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Analysis of differential protein expression in normal and neoplastic human breast epithelial cell lines

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Abbreviations: HMEC, human mammary epithelial cell; 2DE, two-dimensional gel electrophoresis, TPA, 12-*O*-tetradecanoyl-13-phorbol acetate; PMSF, phenylmethylsulfonyl fluoride.

Abstract

High resolution two dimensional gel electrophoresis (2DE) and database analysis was used to establish protein expression patterns for cultured normal human mammary epithelial cells and thirteen breast cancer cell lines. The Human Breast Epithelial Cell database contains the 2DE protein patterns, including relative protein abundances, for each cell line, plus a composite pattern that contains all the common and specifically expressed proteins from all the cell lines. Significant differences in protein expression, both qualitative and quantitative, were observed not only between normal cells and tumor cells, but also among the tumor cell lines. Eight percent (56/727) of the consistently detected proteins were found in significantly ($P < 0.001$) variable levels among the cell lines. Using a combination of immunostaining, comigration with purified protein, subcellular fractionation, and amino-terminal protein sequencing, we identified a subset of the differentially expressed proteins. These identified proteins include the cytoskeletal proteins actin, tubulin, vimentin, and cytokeratins. The cell lines can be classified into four distinct groups based on their intermediate filament protein profile. We also identified heat shock proteins; hsp27, hsp60, and hsp70 varied in abundance and in some cases in the relative phosphorylation levels among the cell lines. Finally, we identified IMP dehydrogenase in each of the cell lines, and found the levels of this enzyme in the tumor cell lines elevated 2- to 20-fold relative to the levels in normal cells.

Introduction

Studies of human breast cancer, including the effects of chemotherapeutic drugs, cell biology, and cellular differentiation, typically use cell lines derived from effusions from patients with metastatic carcinoma of the breast. These tumor cell lines are heterogeneous, varying in such diverse aspects as cellular morphology, growth rate, tumorigenicity in nude mice, steroid hormone receptor status, and drug sensitivity. Two of the most commonly used cell lines are the estrogen-receptor-positive MCF7 and the estrogen-receptor-negative MDA-MB231 (1, 2). Cellular responses to external stimuli depend on a variety of cellular properties, including both well characterized properties such as p53 or estrogen-receptor status, and also undefined characteristics particular to a specific cell line. In some cases, the specific property of a cell line can be associated with a response, as in the estrogen-receptor-dependent inhibition of proliferation by retinoic acid treatment (3,4). On the other hand, cell-specific responses to a variety of other stimuli are often less straightforward. For example, epidermal growth factor induces transcription of the *ras*-related *rhoB* gene in MDA-MB231 cells, but not in MCF7 and ZR75-1 cells (5). In MDA-MB231 cells, expression of acidic fibroblast growth factor mRNA is decreased in the absence of serum, but expression is unaffected in MCF7 cells (6). The protein kinase C activators TPA and bryostatin 1 also have cell-specific effects on growth in human breast cancer cell lines (7). Such differences in cellular responses lead to the conclusion that there is significant and complex variability among human breast carcinoma cells and a more complete characterization of these cell lines will help identify the cellular factors responsible for such differences.

High-resolution two-dimensional gel electrophoresis (2DE) can be used to measure the abundance of over 500 polypeptides in a single sample. Although numerous comparisons have been made between pairs of normal and neoplastic cells using 2DE, a global comparison of the proteins present in normal human breast epithelial cells and those in the most widely used breast tumor cell lines has not been accomplished. We describe the comparative mapping by 2DE of proteins found in normal breast epithelial cells (cultured from reduction mammoplasty and grown in short-term culture) and proteins found in 13 tumor cell lines (originally derived from patients

with breast cancer and maintained in long-term culture). The goals of this research are to define patterns of protein expression characteristic of normal and neoplastic cells and to characterize differences in protein expression among tumor cell lines that could influence their response to external stimuli such as chemotherapeutic drugs and be predictors of therapeutic response. We found significant differences in protein expression, both qualitative (i.e., presence or absence) and quantitative, not only between normal cells and tumor cells but also among the tumor cell lines. A composite human breast cell protein pattern has been created that includes all the common and specifically expressed proteins in the normal and neoplastic cells analyzed. We have identified a subset of the differentially expressed proteins that includes proteins known to be important in proliferation, differentiation, and cellular responses to chemotherapeutic chemicals. This subset includes components of the cytoskeleton, stress proteins, and the enzyme inosine-5'-monophosphate (IMP) dehydrogenase.

Materials and Methods

Cells and cell culture. The tumor cell lines used in this study were originally obtained from American Type Culture Collection, Rockville, MD (BT549, DU4475, HS578T, MCF7, MDA-MB157, MDA-MB231, MDA-MB435s, MDA-MB436, MDA-MB453, MDA-MB468, ZR75-1) or Naval Biosciences Laboratory, Oakland, CA (AU565, BJ015) and are now routinely cultured at Argonne National Laboratory. Normal human mammary epithelial cells (HMEC), obtained from reduction mammoplasty tissue, were purchased at passage 7 from Clonetics (San Diego, CA). The tumor cell lines were cultured in RPMI 1640 supplemented with 15% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and 2 mM l-glutamine (Gibco-BRL, Gaithersburg, MD). HMEC were maintained (passages 8-10) in serum-free MEGM (Clonetics). Cells were cultured at 37 °C in a humidified atmosphere of 6% CO_2 in air. Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium, pH 7.5, was used for all washes.

Sample Preparation. For whole lysates, cultured cells were harvested by treatment with trypsin (0.05% trypsin and 0.2% EDTA; Gibco), washed three times in PBS, and counted using a hemocytometer. The cell pellets were solubilized in NP40-urea mix containing 9 M urea, 4%

Nonidet P-40, 2% ß-mercaptoethanol, and 2% ampholytes (pH 8-10) at a concentration of 6 x 10^4 cells/µl. Pure proteins were dissolved in NP40-urea mix to obtain a final protein concentration of 1 mg/ml. Protein concentrations were determined using a modified Bradford protein assay (8).

Cytoskeleton Preparation. Cells were plated at 5 x 10^5 per well in 24 well plates 24 h. prior to the experiment. Complete cytoskeletons were isolated by incubating the cells in 100 mM PIPES, pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA, 2 M glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM pepstatin, and 0.5 % NP-40 at room temperature for 5 min. The buffer was removed and the wells were washed with the buffer minus NP-40. Cytoskeleton preparations were solubilized in 50 µl NP40-urea mix per well. Cytoskeletons without microtubules were prepared by incubating the cells for 5 min at room temperature in 100 mM PIPES, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 2 M glycerol, 1 mM PMSF, 1 mM pepstatin, and 0.5% NP40. The wells were rinsed in the buffer minus NP-40 and proteins were solubilized in 50 µl of NP40-urea mix. Intermediate filaments were isolated by incubation in 0.1 M PIPES, 1 mM $MgSO_4$, 2 mM EDTA, 2 M glycerol, 1 mM PMSF, 1 mM pepstatin, and 0.5% NP40, followed by washing with low-salt solution (60 mM KCl, 1 mM EDTA, 2 mM EGTA, 1 mM cysteine, 10 mM ATP, 40 mM imidizole) for 1 min, high-salt solution (0.6 M KCl, 1 mM EDTA, 1 mM cysteine, 2 mM ATP, and 40 mM imidizole) for 1 min and then low-salt solution for 1 min prior to solubilization in 50 µl of NP40-urea mix per well.

Two-dimensional Gel Electrophoresis. Samples containing 35 µg of breast cell lysate proteins or 20 µl of cytoskeletal proteins were separated by isoelectric focusing (IEF) in rod gels containing 50% pH 5-7 and 50% pH 3-10 ampholytes, as described previously (9). The second-dimension separation was done using 10-17% linear gradient SDS-PAGE (10). Proteins were detected by silver staining (11). For comigration experiments, 50 ng of purified protein was loaded alone or in combination with 35 µg of cell lysate proteins onto an isoelectric focusing gel.

Data Acquisition and Analysis. Silver-stained gels were digitized using an Eikonix 1412 CCD scanner. The images were processed and spot files were created using Tycho II software (12).

The class statistics capability of the GR42x software was used to detect significant differences in protein abundance between groups.

Protein identification. Proteins were separated by 2DE as described above, except that 300 µg of protein was used per gel. For immunostaining, gels were equilibrated in 10 mM CAPS buffer, 10% methanol for 10 min and the proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) via electroblotting (13). Anti-vimentin (V-5255), anti-pan-cytokeratin (C2562), and anti-cytokeratin 19 (C-6930) were from Sigma (St. Louis, MO). Anti-hsp27 was from Calbiochem (Cambridge, MA). Enhanced chemiluminescent detection (Boehringer-Mannheim, Indianapolis, IN) and colorimetric detection using 4-chloro-1-naphthol (Sigma) were performed according to the manufacturer's protocols. Purified human inosine 5'-monophosphate dehydrogenase was kindly provided by Frank Collart, Argonne National Laboratory. For amino-terminal sequencing, proteins were electroblotted to ProBlott membranes (Applied Biosystems, Foster City, CA) and stained with Coomassie Brilliant Blue R-250. The regions of the membrane containing the protein of interest were excised and sequencing was performed by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University, New Haven, CT.

Results

The 2DE image shown in Figure 1 is the human breast epithelial cell master pattern. The 2DE pattern of proteins found in HMEC whole cell lysates was selected as the basis for this master pattern. The proteins found in replicate patterns of each tumor cell line were then matched to the master pattern. When a protein was found in the 2DE patterns of a tumor cell line that was not in the master pattern, it was added. Thus, the human breast epithelial cell master pattern is a composite, showing the proteins found in normal HMEC and in the breast tumor cell lines AU565, BJ015, BT549, DU4475, HS578t, MCF7, MDA-MB157, MDA-MB231, MDA-MB435s, MDA-MB436, MDA-MB453, MDA-MB468, and ZR75-1. Each individual pattern comprises over 1200 protein spots, with an average of 727 of these spots matched to the master pattern.

The class statistics capability of the GR42x software (12) was used to detect significant differences in protein abundance between cell lines. A comparison of the protein expression profiles of normal and neoplastic cells revealed 56 proteins that varied significantly ($P < 0.001$) in abundance between the cell lines. These proteins are highlighted in Figure 1. Eight proteins (MSNs 165, 240, 600, 943, 944, 1018, 3067, and 3226) consistently detected in normal mammary epithelial cell lysates were undetectable in any of the tumor cell lines (Figure 1, circles). A second subset of proteins (MSNs 3729, 3750, 3751, 3752, and 3753) were found in some tumor lines but were absent in normal cells (Figure 1). The remaining differentially expressed proteins were found in normal cells and some of the tumor cell lines. The relative abundances of this latter group of proteins in each cell line are shown in Figure 2.

Cytokeratins, a family of intermediate filament proteins, are components of the cytoskeleton in virtually all epithelial cells. The expression pattern of cytokeratins can be used to characterize a specific epithelial cell type (14). In the human mammary gland, the cytokeratin profile has been used to distinguish luminal cells from basal cells. Cytokeratins 7, 8, 18, and 19 are luminal cell markers, and cytokeratins 5, 14, and 17 are basal cell markers (15). On a two-dimensional gel, the cytokeratin polypeptides are usually seen as a series of isoelectric variants representing phosphorylated forms of the protein (14, 16, 17). We used two methods to identify the cytokeratins in the human breast cell 2DE patterns. In one method, we isolated cytoskeletons enriched for intermediate filaments from each cell line and separated the proteins using 2DE. In these intermediate filament preparations, specific cytokeratins were identified by comparison with the 2DE migration patterns of cytokeratins described by Moll (18). In the second method, we used a monoclonal antibody recognizing cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19 to identify cytokeratins on 2DE Western blots of cell lysates.

The expression of cytokeratin proteins was highly variable in the human breast cell lines. While the HMEC cells contain abundant cytokeratins 14 and 17 (basal markers) and 8 and 18 (luminal markers), cytokeratins 14 and 17 were undetectable in any of the breast carcinoma cell lines (Figure 3). The cytokeratins 8, 18, and 19 were extremely abundant in MCF7, MDA-MB468, AU565, and BJ015. Cell lines MDA-MB157, MDA-MB231, MDA-MB453, and

ZR75-1 expressed cytokeratins 8 and 18 also, but at significantly lower levels. Of these four cell lines, MDA-MB453 and ZR75-1 also expressed cytokeratin 19. Four cell lines (BT549, HS578t, MB435s, and MB436) did not contain any detectable cytokeratin protein, as determined by both gel analysis and immunostaining. A representative of each of these four classes is shown in Figure 3. The tumor cell lines also varied greatly in the total amount of cytokeratin protein present (Figure 4).

The intermediate filament protein vimentin is primarily expressed in cells of mesenchymal origin and in a variety of tumors and transformed cell lines (19), including some breast cell lines (20). Immunostaining with a monoclonal antibody to vimentin and comparison with the 2DE migration patterns of intermediate filament preparations (18) identified protein MSN 2207 as vimentin. A subset of the tumor cell lines expressed vimentin. The four cell lines which did not contain any cytokeratins (BT549, HS578t, MB435s, and MB436) all expressed vimentin. Vimentin was also expressed in normal HMEC and in the tumor lines MB157 and MB231. The abundance of vimentin protein within these cell lines varied significantly ($P < 0.001$, Figure 4). Vimentin was approximately twice as abundant in HS578t as in the other vimentin-positive tumor cell lines. Without exception, cell lines that expressed cytokeratin 19 did not express vimentin. Cell lines expressing cytokeratin 19 appeared rounded, whereas those lacking cytokeratin 19 appeared more elongated and spindle-shaped. DU4475, which grows in suspension rather than as attached cells, was unique among the cytokeratin-expressing tumor cell lines in that it contained cytokeratin 8 and 18 but not cytokeratin 19 or vimentin.

Tubulin (Tub, Figure 1) was identified by its localization in cytoskeleton fractions enriched for microtubules (rather than for intermediate filaments) and by comparison with published 2DE patterns (21). Actin (Act, Figure 1), the major component of microfilaments, was identified based on its enrichment in microfilament preparations and by comparison with published 2DE patterns. In contrast to the intermediate filament proteins vimentin and cytokeratin, tubulin and actin did not differ significantly ($P < 0.001$) in abundance among the cell lines (Figure 4).

Inosine-5'-monophosphate dehydrogenase, the rate limiting enzyme in the *de novo* synthesis of guanine nucleotides. is often present at elevated levels in cancer cells. IMP dehydrogenase was identified in the 2DE patterns of breast cells by comparing the migration of purified IMP dehydrogenase with that of proteins in MCF7 lysates. Figure 5 shows the silver-stained pattern obtained from an MCF7 lysate, purified IMP dehydrogenase, and the comigration of pure IMP dehydrogenase plus MCF7. The corresponding protein spot numbers in the master pattern are MSN 2982 and MSN 2312. The more basic form of the protein, MSN 2312, could not be reliably detected in the patterns of all cell lines because of the close proximity of another protein which obscured this spot in some patterns. However, comparison of the levels of MSN 2982 in the different cell lines revealed a dramatic variation in the abundance of IMP dehydrogenase (Figure 6). The level of IMP dehydrogenase was elevated in all of the tumor cell lines relative to HMEC. Two cell lines, BJ015 and BT549 contained approximately twice the amount of IMP dehydrogenase found in HMEC. ZR75-1 contained the highest level in the cell lines analyzed, with almost 20 times as much IMP dehydrogenase as HMEC. The other 10 tumor cell lines had IMP dehydrogenase protein levels varying from 4- to 10-fold that of HMEC.

Three highly expressed proteins found to vary significantly in abundance, MSNs 25, 42, and 135, were excised from PVDF membranes and subjected to N-terminal sequencing. The N-terminal amino acid sequences of MSN25: AKDVKFGABA(B)(A)LMX(Q)XV and MSN 42: AKDVKFGABA(B)(A)LXLQ(G)V were almost identical. A protein database search indicated a match to heat shock protein 60 (hsp60) from various sources. The levels of each of the two isoforms of hsp60 varied three-fold among the cell lines (Figure 7). However, in each cell line the predominant form of hsp60 is the basic isoform. MSN 135 failed to yield sequence information.

Hsp27 was identified by immunostaining a 2DE western blot of a ZR75-1 lysate using an anti-hsp27 antibody. Three protein spots (Figure 8), corresponding to MSN 135, MSN 133, and MSN 2066 in the master pattern, were identified. The charge variants observed are most likely the result of varying degrees of phosphorylation (22) and are denoted by primes (',"). The most acidic form, hsp27" (MSN 2066), was found in low abundance in a subset of cell lines and could not be consistently detected in silver-stained gels. The two additional isoforms, hsp27 (MSN

133) and the hsp27' (MSN 135) were found to vary in abundance up to 25-fold among the cell lines analyzed (Figure 7). MSN 135 was undetectable in MDA-MB453, and the level of MSN 133 in this line was much lower than seen in any of the other cell lines. HMEC showed an intermediate level of both isoforms relative to the tumor cell lines. The ratio of hsp27 to hsp27' was not consistent. Seven of the cell lines, including HMEC, contained similar levels of each isoform. The remaining six cell lines contained two to three times more of hsp27, the more basic form, than hsp27'.

Quantitative analysis of hsp70, previously identified by N-terminal sequence analysis to be MSN 14 (21), revealed significant ($P < 0.005$) differences in its abundance between some of the cell lines (Figure 7). Hsp73, also known as hsc70, was identified in the master pattern as MSN 13 by comparison to a published 2DE image of MDA-MB231 cell proteins (23). We found no significant differences in the abundance of hsp73 among any of the cell lines.

Discussion

We investigated the differential expression of proteins in human breast epithelial cells in order to define and characterize differences that could influence cellular responses to various stimuli and that may ultimately be important in the selection of the most useful or appropriate cell lines in breast cancer research. Protein expression in normal breast epithelial cells and 13 breast tumor cell lines was analyzed using 2DE combined with immunostaining, protein sequencing, and database analysis. We found significant differences in protein expression, both qualitative and quantitative, not only between normal cells and tumor cells but also among the tumor cell lines. Almost 8% of the matched proteins were found in significantly ($P < 0.001$) different levels among the cell lines. Only two proteins could be consistently detected in some tumor cell lines but were absent in the normal HMEC: cytokeratin 19 (MSNs 3750, 3751, 3752, 3753) and MSN 3729. Eight proteins were HMEC-specific and may be specific to basal epithelial cells. Human breast tissue is comprised of both basal and luminal epithelial cells. The HMEC cultures used in this study are a mixed population of both cell types, and at the passages used (8-10) they display a predominantly luminal phenotype. Invasive breast cancer cells from primary cancers appear to have the same phenotype as the differentiated luminal epithelial cell

(15). Kao *et al.* (24) have described a method to separate normal breast cells from reduction mammoplasty into basal and luminal cell types in culture. Using this technique, protein patterns from basal cells and luminal cells will be compared in future studies to verify the basal-cell specificity of the eight HMEC-specific proteins.

The majority of the differences in protein expression detected, however, were quantitative rather than qualitative. These differences, in contrast to the small number of qualitative differences observed, did not distinguish normal cells from tumor cells but were found among the tumor cell lines. In some cases, e.g., the intermediate filament proteins, cell lines could be grouped according to their protein expression profiles. However, we detected no obvious trends in the distribution of the forty-two as yet unidentified proteins that are found in significantly different levels among the cell lines. These differences in protein expression underscore the heterogeneous nature of breast tumor cell lines.

We identified components of the cytoskeleton by using antibodies against intermediate filament proteins and also by subcellular localization. The cytoskeleton, in addition to its structural function, also interacts with elements of second-messenger pathways (25), altering the activity of proteins or sequestering proteins in the cytoplasm. The abundance of intermediate filament proteins in the breast tumor lines, as well as their composition, varied dramatically, and the cell lines could be classified into four groups. The HMEC contained cytokeratins characteristic of both basal cells (cytokeratins 14 and 17) and luminal cells (cytokeratins 8 and 18), as has been previously reported (15). A second group of cell lines (MCF7, MDA-MB468, AU565, BJ015) contained high levels of cytokeratins 8, 18, and 19. Four cell lines (BT549, HS578t, MDA-MB435s, MDA-MB436) did not contain any detectable cytokeratin. The fourth group (MDA-MB453, ZR75-1, MDA-MB157, MDA-MB231) expressed intermediate levels of cytokeratins.

Each of the cytokeratin-negative cell lines contained the intermediate filament protein vimentin. MDA-MB157 and MDA-MB231 were the only cytokeratin-containing tumor lines that also expressed vimentin. We found cytokeratin protein in MDA-MB157 and MDA-MB231, in contrast to a report by Sommers *et al.* (20) who detected no cytokeratin in those cell

lines using immunofluorescence. The pan-cytokeratin antibodies used in their study did not recognize cytokeratin 18, only cytokeratins 8 and 19. In our work, 2DE of intermediate filament preparations as well as immunostaining of 2DE western blots with a pan-cytokeratin antibody recognizing cytokeratins 8, 18, and 19 revealed low levels of cytokeratins 8 and 18 in these two cell lines. The absence of vimentin expression was correlated with cytokeratin 19 expression: cell lines that expressed cytokeratin 19 did not express vimentin. In accordance with Sommer's data, cell shape was correlated with vimentin, and therefore cytokeratin 19, expression. Cell lines expressing cytokeratin 19 appeared rounded, whereas those lacking cytokeratin 19 appeared more elongated and spindle-shaped. The DU4475 cell line, which grows in suspension, was unique among the cytokeratin-expressing tumor cell lines in that it contains cytokeratins 8 and 18, but not cytokeratin 19 or vimentin.

The capability of intermediate filaments to alter the effects of chemotherapeutic agents is becoming increasingly clear. The DNA-binding anticancer drug cisplatin has been found to interact with microtubular and intermediate filament portions of the cytoskeleton. A cisplatin-resistant human ovarian adenocarcinoma cell line contained markedly lower levels of cytokeratin 8 than the parent cell line. Restoration of cytokeratin 8 by transfection with a cytokeratin 8 cDNA clone resulted in increased sensitivity to cisplatin (26). Transfection of a mouse fibroblast cell line with cytokeratins 8 and 18 conferred multidrug resistance in the absence of altered drug accumulation in the cell (27). In addition to its structural role as part of the cytoskeleton, cytokeratin 8 has recently been found to be expressed on the external cell surface, to possess plasminogen-binding activity, and to promote plasminogen activation (28, 29).

IMP dehydrogenase is the rate limiting enzyme in the *de novo* synthesis of guanine nucleotides and therefore is involved in the regulation of cell growth. IMP dehydrogenase inhibitors induce cellular differentiation and inhibit cell growth and are thus targeted as potential chemotherapeutic agents (30). We identified IMP dehydrogenase in 2DE patterns of human breast epithelial cells and found the levels of this enzyme elevated in each of the breast cancer cell lines relative to HMEC. The levels of this enzyme in the tumor cell lines is heterogeneous, varying up to 20-fold. Cell lines with more rapid doubling times, e.g. AU565, MDA-MB231,

MCF7, and ZR75-1, all have highly elevated levels of IMP dehydrogenase. This finding may be of use in the clinical setting for differentiating slow-growing from fast-growing tumors.

The members of the heat shock, or stress, protein family are highly conserved in structure and function and have diverse roles in protein import, folding, and assembly, immunity, and cancer. We have identified the prominent heat shock proteins in the 2DE patterns of breast cells and quantified the levels of expression of these proteins in unstressed normal and neoplastic cells. Hsp70, one of the most inducible of the heat shock proteins, is involved in the chaperoning of nascent polypeptides and changes in conformation necessary for translocation to the endoplasmic reticulum and mitochondria. The basal levels of this protein showed quantitative differences in only two cell lines, AU565 and MDA-MB231. The levels of hsp73, the constituitively expressed cognate of hsp70, did not vary significantly among any of the cell lines. Hsp60 is a mitochondrial matrix protein that functions in protein folding and assembly (31). The two isoforms of this protein varied threefold among the cell lines. However, in each cell line the predominant form of hsp60 is the basic isoform, which is likely the unphosphorylated form.

Hsp27 is a cytoplasmic protein with roles in stress resistance, microfilament organization, chaperone activity, and thermotolerance (22). It is induced by environmental stresses (such as heat shock and toxic chemicals) and by estrogen stimulation in MCF7 cells (32). Elevated hsp27 levels in breast cancer have been correlated with estrogen receptor content (33). In this study of 13 breast carcinoma cell lines, the two estrogen-receptor positive cell lines, MCF7 and ZR75-1, contained the highest levels of hsp27. However, two estrogen-receptor negative cell lines, AU565 and BJ015, contained approximately the same amount of hsp27 protein as MCF7 cells. Hsp27 is phosphorylated in response to environmental stress, mitogens, tumor promoters such as phorbol esters, and cytokines (22, and references therein). The overexpression of hsp27 confers doxorubicin resistance in breast cancer cells (34). Knowledge about the differential basal expression and phosphorylation of hsp27 in these cell lines may be useful in selecting appropriate cell lines for studying cellular responses to anticancer drugs and drug resistance.

The human breast epithelial cell master pattern includes common and specifically expressed proteins from normal breast epithelial cells and from a panel of breast carcinoma cell lines. Comparison of 2DE protein patterns provides information about many proteins simultaneously, including proteins that may be involved in neoplastic transformation, signal transduction pathways, and in the cellular response to chemicals. The global surveillance of protein synthesis, modification, and degradation using 2DE promises to be useful in identifying significant differences in protein expression and in monitoring the effects of chemotherapeutic agents in breast cancer cells.

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References

1. Soule, H. D., Vasquez, J., Long, A., Albert, S., and Brennan, M. A human cell line from a pleural effusion derived from a breast carcinoma. J. Natl. Cancer Inst., *51:* 1409-1416, 1973.

2. Cailleau, R., Young, R., Olive, M., and Reeves, W. J.: Breast tumor cell lines from pleural effusions. J. Natl. Cancer Inst., *53:* 661-674, 1974.

3. Marth, C., Mayer, I., and Daxenbichler, G.: Effect of RA and 4-hydroxytamoxifen on human breast cancer cell lines. Biochem. Pharmacol., *33:* 2217-2221, 1984.

4. Fontana, J.A.: Interaction of retinoids and tamoxifen of the inhibition of human mammary carcinoma cell proliferation. Exp. Cell Biol., *55:* 136-144, 1987.

5. de Cremoux, P., Gauville, C., Closson, V., Linares, G., Calvo, F., Tavitian A., Olofsson, B.: EGF modulation of the *ras*-related *rhob* gene expression in human breast cancer cell lines. Int. J. Cancer, *59:* 408-415, 1994.

6. Renaud, F., El Yazidi, I., Boilly-Marer, Y., Courtois, Y., and Laurent, M.: Expression and regulation by serum of multiple FGF1 mRNA in normal transformed and malignant human mammary epithelial cells. Biochem. Biophys. Res. Comm., *219:* 679-685, 1996.

7. Kennedy, M. J., Prestigiacomo, L. J., Tyler, G., Stratford, M., and Davidson, N. E.: Differential effects of bryostatin 1 and phorbol ester on human breast cancer cell lines. Cancer Res., *52:* 1278-1283, 1992.

8. Ramagli, L. S. and Rodriguez, L. V.Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis *6:* 559-563, 1985.

9. Anderson, N. L. and Anderson, N. G. Analytical techniques for cell fractions: XXI. Two-dimensional analysis of serum and tissue proteins: multiple isoelectric focusing. Anal. Biochem., *85:* 331-340, 1978.

10. Anderson, N.L. and Anderson, N.G. Analytical techniques for cell fractions: XXII. Two-dimensional analysis of serum and tissue proteins: multiple gradient slab-gel electrophoresis. Anal. Biochem., *85:* 341-354, 1978.

11. Giometti, C. S., Gemmell, M. A., Tollaksen, S. L., and Taylor, J. Quantitation of human leukocyte proteins after silver staining: a study with two-dimensional electrophoresis. Electrophoresis, *12:* 536-543, 1991.

12. Giometti, C.S. and Taylor, J. : The application of two-dimensional electrophoresis to mutation studies. *In:* A. Chrambach, M. J. Dunn, and B. J. Radola (eds.), Advances in Electrophoresis, pp. 359-389. Advances in Electrophoresis. New York: VCH Publishers, 1991.

13. Matsudaira, P. J. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. Biol. Chem. *262:* 10035-10038, 1987.

14. Franke, W. W, Schiller, D. L., Moll, R., Winter, S., Schmid, E., Engelbrecht, I., Denk, H., Krepler, R., and Platzer, B. Diversity of cytokeratins: differentiation specific expresson of cytokeratin polypeptides in epithelial cells and tissues. J. Mol. Biol., *153:* 933-959, 1981.

15. Taylor-Papadimitriou, J., Stampfer, M., Bartek, J., Lewis, A., Boshell, M., Lane, E. B., Leigh, I. M. Keratin expression in human mammary epithelial cells cultured from normal and malignant tissue: relation to *in vivo* phenotypes and influence of medium. J. Cell Sci., *94:* 403-413, 1989.

16. Sun, T-T., and Green, H. Keratin filaments of cultured human epidermal cells. Formation of intermolecular disulfide bonds during terminal differentialtion. J. Biol. Chem., *253:* 2053-2060, 1978.

17. Gilmartin, M. E., Culbertson, V. B. and Freedberg, I. M. Phosphorylation of epidermal keratins. J. Invest. Dermatol. ,*75:* 211-216, 1980.

18. Moll, R., Franke, W. F., and Schiller, D. L. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell, *31:* 11-24, 1982.

19. Fuchs, E., and Weber, K. Intermediate filaments: structure, dynamics, function, and disease. *In:* C. C. Richardson (ed.), Annual Review of Biochemistry, pp. 345-82. Palo Alto, CA: Annual Reviews Inc., 1994.

20. Sommers, C. L., Walker-Jones, D., Heckford, S. E., Worland, P., Valverius, E., Clark, R., McCormick, F., Stampfer, M., Abularach, S., and Gelmann, E. P. Vimentin rather than keratin expression in some hormone-independant breast cancer cell lines and in oncogene-transformed mammary epithelial cells. Cancer Res., *49:* 4258-4263, 1989.

21. Giometti, C. S., Tollaksen, S., Chubb, C., Williams, C., and Huberman, E. Analysis of proteins from human breast epithelial cells using two-dimensional gel electrophoresis. Electrophoresis, *16:* 1215-1224, 1995.

22. Arrigo, A.-P. and Landry, J. Expression and function of the low-molecular weight heat shock proteins. *In:* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (eds.), The Biology of Heat Shock Proteins and MolecularChaperones, pp 335-374. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1994.

23. Franzen, B., Linder, S., Akaiya, A. A., Eriksson, E., Uryu, K., Hirano, T., Okuzawa, K., Kato, H., and Auer, G. U. Analysis of polypeptide expression in benign and malignant human breast lesions: down-regulation of cytokeratins. Brit. J. Cancer, *74:* 1632-1638, 1996.

24. Kao, C., Nomata, K., Oakley, C. S., Welsch, C. W., and Chang, C. Two types of normal human breast epithelial cells derived from reduction mammoplasty: phenotypic characterization and response to SV40 infection. Carcinogenesis, *16:* 531-538, 1995.

25. Rosales, C., O'Brien, V., Kornberg, L., and Juliano, R. Signal transduction by cell adhesion receptors. Biochim. Biophys. Acta, *1242:* 77-98, 1995.

26. Parekh, H. K. and Simpkins, H.: The differential expression of cytokeratin 18 in cisplatin-sensitive and -resistant human ovarian adenocarcinoma cells and its association with drug sensitivity. Cancer Res., *55:* 5203-5206, 1995.

27. Bauman, P. A., Dalton, W. S., Anderson, J. M., and Cress, A. E. Expression of cytokeratin confers multiple drug resistance. Proc. Natl. Acad. Sci., *91:* 5311-5314, 1994.

28. Hembrough, T. A., Vasudevan, J., Allietta, M. M., Glass W. F., Gonias, S. L. A cytokeratin 8-like protein with plasminogen-binding activity is preasent on the external surfaces of hepatocytes, HepG2 cells and breast carcinoma cell lines. J. Cell Sci., *108:* 1071-1082, 1995.

29. Hembrough, T. A., Kralovich, K. R., Li, L., and Gonias, S. L. Cytokeratin 8 released by breast carcinoma cell *in vitro* binds plaminogen and tissue-type plasminogen activator and promotes plasminogen activation. Biochem. J., *317:* 763-769, 1996.

30. Kiguchi, K., Collart, F. R., Henning-Chubb, C., and Huberman, E. Induction of cell differentiation in melanoma cells by inhibitors of IMP dehydrogenase: altered patterns of IMP dehydrogenase expression and activity. Cell Growth Differ., *1:* 259-270, 1990.

31. Langer, T. and Neupert, W.: *In:* R. I. Morimoto, A. Tissieres, and C. Georgopoulos (eds.), The Biology of Heat Shock Proteins and Molecular Chaperones, pp 53-84. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1994.

32. Fuqua, S. A. W., Blum-Salingaros, M., and McGuire, W. L. Induction of the estrogen-regulated "24K" protein by heat shock. Cancer Res., *49:* 4126-4129, 1989.

33. Seymour, L., Bezwoda, W. R., Meyer, K., and Behr, C. Detection of P24 protein in human breast cancer: influence of receptor status and oestrogen exposure. Br. J. Cancer, *61:* 886-890, 1990.

34. Oesterrich, S., Weng, C. N., Qui, M., Hilsenbeck, S. G., Osborne, C. K., and Fuqua, S. A. The small heat shock protein hsp27 is correlated with the growth and drug resistance in human breast cancer cell lines. Cancer Res., *53:* 4443-4448, 1993.

Figure Legends

Figure 1. The two-dimensional gel electrophoresis master pattern of human breast epithelial proteins. This computer image shows the composite pattern containing all the common and specifically expressed proteins found in the cell lines used in this study. Proteins that vary significantly ($P < 0.001$) in abundance between the cell lines are highlighted in orange and yellow. Proteins found only in HMEC cells are circled. Act, actin; Tub, beta-tubulin; Vim, vimentin; ck, cytokeratin; hsp, heat shock protein; IMPDH, inosine-5'-monophosphate dehydrogenase; MSN, master spot number.

Figure 2. Histograms showing the abundance of specific breast cell proteins in each of the cell lines. Each box represents a different protein, highlighted in Figure 1, that varied in abundance between the cell lines. The average integrated density for the spot is shown along the Y-axis, which is different for each protein. The bars of each histogram represent (from left to right): HMEC, AU565, BJ015, BT549, DU4475, HS578t, MDA-MB157, MDA-MB231, MDA-MB435s, MDA-MB436, MDA-MB453, MDA-MB468, MCF7, and ZR75-1. Values for the intermediate filament proteins, the stress proteins, and HMEC-specific proteins are omitted.

Figure 3. Identification of intermediate filament proteins in two-dimensional gel patterns of human breast epithelial cells. Cytoskeleton proteins enriched for intermediate filaments were separated by 2DE and detected using silver stain. Each panel shows a representative pattern from each class of intermediate filament composition discussed in the text. (*A*) HMEC; (*B*) AU565; (*C*) MDA-MB157; (*D*) BT549 . The cytokeratins are designated by numbers as described in the text. a, actin; v, vimentin;

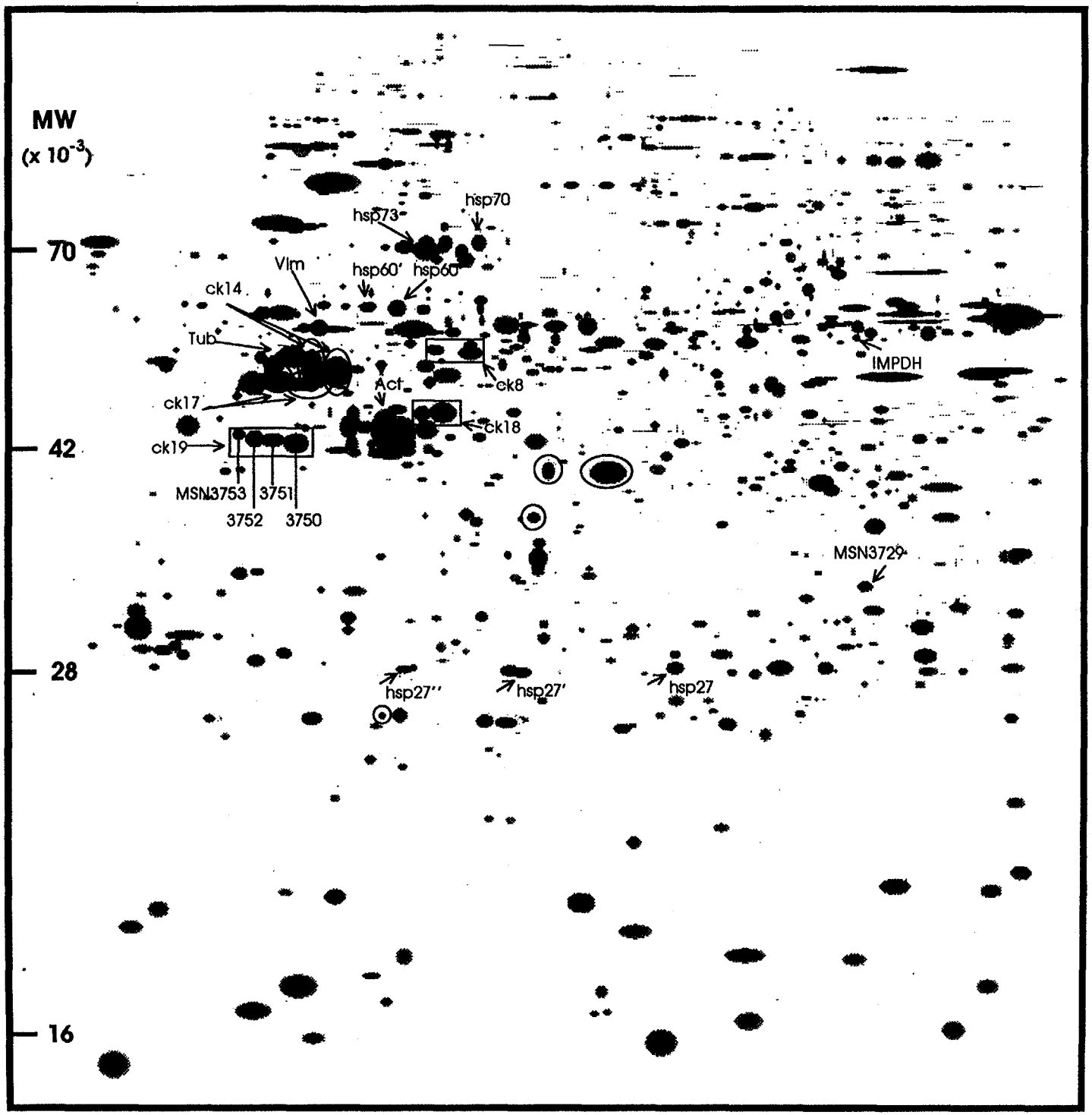
Figure 4. Intermediate filament protein levels. Histograms showing the abundance of intermediate filament proteins in breast cells. For the cytokeratins, the bars show the sum of the average integrated density (Y-axis) of the isoelectric variants of each protein.

Figure 5. Identification of IMP dehydrogenase in the MCF7 protein pattern. (*A*), Silver-stained 2DE gels of MCF7; (*B*) purified IMP dehydrogenase; (*C*) MCF7 plus purified IMP dehydrogenase. The position of the enzyme in the MCF7 patterns is indicated by the arrows. The lower molecular weight spots, seen most clearly in the purified sample, are degraded forms of the protein.

Figure 6. Differential expression of IMP dehydrogenase in human breast cells. The histogram shows the quantitative differences in the abundance of IMP dehydrogenase (MSN 2982). The values are expressed relative to the amount of protein in HMEC, which is given a value of 1.

Figure 7. Comparison of heat shock protein abundance in human breast cells. The histograms show the levels of heat shock proteins in breast cancer cells. The average integrated density for each protein spot is shown on the Y-axis. Note that the Y-axis for hsp70 is different than the others.

Figure 8. Identification of hsp27 isoelectric variants in whole cell lysates. Proteins from whole cell lysates were separated using 2DE, transferred to PVDF membranes, and probed with an anti-hsp27 antibody. (*A*) Silver-stained 2DE gel of ZR75-1 lysate; (*B*) 2DE western blot of ZR75-1 lysate reacted with monoclonal anti-hsp29 antibody. Only a portion of the gel is shown.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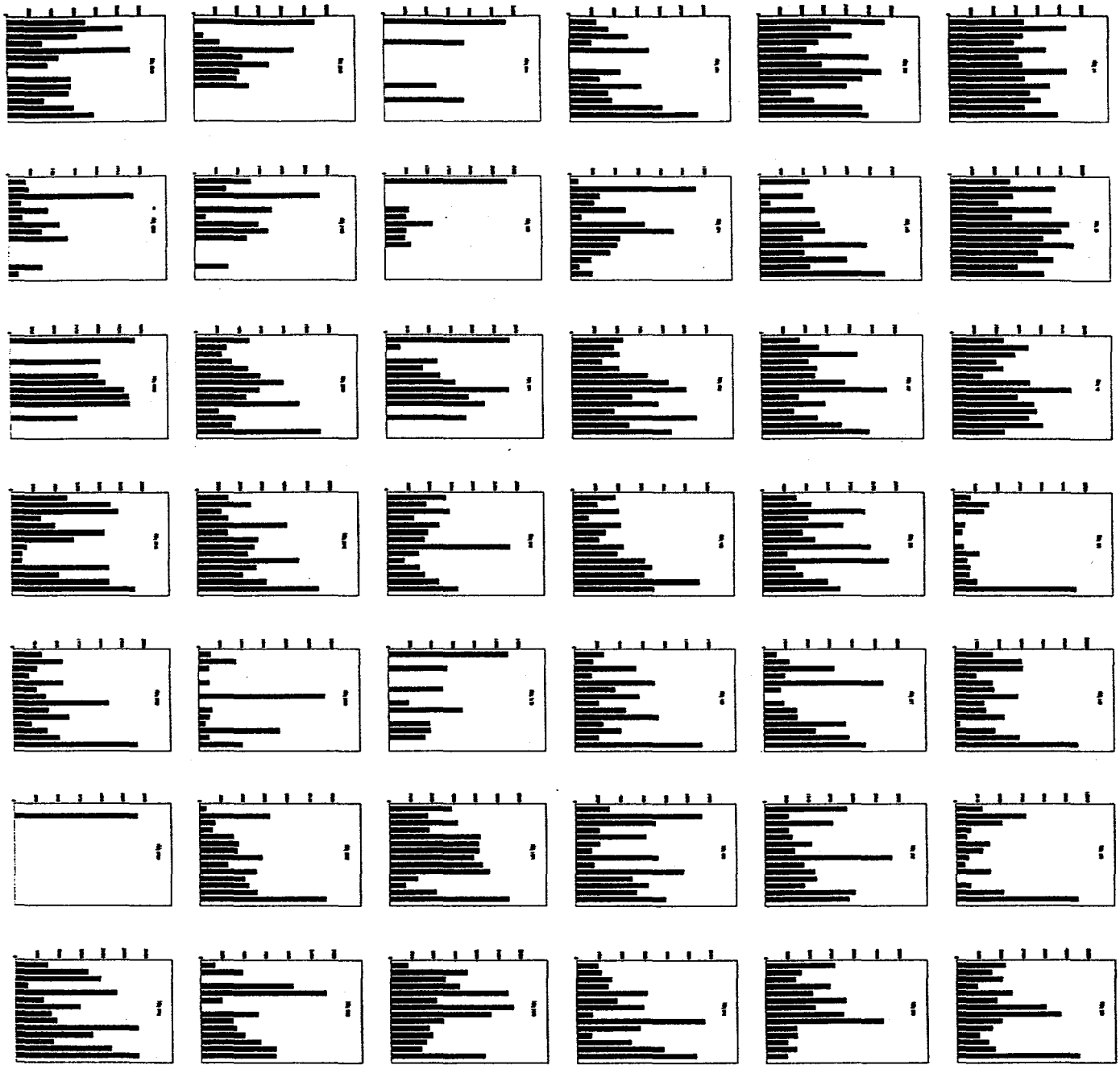

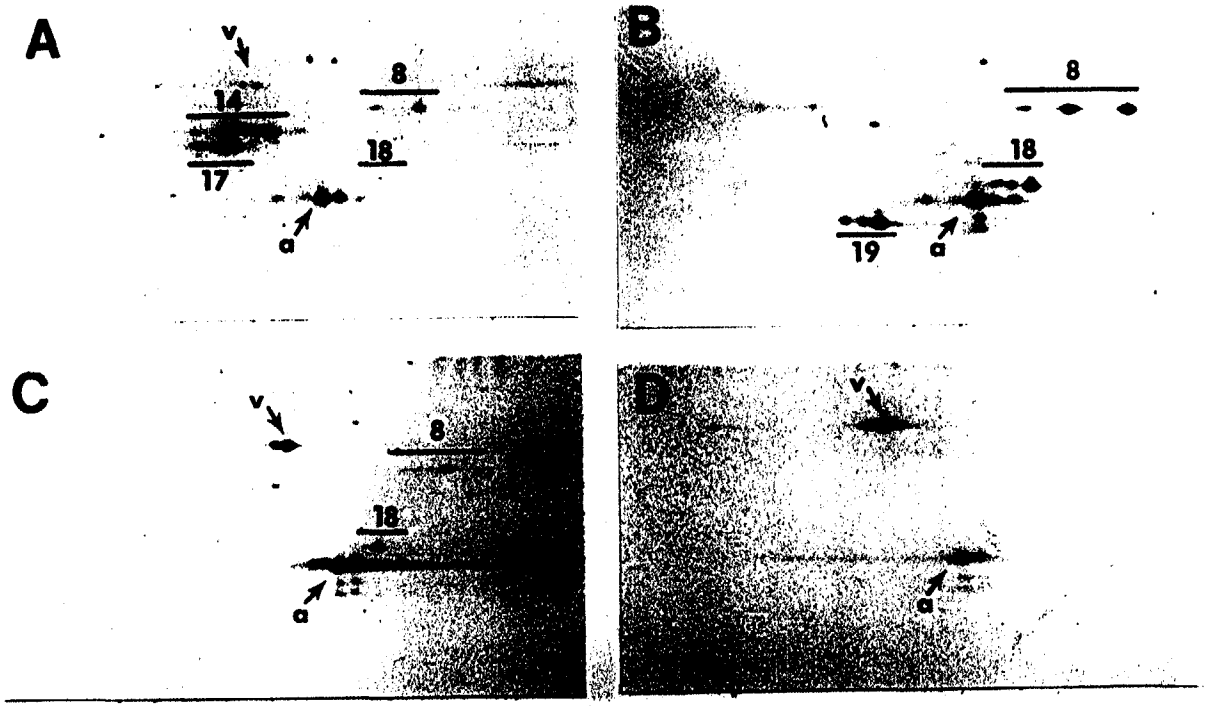
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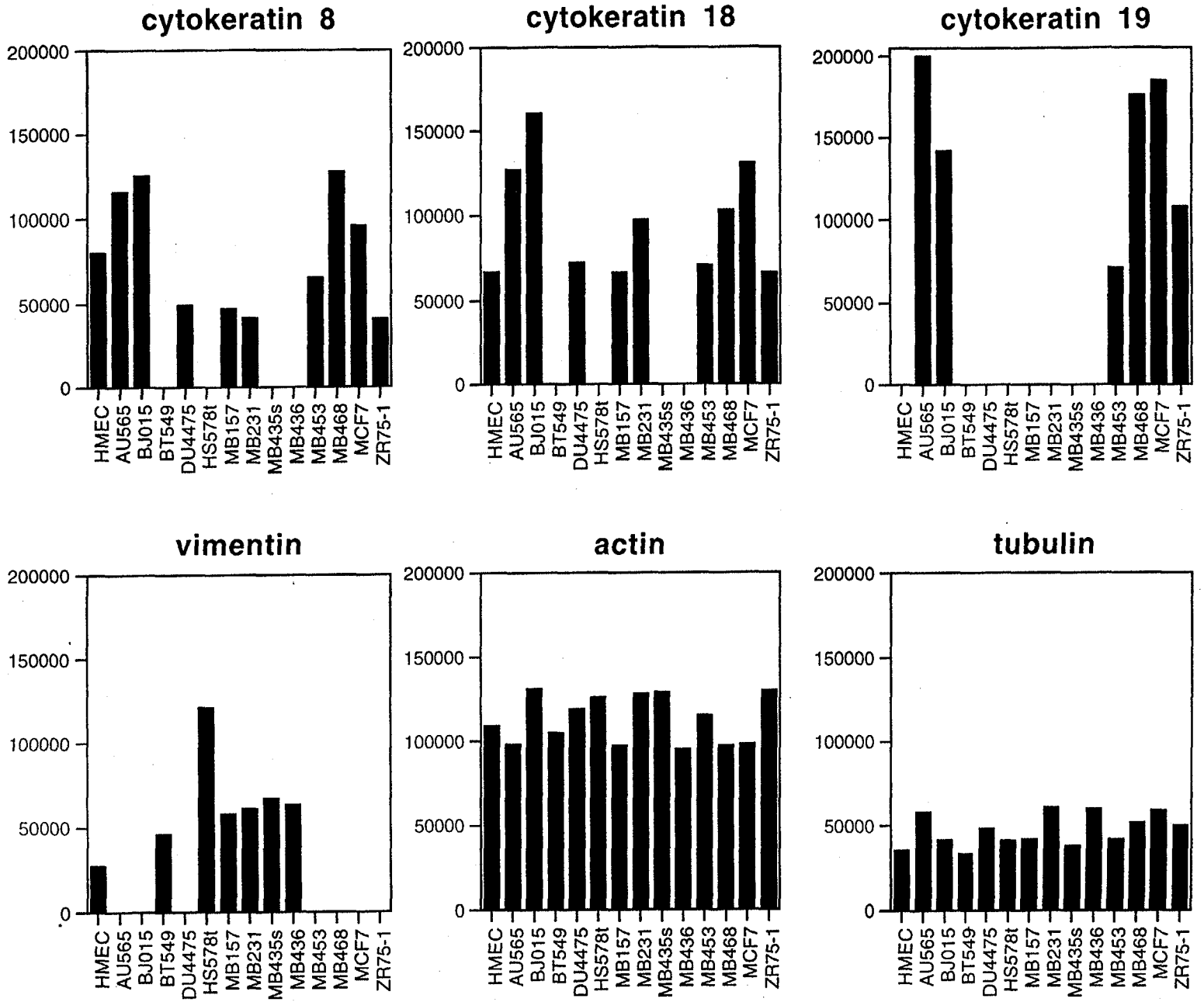
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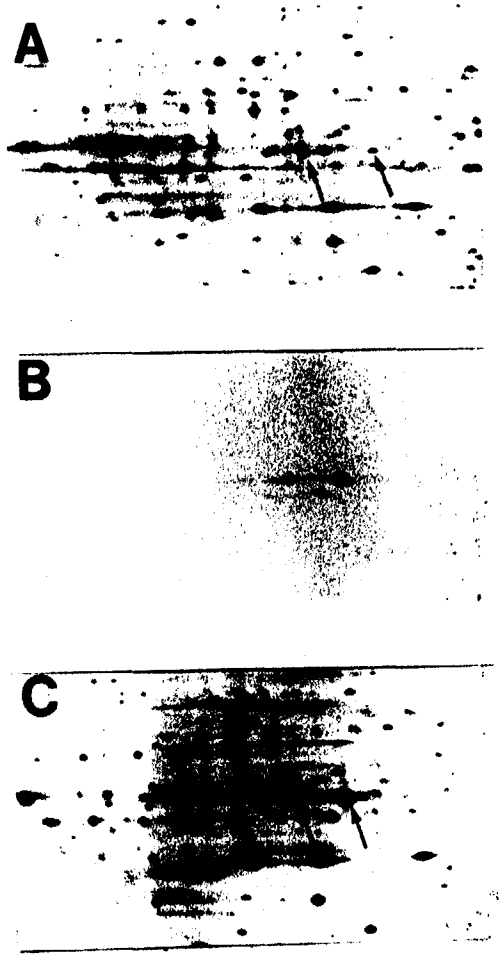
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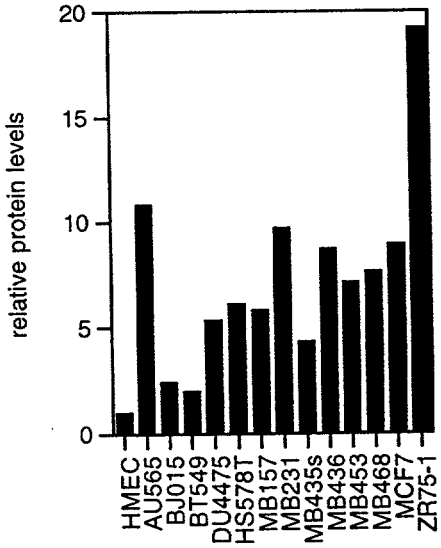
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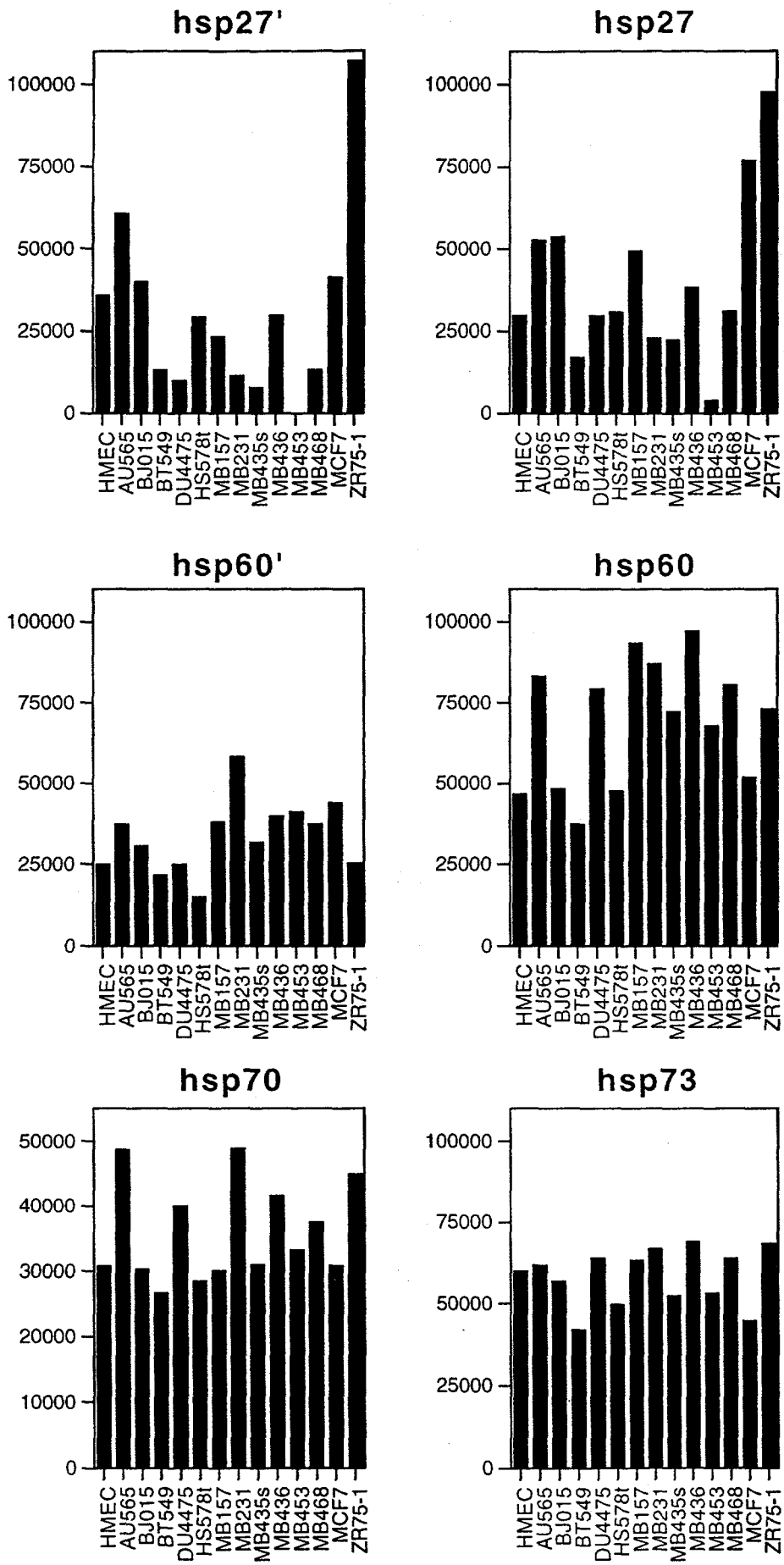
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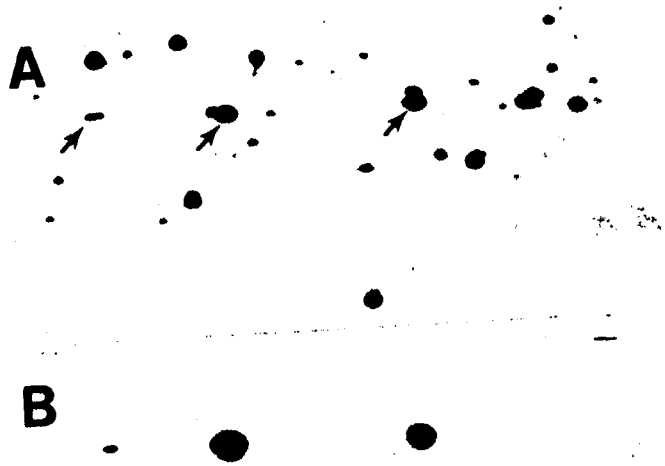
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