cells defective in GAG synthesis. These results suggest that the major PrP binding site in N2a cells is a proteoglycan. It will be important now to identify this PrP binding protein, and to determine whether it is involved in the endocytosis of PrP^c.

PROTEASOME MEDIATED CYTOSOLIC DEGRADATION OF A BACTERIAL ANTIGEN IS NECESSARY BUT NOT SUFFICIENT FOR CTL EPITOPE GENERATION.

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The antigenic peptides presented by MHC class I molecules are the products of cytosolic protein degradation. The intracellular bacterium *Listeria monocytogenes* secretes p60, a murein hydrolase involved in septation, into the cytosol of infected cells. In mouse BALB/c cells, p60 is degraded into the nonamer peptide p60 217-225 which is presented to CTL by the H-2 K^d class I molecule. We investigated the role of proteasomes, the major mediators of cytosolic proteolysis, in the generation of p60 217-225 using two membrane permeable peptide aldehyde protease inhibitors, LLnL and Z-LLF. When applied to infected cells, we found that both these compounds completely prevented p60 degradation. In contrast, leupeptin and E64 which are inhibitors of lysosomal and cysteine proteases respectively did not affect the p60 degradation. On the basis of the minimal concentrations required for inhibition by LLnL and Z-LLF, we concluded that proteasomes degrade p60. The effect of LLnL and Z-LLF on p60 epitope generation was determined by acid eluting, HPLC purifying and quantifying p60217-225 from infected cells. Both aldehydes completely inhibited the production of p60 217-225, and at lower inhibitor concentrations than required to achieve p60 stabilization. These findings indicate that proteasomes are implicated in the earliest stages of antigen degradation and are essential for epitope production, but implicate additional proteolytic steps in the final sculpture of the epitopes.

THE 11-CIS RETINOL DEHYDROGENASE - A MEMBRANE BOUND RETINOID PROCESSING ENZYME INVOLVED IN THE VISUAL PROCESS

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The universal chromophore of all visual pigments in higher animals is 11-cis retinaldehyde. It is synthesized in the retinal pigment epithelium (RPE) of the eye and subsequently transferred to the photoreceptor cells in the neural retina. The molecular and cellular mechanisms involved in synthesis and intercellular transport of 11-cis retinaldehyde and related compounds in the visual cycle are not well defined at present.
In RPE, a membrane bound enzyme, 11-cis retinol dehydrogenase, converts 11-cis retinol into 11-cis retinaldehyde, in the presence of NAD^+. In the presence of NADH, the enzyme catalyses the opposite reaction. We have isolated a cDNA clone encoding an 11-cis retinol dehydrogenase and carried out a structural and functional analysis of the protein. The cDNA encodes a 318 amino acid long polypeptide and expression of the gene is restricted to the RPE of the eye. The enzyme belongs to the family of short chain alcohol dehydrogenases and this is the first membrane bound retinol dehydrogenase which has been cloned. The hydrophobic N-terminal amino acid sequence, probably acting as signal sequence, is followed the catalytic domain with the cofactor binding site and the active site of the enzyme. A hydrophobic domain near the C-terminus anchors the protein in the membrane. In vitro translation in the presence of membranes followed by proteolytic protection experiments show that the catalytic domain has a luminal localization and that the 7 amino acids in the extreme C-terminus is facing the cytoplasm. The short C-terminal amino acid sequence shows some resembles of the tyrosine containing motif , N-X-X-Y, previously shown to be involved in intracellular sorting of several membrane proteins into the endocytic pathway. Overexpression of the enzyme in Cos cells followed by indirect immunofluorescense localization showed that the protein accumulated in large intracellular vesicular structures. Colocalization studies with an lysosomal marker protein (Lamp 1) revealed only minor overlapping localization suggesting that the enzyme does not accumulate in lysosomes. Our current aims are to identify the vesicular structures in which the enzyme accumulates and to investigate whether the extreme C-terminal tyrosine containing cytoplasmic tail carries the information for its intracellular sorting into these structures.

THE GENERATION OF POST GOLGI VESICLES CARRYING VIRAL GLYCOPROTEINS FROM AN MDCK GOLGI FRACTION.

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We have developed an *in vitro* system for the generation of post Golgi vesicles from an isolated Golgi fraction prepared from VSV or Influenza virus-infected MDCK or HepG2 cells. In this system, vesicle generation is temperature and ATP-dependent, requires cytosolic proteins, including an NEM-sensitive factor distinct from NSF, and is inhibited by BFA and primaquine. The vesicles (50-100 nm)—which are recovered after sedimentation of the residual Golgi—are depleted of the *trans* Golgi marker sialyltransferase, contain the sialylated viral glycoprotein molecules with their cytoplasmic tails exposed, and do not show an obvious protein coat, probably because they become uncoated soon after they are produced. Vesicles generated in the presence of GTPγS sediment more rapidly in a sucrose gradient than those produced in the absence of the analogue and when examined by EM, were found to bear a nonclathrin protein coat. The GTPγS and BFA effects suggest that vesicle generation from the TGN is an ARF-mediated coat-dependent process. Vesicle formation can also be supported by yeast cytosol, which has the same requirements as mammalian cytosol. Vesicle release from the TGN appears to require the activity of a protein kinase C-like protein since several PKC inhibitors, including the highly specific pseudosubstrate synthetic peptide and dimethylsphingosine suppress the formation of vesicles and the effect of the latter is counteracted by TPA, which by itself stimulates vesicle release.

An antibody to the cytoplasmic tail of VSV G selectively precipitates vesicles containing viral glycoproteins from a mixed vesicle population that also contains the influenza HA and is derived *in vitro* from Golgi fractions obtained from doubly infected MDCK or HepG2 cells. This indicates that the viral glycoproteins are sorted into separate vesicles, even in cells such as HepG2 that do not target the two proteins to different surfaces. This raises the possibility that homotypic interactions between the viral glycoprotein molecules themselves contribute to their segregation into different vesicles.

In an effort to purify cytosolic proteins that participate in the process of vesicle generation we obtained a subfraction that is capable of supporting the release of the viral glycoproteins in the absence of ATP. The complementary cytosolic fraction, which by itself does not have any vesicle generating activity, when added to this subfraction, i) suppresses the ATP-independent VSV G release activity and ii) restores the ATP requirement for vesicle generation. NEM-treatment of the cytosolic protein subfraction inhibits both its capacity to support an ATP-independent release and to restore the ATP-dependent process of vesicle generation when added to the complementary cytosolic protein fraction. This would be the case if the ATP-independent release activity is an essential component of the molecular machinery involved in vesicle formation from the TGN. Further purification by column chromatography of the cytosolic activity that promotes the ATP-independent VSV-G release showed that it is present in a high molecular weight (~800-1000 kDa) protein complex that is essentially depleted of both clathrin light chains and β-COP.

MYOSIN Iβ IS LOCALIZED ON LATE ENDOSOMES

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Myosins I, a family of actin-dependent molecular motors, are thought to be involved in cellular organelle trafficking by virtue of their ability to bind to membranes and to promote vesicle movement in *in vitro* motility assays. The significant amino acid sequence divergence among the myosins I has led to speculation that different myosins may by targeted to specific organelles. We have purified a mammalian myosin I (MMIβ) and have shown that this form of myosin I is widely expressed among cells and tissues. Here we present data indicating that MMIβ is associated with elements of the endocytic pathway in cultured cells. MMIβ co-localized extensively with fluorescent LDL and with a fluid phase endosomal marker, 40kDa FTC-dextran, following steady-state uptake for one hour. In contrast, there was no detectable co-localization with β-COP, a marker for the trans-Golgi network and ER-Golgi transport vesicles. MMIβ does not appear to play a role in the earliest steps of receptor-mediated endocytosis. Pulse-chase experiments indicate that MMIβ co-localizes with fluorescent LDL only after at least 2 min of uptake, i.e. at the time of transition from early to late endosomes. At steady state, the antigen co-localizes extensively with cathepsin D, which is found primarily in lysosomes and late endosomes. Besides strengthening the view that specific isoforms of myosin I bind to specific organelles, our data point to a relatively unexplored role for actomyosin interactions in endocytosis.

POM121, AN INTEGRAL PROTEIN FROM THE NUCLEAR PORE MEMBRANE WITH A POSSIBLE ROLE IN NUCLEAR PORE BIOGENESIS.

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The pore membrane domain of the nuclear envelope contains a distinct set of integral membrane proteins, whose functions potentially are to anchor the nuclear pore complex to the nuclear membrane and to function in post mitotic assembly of the nuclear envelope. The cDNA of POM121, a novel integral pore membrane protein that binds wheat germ agglutinin, has been cloned and sequenced (Hallberg *et al.*, 1993). Its primary structure displays a hydrophobic segment close to the N-terminus and a nucleoporin-like domain with XFXFG repeats in its C-terminal third.

Experimental evidence employing domain specific antibodies show that the large C-terminal region of POM121, containing more than 90 % of its total mass, is adjoining the nuclear pore complex. The data also provides strong evidence that the small N-terminal portion is projecting into the perinuclear space and thus, that POM121 is a type I bitopic membrane protein with a single transmembrane segment (Söderqvist and Hallberg, 1994). The location of the nucleoporin-like domain suggests that the C-terminal portion of POM121 is part of and interacts with the nuclear pore complex. This idea is further supported by recent data showing that the C-terminal portion is sufficient to target POM121 to the nuclear pores. Furthermore, *in vitro* data (Hallberg *et al.*, 1993) suggest that POM121 interacts with another pore membrane protein, gp210, which has most of its mass exposed on the luminal side of the nuclear membrane. Thus, POM121 appears to be well suited for anchoring the nuclear pore complex at the pore membrane.

Recent results employing FDIM, Fluorescent Digital Image Microscopy (Fay *et al.*, 1989), show that the C-terminal portion of POM121 is also able to mediate self assembly of POM121 into cylindrical nuclear bodies when extensively overexpressed in COS cells. These intranuclear bodies showed a distinct localization at or in the immediate vicinity of the inner nuclear membrane. Furthermeore, these structures displayed a distinct cylindrical shape with a central hole oriented perpendicular to the plane of the nuclear envelope. The observations may reflect a functional property common to other pore proteins, and which is necessary for nuclear pore complex assembly.

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THE B CELL ANTIGEN RECEPTOR REGULATES THE INTRACELLULAR TRANSPORT OF MHC CLASS II MOLECULES DURING ANTIGEN PROCESSING

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B lymphocytes process and present antigen to helper T lymphocytes. Processing involves the binding of protein antigens to the B cell antigen receptor (BCR) and internalization of the bound antigen into an endocytic compartment where the antigen is proteolytically degraded and the resulting peptides are bound to the MHC class II molecules. The peptide-class II complexes are subsequently transported to the cell surface for recognition by helper T cells. Previous studies showed that the BCR serves both to internalize antigen into the B cell for subsequent processing and to signal for enhanced processing, although the mechanisms underlying these functions of the BCR were not elucidated. Recently, we succeeded in isolating the subcellular compartment in which functional peptide-class II complexes are first formed, termed the peptide-loading compartment (Qiu, *et al.* 1994. *J.Cell Biol.* 125:595). We also identified the transport vesicles in which class II molecules are moved from the trans-Golgi network to the peptide-loading compartment. We now show that in the absence of antigen, the BCR is internalized into the peptide-loading compartment. Cross-linking the surface BCR, using BCR-specific antibodies mimicking antigen binding, results in more rapid movement of a larger number of BCR to the peptide-loading compartment where bound antigen is degraded. Cross-linking the BCR also increases the rate of movement of class II molecules in transport vesicles from the trans-Golgi network to the peptide-loading compartment. The increased rate is correlated with changes in both the phosphoproteins and GTP-binding proteins associated with the peptide-loading compartment. Studies are in progress to elucidate the BCR signaling mechanism responsible for these changes.

ISOLATION AND CHARACTERIZATION OF NUCLEAR ENVELOPES FROM THE YEAST *SACCHAROMYCES*

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The nuclear envelope (NE) is a double membraned organelle that controls the exchange of material between the nucleus and cytoplasm and is thought to be involved in the organization of chromatin and in the regulation of its metabolism. In addition, the NE of the yeast *Saccharomyces* forms a significant part of the ER. We have developed a method which produces large quantities of NEs from yeast. This material is proving useful in ultrastructural studies and the localization of previously identified NE components. The stripping of this NE fraction with heparin yields a heparin-extracted NE (H-NE) fraction highly enriched in integral NE membrane proteins. Both the NEs and H-NEs appear to be morphologically intact and 80-90% pure on the basis of microscopical examination, the removal of potential contaminants, the coenrichment of NE components and the partial coenrichment of ER proteins. As part of the ER, it was found that both NEs and H-NEs are competent for the import of prepro-a-factor *in vitro*, and that they have a significantly higher specific activity than previously described translocation-competent "microsomal" membranes. The NE fractions were injected in mice to raise mAbs which will be used to define and localize novel components of the NE. The preliminary characterization of some of these monoclonals will be presented.

IDENTIFICATION OF AN INTRAPEROXISOMAL COMPONENT OF THE PEROXISOMAL TRANSLOCATION APPARATUS

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Peroxisomes are ubiquitous organelles involved in many biochemical functions, most notably the β-oxidation of fatty acids. We have taken a genetic approach to the study of peroxisome biogenesis by making peroxisome assembly mutants in the yeast *Yarrowia lipolytica* (*pay* mutants). These mutants fail to assemble peroxisomes according to biochemical and morphological criteria.

The mutant *pay32* was identified by its inability to use oleic acid as a carbon source. Electron microscopy demonstrated that *pay32* cells have abnormally small peroxisomes, often found in clusters surrounded by membranous material. In normal cells the ratio of peroxisomal matrix protein to peroxisomal membrane protein is approximately 3:1. This ratio is reversed in peroxisomes of *pay32* cells. A subset of matrix proteins including thiolase, catalase and malate synthase are found in peroxisomes of *pay32* cells at levels found in peroxisomes of wild type cells. One matrix protein recognized by antiserum to a type 1 peroxisomal targeting signal (anti-SKL) is completely mislocalized to the cytosol. Another anti-SKL-reactive protein normally found in the matrix is associated with the membrane of peroxisomes of *pay32* cells and is resistant to extraction by various agents. This protein may be trapped in an intermediate stage of translocation.

The *PAY32* gene functionally complements the mutation in *pay32* cells. It encodes a protein, Pay32p, of 598 amino acids (66 kDa) that is a member of the tetratricopeptide repeat family. Pay32p shows homology to proteins from other yeasts which have been shown to bind to SKL tripeptides and are required for peroxisome assembly. Pay32p is primarily associated with the peroxisomal membrane in wild type cells, but is exclusively localized to the matrix of peroxisomes in the *pay32* mutant. Pay32p appears to have essential roles in both targeting and translocation into peroxisomes of proteins targeted by a peroxisomal targeting signal type 1. While previous studies have revealed different peroxisomal targeting mechanisms, this is the first evidence for multiple pathways of translocation across the peroxisomal membrane.

THE TRIPARTITE STRUCTURE MODEL OF CALPAIN INHIBITION BY CALPASTATIN

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Both calpain (Ca^{2+}-dependent cysteine protease) and its specific endogenous inhibitor protein calpastatin are known to be widely distributed in animal cells. Calpain-calpastatin system is suggested to be involved in various cellular responses coupled with calcium immobilization. Calpain requires Ca^{2+} both for proteolysis of its substrates and for interaction with calpastatin. The detailed inhibition mechanism of calpain by calpastatin has remained unsolved.

Calpastatin molecule contains four repetitive inhibition domains, each having highly conserved internal regions A, B and C. Structure-function studies revealed that the peptide fragments containing only region B retained activity, but its inhibition potency was lower than one domain. Recently, we found that region A and C were not essential for inhibitory activity, but that these regions were suggested to potentiate the inhibitory function of calpastatin. Real-time specific interaction analysis using a BIAcore™ instrument revealed that bacterially expressed calmodulin-like domain (CaMLD) of the μ-calpain large subunit (L-CaMLD) and of the small subunit (S-CaMLD) interacted specifically with a synthetic oligopeptide of region A and region C, respectively, in a Ca^{2+}-dependent fashion. Taken together, the tripartite structure of the calpastatin functional domain may confer specific interactions with the proteinase domain and the two CaMLDs of calpain to form a tight enzyme-inhibitor complex.

ANALYZING CALNEXIN AND ITS SUBSTRATE COMPLEXES IN THE ER OF CHO CELLS.

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Calnexin is a membrane-bound lectin and a molecular chaperone (MW 65 kD) in the ER. It specifically binds to newly synthesized glycoproteins with core N-linked glycans trimmed to their monoglucosylated form. Calnexin's role is to promote correct folding and assembly and retain incompletely folded and assembled proteins in the ER. To analyze the oligomeric status of calnexin and calnexin-substrate complexes, we used sucrose velocity gradient centrifugation and chemical crosslinking. In cells that lack calnexin ligands (i.e. Lec23 mutant cells devoid of glucosidase I, wt CHO cells after prolonged cycloheximide treatment, and cells treated with castanospermine), the CHAPS solubilized lectin sedimented at $5S_{20,w}$. When the endogenous substrates were bound, the complexes range between 5-15$S_{20,w}$ with the size increasing with MW of the substrate. Influenza HA, a well characterized glycoprotein substrate (MW 84kD), was present in 5-5.5$S_{20,w}$ complexes. These could be crosslinked to a 150 kD species suggesting a 1:1 complex. While rigourous molecular weight determinations are yet to be completed, we tentatively conclude that calnexin binds to HA and most of its substrates as a monomer. When unoccupied, it may be either a dimer or a monomer.

When crosslinking with DSP was performed at the time of CHAPS lysis instead of gradient fractions, the calnexin-HA complexes and calnexin itself was found to be part of a much larger protein network which did not enter SDS gels, and included many different proteins. HA was part of this network only during early stages of folding but not when it reached its mature homotrimeric state. This suggested that HA-calnexin complexes are part of a larger but less stable protein matrix in the ER lumen which does not withstand CHAPS solubilization and centrifugation conditions. This matrix may serve as an immobile phase that restricts diffusion of chaperones and folding intermediates in the ER and helps in the quality control process.

SEC6, SEC8, AND SEC15 ARE COMPONENTS OF A MULTISUBUNIT COMPLEX WHICH LOCALIZES TO SMALL BUD TIPS IN *SACCHAROMYCES CEREVISIAE*

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In the yeast *Saccharomyces cerevisiae*, the products of at least 14 genes are specifically involved in vesicular transport from the Golgi apparatus to the plasma membrane. Two of these genes, *SEC8* and *SEC15*, encode components of a multisubunit complex that is found both in the cytoplasm and associated with the plasma membrane. In this study, oligonucleotide directed mutagenesis was used to alter the C-terminal portion of Sec8 with a 6-histidine tag, a 9E10 c-myc epitope, or both to allow the isolation of the Sec8/15 complex from yeast lysates by immobilized metal affinity chromatography or by immunoprecipitation. Sec6 co-fractionates with Sec8/15 by immobilized metal affinity chromatography, gel filtration chromatography, and by sucrose velocity centrifugation. Sec6 and Sec15 co-immunoprecipitate from lysates with c-myc-tagged Sec8. These data indicate that the Sec8/15 complex contains Sec6 as a stable component. Additional proteins associated with Sec6/8/15 were identified by immunoprecipitations from radiolabeled lysates. The entire Sec6/8/15 complex contains 8 polypeptides which range in molecular weight from 70 kD to 144 kD and total 832 kD (assuming single copy for each protein). Crosslinking experiments using DTSSP and DSP have identified 3 additional membrane-associated proteins which may be the receptor for the Sec6/8/15 complex. Temperature sensitive *SEC* strains containing *SEC8-c-myc* were constructed and the composition of the Sec6/8/15 complex in these strains was analyzed by immunoprecipitation. A distinct subset of the 8 polypeptides are absent when the Sec6/8/15 complex is isolated from the *sec3-2*, *sec5-24*, and *sec10-2* strains. Sec3 and Sec5 are not integral components of the Sec6/8/15 complex when analyzed by western blot and Sec10 is unlikely to be an integral complex component since its predicted molecular weight differs by 35 kD from the nearest subunit. Therefore, the specific strain effects suggest that the wild type gene products for *SEC3*, *SEC5* and *SEC10* are involved in assembly or in maintaining the integrity of the Sec6/8/15 complex. The temperature sensitive block in secretion in these strains may be a consequence of the loss of functional Sec6/8/15 complex. The c-myc-Sec8 protein is localized by immunofluorescence to small bud tips. Since Sec6, Sec8 and Sec15 always co-fractionate, we believe the immunofluorescence localization is indicative of the Sec6/8/15 complex as a whole. Thus, the Sec6/8/15 complex may perform its necessary function at the predominant site of exocytosis in *S. cerevisiae*.

THE CATALYTIC CYCLE OF THE UNSTIMULATED ATP HYDROLYSIS REACTION OF THE MOLECULAR CHAPERONE DNAK: A SINGLE STEP KINETIC ANALYSIS.

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The molecular chaperone DnaK is the major hsp70 analogue from the prokaryote E. coli. The function of this class of proteins in the cell is presumably to assist protein folding at the expense of the chemical energy that is available upon the hydrolysis of the nucleotide ATP to the products ADP and inorganic phosphate (P_i).

In order to investigate the nucleotide binding properties of DnaK and to perform experiments under single turnover conditions we prepared nucleotide-free DnaK by enzymatic degradation of ATP and ADP which were bound to DnaK after the usual purification procedure. The binding properties of nucleotides could then be studied either with the intrinsic fluorescence signal of the single tryptophan residue (Trp102) of DnaK or with a novel fluorescence nucleotide analogue of ADP, N_8-(4-N´-methylanthraniloyl-aminobutyl)-8-aminoadenosine 5´-diphosphate (MABA-ADP) as spectroscopic probes. MABA-ADP binds with a stochiometry of 1:1 to DnaK and the dissociation constant (K_d) at 25 °C, pH 7.5 is 0.09 ± 0.01 µM close to the K_d of ADP of 0.1 ± 0.02 µM.

The rate of hydrolysis of ATP or MABA-ATP in a single turnover experiment is 1.5 ± 0.1 x 10^{-3} s^{-1} and is thus virtually the same as the rate of 1.5 ± 0.17 x 10^{-3} s^{-1} obtained under steady state conditions. Furthermore the rate constant of dissociation of ADP is 35 ± 7 x 10^{-3} s^{-1} in the absence or 15 ± 5 x 10^{-3} s^{-1} in the presence of 2 mM P_i and thus some 10-20 times faster than the rate of hydrolysis. These results show directly that hydrolysis of ATP or a conformational change that preceedes hydrolysis is the rate limiting step of the overall ATPase activity of DnaK and that the unstimulated enzyme is mainly in its ATP bound state.

We observed three different fluorescent states of the single Trp residue: The binding of ATP gave a decrease of 15 % compared to the nucleotide-free state, and subsequent hydrolysis or the simultaneous addition of ADP and P_i then increased the fluorescence 7 % above the level of the nucleotide-free protein; the addition of ADP, Pi or the non-hydrolyzable nucleotide analogs AMPPNP (Kd = 1.62 ± 0.1 µM) or ATPγS (Kd = 0.044 ± 0.003 µM) did not cause any observable change of the Trp fluorescence.

We propose that depending on the occupancy of the nucleotide binding site, DnaK exists in at least 3 different conformational states that are likely to be of functional relevance. AMPPNP or ATPγS are not able to induce the conformational change that is observed upon binding of ATP, so that they appear not to mimic the binding of ATP and are not able to induce the conformational change of DnaK that presumably causes peptide release.

IDENTIFICATION AND CHARACTERIZATION OF COMPONENTS OF THE VESICULAR DOCKING AND FUSION MACHINERY IN HUMAN EOSINOPHILS. N. Thompson, J. Armstrong and R. Solari. Glaxo Research and Development Ltd., Greenford, Mddx, UB6 OHE

In an attempt to identify potential key regulators of exocytosis in eosinophils, a subcellular fractionation protocol has been developed for human eosinophils purified from blood. This method allows the partial purification of subcellular organelles from mechanically sheared cells by density gradient centrifugation using a 0-45% linear nycodenz gradient. Western blotting and GTP-overlay studies have identified a number of small GTP-binding proteins throughout the gradient, including the granule enriched fractions, where proteins of approximately 20kDa, 25kDa and 26kDa were observed after GTP-overlay. These proteins have been futher characterized by two-dimensional electrophoresis.

Other components of the proposed vesicular docking and fusion machinery have also been identified in human eosinophils by PCR screening and cDNA cloning.

IDENTIFICATION OF A NEW COMPONENT OF THE YEAST MITOCHONDRIAL INNER MEMBRANE TRANSLOCATION MACHINERY BY A CROSSLINKING APPROACH.

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A crosslinking study was undertaken to identify new components of the translocation and sorting machinery of the yeast mitochondrial inner membrane. We used a hybrid precursor consisting of an intermembrane space targeting sequence attached to mouse dihydrofolate reductase. This artificial precursor becomes arrested across the inner membrane upon import into isolated mitochondria. The arrested precursor can be crosslinked to a neighboring protein of about 10 kDa with a crosslinking efficiency of up to 80%. The crosslinked product is not extractable by sodium carbonate treatment, suggesting that it is an integral membrane protein. The hybrid precursor could be overproduced and purified from *E. coli* as a 6His-tagged version, imported in chemical amounts into isolated mitochondria, and crosslinked to the 10 kDa protein with the same efficiency as the untagged radiolabeled precursor. We are currently trying to identify this protein by microsequencing.

NUCLEAR IMPORT OF TRANSCRIPTION FACTORS NF- κB, AP-1, AND NF-AT IN HUMAN T-LYMPHOCYTES: INHIBITION BY CELL-PERMEABLE PEPTIDES CONTAINING THE NUCLEAR LOCALIZATION SEQUENCE OF NF- κB p50.

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NF- κB, AP-1, and NF-AT are transcription factors which play a vital role in the activation of T-lymphocytes in response to antigenic stimuli. Each of these factors contains a distinct class of nuclear localization signals that mediate its nuclear import upon activation of T-cells with agonists.

To investigate whether a common or distinct mechanism is involved in nuclear import of these three transcription factors, we have designed cell-permeable peptides. These peptides contain a nuclear localization sequence attached to the cell membrane-permeable sequence that is derived from the hydrophobic region of signal peptides. Such cell-permeable peptides enter intact cells and permit analysis of nuclear import *in vivo*. Our results indicate that cell-permeable peptides containing the nuclear localization signal of NF- κB p50 inhibit the nuclear import of NF- κB (61.0 $\pm$ 6.6 % inhibition) in T-lymphocytes treated with PMA/Ionomycin, a combination that simulates activation through the T-cell receptor. Surprisingly, the same peptide efficiently inhibited the nuclear import of AP-1 (89.7 $\pm$ 2.6 % inhibition) and NF-AT (97.2 $\pm$ 2.4 % inhibition), both of which are known to contain components of the Fos and Jun protein families. Control peptides bearing the NF- κB p50 NLS but lacking the hydrophobic cell membrane translocating sequence, or a cell-permeable peptide containing a mutated p50 NLS failed to inhibit nuclear import of NF- κB, AP-1 and NF-AT. The cell-permeable peptide containing the NF- κB NLS did not inhibit proteolysis of I κBα, the major inhibitor of NF- κB responsible for its cytosolic retention in non-activated cells. Taken together, these data indicate that NF- κB, AP-1, and NF-AT utilize similar mechanisms for signal-dependent nuclear import involving the action of a common NLS binding protein(s).

IDENTIFICATION AND ANALYSIS OF COMPONENTS OF THE CHLOROPLASTIC PROTEIN IMPORT APPARATUS: NOVEL TARGETING AND INSERTION PATHWAY OF ONE OF THE COMPONENTS.

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Most nuclear-encoded chloroplastic proteins are translocated across the chloroplastic envelope via a common import apparatus. We used a chemical cross-linking strategy to identify two outer envelope membrane proteins (OEP75 and OEP86) of the import apparatus from pea chloroplasts, and are employing similar strategies to identify additional components of the apparatus. We are also analyzing the roles of hsp70 homologues in the import process. Current hypotheses and supporting evidence for the functions of these identified import components will be presented.

Additionally, we have begun investigating the biogenesis of the import apparatus. A cDNA clone encoding a precursor protein to OEP75 (prOEP75) was isolated and we have extensively characterized the import pathway of this protein. Results from *in vitro* import experiments indicate that prOEP75 is targeted to chloroplasts by its N-terminal transit peptide and is stepwise processed to the mature form (mOEP75). Two proteins intermediate in size between prOEP75 and mOEP75 are observed in the *in vitro* assay; one of these intermediates is abundant in young pea leaves and declines in abundance with leaf age. External treatment of chloroplasts with protease abolishes subsequent binding and import of prOEP75. prOEP75 likely uses the general import apparatus as it competes for import with precursor to small subunit of ribulose bisphosphate carboxylase/oxygenase (prSSU). Furthermore, the first step of prOEP75 processing can be reconstituted by incubation of prOEP75 with stromal extract, and the processing is competed by addition of prSSU. We propose a model in which the N-terminus of the prOEP75 transit peptide directs the protein to the general chloroplastic import apparatus whereupon the precursor is partially translocated, exposing the N-terminus to the stroma. A hydrophobic region near the center of the transit peptide may prevent complete translocation into the stroma and thereby allow the mature region of the protein to assemble in the outer membrane. Our proposed route of translocation and assembly of OEP75 into the outer membrane is unique among outer membrane proteins of both chloroplasts and mitochondria.

RAB3 PROTEIN EXPRESSION IN RBL-2H3 CELLS

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The Rat Basophilic Leukemia (RBL-2H3) cell line serves as a useful model for the tissue mast cell, and exocytotic granule release has been extensively studied in this cell line. By analogy with synaptic vesicle release from neurons, we have hypothesized that a Rab3 protein regulates some aspect of RBL-2H3 granule release. To identify Rab3 isoform(s) in RBL-2H3 cells, we previously designed Rab3-specific PCR primers that averaged 86% identity with the four known Rab3 cDNAs (A-D), but only about 40% identity with irrelevant Rabs. PCR amplification of RBL-2H3 reverse transcripts, followed by cloning and sequencing, produced 370 base pair cDNA fragments of Rab3A, 3B, and 3D isoforms (Tuvin et al; J. Cell. Biochem., 1994, 18B: 270). Quantitative competitive RT-PCR was attempted to estimate the amounts of respective messages using both double- and single-stranded competitors, but reliable quantitation was not acheived. We then turned to Northern blotting using a 370 base Rab3B ^{32}P-labelled cRNA probe. This confirmed the presence in RBL-2H3 cells of multiple Rab3 transcripts of ≈3.2, 2.0 and 1.1 kb. Two of these correspond to sizes previously described for Rab3B (1.1 kb in rat pituitary) and Rab3D (2.0 kb in rat brain). The abundance of each Rab3 transcript was estimated at ~1 femtomole per μg of RBL-2H3 mRNA using cRNA serial dilutions as a standards. There was no hybridization to RBL-2H3 mRNA at 1.4 kb (the reported size of rat brain Rab3A), although there was a strong band in control brain mRNA. Western blots using monoclonal antibody 42.1, which recognizes all four Rab3 isoforms, failed to demonstrate Rab3 protein in RBL-2H3 cells at an abundance even $1/100_{th}$ that in a rat brain (2 ng total Rab3 per 1 μg brain). Together, these data suggest that several Rab3 isoforms are expressed in RBL-2H3 cells, but that considerably less Rab3 is required for the vesicle transfer process regulated in RBL-2H3 cells than is required for the regulation of synaptic vesicle release in brain. Current work is directed toward definitive identification of the RBL-2H3 transcripts using non-coding riboprobes, and toward subcellular localization of Rab3 proteins in RBL-2H3 cells.

REQUIREMENTS FOR PEPTIDE BINDING TO THE MHC-ENCODED ABC-TRANSPORT COMPLEX (TAP) - A COMBINATORIAL APPROACH

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Antigenic peptides are translocated into the ER-lumen where they are needed for correct assembly of MHC molecules. The transport function was reconstituted in insect cells by expression of both TAP-genes indicating that additional cofactors of a highly developed immune system are not essential for peptide translocation. On basis of this overexpression system, requirements for substrate selection were analyzed in a bimolecular mode. Binding and competition assays with peptide variants, including substitutions of residues with alanine, structurally related or bulky hydrophobic side chains, elongated and randomized peptides underline the broad peptide specificity of the human TAP-complex. Evidence is given for a stabilizing effect of the N-terminal arginine and hydrophobic C-terminus on peptide binding to TAP. No length selectivity was observed within the range of 9-15 amino acids, since the longest peptide tested decreased the affinity only 1.2-fold.

Steric requirements of the substrate binding pocket were mapped by scans of hydrophobic, bulky amino acids. Surprisingly, these substitution strongly stabilize peptide binding at almost every position.

Complex nonapeptide libraries (X_8O) were used to determine the contribution of each residue to stabilize peptide binding. Stabilizing and destabilizing residues of sublibraries (X_8O) were identified with reference to the totally randomized nonapeptide library (X_9). The experimental data demonstrate the role of the human TAP-complex in the overall process of antigen presentation, the broad substrate specificity, and the electrostatic as well as steric requirements for peptide binding.

IDENTIFICATION OF AUXILIN AS THE 100 KDa COFACTOR REQUIRED FOR HSP70 TO BIND TO AND UNCOAT CLATHRIN BASKETS WITH OR WITHOUT LIGHT CHAINS. E. Ungewickell[1], H. Ungewickell[1], K. Prasad[2], W. Barouch[2], L. Greene[2] and E. Eisenberg[2], [1]Dept. of Path., Washington U School of Medicine, St. Louis MO 63110; [2]Lab of Cell Biol., NHLBI, NIH, Bethesda, MD. 20892-0301

We previously found that hsp70 required the presence of a 100 kDa cofactor to uncoat clathrin baskets prepared with various assembly proteins (J. Biol Chem 268, 23758 (1993)). We have now found that this cofactor reacts on immunoblots with an auxilin-specific monoclonal antibody. In addition, microsequencing showed that 9 peptides of the cofactor have auxilin specific sequences, and recombinant auxilin substituted for the cofactor in uncoating experiments. Therefore, we have identifed the 100 kDa cofactor as auxilin. Since it has been demonstrated that auxilin binds to light-chain free clathrin (Biochemistry 30, 9097 (1991)), we reinvestigated the question of whether light chains are required for clathrin uncoating by hsp70. In contrast to the earlier results of Rothman and coworkers (Nature 311, 228 (1984)), we found that clathrin baskets prepared with clathrin stripped of its light chains, either by proteolytic digestion or thiocyanate, were uncoated normally by hsp70. In fact, even clathrin baskets composed of truncated clathrin, in which both the terminal domains and light chains are removed by digestion, were uncoated normally. However, as we found for baskets prepared with intact clathrin containing a normal complement of light chains, auxilin was always requried for uncoating. These data strongly suggest that light chains are not required for the uncoating of clathrin baskets by hsp70 whereas auxilin is always required whether or not light chains are present. Analysis of the carboxyterminal domain of auxilin suggested that it has some homology to the J-domain of DnaJ from *E. coli*. In this regard, we found that auxilin not only interacts with clathrin baskets but also activates the ATPase activity of hsp70 in the absence of clathrin, suggesting that it directly interacts with hsp70. In addition, in the presence of ATP, substoichiometric amounts of auxilin support the binding of hsp70 to clathrin baskets at low pH where uncoating does not occur. Taken together, these data suggest that auxilin may present clathrin baskets to hsp70 just as DnaJ presents substrates to DnaK, the hsp70 protein present in *E. coli*.

IMPORT OF STABLY-FOLDED PROTEINS INTO PEROXISOMES.

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By virtue of their synthesis in the cytoplasm, proteins destined for import into peroxisomes are obliged to traverse the single membrane of this organelle. Because the targeting signal for most peroxisomal matrix proteins is a carboxy-terminal tripeptide sequence (SKL or its variants), these proteins must remain import-competent until their translation is complete. We sought to determine whether stably-folded proteins were substrates for peroxisomal import. A hybrid peroxisomal protein stabilized with 17 disulfide bonds, which were not reduced in the cytosol, was shown to be a substrate for peroxisomal import following microinjection into human fibroblasts. With stable disulfide bonds, the microinjected albumin molecules are expected to have retained much of their tertiary structure during the import assays. A prefolded protein, stabilized with chemical crosslinkers was shown to be a substrate for peroxisomal import, as were mature folded and disulfide-bonded IgG molecules containing the peroxisomal targeting signal. In addition, colloidal gold particles conjugated to proteins bearing the peroxisomal targeting signal were translocated into the peroxisomal matrix. These results support the concept that proteins may fold in the mammalian cytosol, prior to their import into the peroxisome, and that protein unfolding is not a prerequisite for peroxisomal import. The translocation of folded proteins and protein complexes into the peroxisome is quite unlike the process of import across the membranes of the mitochondrion or chloroplast. It is possible that organelles that possess ionic or chemical gradients across their membranes require a more restrictive form of protein translocation. As stably-folded proteins can be imported into the matrix of the peroxisome, these proteins may make use of cytosolic chaperones to facilitate their folding, prior to import.

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REGULATION BY FATTY ACIDS OF HEPATIC LIPOPROTEIN ASSEMBLY AND APOB DEGRADATION

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Very low density lipoproteins (VLDL) are assembled in the liver from lipids and apoproteins (including one known as apoB) and are secreted into the plasma of most animal species. VLDL has two important metabolic roles: 1) as the major transporter of the endogenously synthesized hepatic lipids to peripheral tissues; 2) as the precursor of LDL, a major risk factor for atherosclerosis in people. To assemble and secrete VLDL, apoB is absolutely required, and factors affecting cellular apoB steady state levels can thereby influence VLDL and LDL production. Unlike most secretory proteins, the net output of apoB is controlled not by synthesis but by pre-secretory degradation. We have shown (Wang et al., J. Clin. Invest. 91:1380, 1993) that apoB degradation can be regulated by the type of fatty acid given to the cells. Specifically, the provision of n-3 polyunsaturated fatty acids (DHA, EPA) found in fish oils induces apoB degradation and decreases VLDL production by rat primary hepatocytes. Using a transfection model (rat hepatoma cells expressing carboxyl-truncated apoB species) we have recently shown (Wang et al., J. Biol. Chem. 269:18514, 1994) that the induced degradation depends on the degree of lipidation of apoB and most likely occurs after the earliest ER steps of apoB translation and translocation. Our most recent studies, returning to the primary hepatocyte system, confirm this and suggest that entire VLDL particles, at least partially assembled, are lost from the secretory pathway when n-3 fatty acids are present. Current efforts are directed at applying cell biological techniques to define the underlying mechanisms for these effects.

IDENTIFICATION OF hSRP1α AS A FUNCTIONAL NLS RECEPTOR

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Import of proteins into the nucleus is an active process consisting of two separable steps: initially, the energy independent docking of the substrate to the nuclear envelope and, subsequently, translocation through the nuclear pore complex, which requires NTP hydrolysis. A specific nuclear localisation sequence (NLS) in the imported protein is a prerequisite for both steps. In most cases the NLS consists either of a short domain of basic amino acids , e.g. the classical NLS of SV40 T antigen (PKKKRKV), or two stretches of basic residues separated by a spacer of about ten amino acids (e. g. in nucleoplasmin). It has been shown that the two steps of nuclear import can be mimicked in an in vitro transport system (Adam et al. (1990) *JCB* 111:807) by two cytosolic fractions called A and B (Moore and Blobel (1992) *Cell* 69:939). Fraction B mediates the translocation step and consists of two protein components: Ran/TC4 and PP15 (Moore and Blobel (1993) *Nature* 365:661; Moore and Blobel (1994) *PNAS* 91:10212). Recently, importin has been identified as a Xenopus factor required for the first step of nuclear import (Görlich et al. (1994) *Cell* 79:767). Importin is homologous to the yeast protein SRP1p.

We show that a human protein, hSRP1α, binds in vitro specifically and directly to substrates containing either a simple or bipartite NLS motif. It promotes docking of import substrates to the nuclear envelope and together with recombinant human Ran reconstitutes complete nuclear protein import. Thus hSRP1α has the properties of a cytosolic receptor for both simple and bipartite NLS motifs. hSRP1α is a member of a large multigene family and is complexed in vivo with a conserved protein of 90kD. The affinity of hSRP1α towards different types of NLS motifs has been compared and the NLS binding site has been characterized. The results of this analysis will be presented.

ENDOCYTOSIS IN YEAST: ISOLATION OF MUTANTS USING A NEW VITAL STAIN

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We have used a lipophilic styryl dye, N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide (FM 4-64), as a fluorescent vital stain to follow bulk membrane internalization and transport to the vacuole in yeast. As a vital stain, FM 4-64 allows us to follow transport from the PM to the vacuole membrane in real time. We show that FM 4-64 initially stains the yeast PM, then small cytoplasmic compartments, and finally the vacuolar membrane. This process is time-, temperature-, and energy-dependent. Under steady state conditions, FM 4-64 staining was specific for vacuolar membranes; other membrane structures were not stained. Interestingly, unlike Lucifer yellow uptake, vacuolar labeling by FM 4-64 was not blocked in several *sec* and *end* mutants. To isolate new yeast mutants defective for uptake of FM 4-64, EMS mutagenized cells were labeled at 38° C, chased for 60 minutes and washed. Poorly labeled, uptake deficient cells, were enriched by fluorescence activated cell sorting. Direct microscopic screening of the candidate mutants showed that ten percent of the enriched cells (10/100) exhibited reduced fluorescence. Two of these "dim" mutants were found to be temperature sensitive for growth. Electron microscopic analysis showed an accumulation of vesicles in these mutants. However, neither of these mutants exhibit defects in secretory protein traffic or vacuolar protein sorting. Characterization of candidate complementing clones will be presented.

T. A. Vida and S. D. Emr. 1995. A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.* **128**: 779-792.

CELL SPREADING MEDIATED BY COMBINATORIAL RECEPTOR ENGAGEMENT. Tracy White, Qiang Zhu, and Marvin L. Tanzer. Department of BioStructure and Function, University of Connecticut Health Center, Farmington, CT 06030.

B16 mouse melanoma cells adhere to and spread on laminin. Cell spreading is uncoupled from adhesion when unglycosylated laminin is used as a substratum, and is specifically restored by the oligomannosides mannan, Man9, and Man6, but not Man3. Laminin itself contains oligomannosides Man6-8. Effector oligomannosides bind to a cell surface receptor as shown by visualization of oligomannoside probes, by binding studies with radiolabeled Man9, and by competition assays. The oligomannoside receptor is a 60 kDa protein which becomes labeled with a reactive cell surface probe, sulfo-NHS-biotin. The receptor protein was isolated by mannan affinity chromatography and its derived tryptides were fractionated, then characterized by microsequence analysis. Three fragments, FYALSAK, KVHVIFNYK and IDNSQVES, are identical to sequences in the N-terminal domain of mouse calreticulin. The melanoma cell surfaces become specifically labeled with anti-calreticulin antibodies; such antibodies also inhibit cell spreading on laminin but cell adhesion is unaffected. Western blots demonstrate specificity of the antibodies; calreticulin standards migrate adjacent to the melanoma protein. Melanoma calreticulin, previously adsorbed to a glycosylated laminin surface, prevents cell spreading by competing with cell surface calreticulin, but does not affect cell adhesion to laminin. Controls show that melanoma calreticulin does not adsorb to unglycosylated laminin. Previously, cell surface calreticulin of B16 mouse melanoma cells had been identified as an immunogen when whole cells were injected into host animals (Gersten, et al., 1990. *Biochim Biophys Acta* 1096:20). The present results implicate this surface calreticulin as the oligomannoside receptor responsible for triggering cell spreading of laminin-adherent B16 melanoma cells. Although calreticulin has not been previously identified as a lectin, a closely related protein, calnexin, seems to be a lectin which recognizes $Man_{8-9}Glc_1$ residues of intracellular glycoproteins in the endoplasmic reticulum lumen (Hammond, et al., 1994. *Proc Natl Acad Sci* 91:913). In contrast to calnexin, calreticulin does not have a defined transmembrane domain; how it reaches the cell surface and is adherent to that surface is unknown.

CHARACTERIZATION OF TRANSPORT INTERMEDIATES ALONG THE VACUOLAR BIOGENESIS PATHWAY. Eric A. Whitters, Robert C. Piper and Tom H. Stevens. Molecular Biology Institute. University of Oregon. Eugene, OR 97403

Over 50 Vacuolar Protein Sorting (*VPS*) genes have been identified in *Saccharomyces cerevisiae* that function in either the correct sorting or delivery of vacuolar hydrolases. Cells carrying null mutations in these various *vps* genes have been subdivided into six separate groups (Class A-F). Members within each of these groups share gross morphological features that suggest each may be involved in a common site of action along the vacuolar sorting pathway. Class C *vps* mutants appear devoid of a central vacuole (despite their ability to carry out complex vacuolar functions). In contrast, class E *vps* mutants exhibit normal vacuolar morphology but accumulate a novel prevacuole-like organelle in a region proximal to the vacuole. Finally, class D *vps* mutants display a large central vacuole and appear defective in providing vacuolar material to the emerging bud. In an effort to determine the site of action of these individual classes, we have analyzed rapid onset temperature-sensitive alleles of *VPS27* (class E), *VPS33* (class C) and *VPS45* (class D). Furthermore, to dissect the progression of membrane traffic to the vacuole, we have generated an inducible copy of the gene encoding an integral membrane protein of the vacuole, Vph1p. Indirect immunofluorescence microscopy on yeast cells induced for Vph1p synthesis demonstrated that each *vps* conditional mutation results in a rapid block in the progression of Vph1p to the vacuole at the restrictive temperature. However, this block could be fully reversed upon return of the cells to permissive conditions. Analysis of these strains under restrictive conditions by immunogold electron microscopy and biochemical fractionation demonstrated that Vph1p was captured in distinct transport intermediates. We have used the accumulated Vph1p in these strains as a marker for the isolation of these membranes and have obtained significant enrichment of each transport intermediate. Detailed analysis of these mutants and their membrane intermediates provides the experimental means for analyzing the functional relationships between the various *VPS* gene products.

DETERMINATION OF THE MEMBRANE TOPOLOGY OF THE YEAST SEC61 PROTEIN REQUIRED FOR TRANSLOCATION ACROSS THE ENDOPLASMIC RETICULUM MEMBRANE.

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Translocation of proteins across the endoplasmic reticulum (ER) membrane is a decisive step in the biosynthesis of many classes of proteins in all eukaryotes. The mechanism by which this occurs is becoming increasingly understood at the molecular level through a combination of biochemical and genetic approaches. The *SEC61* gene of the yeast *Saccharomyces cerevisiae* encodes an essential protein (Sec61p), which has been shown to play a direct role in the translocation process. Sec61p is a 53kD integral membrane protein consisting of 10 hydrophobic stretches some containing hydrophilic and charged amino acids.

We have used protein fusions to study the topology and membrane assembly of Sec61p. A series of fusions comprising a Suc2p (invertase) C-terminal reporter domain fused in frame to N-terminal portions of Sec61p were constructed. The topology of these fusions expressed in yeast was determined by analysis of their glycoprotein status. In addition, a highly specific protease cleavage site was inserted into a number of hydrophilic domains to create a set of insertion fusions. *In situ* cleavage of these fusions in the absence and presence of detergent mediated membrane permeabilization also provided topological information. The data we have obtained is consistent with a 10 transmembrane structure with the N- and C-termini located on the cytoplasmic side of the membrane. Crucially, this positions some hydrophilic and charged residues in a transmembrane location where they may contribute to the formation of a hydrophilic protein conducting channel. Also, the data suggests that some transmembrane domains require C-terminal sequences for their correct membrane assembly, indicating that several mechanisms may be required for the integration of multispanning membrane proteins into the ER.

PROTEIN FOLDING AND ASSEMBLY IN SEMI-PERMEABILISED CELLS

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Optimized cell-free translation systems have proved useful in determining the initial requirements for protein translocation, folding and assembly. A study was undertaken to investigate whether semi-permeabilised cells (SP-cells) could be used in place of microsomal membranes as an *in vitro* translation/translocation system. The detergent digitonin was used to prepare SP-cells. That the plasma membrane was selectively permeabilised was demonstrated by the fact that the endoplasmic reticulum (ER) remained intact, as shown by direct immunofluorescence.

A comparison was made between the folding and assembly of influenza virus haemagglutinin in dog pancreas microsomes and SP-HT1080 cells. Translation of HA produced native trimeric molecules, as recognised by a conformation-specific antibody, only in the presence of SP-cells.

We have also studied the folding and assembly of type X collagen. In contrast to microsomes, SP-HT1080 cells yielded temperature-stable, trypsin/chymotrypsin-resistant collagen. These cells are therefore able to carry out the translocation, signal peptide cleavage, hydroxylation, folding and assembly of correctly aligned collagen (X) triple helices.

MECHANISMS OF MAINTAINING DIFFERENTIAL MEMBRANE PROTEIN DISTRIBUTION IN HIPPOCAMPAL NEURONS IN CULTURE

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Cellular polarity is crucial for the proper functioning of many cell types. For instance, the maintenance of distinct axonal and somatodendritic compartments that differ in protein composition ensures the correct synaptic polarity of vertebrate neurons. As a model system, we are studying the maintenance of the differential distribution of membrane proteins in cultured hippocampal neurons.

Kabayashi et al. (Nature 359:647 1992) proposed that a barrier to lipid diffusion exists at the axon hillock, analogous to the tight junction in epithelial cells. This diffusion barrier might serve to maintain differential protein distribution.

Using a local perfusion technique, we labeled a short segment of the axon or of a dendrite with DiI or NBD-PE and imaged the diffusion of the label over time using a cooled CCD camera. We show that no diffusion barrier to these lipids exists in neurons cultured for two weeks. The fluorescent lipid diffuses across the axon hillock from both the axonal side and from the soma. Immunostaining against MAP2 confirmed that the individual neurons labeled maintain distinct axonal and somatodendritic compartments. We use fluorescence polarization to show that the lipid label diffuses across the axon hillock in the plasma membrane rather than internally.

We are now studying the distribution of membrane proteins to investigate other possible mechanisms of maintaining distinct membrane compartments. In particular, we are testing whether the axon hillock contains a barrier to protein diffusion or whether membrane proteins are restricted in their diffusion, possibly by attachments to the cytoskeleton.

ALPHA$_2$-ADRENERGIC RECEPTOR SUBTYPES ACHIEVE STEADY-STATE BASOLATERAL LOCALIZATION VIA DISTINCT TARGETING MECHANISMS. ((M. Wozniak, J.R. Keefer and L.E. Limbird)) Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232.

Subtypes of the α_2-adrenergic receptor (α_{2A}, α_{2B}, and α_{2C}AR) couple to the pertussis toxin-sensitive G_i and G_o subpopulations of G proteins and mediate a variety of physiological effects, including transcellular ion transport in renal epithelial cells. Previous studies have revealed that the α_{2A}AR is directly targeted to the lateral subdomain of polarized, cultured Madin-Darby Canine Kidney (MDCK) cells via a pertussis toxin-insensitive mechanism; mutational analysis suggests that regions in or near the bilayer are critical for direct targeting of the α_{2A}AR whereas endofacial domains contribute to α_{2A}AR retention in the lateral surface (*J. Biol. Chem.* 268: 11340-11347, 1993 and 269: 16425-16432, 1994). Unique structural characteristics in the α_{2A}, α_{2B} and α_{2C}AR subtypes led us to evaluate their targeting and retention in polarized MDCK cells. Although the α_{2B}AR, like the α_{2A}AR, achieves 85-95% basolateral localization at steady-state, this polarization occurs after initial random insertion into both apical and basolateral surfaces, followed by selective retention and/or rerouting to the lateral subdomain; pulse-chase studies confirm that the half-life of the α_{2B}AR on the apical surface is markedly shorter than on the basolateral surface of MDCK cells. The α_{2C}AR also is expressed on the lateral subdomain and, like the α_{2A}AR, achieves its localization via direct delivery to the basolateral surface. However, the α_{2C}AR also demonstrates significant intracellular localization, which others have interpreted as a precursor receptor pool. The findings indicate that the α_{2A}, α_{2B} and α_{2C}AR subtypes, which possess highly homologous structures, nonetheless manifest distinct delivery mechanisms, thus providing useful models for revealing structural motifs that confer unique trafficking information. (These studies were supported by NIH DK 43879 and a postdoctoral fellowship award from the National Kidney Foundation to Magda Wozniak).

REGULATED FORMATION OF SECRETORY VESICLES CONTAINING ALZHEIMER ß-AMYLOID PRECURSOR PROTEIN

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Alzheimer disease is a common neurodegenerative disorder characterized by progressive dementia and accumulation of abnormal proteinaceous structures in brain, including extracellular deposits composed of the β-amyloid protein (Aß). Signal transduction via protein phosphorylation determines the rate of generation of the βAPP fragments, soluble βAPP (sβAPP) and Aβ, by governing the relative utilization of competing pathways for βAPP metabolism. Activated protein kinase C (PKC) stimulates nonamyloidogenic "α-secretase" type βAPP cleavage, generating sβAPP at the expense of other pathways, including potentially amyloidogenic pathways such as that specified by "ß-secretase" . The molecular and cellular bases of this phenomenon of PKC-dependent "regulated βAPP cleavage" are poorly understood. PKC-dependent regulated βAPP cleavage does not require changes in phosphorylation state of the βAPP cytoplasmic tail , suggesting that some molecule(s) of the βAPP trafficking and processing apparatus may be the relevant PKC target mediating regulated cleavage.

Of note, βAPP resides preponderantly intracellularly, codistributing with TGN38, a marker of the *trans*-Golgi network (TGN), where no processing activities for wildtype βAPP are known to reside. Thus, it seems possible that PKC might exert some or all of its actions on regulated βAPP cleavage by redistributing βAPP out of its usual residence in the TGN and toward other post-TGN compartments where βAPP can undergo processing. This possibility was supported by other studies demonstrating PKC-dependent stimulation of the release of sulfated glycosaminoglycans and heparan sulfate proteoglycan, intraluminal molecules of the constitutive secretory pathway. Other groups have also obtained evidence for an involvement of protein phosphorylation in secretory vesicle formation from the TGN.

To test the possibility that an important component of regulated βAPP cleavage was PKC-stimulated formation of TGN constitutive secretory vesicles containing and transporting mature βAPP, we have developed an *in vitro* TGN budding assay and characterized the regulation of formation of βAPP-containing vesicles using various signalling compouds. Current progress in using these systems for clarifying the molecular and cellular basis of regulated cleavage of APP will be discussed.

BASOLATERAL TRAFFICKING OF THYROTROPIN-RELEASING HORMONE RECEPTORS IN MDCK CELLS OCCURS INDEPENDENTLY OF SIGNALS MEDIATING RECEPTOR ENDOCYTOSIS AND G-PROTEIN COUPLING.

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Very little is known about the molecular trafficking of G-protein-coupled receptors in polarized epithelial cells. Using adenovirus-mediated gene transfer, we have expressed the receptor for thyrotropin-releasing hormone (TRH) in Madin-Darby canine kidney (MDCK) cells to define its distribution and to begin analyzing the molecular information responsible for targeting this protein to discrete plasma membrane domains. Ectopically expressed TRH receptors bind ligand and are functionally coupled to the formation of inositol phosphate second messengers. Equilibrium binding of [*methyl*-^{3}H]TRH to apical and basolateral surfaces of polarized MDCK cells reveals that TRH receptors are expressed predominantly (> 80 %) on the basolateral cell surface. In striking contrast to the observed polarity of TRH receptor expression, the magnitude of stimulation of inositol phosphate formation is similar for apical and basolateral receptors at all concentrations of *methyl*-TRH, possibly reflecting a polarized distribution of downstream effector molecules. TRH receptors undergo rapid endocytosis following agonist binding; up to 80 % of the receptors are internalized in 60 minutes. A mutant receptor missing the last 59 residues, C335Stop, is poorly internalized (< 10 %) but is nevertheless basolaterally expressed (> 85 %). A second mutant TRH receptor, Δ218-263, lacks essentially all of the third intracellular loop and is not coupled to G proteins upon binding agonist. This receptor internalizes TRH approximately half as efficiently as wild type TRH receptors, but is nevertheless strongly polarized (> 90 %) to the basolateral surface. These results indicate that molecular sequences specifying basolateral targeting of multi-spanning membrane proteins can be segregated from signals for ligand-induced receptor endocytosis and coupling to heterotrimeric G proteins. (Supported by Public Health Service Grants DK43046 (to M. C. G.), GM34107-11 (to E.R.-B.)).

MOLECULAR CLONING OF A GIANT NUCLEOPORIN WHICH BINDS RAN-GTP

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A small nuclear G protein Ran and its guanine nucleotide releasing protein RCC1, which locates on the chromatin, are well conserved from lower eukaryotes to vertebrates, suggesting that they are essential for nuclear functions in eukaryotes. Indeed, loss of RCC1 causes pleiotpropic defects in nuclear functions including mRNA export and protein import. In order to clarify Ran-mediated nuclear processes, we searched for Ran-interacting proteins using a yeast two-hybrid system and obtained ten cDNA fragments from a human cDNA library. One of the fragments encoded the human homologue of RanBP1 (Ran-binding protein 1). Seven out of the ten cDNA fragments covered 5.1 kb of a continuous open reading frame that derived from the 3' half of a mRNA of about 10 kb as judged by Northern blotting. Using the most 5' clone of obtained cDNAs as a probe of another cDNA library, we isolated a cDNA of 10 kb. The complete sequence of the cDNA encodes a novel protein of predicted molecular weight of 358 kDa. The primary sequence of the molecule revealed various characteristic structures; an N-terminal 700-residue leucine rich region, eight zinc finger motifs in the middle of the molecule similar to those of NUP153, four domains homologous to RanBP1, degenerate XFXFG motifs suggesting that the molecule is a nuclear pore complex protein, and an C-terminus with high homology to cyclophilins. The four RanBP1 homologous domains expressed in *E. Coli* bound to Ran-GTP but not to Ran-GDP, hence we designated this giant protein as RanBP2. Immunofluorescence staining of digitonin-permealized HeLa cells by antibodies against RanBP2 showed punctuate staining of the nuclear membrane that co-localized with epitopes recognized by anti-nucleoporin monoclonal antibodies, suggesting that RanBP2 localized to the cytoplasmic side of the nuclear pore. In immuno-EM study on rat liver nuclear envelope, all three antibodies labeled the cytoplasmic side of the nuclear pores and two of three antibodies also labeled the nucleoplasmic side. Since it has been suggested that GTP hydrolysis by Ran is required for signal-mediated nuclear protein import, it is reasonable to consider that the binding of Ran-GTP to RanBP2 at the nuclear pore is a critical step for protein import through the nuclear pore.

TRANSLATION PAUSING AT AN mRNA SEQUENCE RESEMBLING A FRAMESHIFT SITE FACILITATES SRP RECEPTOR MEMBRANE ASSEMBLY. Jason C. Young and David W. Andrews, Dept. of Biochemistry, McMaster University, 1200 Main St. W. Hamilton, Ontario, Canada L8N 3Z5.

Many proteins including the α subunit of the signal recognition particle receptor (SRα) are targetted within the cell by poorly defined mechanisms. A 140 residue amino terminal domain of SRα targets and anchors the polypeptide to the endoplasmic reticulum membrane by a mechanism independent of the pathway involving the signal recognition particle. To investigate the mechanism of membrane anchoring, translation pause sites on the SRα mRNA were used to examine the targetting of translation intermediates. A strong pause site at nucleotide 505 of the mRNA open reading frame corresponded with the shortest nascent SRα polypeptide able to assemble on membranes. An mRNA sequence at this pause site that resembles a class of viral -1 frameshift sequences caused translation pausing when transferred into another mRNA context. Site-directed mutagenesis of the mRNA greatly reduced translation pausing without altering the polypeptide sequence, demonstrating unambiguously a role for this mRNA sequence in translation pausing. SRα polypeptides synthesized from the non-pausing mRNA were impaired in cotranslational membrane anchoring. Furthermore, cotranslational membrane assembly of SRα appears to anchor polysomes translating SRα to membranes.

ALTERING THE EXPRESSION LEVEL OF MAMMALIAN PHOSPHATIDYLINOSITOL TRANSFER PROTEIN IN PC12 CELLS

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Regulated secretion in neuroendocrine cells requires calcium and ATP. Reconstitution studies in permeabilized PC12 cells have resolved the secretion event into sequential ATP-dependent priming step and Ca^{2+}-activated triggering steps. Phosphatidylinositol transfer protein (PITP) and PI4P-5 kinase have been identified as cytosolic factors that participate in the priming step. In vitro studies illustrated that the major function of PITP is to transfer phospholipids between different membrane compartments. However, there has been little study of the in vivo function of mammalian PITP and its role in regulated secretion. We took the approach of altering PITP level in intact PC12 cells by overexpressing PITP coding sequence and PITP antisense RNA, using inducible or constitutive vectors. Multiple cell clones stably transfected with PITP coding and antisense sequences have been established, as confirmed by Southern analysis of genomic DNA isolated from PC12 transfectants. PITP expression level in these transfectants is assayed by immunoblot analysis. PC12 cells transfected with PITP coding sequence are screened for an increase in PITP protein level, while cells transfected with antisense PITP sequence are screened for reduction of PITP protein level. These PC12 cells with altered PITP levels will be used to study PITP in vivo function in regulated secretion.

SEQUENCES AND REQUIREMENTS FOR THE NUCLEAR IMPORT, EXPORT, AND RETENTION OF hnRNP PROTEINS

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The hnRNP proteins are the major pre-mRNA/mRNA binding proteins in eukaryotic cells and are some of the most abundant proteins in the nucleus. They are localized to the nucleoplasm and a subset shuttles between the nucleus and the cytoplasm. Their intracellular transport properties are therefore of considerable interest as they appear to be important for mature mRNA formation and nuclear export. We are engaged in the identification of the signals within the proteins as well as the machineries involved in these intracellular transport pathways. The hnRNP A1 protein localizes primarily in the nucleus and also shuttles between the nucleus and cytoplasm. Unlike most nuclear proteins characterized so far, A1 does not contain a recognizable nuclear localization signal (NLS). We have found that a segment of ca. 40 amino acids near the carboxyl end of the protein (designated M9) is necessary and sufficient for nuclear localization; attaching this segment to heterologous proteins completely localized these otherwise cytoplasmic proteins to the nucleus. The RNA-binding motifs of A1 are not required for its nuclear localization. M9 is a novel type of nuclear localization domain as it does not contain sequences similar to classical basic-type NLSs. Interestingly, sequences similar to M9 are found in other nuclear RNA-binding proteins. We have also identified specific amino acids in M9 which are critical for nuclear localization, as well as requirements and sequences within A1 which confer nuclear export. The hnRNP C proteins differ from A1 in that they do not shuttle, and we have delineated the sequences within C1 which target it to the nucleus as well as sequences that retain it in the nucleus.

A NUCLEAR CAP-BINDING PROTEIN COMPLEX INVOLVED IN PRE-mRNA SPLICING AND mRNA EXPORT FROM THE NUCLEUS

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The cap structure, $m^7G5'ppp5'N$, has been suggested to play an important role in both pre-mRNA processing and RNA nuclear export. Previous investigations of the transport of U snRNAs have shown that a cap binding activity was the limiting factor for the export of these RNAs. By examining the sensitivity of export of U1 snRNA to inhibition by chemically modified cap analogues, it was possible to define the binding specificity of the putative cap binding activity involved in this process. Using this cap competition approach we have shown that the putative cap binding activity involved in pre-mRNA processing exhibited similar specificity.

A cap binding activity found in HeLa nuclear extracts having the binding properties expected for a mediator of U snRNA export and pre-mRNA processing was characterised, purified and cloned. This activity consist of a heterodimeric complex between two Cap Binding Proteins: CBP80 and CBP20. Both subunits are required for binding and both are evolutionarily conserved.

By immuno-depletion of HeLa nuclear extracts, using antibodies raised against the large subunit of the Cap Binding Complex, CBC, we were able to show the role of the complex in splicing (1). Antisera raised against the small subunit of the complex, CBP20, prevent CBC binding to a capped RNA *in vitro*. Affinity purified anti-CBP20 antibodies also inhibit splicing both *in vitro* in HeLa nuclear extracts, and *in vivo*, after microinjection into Xenopus oocytes. Microinjection of these antibodies into the oocyte nuclei also results in a strong specific inhibition of U snRNA export. Export of tRNA, which as a polymerase III transcript does not carry a cap structure, is not affected. The antibodies are now being used to examine the role of CBC in mRNA transport. These antibody inhibition experiments provide strong evidence for the role of CBC as an RNA export factor.

1. Izaurralde et al. (1994). Cell 78, 657-668.

GENETIC AND BIOCHEMICAL ANALYSIS OF THE NUCLEAR PORE COMPLEX

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A synthetic lethal approach was applied to identify the components of the nuclear pore complex. Twenty-nine synthetic lethal mutants were isolated, which belong to at least ten different complementation groups and all correspond to nuclear pore proteins including GLFG nucleoporins Nup49p, Nup57p, Nup116p and Nup145p, and nucleoporins that lack repeat sequences Nic96p, Nup84p, Nup85p, Nup133p and Nup188p.

Complementary to the genetic screen, biochemical approaches allowed the identification and isolation of several hetero-oligomeric NPC subcomplexes, including the Nsp1p/Nup49p/Nup57p core nucleoporin complex to which also Nic96p is bound, and a Nsp1p/Nup82p complex. Nup84p also forms a stable complex with at least 5 other proteins. Peptide sequences of all five proteins could be obtained. We speculate that Nup84p together with its interacting proteins has a role in nuclear envelope and nuclear pore biogenesis.

We have further identified a genetic network of interaction between Nsp1p, Nup126p which also localizes to the nuclear pore complex and is identical to Los1p (a protein required for *in vivo* tRNA splicing [1]), and a novel protein which shares homology to *E. coli* tRNA pseudouridine synthetase. This raises the interesting possibility that tRNA biogenesis steps are coupled to the translocation through the NPC.

[1] Hopper, A.K. and Schultz, L.D. (1980). *Cell* **19**, 741-751

NUCLEAR TRANSPORT FACTORS IN NUCLEAR LOCALIZATION SEQUENCE-MEDIATED TARGETING TO THE PORE COMPLEX

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The association of nuclear proteins with the nuclear pore complex during transport across the nuclear envelope is mediated by short basic amino acid sequences called nuclear localization sequences (NLSs). We have identified soluble protein factors required for pore complex association in a permeabilized cell nuclear protein import assay. These factors include the 60 kDa NLS receptor and a 97 kDa protein (NTF97). cDNA cloning of the human NLS receptor identifies it as a member of a family of proteins closely related to the yeast SRP1 protein. Members of this family contain 8 conserved armadillo repeats and are specific NLS-binding proteins. NLS-peptide crosslinking experiments demonstrate that two members of the human group, Rch1 and hsrp1, have differential specificities for various NLSs, suggesting multiple pathways of protein import. Affinity purified polyclonal antibodies to the NLS receptor localize the protein to the cytoplasm, nucleus and nuclear envelope.

A monoclonal antibody localizes NTF97 to both a soluble cytoplasmic pool and the nuclear envelope. The antibody also inhibits translocation of proteins across the nuclear envelope, but does not affect NLS-mediated association with the pore complex. Immunoadsorption of soluble NTF97 from digitonin extracts of cells or reticulocyte lysates identifies proteins of 23 kDa, 33 kDa, 90 kDa, 116 kDa and 230 kDa that form complexes with NTF97. A group of proteins between 50 and 60 kDa corresponding to the NLS receptor proteins are also specifically co-adsorbed. Three NTF97-containing complexes are evident by size fractionation, each forming with a subset of the proteins. Only one of these complexes is capable of targetting a NLS-containing protein to the nuclear pore complex. NTF97 is extracted from nuclear envelopes under the same conditions as nucleoporins, suggesting a strong association of NTF97 with pore complex proteins. Immunoadsorption of extracted NE-associated NTF97 shows that Ran/TC4 and Nup153 are closely associated with this fraction of NTF97. Two additional proteins of 30 kDa and 300 kDa are also associated with NTF97.

The localization of the NLS receptor and NTF97 and the biochemical characteristics of each suggest probable models for NLS-mediated targetting to the pore complex and the role of each component in the transport pathway.

TWO DIFFERENT SUBUNITS OF IMPORTIN CO-OPERATE TO RECOGNIZE NUCLEAR LOCALIZATION SIGNALS AND BIND THEM TO THE NUCLEAR ENVELOPE.

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Selective protein import into the cell nucleus occurs in two steps, binding to the nuclear envelope followed by energy-dependent transit through the nuclear pore complex. Both steps are dependent on cytosolic factors.

We have purified, cloned and bacterially expressed a 60kD protein, importin 60, that is essential for the first nuclear import step. It is a homologue of the yeast nuclear pore protein SRP1p. Subsequently a second importin subunit of 90kD (importin 90) was identified and cloned from a human cDNA library. Yeast SRP1p was found to be associated with a homologue of importin 90. Importin 90 potentiates the effects of importin 60 on nuclear protein import, indicating that the importin-complex is the physiological unit responsible for import. To test whether nuclear localization sequences (NLS) are recognized by cytosolic receptors, a biotin-tagged BSA-NLS-conjugate was allowed to form complexes in Xenopus egg extract and retrieved with streptavidin agarose. The pattern of bound proteins was surprisingly simple and showed only two predominant bands: those of the importin complex, indicating that the two importin subunits constitute a cytosolic receptor for nuclear localization signals, enabling import substrates to bind to the nuclear envelope.

SOLUBLE FACTORS REQUIRED FOR NUCLEAR PROTEIN IMPORT

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We have identified two soluble protein complexes present in Xenopus ovarian cytosol that are required sequentially to support import of a nuclear localization sequence (NLS)-containing substrate into the nuclei of digitonin-permeabilized cells. The first protein complex (called karyopherin) is required for NLS-recognition and docking at the nuclear envelope and consists of a doublet of proteins of 54 and 56 kD (called karyopherin α-1 and α-2) and a protein of 97 kD (called karyopherin-β). Karyopherin-β has been cloned and sequenced and is a unique protein. The karyopherin α subunits appear to be equivalent to the previously purified bovine NLS receptors (Adam and Gerace, Cell 66, 837-847, 1991) and the recently cloned Xenopus importin (Görlich et al., Cell 79, 767-778, 1994).

We have purified two additional proteins which are required for the second stage of nuclear import, namely translocation of the docked substrate into the nuclear interior. The first of these is Ran (Ras-related nuclear protein) which is a member of the Ras superfamily of small GTP-binding proteins. The second is a protein we called p10 based on its apparent molecular weight by SDS-PAGE. p10 is highly homologous to a previously identified human protein of unknown function called placental protein 15 (pp15). Based on their cofractionation, these two proteins (Ran and p10) appear to form an active complex consisting of one molecule of Ran and one p10 dimer. Preliminary experiments have indicated that pp15, like Ran, is located primarily inside the nucleus. Karyopherin, Ran, and p10 appear to supply most (if not all) of the import activity found in crude cytosol indicating that these may be the only import factors that leak out from the digitonin-permeabilized cells in sufficient quantitites such that their loss can be detected.

BIOCHEMICAL DISSECTION OF NUCLEAR PROTEIN IMPORT

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We have been analyzing nuclear protein import mediated by the nuclear pore complex (NPC) using an *in vitro* assay based on digitonin-permeabilized mammalian cells (JCB 111:807-816). Multiple cytosolic factors are required for nuclear import, and we have characterized three of these in detail: the NLS receptor, the small GTPase Ran/TC4, and the 14 kD protein NTF2. The NLS receptor is an NEM-sensitive protein that specifically binds basic-type nuclear localization sequences (NLSs) and is suggested to be a shuttling carrier for transport of NLS-containing ligands to the nuclear interior. According to our working model, the NLS receptor first interacts with NLS ligands in the cytoplasm. After this, the receptor-ligand complex associates with peripheral fibrils associated with the cytoplasmic surface of the NPC, is moved to secondary accumulation sites near the central gated channel, and finally is translocated through the central gated channel of the NPC to the nuclear interior.

The cytosolic factors and NPC components that mediate these steps are being analyzed by biochemical and structural approaches. We have found that GTP-bound Ran specifically associates with the cytoplasmic surface of the NPC at peripheral regions involved in binding of NLS-containing transport ligands, and have identified an ~350 kD protein that is likely to represent this Ran binding site. Our data suggest that hydrolysis of GTP by Ran after the NLS ligand initially binds to the NPC serves to commit the ligand to more distal transport steps. The accumulation of transport ligands near the central gated channel is suggested to involve interaction with a complex of four proteins containing O-linked N-acetylglucosamine (the p62 complex) located in this region. The p62 subunit of this complex binds the cytosolic transport factor NTF2, which may be involved in targeting the NLS ligand complex to this structure. We have determined that the isolated p62 complex has a torus-like structure, consistent with its being an "adaptor" for delivering ligand to the central gated channel.

MITOTIC DISASSEMBLY AND REASSEMBLY OF GOLGI STACKS *IN VITRO*

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When rat liver Golgi stacks are incubated with mitotic cytosol, fragmentation occurs by two overlapping pathways. The first involves the continued budding of COP I-coated transport vesicles which uncoat and accumulate because fusion with the target membrane is inhibited (Misteli and Warren, 1994). The proteins responsible for this inhibition of fusion are currently being characterised. The other is a COP I-independent pathway that appears to involve tubular networks as intermediates. These appear at early times during fragmentation and then disappear as they break down into smaller fragments.

Re-isolation and re-incubation of these fragments with interphase cytosol triggers the reassembly process. Cisternal remnants appear to grow by the rapid fusion of vesicles which results in extensive tubular networks emanating from the cisternal rims. Cisternae stack and continue to grow in part by lateral fusion of pre-existing stacks. The tubular networks become flattened and more cisternal-like. The final product is a cup-shaped stack of 4-5 cisternae surrounding an electron-lucent space that is largely devoid of membranes. Stacking is enhanced by GTPγS and inhibited by microcystin (Rabouille, et al., 1995). The proteins involved in both fusion and stacking are currently being characterised.

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FORMATION OF GOLGI STACKS FROM VESICULATED GOLGI MEMBRANES

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The stacks of Golgi cisternae in mammalian cells vesiculate into 60-90 nm size vesicles upon treatment with the sponge metabolite ilimaquinone (IQ). The change in the relative distribution of vesiculated Golgi membranes (VGMs) and stacks of cisternae is easily distinguished by immunofluorescence microscopy using Golgi specific antibodies. VGMs are diffusely dispersed throughout the cytoplasm while the stacks appear as large and discrete aggregates. This differential organization of Golgi membranes has been used to reconstitute the process of stack formation from VGMs in permeabilized cells. This is carried out by treating normal rat kidney (NRK) cells with IQ to vesiculate Golgi stacks. The cells are washed to remove IQ and then permeabilized by a rapid freeze-thaw procedure. VGMs fuse and assemble into stacks of cisternae by a process that is temperature dependent, requires ATP and a high speed supernatant from cell extract (cytosol). The newly formed stacks are functionally active in protein transport and Golgi specific glycosylation reactions. Immunoelectron microscopy using Golgi specific anti-ManII antibody reveals that VGMs first fuse to form larger vesicles of about 200-300 nm average diameter. These larger vesicles then fuse and assemble into tubulo-reticular elements which subsequently assemble into stacks of cisternae.

PLASMA MEMBRANE IN-PLANE TENSION DECREASES DURING SECRETION. Jianwu Dai & Michael P. Sheetz, Department of Cell Biology, Duke University Medical Center, Durham, NC 27710

After exocytosis, membrane area increases and endocytosis retrieves this excess membrane but no more. Is there a membrane parameter that sets the correct membrane area? For many cells, the plasma membranes are stretched tightly over the cellular cytoskeleton much like a tightly fitting glove. Because the plasma membrane bilayer is fluid and continuous, such stretching produces an in-plane tension distributed throughout the membrane. Using the laser tweezers to measure tether forces, we have shown that a significant tension (~0.003 mN/m) exists in the plasma membranes of neuronal growth cones (Dai and Sheetz, Biophys. J. 68:988-996 (1995)). Since stimulated secretion can temporarily increase the plasma membrane area, there may be a concomitant decrease in the in-plane tension. During stimulated secretion in rat basophilic leukemia (RBL) cells by the crosslinking of IgA molecules, there is a transitory increase in the plasma membrane area that is corrected by increased endocytosis. We observed that membrane tubes or tethers drawn from rat basophilic leukemia (RBL cells) exert considerable retractile force (20.8 ± 2.2 pN) that is related directly to the in-plane membrane tension. Tension does not depend significantly upon tether length. Upon stimulation of secretion by DNP-BSA binding to anti-DNP-IgA, retractile force drops and that drop corresponds to a decrease in in-plane tension to ~16% of the resting value. The tension decreases within 20 sec after addition of the antigen (similar to the delay before the first observed secretion of [^{3}H] serotonin) and recovers to the original level when secretion is terminated. Blocking secretion by removing Ca^{++} or adding DNP-Lysine also prevents the drop in tension. Because the in-plane membrane tension correlates inversely with endocytosis rate, we postulate that cells normally balance exo- and endocytosis by sensing the plasma membrane in-plane tension.

MEMBRANE FUSION BY INFLUENZA HAEMAGGLUTININ

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Haemagglutinin is the receptor binding and membrane fusion glycoprotein of influenza virus. Membrane fusion potential is activated at endosomal pH in a process that involves extensive re-folding and re-arrangement of the molecule. Our studies address the nature of these changes in structure and the requirement for HA fusion activity. X-ray analysis of crystalline fragments of HA in the fusion pH conformation and studies of mutant HAs indicate that fusion requires dissociation of the globular membrane distal domain with retention of monomer structure and involves re-location of 145 of the 175 residues of the central fibrous domain including the "Fusion Peptide". The significance of these changes for possible mechanisms of HA-mediated fusion will be considered.

MEMBRANE FUSION: THE INFLUENZA PARADIGM

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One of the best characterized membrane fusion events is that mediated by the influenza virus hemagglutinin (HA). The process of HA-mediated fusion is initiated by a conformational change in HA that releases the fusion peptides from the interior of the HA trimer. Thus exposed, the hydrophobic fusion peptides associate with the target, and perhaps, viral bilayers. The next stages of fusion involve creation of a fusion site, formation of a hemifusion intermediate, (reversible) opening of a narrow fusion pore, and irreversible dilation of the fusion pore. Recent work on fusion pore formation will be discussed.

Following the hydrophobic association of HA with the target bilayer, there is a lag phase before the onset of lipid mixing. It has been hypothesized that during this lag, several HAs laterally associate to establish a fusion site. If this is the case, then the process of fusion pore formation might be cooperative with respect to HA trimers. To test this possibility, we analyzed the fusion kinetics of stable cell lines that express HA at nine different defined densities. The observed relationship between the lag phase and HA surface density was clearly sigmoidal. A Hill plot analysis of the data suggested that three to four HA trimers are necessary to form a fusion site.

We recently provided evidence supporting the notion that HA-mediated fusion proceeds through a hemifusion intermediate. Our evidence was that GPI-anchored HA mediates (outer leaflet) lipid, but not content, mixing with bound red blood cells (Kemble et al. 1994. Cell. 76:383-391). Follow-up studies have confirmed and extended these findings. Collectively, our results indicate that the transmembrane domain of HA is necessary to initiate very early (and apparently reversible) events in fusion pore opening.

These new findings will be discussed in the context of models for the structure of the HA fusion pore.

INTERMEDIATES IN MEMBRANE FUSION.

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To understand the pathway by which membrane components fuse, we have studied fusion protein conformation, lipid and aqueous dye mixing, and fusion pore conductance. The docked, calcium-triggered exocytosis of sea urchin egg granules require proteins in at least one of the fusing membranes, but not ATP or cytosol. Reversible thiol reagents, but not non-hydrolyzable ATP analogues, block fusion by preventing activation of fusion proteins.

After protein activation, in sea urchin exocytosis and baculovirus gp64-induced cell fusion, an intermediate is revealed by adding inhibitory exogenous lipids or enzymatically producing them in-situ. These inhibitory lipids increase the free energy of contacting membrane monolayers bending towards each other. In the presence of inhibitory lipids, monoclonal antibodies show the fusogenic gp64 conformational change, but there is no fusion. Removal of these inhibitory lipids allows fusion to continue. Fusion is promoted by lipids of opposite curvature, suggesting that a bent membrane fusion intermediate can be rate-limiting. Promotion and inhibition are also found in the fusion of phospholipid membranes with lipids of curvatures which promote or inhibit biological fusion. After docking and triggering, fusion proteins may work by lowering a barrier whose energy is lipid-dependent so that fusion may proceed via at least partially lipidic intermediates, such as hemifusion.

Following this lipid-sensitive step, ionic contact between aqueous compartments separated by fusing membranes is achieved through the fusion pore. For influenza HA, fusion pore formation involves the formation of multiple, small pores which initally impede lipid and aqueous dye mixing and later grow to allow macromolecular mixing. In baculovirus fusion, pore formation is abrupt (within hundreds of microseconds), large, and highly variable in initial size (0.2-3 nS). Within hundreds of microseconds of its formation, the fusion pore may reflect the conductivity of a pore formed from coplanar membranes whose high curvature is stabilized by the length of the pore.

ATTRACTIVE AND REPULSIVE INTERACTIONS BETWEEN LIPID BILAYERS. T.J. McIntosh[1] and S.A. Simon[2] Departments of Cell Biology[1], Neurobiology[2] Duke University Medical Center, Durham, NC 27710.

Due to the inherent structural complexity and the heterogeneity of lipids and proteins of biological membranes it is often difficult to obtain molecular information regarding the details of membrane adhesion. To partially overcome this difficulty, adhesion is often studied between well-characterized phospholipid bilayer vesicles. In these systems the adhesion energy arises from a balance between a number of non-specific repulsive (electrostatic, steric, hydration) and attractive interactions (van der Waals, H-bonds, bridging). Our goals are to measure the range and magnitude of these interactions and to understand their origin. This is achieved by directly measuring the interactions between bilayers using an osmotic stress/X-ray diffraction method. Here we focus on one of these interactions, a strong, ubiquitous repulsive pressure called the hydration pressure (P_h). Overcoming this pressure by the addition of exogenous compounds can cause bilayers to increase their adhesion

P_h arises from polarization of water molecules by the hydrophilic regions of macromolecules. For a variety of bilayer systems P_h decays exponentially with a decay length $\lambda \approx 1.5$ Å and a magnitude that is proportional to $(V/\lambda)^2$, where V is the potential between bulk water and the middle of the membrane. For many lipids, V is several hundred millivolts (positive) and arises from the dipoles and multipoles of the lipid and polarized water. It has been found that P_h has a range of one to two water molecules. Decreasing V, for example by the addition of selected polyphenols, causes uncharged vesicles to adhere. In contrast, increasing V increases the fluid spacing between bilayers and decreases their adhesion. This very large, short-range electric field (V/λ) provides a mechanism by which the adsorption to bilayers is greater for anions than for cations. Moreover, this field causes a preferential orientation of proteins or peptides with large dipole moments that adsorb to the membrane-water interface.

IMPORT AND FOLDING OF PROTEINS BY YEAST MITOCHONDRIA

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Protein import into mitochondria is initiated by binding of precursors to the four outer membrane receptor subunits Mas70p, Mas37p, Mas22p and Mas20p. Mas70p and Mas37p accept precursors from ATP-requiring cytosolic chaperones and may recognize the unfoldedd precursor chain. The highly acidic Mas20p subunit recognizes mainly the basic and amphiphilic mitochondrial targeting signal. Mas22p is the only essential receptor subunit; it has acidic presequence-binding domains on both sides of the outer membrane and may mediate transfer of the precursor's N-terminus across the outer membrane.

Transport across the mitochondrial membranes is effected by a distinct, hetero-oligomeric transport system in each membrane. These two systems interact dynamically with each other for protein transport into the matrix, but can operate independently for protein sorting to the outer membrane or the intermembrane space. The inner membrane system is powered by the transmembrane protein Isp45p (MIM44) and the chaperone proteins mhsp70 and GrpEp. Interaction among these three proteins is disrupted by binding of ATP to mhsp70. We propose that these three proteins act as a force-generating "motor unit" that pulls proteins into mitochondria by ATP-driven conformational changes of mhsp70 which are controlled by GrpEp.

Refolding of proteins within the matrix is mediated by mitochondrial cyclophilin (Cpr3p) and hsp60/cpn10. The cyclophilin functions as a proline rotamase because its effect on the refolding of an artificial precursor requires rate-limiting proline peptide bond isomerizations in the precursor protein. The requirement for hsp60/cpn10 system is precursor-specific and bypassed by artificial precursors containing DHFR as the "mature" moiety.

PROTEIN TRANSLOCATION AND PROTEIN FOLDING IN MITOCHONDRIA

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Mitochondria contain import machineries for nuclear-encoded proteins in the outer and inner membranes of mitochondria. These machineries, termed MOM complex and MIM complex, usually cooperate in the translocation of preproteins into the matrix but they can be physically separated and their functions studied independently. With the MOM complex a *cis*-site and a *trans*-site for preprotein binding have been determined and the contributions of the individual MOM components (in particular MOM19, MOM22 and MOM72) have been studied. MOM19 and MOM22 cooperate in the binding of the targeting signals which then permits movement through a channel and binding to the *trans*-site. These reactions serve to initiate translocation and position the targetting signal such that it can be taken over by the import machinery of the inner membrane.

The MIM complex is believed to provide a channel which allows reversible sliding of the unfolded preprotein through the inner membrane. Insertion into this channel requires that the N-terminal targeting signal has moved into or across the inner membrane, triggered by $\Delta\Psi$. The MIM complex is coupled to the mt-Hsp70/MGE/ATP system in the matrix, thereby generating an energetic driving device. An important role is exerted by MIM44 which targets mt-Hsp70 to the incoming polypeptide chain. Binding to MIM44 and to the preprotein in transit requires the ATP form of mt-Hsp70; upon ATP hydrolysis mt-Hsp70 binds firmly to the preprotein but not to MIM44. MGE has a function in releasing ADP from mt-Hsp70 thereby regenerating binding competent mt-Hsp70. This machinery consisting of a "passive channel" linked to a system that renders movement unidirectional has been proposed to represent a "molecular ratchet".

The mitochondrial chaperone system mt-Hsp70, MDJ and MGE mediates folding of newly imported proteins in the matrix either by keeping them in a folding-competent state or/and by transferring them to the chaperonin system Hsp60/Hsp10 which actively supports folding.

Finally, preproteins must be sorted to the various subcompartments of the mitochondria. Several proteins of the inner membrane have been studied in some detail in this regard. These proteins are first imported through both MOM and MIM complexes and are then inserted into the inner membrane from the matrix side. This pathway, as well as the energetic requirements for membrane insertion, suggest that this sorting follows a "conservative mechanism": in several aspects it resembles sorting of plasma membrane proteins in prokaryotes, from which mitochondria presumably have evolved by endosymbiosis. This sorting of inner membrane proteins also resembles the sorting of some, but definitely not all, proteins of the intermembrane space, for which a "detour" through the matrix has been proposed.

IDENTIFICATION OF THE PROTEIN IMPORT MACHINERY OF CHLOROPLASTS

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A majority of chloroplast proteins are synthesized in the cytoplasm and posttranslationally imported into the organelle across the double membrane of the chloroplast envelope. We have identified a set of six envelope proteins that associate with distinct import intermediates generated during the import of a hybrid precursor protein. We refer to these proteins as IAPs (for import intermediate associated proteins). A subset of four of the six IAPs (IAP34, IAP75, hsp70 IAP and IAP86) form an outer envelope import complex that associates with precursors at an early stage in the import process. IAP86 and IAP34 are GTP-binding proteins. Precursor binding to the outer envelope requires GTP hydrolysis, suggesting a role for these two proteins in precursor recognition. IAP75 is an integral membrane protein with a structure suggestive of a channel protein. The hsp70 IAP is a molecular chaperone of the hsp70 family and is localized in the intermembrane space of the envelope. Two additional IAPs (IAP36 and IAP100) copurified with a late import intermediate that had been partially translocated and spanned both the outer and inner envelope membranes en route to the stroma. These two "late" IAPs are candidates for components of the import machinery of the chloroplast inner membrane.

ROLE OF A PEROXISOMAL TARGETING SIGNAL RECEPTOR IN PEROXISOMAL PROTEIN IMPORT AND IN HUMAN PEROXISOMAL DISORDERS.

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The matrix and membrane proteins of peroxisomes are encoded by nuclear genes, and most of these proteins are imported post-translationally. At least two peroxisome targeting signals (PTSs) are known to direct proteins to the peroxisome matrix. The major targeting signal, PTS1, is a conserved, C-terminal tripeptide (SKL or variants). A second targeting signal, PTS2, is an N-terminal, conserved peptide used by a smaller subset of proteins. There is both biochemical and genetic evidence in yeast and human cells for PTS1 and PTS2-dependent pathways of import. The PTS1 receptor of *Pichia pastoris* is encoded by the *PAS8* gene which belongs to the tetratricopeptide repeat (TPR) family. Yeast cells lacking PAS8p are deficient in the PTS1 import pathway but are competent for import via the PTS2-dependent pathway. The PAS8p protein is largely peroxisomal, behaves like an integral-membrane protein, faces the cytoplasmic side of the peroxisomes and binds directly to a PTS1 peptide. Three of the seven TPR motifs of PAS8p are sufficient to bind the PTS1 peptide. The human counterpart of PAS8p, called PTS1R, is primarily cytosolic but some is also associated with peroxisomes. The PTS1R protein binds the PTS1 peptide and is required for peroxisomal protein import. This gene also complements the PTS1-specific protein import defect in one complementation group (group 2) of fatal human peroxisomal disorders. Although cells from two patients tested in this group are defective in PTS1-specific import, cells from one of these patients (which do not contain any detectable PTS1R protein) are also impaired in PTS2 import, suggesting a possible connection between the PTS1 and PTS2-import pathways. This observation is intriguing in light of the ability of TPR proteins to interact with WD40 proteins, one of whose members (*S. cerevisiae* PAS7p, Marzioch et al., EMBO J. 13: 4908-18, 1994) is an excellent candidate for the PTS2 receptor. Some of the other components required for peroxisomal protein import have also been defined and will be discussed.

TRAFFIC ACROSS THE IMPERMEABLE PEROXISOMAL MEMBRANE IN *SACCHAROMYCES CEREVISIAE*

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Peroxisomes are small organelles of eukaryotic cells with distinct contributions to cellular metabolism. They are bounded by a single impermeable membrane, which allows formation of a specific niche for the enzymes residing in the peroxisomal matrix. In *S. cerevisiae* most peroxisomal matrix proteins are recognized in the cytoplasm by the PAS10 protein and subsequently targeted to the peroxisomal membrane for import. PAS10p interacts with peroxisomal targeting signals located at C-termini (PTS1). A few proteins contain a different, N-terminally located PTS2, the import of which is mediated by PAS7p.

A number of human diseases is caused by abnormalities in the biogenesis or function of peroxisomes. We investigated whether a deficiency to import proteins into the organelles could be the molecular basis for some of the observed cellular phenotypes. The protein import capability was tested by microinjecting plasmids encoding PTS1 and PTS2 specific reporter proteins into patient fibroblasts. We found a Zellweger patient cell line deficient in PTS1 specific protein import and rhizomelic chondrodysplasia punctata cell lines with a deficiency in PTS2 specific protein import. This similarity between yeast and fibroblast mutant phenotypes illustrates the usefulness of yeast to study this group of diseases in molecular detail.

Traffic across the peroxisomal membrane not only concerns proteins but also includes substrates for ß-oxidation and its products. Long chain and short chain fatty acids enter peroxisomes in different ways. Long chain fatty acids or their CoA\carnitine esters are actively transported across the peroxisomal membrane by the ABC transporter protein encoded by PAT1 (a putative homolog of the human X-ALD gene). We found no evidence for the presence in peroxisomes of an acyl-CoA synthetase with preference for long chain fatty acids. Hence, activation may occur already in the cytoplasm. Transport of short chain fatty acids is not dependent on PAT1p and activation occurs within peroxisomes by the matrix located acyl-CoA synthetase specified by FAA2.

The products of fatty acids ß-oxidation, NADH and acetyl-CoA, cannot leave the peroxisome as such. Peroxisomal malate dehydrogenase (MDH3) is essential to regenerate NAD^+ for continued ß-oxidation. Acetyl-CoA is converted to succinate via the glyoxylate cycle or is esterified to carnitine by peroxisomal carnitine acetyltransferase.

Combined, these results suggest that translocation carriers must exist in the peroxisomal membrane to allow proteins and long chain fatty acids to be imported and to allow malate, succinate and acetylcarnitine to be exported.

PROTEIN IMPORT INTO PEROXISOMES OF *SACCHAROMYCES CEREVISIAE*

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Isolation of *S. cerevisiae* mutants (15 complementation groups) (1, 2, 3) impaired in peroxisomal biogenesis has allowed to clone genes encoding proteins essential for this process. At present identification and location of their predicted gene products as well as their structure-function analysis are major points of interest. Three of these PAS proteins will be discussed.

- Pas7p (4): Is this protein the PTS2-receptor?
- Pas3p (5): An integral peroxisomal membrane protein-structure-function analysis.
- Pas9p : A novel peroxisomal membrane protein required for the biogenesis of the organelle.

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MOLECULAR-SCALE ANALYSIS OF KINESIN MOTOR MOVEMENT

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By marrying optical tweezers with a sensitive laser-based interferometer capable of determining position with subnanometer accuracy, we produced a device with the spatial and temporal sensitivity required to record movements of motor proteins on a molecular scale[1]. Using this instrument in conjunction with an *in vitro* motility assay[2], stepwise displacements are seen for single molecules of kinesin moving along microtubules[3]. Steps average ~8 nm in length and are completed in under 1 ms. By characterizing the restoring force generated by the optical trap, we were able to subject single proteins to calibrated, piconewton-sized forces. In this fashion, we measured forces generated by kinesin as functions of speed and ATP level[4]. The kinesin force-velocity relationship is roughly linear at both high and low ATP, producing a peak force of 5-6 pN that is roughly independent of ATP concentration. This finding suggests that kinetically-important rates in the mechanochemical cycle (e.g., the K_m for movement) may be load-independent, and that the coupling of motion to ATP hydrolysis may be variable. At low mechanical loads and high ATP levels, kinesin stepping is rapid (v~1000 nm/s) and individual steps cannot be discerned. In this regime, records of displacement were subjected to a variance analysis[5]. Surprisingly, the variance is smaller by a factor of ~2 than that anticipated for an exponential stepper moving on a regular 8 nm lattice. This result may indicate that the kinesin stepping motion involves two (or more) sequential, rate-limiting processes.

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3. Svoboda, K., *et al.*, *Nature* **365:** 721-727, 1993.
4. Svoboda, K. & S.M. Block, *Cell* **77**: 773-783, 1994.
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TARGETING OF CYTOPLASMIC DYNEIN WITHIN THE CYTOPLASM.

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Cytoplasmic dynein is a multi-subunit minus end-directed microtubule motor protein thought to be responsible for organelle translocation and some aspects of chromosome movement. Biochemical evidence has indicated that another multi-subunit complex, dynactin, stimulates dynein-driven organelle movement *in vitro*, and recent genetic evidence has suggested that cytoplasmic dynein and dynactin are on the same functional pathway. However, a direct interaction between the two complexes has not been observed, and the role of dynactin in dynein function remains uncertain.

To begin to understand the structural organization of the cytoplasmic dynein molecule, and to identify its functional components, our laboratory has cloned cDNAs encoding all of what we now understand to be three classes of cytoplasmic dynein subunit, the heavy chains (HCs, M_r = 532,000), intermediate chains (ICs, M_r = 74,000), and light intermediate chains (LICs ,M_r = 55 and 57 kD). The HCs are closely related to those of the axonemal dyneins and are responsible for catalytic activity. The ICs are related to the axonemal dynein ICs. The latter are located at the base of the dynein molecule and have been implicated in anchoring dynein to the A microtubules of the axonemal outer doublets. By analogy with the axonemal ICs, we have proposed that the cytoplasmic dynein ICs are responsible for organelle and possibly kinetochore binding. The LICs contain a P-loop element and exhibit partial homology to the ABC transporter ATPases in this region.

To identify potential organelle and kinetochore surface receptors for cytoplasmic dynein within the cell, we have expressed two forms of IC in *E. coli*. The recombinant ICs were used to probe total Rat2 cell lysates fractionated by SDS gel electrophoresis and transferred to nitrocellulose. A pair of bands was recognized at the position of the p150^{Glued} doublet. Reciprocal experiments in which blots were probed with the N-terminal half of p150^{Glued} revealed a single major band at the position of the ICs. The components of the dynactin complex were also found to coimmunoprecipitate with recombinant ICs.

These data represent the first evidence of a direct interaction between cytoplasmic dynein and dynactin. However, because both p150^{Glued} and the dynein HCs contain microtubule binding sites, we propose that the interaction must be regulated. We suggest that dynactin serves in docking or recognition between cytoplasmic dynein and membranes or kinetochores, a process which may be regulated by the dynein LICs.

In support of a direct interaction between the two complexes, we have now found marked co-localization of their component polypeptides on kinetochores of mitotic chromosomes. We have also found that overexpression of the prominent p50 subunit of dynactin in cultured cells disrupts the Golgi apparatus and the mitotic spindle. Supp. by GM43474 to RBV.

ANALYSIS OF NUCLEAR MIGRATION IN *ASPERGILLUS NIDULANS*

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Nuclear migration is an essential feature of morphological development in both higher and lower eukaryotes. To analyze this process we have identified and studied temperature sensitive (ts) mutations that prevent nuclear migration in the filamentous fungus *Aspergillus nidulans*. Three genes, *nudA*, *nudC* and *nudF* have been cloned by complementation of their temperature sensitivity. *nudA* encodes a protein with 52% overall sequence identity to the heavy chain of vertebrate cytoplasmic dynein. Antibodies raised against *nudA* fusion proteins stained a large molecular weight protein on Western blots. This protein, which was depleted in extracts from *nudA* mutants, presumably represents the heavy chain of cytoplasmic dynein. Thus, cytoplasmic dynein appears to be the primary motor that mediates nuclear migration in *Aspergillus nidulans*. *nudC* encodes a 22 kDa, evolutionarily conserved protein domain similar to the C-terminal region of a 45 kDa protein found in *Drosophila* and rat. The C-terminal domain of the *Drosophila* protein complemented the temperature sensitivity of the *A. nidulans nudC3* mutation, demonstrating that the domain is functionally conserved. The *nudF* gene was initially identified as an extracopy suppressor of *nudC3*, suggesting that *nudC and* nudF interact in some way. Antibodies raised against *nudF* fusion protein identified a 49 kDa protein on Western blots that was depleted in *nudC3* mutants at restrictive temperature. Ultracentrifugation of *A. nidulans* proteins indicates that *nudF* sediments faster than its molecular weight would suggest. It therefore may be part of a protein complex. It does not cosediment with the NUDC protein. We have isolated extragenic suppressors of *nudC3* that restore the intracellular concentration of NUDF protein and restore growth. These data indicate that maintenance of NUDF protein levels is essential for nuclear migration, but its biochemical effect is unknown. *nudF* encodes a WD-40 protein with an N-terminal coiled-coil motif. It is 42% identical to the human LIS-1 gene, whose deletion causes failure of brain development (Miller-Dieker lissencephaly) due to impaired neuronal migration. A possible relationship between nuclear migration and neuronal migration will be discussed.