

Laminin Mediates Tissue-specific Gene Expression in Mammary Epithelia

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Abstract

Tissue-specific gene expression in mammary epithelium is dependent on the extracellular matrix as well as hormones. There is good evidence that the basement membrane provides signals for regulating β -casein expression, and that integrins are involved in this process. Here, we demonstrate that in the presence of lactogenic hormones, laminin can direct expression of the β -casein gene. Mouse mammary epithelial cells plated on gels of native laminin or laminin-entactin undergo functional differentiation. On tissue culture plastic, mammary cells respond to soluble basement membrane or purified laminin, but not other extracellular matrix components, by synthesizing β -casein. In mammary cells transfected with chloramphenicol acetyl transferase reporter constructs, laminin activates transcription from the β -casein promoter through a specific enhancer element. The inductive effect of laminin on casein expression was specifically blocked by the E3 fragment of the carboxy terminal region of the $\alpha 1$ chain of laminin, by antisera raised against the E3 fragment, and by a peptide corresponding to a sequence within this region. Our results demonstrate that laminin can direct tissue-specific gene expression in epithelial cells through its globular domain.

To understand how cell function is maintained within a tissue, it is necessary to know how the local microenvironment acts on a cell to regulate its phenotype. Most cells are either in a static location in contact with adjacent cells and an extracellular matrix (ECM), or they are migrating to and from different cellular and ECM environments. One conceptual framework for how cells behave in tissues is to consider that cells sense their surroundings through adhesive interactions with neighboring cells and ECM, and that in some instances such interactions control cell phenotype directly by regulating gene expression (Stoker et al., 1990). For example, cells can migrate to different zones of ECM that contribute to their profile of gene expression, as seen in monocyte invasion (Yurochko et al., 1992), neural crest migration (Bronner-Fraser, 1993), and wound repair (Whitby and Ferguson, 1991). In epithelial tissues such as mammary gland (Streuli et al., 1991; Schmidhauser et al., 1992) and liver (Bissell et al., 1987; Caron, 1990; DiPersio et al., 1991), or in terminally differentiating epidermis (Adams and Watt, 1990), cells contact a special ECM known as the basement membrane, which provides extracellular positioning information by defining the basal surface of the cell, and contributes to the differentiated phenotype by regulating transcription of tissue-specific genes.

Basement membranes contain supramolecular networks of laminin and collagen IV, and glycoproteins such as entactin and proteoglycans, which participate in basement membrane architecture (Yurchenco et al., 1992; Aumailley et al., 1993; Yurchenco and O'Rear, 1994). All of these molecules can interact independently with epithelial cells through a variety of cell surface receptors, including the signaltransducing class of heterodimeric receptors known as integrins (Hynes, 1992; Hayashi et al., 1992; Yelian et al., 1993).

It has been clear for some time that basement membranes are involved with the control of mammary gland differentiation. They cooperate with lactogenic hormones to activate transcription from tissue-specific genes such as β -casein. Evidence for this comes from studies which show that: (a) mammary epithelial cells cultured on floating collagen I gels express milk proteins coordinately with the deposition of an endogenous basement membrane (Emerman and Pitelka, 1977; Lee et al., 1985; Streuli and Bissell, 1990); (b) a reconstituted exogenous basement membrane induces milk protein synthesis (Li et al., 1987; BarceUos-Hoff et al., 1989) through the transcriptional activation of milk protein promoters (Schmidhauser et al., 1990, 1992); and (c) basement membrane, in the absence of cell-cell contact, can induce casein synthesis via an interaction with $\beta 1$ integrins (Streuli et al., 1991). We have argued that milk protein expression in the absence of exogenous basement membrane, as seen by other investigators, is caused by the formation of ECM synthesized in situ (Streuli, 1993).

The purpose of the studies reported here was to identify ligands that trigger the molecular signaling pathway between basement membrane and activation of β -casein transcription. We dissected the basement membrane into its component parts to ask whether any specific ECM protein could stimulate differentiation. Mammary epithelial cells cultured on thick gels of purified laminin or a laminin-entactin complex formed multicellular structures similar to those on whole basement membrane matrix, and they synthesized as much milk protein. To confirm that laminin was required for signaling, and to determine whether specific domains of laminin were involved, we developed an overlay assay for mammary differentiation in which cells were seeded on glass or plastic and then treated with soluble ECM proteins. Laminin, but not other ECM components, induced the synthesis of caseins and enhanced the transcriptional activity of

the β -casein promoter. Inhibitor studies with peptides derived from the α and β 1 chains of laminin, with purified laminin fragments, as well as with anti-laminin antibodies, demonstrated that the E3 region, located at the carboxy terminus of the α 1 chain, was required for laminin signaling. However, laminin fragments by themselves were unable to stimulate casein synthesis. We discuss our results in terms of alternative models for the mammary cell interaction with laminin.

Materials and Methods

Substrata

Engelbreth-Holm-Swarm (EHS) matrix was prepared and used as a substratum as described (Barcellos-Hoff et al., 1989; Streuli et al., 1991). This reconstituted basement membrane matrix has the same effect on the morphological and functional differentiation of mammary cells as a matrix depleted of growth factors (Streuli et al., 1991). EHS matrix is equivalent to the commercially available Matrigel. Calcium-free EHS matrix was prepared by dialysis against calcium free DME/F12 medium (Life Technologies, Ltd., Paisley, Scotland).

Purified laminin, laminin bound to entactin, and elastase-generated laminin fragments were prepared from EDTA-extracted EHS tumor as described (Paulsson et al., 1987; Yurchenco et al., 1992). Collagen I was prepared from rat tails (Lee et al., 1984), collagen IV was from Life Technologies Ltd., fibronectin was from Bioproducts Laboratory (Elstree, UK), vitronectin was from Sigma Chemical Co. (Poole, UK), and perlecan was from Becton Dickinson Labware (Bedford, MA).

Elastase-digested whole EHS matrix was prepared by incubating the matrix with 1:560 (wt/wt) enzyme/substrate ratio of porcine elastase (Sigma) in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, for 5 h on ice, followed by 25 h at 25°C, then inhibiting the digestion with 1 mM PMSF and dialysis against culture medium at 4°C. Pepsin-digested whole EHS matrix was prepared by incubating the matrix with 1:13 (wffwt) enzyme/substrate ratio of pepsin (Sigma) in 10% acetic acid for 5 h on ice, followed by 25 h at 15°C, inhibiting the reaction by adjusting the pH to 7.4 with NaOH, and then dialysis against culture medium at 4°C (both methods adapted from Schittny and Yurchenco, 1990). The extent of digestion was monitored by SDS-PAGE followed by silver staining.

Cell Culture

Most experiments were done with first passage (secondary) mammary epithelial cells derived from 14.5-16.5-d pregnant ICR mice. Primary epithelial cultures were prepared from isolated mammary alveoli (Lee et al., 1985; Barcellos-Hoff et al., 1989; Streuli et al., 1991) and plated at $2.5\text{--}5.0 \times 10^5$ cells per cm^2 on tissue culture plastic in F12 medium (Sigma) containing 10% heat-

inactivated FCS (batch tested from Life Technologies), 1 mg/ml fetuin (Sigma), 5 µg/ml insulin (Sigma), 1 µg/ml hydrocortisone (Sigma), 10 ng/ml mouse EGF (Promega Corp., Southampton, UK), and 50 µg/ml gentamycin (Life Technologies). After 48 h, the medium was changed to FI2 medium containing only insulin, hydrocortisone, EGF, and gentamycin. After an additional 12-36 h, the cell monolayers were trypsinized to single cell suspensions, washed with serum-containing medium, and either used directly or stored in 10% FCS, 10% DMSO in liquid nitrogen. The first-passnge cultures were plated at $0.5-1.0 \times 10^5$ cells per cm² initially in DME/FI2 medium containing 10% FCS, 5 µg/ml insulin, 1 µg/ml hydrocortisone, 10 ng/ml EGF, and 50 µg/ml gentamycin, and the medium was subsequently changed to DME/FI2 containing insulin, hydrocortisone, and 3 µg/ml wine prolactin (Sigma), but no serum or EGF.

The mouse mammary cell strains, CID-9 and its transfected derivatives (Schmidhauser et al., 1990) and SCp2 (Desprez et al., 1993), were passaged in DME/Ft2 medium containing 5% FCS, 5 µg/mi insulin, and 50 µg/ml gentamycin.

Overlay Assay

First-passage mammary epithelial cells, or transfected CID-9 cell populations, were seeded onto tissue culture plastic or onto acid-etched glass coverslips that were either precoated with 10% serum or 3 µg/cm² vitronectin (1 h, 37°C). To obtain an even plating of cells, a number of coverslips were placed in 50- or 85-ram diameter dishes for initial cell attachment and spreading, and then transferred to four-well dishes for the overlay experiment. SCp2 ceils were plated on tissue culture plastic or on plates treated with poly 2-hydroxyethylmethacrylate (Roskelley et al., 1994). 24- to 36 h after plating, cells were washed three times, and the medium was replaced with DMEtFI2 containing insulin, hydrocortisone, prolactin, and the relevant ECM proteins. The ECM proteins were diluted directly into medium that had been previously chilled on ice, and the complete overlay medium was then warmed to room temperature before being added to the ceils.

All concentrated ECM stock solutions and laminin fragments (except collagen) were dialyzed at 4oc against DME/FI2 medium, and they were stored in aliquots at -70°C. These stock solutions were then thawed on ice before use in the overlay experiments. Collagens I and IV were dialyzed at 4°C against water, and then diluted directly into the overlay medium together with an appropriate amount of 5 x concentrated DME/FI2 medium. Peptides were reconstituted to 2 mg/ml in distilled deionized water and stored in aliquots at -70°C. Antibodies were dialyzed against DME/F12 and filter sterilized.

In most experiments, the medium was changed 48 h after adding the overlay medium, and the ceils were harvested after a further 36 h. To control for the quantity and the type of ECM addition, an aliquot of complete overlay medium was always retained at the time of addition to the cells, then mixed with an equal volume of sample buffer, separated by SDS-PAGE, and the gel was silver stained.

Immunohistory

Cells were washed three times with warm DEM/F12 medium, fixed for 3 min at -20°C with 1:1 methanol/acetone, and air-dried. Staining with anti-E-casein antibodies and counterstaining with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) was carried out as described (Streuli et al., 1991).

Immunoprecipitation

Cultures were washed three times with methionine-free DME/F12 medium (Sigma) containing appropriate hormones, and after 60-90 min, this was replaced with similar medium containing 0.25 mCi/ml [³⁵S]methionine (Trans³⁵S-label; ICN Biomedicals Ltd., High Wycombe, UK) for 75-105 min. To harvest cell proteins, cultures were washed three times with warm medium, chilled on ice, extracted with radioimmune precipitation buffer containing proteinase inhibitors, and the detergent-insoluble proteins were cleared by centrifugation, all as described (Streuli et al., 1990). Metabolic incorporation into newly made protein was measured by precipitation with trichloroacetic acid and aliquots representing equal numbers of precipitable counts were used for immune precipitation reactions in a total volume of 700 µl. As a control that equal amounts of newly synthesized cell proteins were being used, 10 µl of each immune precipitation reaction was separated by SDS-PAGE and analyzed by fluorography. Milk proteins were precipitated with a rabbit anti-mouse milk antiserum, followed by protein A-Sepharose (Zymed Laboratories Inc., South San Francisco, CA), before separation on 13 % polyacrylamide gels under reducing conditions. The levels of newly synthesized caseins were analyzed by fluorography. In some cases, digitized images were obtained from dried gels using storage phosphor imaging plates and a Fujix Bas 2000 Bio Imaging Analyzer (Fuji Photo Film Co. Ltd., Tokyo, Japan). The levels of casein in immune precipitates was quantitated with Fuji software.

Chloramphenicol Acetyl Transferase (CAT) Assays

To measure CAT enzyme levels as an indicator of β-casein promoter activity in transfected cells cultured in the overlay assay, cells were harvested, the extracts normalized, and the CAT activity measured exactly as described (Schmidhauser et al., 1992; Streuli et al., 1993).

Results

A Laminin Gel Promotes the Differentiation of Mammary Epithelial Cells

The EHS tumor basement membrane contains ECM signaling molecules that can induce differentiation of mammary epithelial cells and transcription of tissue-specific genes (Barcellos-Hoff et al., 1989; Streuli et al., 1991; Schmidhauser et al., 1992). Since 85-90% of the protein in the EHS basement membrane is laminin, we reasoned that laminin was a good candidate for promoting differentiation in these cells. We therefore purified laminin with or without associated entactin (Fig. 1 a in this paper; Yurchenco et al., 1992), and we cultured mouse mammary cells on gels of EHS matrix or purified laminin, in the presence of lactogenic hormones. At a plating density of 5×10^4 cells/cm², the cells aggregated and formed small spherical structures on

all three substrata (Fig. 1 b), many of which contained hollow lumina (not shown). Pulse labeling with immune precipitation (Fig. 1 c), and immunofluorescence studies using anti- β -casein antibodies (not shown) demonstrated that on all three types of gel, differentiation was induced. Cells cultured with identical media, but on tissue culture plastic, synthesized no β -caseins (Fig. 1 c).

These results provided the first indication that laminin could cooperate with lactogenic hormones to drive the morphological and functional differentiation of mammary epithelial cells. However, large quantities of laminin were required for this type of experiment, and mammary cells cultured in basement membrane gels can synthesize their own ECM proteins (Petersen et al., 1992) that may provide independent differentiation signals. We therefore used a totally different culture system to obtain further evidence that laminin could trigger mammary-specific gene expression.

An Overlay Assay for Mammary Epithelial Differentiation

In well-established culture models for mammary differentiation, epithelial cells are plated on top of basement membrane gels such as the one described above (Li et al., 1987; Barcellos-Hoff et al., 1989; Schmidhauser et al., 1990), or on collagen I gels where the cells deposit their own basement membrane (Emerman et al., 1977; Streuli and Bissell, 1990). Alternatively, cells are plated on plastic dishes thinly coated with specific substrata (Blum et al., 1987; Li et al., 1987), although under these conditions, not much of the ECM proteins remain tightly bound to the dish (Blum and Wicha, 1988) and thus the levels of mammary-specific gene induction are low. In this study, a method for inducing differentiation was used in which mammary cells were seeded on glass or plastic, then treated with purified basement membrane components by dilution into the culture medium (Fig. 2 a). This type of assay was previously described for inducing differentiation in hepatocytes (Caron, 1990) and a similar method has been adapted for studying tubule morphogenesis in human mammary luminal epithelium (Berdichevsky et al., 1992).

The technique was initially developed using complete basement membrane from the EHS tumor, which was diluted to 20-200 μ g/ml into medium containing lactogenic hormones (cf. the protein concentration in an EHS gel is 14-16 mg/ml). Mammary cells that had already formed small epithelial islands (Fig. 2 b, *top panel*) often aggregated, appeared more refractile by phase contrast microscopy (Fig. 2 b, *lower panel*), and the nuclei were closer together (not shown). These cells now expressed β -casein, demonstrated by immunofluorescence (Fig. 2 c) and immune precipitation (Fig. 2 d). Although shape changes often occurred, they did not appear to be a prerequisite for differentiation in these first passage cultures since the cells expressing casein were often flattened in appearance, especially towards the edge of islands of cells (Fig. 2 c). The induction of casein synthesis was more dramatic in sparse culture than under confluent conditions. Casein expression was maximal at 4 d, and the levels of protein synthesis were similar to those in cells cultured as spheres on top of the EHS matrix (Fig. 2 d). Although 1-2 d were sufficient to promote a response (Fig. 2 d), the maximal expression was usually seen at 4 d. Similar results were obtained from cells cultured on thin layers of collagen I, fibronectin, or vitronectin, with soluble basement membrane added to the medium (not shown).

To see whether cell aggregation was required for casein expression, cells cultured in different concentrations of calcium ions were stimulated with lactogenic hormones and diluted basement membrane. Even in 5 μM Ca^{++} , when the cells were isolated from each other by phase contrast microscopy and no E-cadherin was seen at cell borders (not shown), casein expression was upregulated by the basement membrane (Fig. 2 e).

Milk Protein Expression in Adherent Cells Is Induced by the Addition of Laminin in Solution

Having established the overlay assay, where the complete basement membrane could be presented in an initially soluble form to induce differentiation, we examined the response of mammary epithelial cells to individual ECM proteins.

Complete basement membrane from the EHS tumor, purified laminin (Fig. 3 a and b), or the laminin-entactin complex (Fig. 3 c) were diluted to different concentrations in culture medium containing lactogenic hormones, and were added to cells in the overlay assay. After 3.5 d, the rate of milk protein synthesis showed a similar concentration dependence in all three conditions. Laminin did not alter the overall profile of newly synthesized cell proteins (Fig. 3 b), indicating that its effects were specific for milk protein expression. In contrast to the effect of laminin, other ECM proteins, such as fibronectin and collagen I (Fig. 3 c), or collagen IV (not shown), induced almost no β -casein production, although it remains possible that the reason for this is that these or other ECM proteins were not being presented to the cells correctly. The low levels of casein synthesis in the collagen cultures (Fig. 3 c) may result from endogenously produced basement membrane (Streuli and Bissell, 1990). Entactin, which normally associates tightly with laminin and interacts with a number of cell types, including mammary epithelia (Paulsson et al., 1987; Chakravarti et al., 1990; Yelian et al., 1993), is also not required since purified laminin is as effective as the native laminin-entactin complex for inducing casein expression (Fig. 3 c). In experiments where prolactin was removed from the overlay assay, no induction of differentiation was seen (Fig. 3 d).

Thus, purified, native laminin can trigger mammary-specific gene expression if presented either in a soluble form or as a gel. Since casein expression was dependent on prolactin, our experiments show that mammary differentiation is coordinately regulated by laminin and lactogenic hormones.

Laminin Activates Mammary-specific Gene Transcription

We next asked whether this laminin-dependent expression of β -casein was mediated through transcriptional regulation. The bovine β -casein promoter contains an enhancer element, BCE1, that is responsive to signals contained within ECM (Fig. 4 a). This enhancer was therefore coupled to a truncated nonactive β -casein promoter linked to the CAT reporter gene, and it was transfected with a vector encoding the neomycin resistance gene into a functional mammary epithelial cell strain, CID-9. Antibiotic-resistant colonies were pooled and assayed. In cells plated on top of an EHS gel, there was a substantial induction of CAT activity (Fig. 4 b), and in a previous study, we showed that this activity was entirely dependent on prolactin (Schmidhauser et al., 1992). The same pool of cells were also cultured on tissue culture

plastic using the overlay assay, with soluble ECM proteins and lactogenic hormones. Both the basement membrane matrix and purified laminin could activate transcription from the β -casein promoter (Fig. 4 b). Other ECM components (e.g., collagen IV, fibronectin, and perlecan) were ineffective, corroborating our previous results with the β -casein protein.

These results indicate that laminin can activate transcription from the β -casein promoter, although at this stage, we do not know whether it can also control mRNA processing or translation. The data provide evidence for a molecular signaling pathway that links specific ECM proteins with the cell's transcriptional machinery.

A Specific Domain within Laminin Is Required for Mammary Differentiation

Laminin is a complex three-chain molecule that interacts with cells through different domains and via several types of receptors. It is already known that mammary cells can bind laminin through integrins since pan-specific anti- β 1 integrin antibodies block adhesion to laminin (Bailey, N., and C. H. Streuli, unpublished data). Since β 1 integrins are required for casein expression (Streuli et al., 1991), the differentiation event is also likely to involve an integrin receptor. However, the antibody GoH3 (the only relevant function-blocking anti-mouse integrin antibody that was readily available) does not inhibit casein induction either by soluble laminin in the overlay assay (not shown) or in single cell assays (Streuli et al., 1991), so the receptor may contain an α chain other than α 6. We therefore chose to dissect the molecular details of laminin-mediated differentiation by first elucidating the specific domain within laminin that contains signaling activity.

In the first series of experiments, peptide sequences corresponding to different regions within the α 1 and β 1 chains of laminin were used as competitive inhibitors of casein synthesis (Table I; Fig. 5 a). Only one of the peptides we used, GD-2, from the globular domain of the laminin α 1 chain, repressed laminin-induced casein expression, whereas peptides from other regions had no effect (Fig. 5 b). Although we have not tested all other functional laminin sequences, we found no effect of RGD or YIGSR peptides on casein expression (unpublished data). This inhibition by peptide GD-2 was effective at low concentrations, with some inhibition occurring at 0.5 μ g/ml, only a twofold molar excess of peptide GD-2 over laminin (Fig. 5 c). Peptide GD-2 also repressed casein expression specifically, since a scrambled version of this sequence did not reverse the induction of casein.

Since the GD-2 peptide maps to the E3 region of laminin, located in the carboxy terminal globular domain of the α 1 chain (Fig. 5 a), we carried out further experiments to confirm that this region is important for differentiation. We first included purified, elastase-generated E8 fragment and the adjacent but nonoverlapping E3 fragment (Fig. 5 a) as inhibitors in the overlay assay. Only E3, but not E8, inhibited laminin-induced casein expression (Fig. 6, a and b). The E3 fragment also blocked casein expression in cells stimulated by the laminin-entactin complex and complete basement membrane (not shown). In addition, affinity-purified anti-bodies raised against the E3 fragment, but not against the E8 fragment, significantly reduced the differentiation induced by laminin (Fig. 6 c). Together, these results indicate that at least one signaling region of laminin is contained in the E3 region of its globular domain, although they do not rule out the possibility that other domains may be required as well.

An expectation from these results is that the E3 fragment might act as a ligand and stimulate mammary differentiation. However, we found that neither this fragment (Fig. 7 a) nor the GD-2 peptide (not shown) were active by themselves. Indeed, when the whole EHS basement membrane matrix was digested with elastase or pepsin to generate a mixture of laminin and other ECM fragments, it completely lost its activity in the overlay assay, even at 1.4 mg/ml (Fig. 7 b) or at 4.76 mg/ml (not shown). We have recently found that in one functional mammary epithelial cell line, SCp2, basement membrane-dependent differentiation can be enhanced by first allowing the cells to undergo shape changes and form clusters (Roskelley et al., 1994). It was therefore possible that although the E3 fragment was unable to induce differentiation in cell monolayers, it might do so in cells that were prerounded. We therefore plated SCp2 cells on tissue culture plastic or on poly 2-hydroxyethylmethacrylate, and we examined whether laminin fragments could affect milk expression. To maximize our chances of inducing differentiation, we used complete elastase-generated EHS matrix. However, although the untreated matrix triggered β -casein expression, and the elastase-digested material inhibited this expression, the fragment mixture was unable to induce differentiation by itself (Fig. 7, c and d). In addition, the digested material did not revert the matrix-dependent cell aggregation and cause the cells cultured on plastic to become flattened (Fig. 7 c, *plastic*), suggesting that these matrix fragments did not block differentiation through an effect on cell shape. These results demonstrate that although short fragments of laminin can block the inductive effect of the intact laminin molecule, they are unable to act by themselves as agonists for differentiation. It therefore appears that β -casein gene expression requires signaling events that can only be induced by intact laminin, but that are lacking in its shorter fragments.

Discussion

In this study, we have demonstrated that laminin can trigger casein expression by activating gene transcription, and that a specific domain within the globular region of the long arm of laminin is required for the production of β -casein. Laminin has profound and dynamic effects on cells, including cell adhesion, cell differentiation, axon guidance, the motility of myoblasts, neurons, neural crest cells, tumor cells, the morphogenesis of endothelium, and lung and kidney epithelium (Goodman et al., 1989; Grant et al., 1989; Sorokin et al., 1990; Calof and Lander, 1991; Schuger et al., 1991; Rivas et al., 1992; Bronner-Fraser, 1993; Matter and Laurie, 1994). Much recent work on understanding how laminin directs cell behavior has been at the level of defining the active sites in laminin and the cell surface receptors involved. Several domains of laminin have function-promoting activity; there are three distinct sites in the short arms of its cruciform structure, as well as sites in the long arm and its terminal globular region (Engel, 1992; Tryggvason, 1993; Matter and Laurie, 1994). This is reflected by a wide spectrum of cell surface receptors for laminin (reviewed in Mercurio and Shaw, 1991; Mecham, 1991; Yamada and Kleinman, 1992). Our work now identifies a region of laminin that affects the production of a tissue-specific protein.

It has been known for some time that basement membrane participates in the control of mammary epithelial phenotype (Li et al., 1987; Barcellos-Hoff et al., 1989; Aggeler et al., 1991). Although we had recently shown that it triggers differentiation directly, rather than by acting through cell-cell adhesion or cell morphology (Streuli et al., 1991), we had not conclusively been able to answer the question of which basement membrane component is

involved. We therefore purified basement membrane proteins and have now shown, using two independent assays (one in which mammary cells were plated on top of a purified laminin gel, and one where cells were overlaid with purified matrix), that laminin can direct casein expression. In similar assays, other ECM components were unable to induce differentiation.

We have also demonstrated that the laminin-cell interaction results in activation of transcription from the β -casein promoter. This is the first recorded incidence of laminin directly regulating gene expression. The lactogenic hormone, prolactin, is required for casein synthesis, indicating that in the mammary system, ECM and soluble factors cooperate to trigger a physiological response. Such dual control of cell behavior also occurs in other systems (Damsky and Werb, 1992), e.g., in neutrophils where the concerted action of ECM and tumor necrosis factor results in rapid protein tyrosine kinase phosphorylation and cell activation (Fuortes et al., 1993), or in normal fibroblasts, where growth factors only stimulate proliferation in cells adherent to a matrix (Schwartz, 1992). Since we had previously shown that β 1-integrins are required for ECM to induce casein expression (Streuli et al., 1991), our results suggest that cooperative signaling through integrins and the prolactin receptor is necessary for the activation of a differentiated phenotype. We are currently assessing whether laminin is involved in this process by restructuring the cytoskeleton or nuclear matrix (Bissell et al., 1982; Ingber, 1993), or by influencing the prolactin-mediated signaling pathway itself (David et al., 1994; Wakao et al., 1994).

The cells used in this work were derived from pregnant mammary epithelium, and they were already committed to an alveolar cell fate with the potential to express casein genes. We therefore believe that laminin may poise the cells to respond to additional lactogenic cues, rather than induce a de novo competence for expression. This type of mechanism would make sense in vivo, where alveolar cells are always associated with basement membranes during the proliferative and lactational phases of mammary development, and where lactation itself is under the temporal control of hormones such as prolactin. Nevertheless, the basement membrane is essential for casein expression in vivo and in culture since in the mammary gland in vivo, dissolution of basement membrane, either naturally during mammary gland involution or by ectopic expression of matrix metalloproteinases in transgenic mice, results in a lost capacity for milk production (Talhouk et al., 1992; Simpson et al., 1994).

The development of our overlay assay has allowed us to ask whether a specific laminin domain is important for differentiation. Two lines of evidence indicate that the E3 fragment, representing the COOH terminal moiety of the carboxy terminal globular domain of the α 1 chain, is required. (a) The complete E3 fragment itself, but not the neighboring E8 fragment, is inhibitory; and (b) antibodies raised against the E3 region, but not against E8, also reduce the production of casein. The observation that the GD-2 peptide sequence from within this region specifically blocks laminin-induced casein expression suggests that this peptide contains the sequence that confers activity within E3. The E3 fragment achieved a 50% inhibition of laminin-induced differentiation at $\sim 1.5 \mu\text{M}$ (i.e., $75 \mu\text{g/ml}$, extrapolated from Fig. 6 b), and since this is a similar concentration to the inhibition observed by the GD-2 sequence ($\sim 0.5 \mu\text{g/ml}$ from Fig. 5 c, or $1.2 \mu\text{M}$), we suggest that this region may be one site, or part of a site, involved in signaling. However, we do not rule out that other sites within E3, and possibly outside of E3, are additionally involved in laminin's capacity to direct casein expression in mammary epithelia.

Three important implications can be drawn from these results. First, we have identified a specific signaling domain within laminin, and since integrins are required for casein expression, it follows that mammary cells must receive their differentiation signals directly from laminin and lactogenic hormones, rather than from growth factors that are associated with the reconstituted basement membrane (Vukicevic et al., 1992). Second, since the laminin $\alpha 1$ chain is synthesized by mouse mammary epithelial cells in culture (Streuli and Bissell, 1990), and since immunostaining experiments with the anti-E3 antibody reveal that it is also present in basement membranes surrounding alveoli in the mammary gland (unpublished data), our new results suggest that laminin may directly control milk protein gene expression in vivo. Third, our study corroborates results from the other cell systems in which the E3 fragment has been shown to influence epithelial cell phenotype. For example, this domain is also involved in kidney morphogenesis, where an epithelium is specified from mesenchymal precursor cells in a process that involves complex cascades of gene induction (Davies, 1993). The induction of polarity in this developing epithelium requires a cell-ECM interaction involving both the laminin E8 region and $\alpha 6\beta 1$ integrin (Klein et al., 1988; Sorokin et al., 1990), as well as the laminin E3 fragment (Sorokin et al., 1992). Thus, although the molecular signaling pathway for kidney induction is not identical to that for mammary differentiation, there may be aspects that are mechanistically related.

Our observation that neither the E3 fragment nor a mixture of elastase-generated fragments from the complete basement membrane matrix could induce casein expression implies that differentiation requires signaling from the intact laminin molecule rather than from its smaller fragments. Since some other functions of laminin, such as self assembly or binding to $\alpha 6\beta 1$ and $\alpha 1\beta 1$ -integrin, entactin, and heparin, are all conformation dependent, it is not unreasonable to suggest that the differentiation function mediated by the E3 domain also requires the correct three-dimensional structure of laminin, which would be destroyed by elastase digestion.

However, there also arise two alternative models for the mammary cell interaction with laminin. One possibility would be that signaling is mediated entirely through the E3 domain, but that polyvalent E3 is required, for example, to induce receptor clustering. Laminin forms intermolecular associations in basement membranes (Yurchenco and O'Rear, 1994), and these could provide the necessary polyvalency for intact laminin, but not for the elastase-generated fragments. Indeed, an extreme view is that one important function of laminin assembly is to provide a mechanism for bringing laminin receptors on the cell surface together. Maximal stimulation of casein expression occurred at 100-200 $\mu\text{g/ml}$ laminin in the overlay assay, when precipitates could be seen on top of cells. This precipitate likely reflects the propagation of a laminin polymer on the cell surface. The critical concentration for laminin to form polymer networks in solution is $\sim 100 \mu\text{g/ml}$ (Yurchenco et al., 1985). When bound to a cell surface, i.e., through a receptor, this critical concentration may be lower (Kalb and Engel, 1991). A polyvalency model consistent with the data is that a surface-bound polymer of laminin is required for cell signaling such that maximal stimulation of casein expression occurs at high polymer densities ($>100 \mu\text{g/ml}$ experimentally), while some effect can be detected at a lower concentration (20 $\mu\text{g/ml}$ experimentally) where the polymer could exist as a surface-bound monomer. To begin to address the issue of polyvalency, we attempted to induce casein expression by first coating dishes with laminin fragments, but to date, we have not found this to be a successful route for differentiation.

An alternative model for the mammary cell interaction with laminin is that multiple domains within laminin are required to trigger differentiation, through both the E3 domain and other cell-binding regions. Such a mechanism is not implausible since some cells are known to bind different sites in laminin simultaneously (Hall et al., 1990; Goodman et al., 1991), and this mechanism includes the possibility that intact laminin forms one stable interaction with a high affinity binding site on the mammary cell surface, thus allowing another part of the molecule to signal through a low affinity receptor. This type of model follows well-established principles for growth factors, where members of the TGF- β and FGF families deliver signals only after being bound by multiple receptors (Wrana et al., 1994). We have already shown that casein expression requires β 1-integrins. Several β 1-integrins are known to be laminin receptors and of these, α 2 β 1, α 3 β 1, and α 6 β 1 are present in primary mouse mammary epithelial cells (Delcommerme, M. and C. H. Streuli, manuscript submitted for publication). α 6 β 1 is unlikely to be part of the laminin-mediated signaling pathway in this system since the function-blocking anti- α 6 antibody GoH3 does not inhibit basement membrane-induced casein expression (Streuli et al., 1991), and α 6 β 1 binds mouse laminin through the E8 rather than the E3 fragment in rat and human mammary cell lines (Sonnenberg et al., 1990). However, α 3 β 1 interacts with the E3 fragment of laminin in a human melanoma cell line (Gehlsen et al., 1992), so this integrin is a good candidate for delivering differentiation signals. E3 may alternatively interact with a different type of receptor since the GD-2 sequence, which represents a heparin-binding domain of laminin within E3, also blocks laminin-mediated differentiation (Skubitz et al., 1991). An intriguing possibility is therefore that laminin interacts with mammary cells through both integrin and proteoglycan receptors, in a mechanism analogous to the interaction of cells with fibronectin (Iida et al., 1992; Woods and Couchman, 1992).

In summary, we have now begun to dissect the molecular details of basement membrane-cell signaling in the tissue specific function of mammary gland epithelium. Laminin contains a domain that is required for differentiation, and together with prolactin, it triggers the expression and synthesis of milk proteins. We are now attempting to identify which laminin receptors are involved and how they deliver their second messenger response.

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Figures and Tables

FIGURE 1

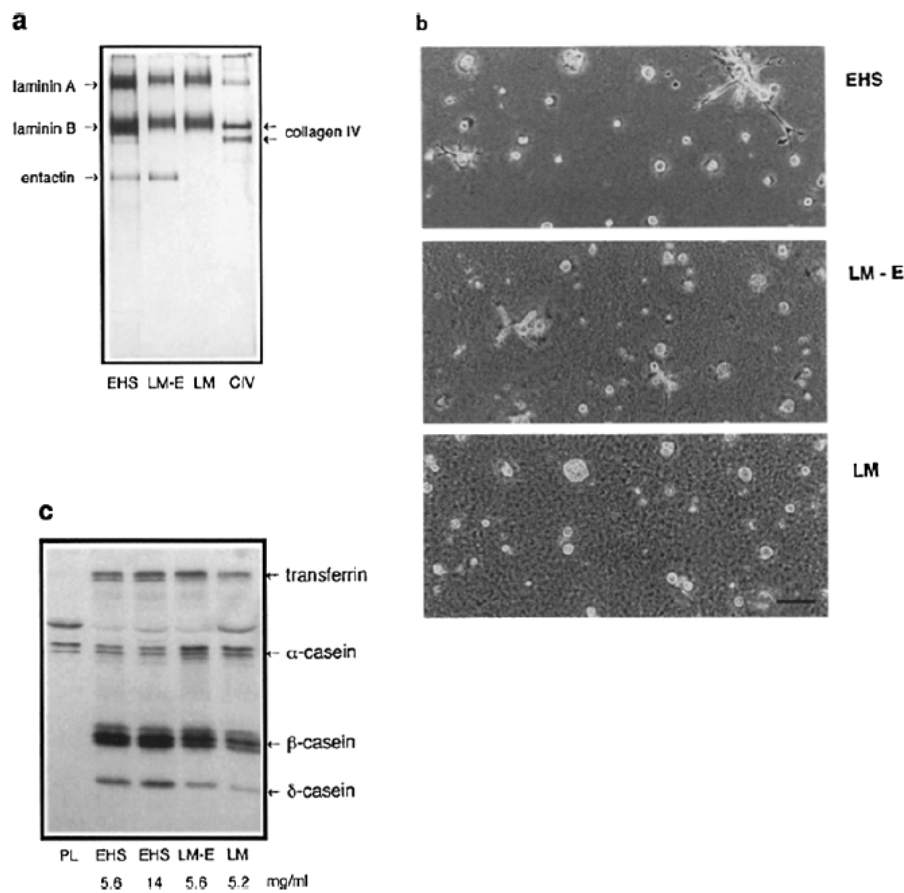


Figure 1. Mammary epithelial cells differentiate on gels of purified laminin, a laminin-entactin complex, or complete basement membrane. (a) Silver-stained polyacrylamide gel of the proteins used to make the gels, with collagen IV (CIV) alongside to show that proteins of this size are present in the EHS (EHS), but not in the laminin (LM) or laminin-entactin (LM-E) preparations. (b) Phase contrast micrographs show that the cells aggregate into spherical structures on all three types of gel. Bar, 100 μ m. (c) Immune precipitation of newly made cellular proteins after pulse labeling 3-d-old cultures with [35 S]methionine for 90 min, using a polyclonal antiserum to mouse milk. This experiment shows that functional differentiation is induced by laminin-containing gels, but not by tissue culture plastic. The protein concentration in each type of gel is indicated. Note that the EHS matrix induces equal amounts of casein synthesis when used at either 14 or 5.6 mg/ml (the latter is the same concentration of protein as in the laminin-entactin gel).

FIGURE 2

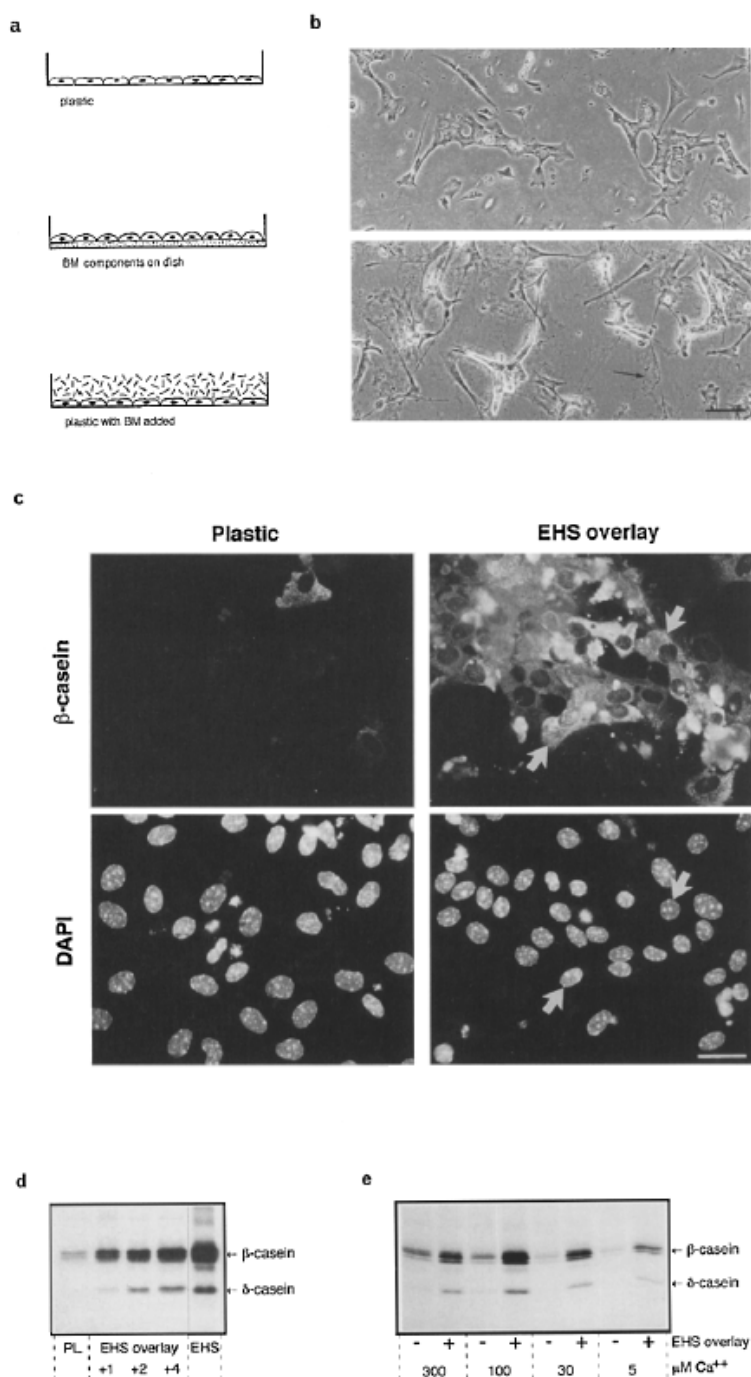


Figure 2. Mammary differentiation can be induced by complete basement membrane in solution. (a) Schematic diagram of the assay. (b) Secondary mouse mammary epithelial cells were plated at a low density (5×10^4 cells/cm²), where they formed small epithelial islands (top panel). The cells often coalesced when soluble basement membrane and lactogenic hormones were added to the medium for 4 d (lower panel). Note that some ECM molecules have precipitated over the dish (arrow). (c) Immunofluorescence with a β -casein-specific antibody shows a dramatic induction of casein synthesis when EHS matrix was added to the culture medium. Some flattened cells towards the edge of epithelial islands also expressed β -casein (cells and corresponding nuclei are arrowed). A rare casein-positive cell is present on tissue culture plastic. Nuclei are stained with DAPI. (d) Immune precipitation with a milk-specific antibody shows a dramatic induction of milk protein synthesis when the cells were cultured with EHS matrix (140 μ g/ml) in the medium (EHS overlay) for 1, 2, or 4 d, compared to culture without added matrix for 6 d (PL). Note that the levels of casein expression approach those seen in cells cultured as spheres for 6 d on top of a gel made with the matrix (EHS). (e) Cells were cultured on coverslips in different concentrations of calcium ions. EHS matrix (200 μ g/ml) was added to the medium of half the cultures for 2 d, before pulse labeling and immune precipitating with a milk-specific antibody. The calcium concentrations are the final concentrations in the medium after adding calcium-free EHS matrix. Bars: (b) 100 μ m; (c) 30 μ m.

FIGURE 3

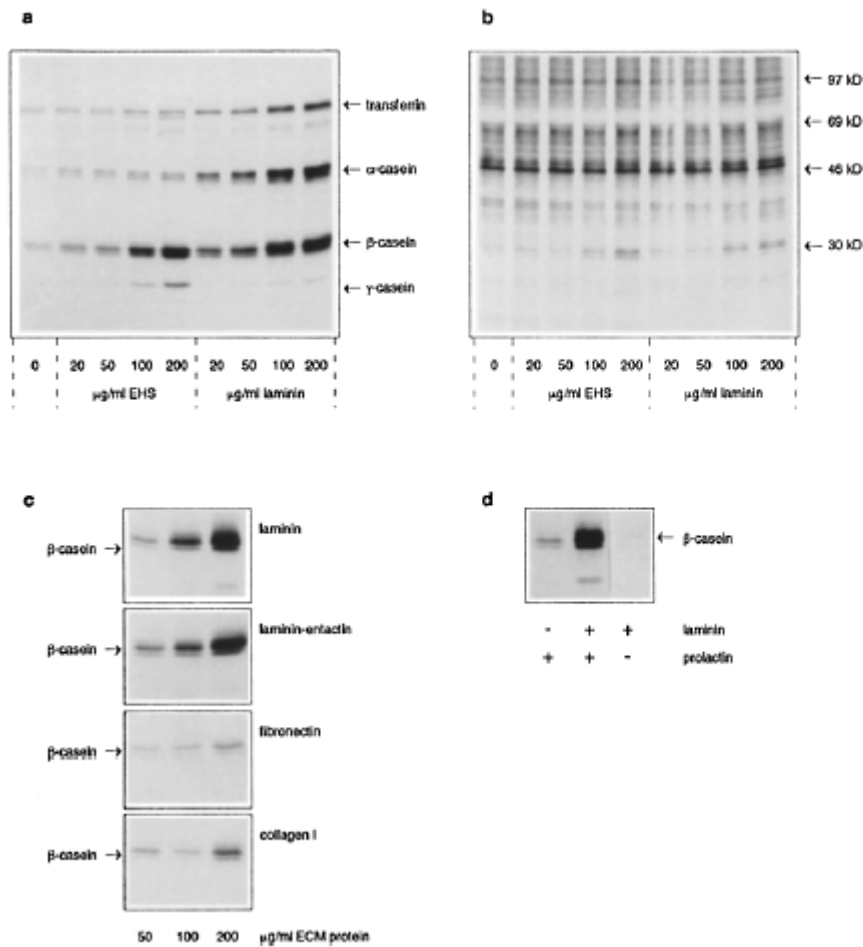


Figure 3. Purified laminin in solution induced substantial levels of milk protein expression. Mammary cells were cultured on coverslips, overlaid for 3.5 d with medium containing hormones and the indicated amounts of soluble EHS matrix or purified ECM proteins, and they were pulse labeled for 90 min with [35 S]methionine. (a) Equal amounts of newly synthesized cell proteins were immune precipitated with a polyclonal antiserum to mouse milk and separated by SDS-PAGE. The fluorograph shows that both EHS matrix and purified laminin induce the synthesis of milk proteins including β -casein. (b) Equal volumes of each immune precipitation reaction mix were separated by SDS-PAGE and analyzed by fluorography to confirm that equal amounts of newly synthesized cell proteins were being used. Note that incorporation of label into most proteins is similar. The 30-kD band that is increased in the overlay cultures is β -casein. (c) Other ECM proteins did not induce β -casein synthesis. (d) Cells cultured in the absence of prolactin (but with insulin and hydrocortisone) did not express β -casein.

FIGURE 4

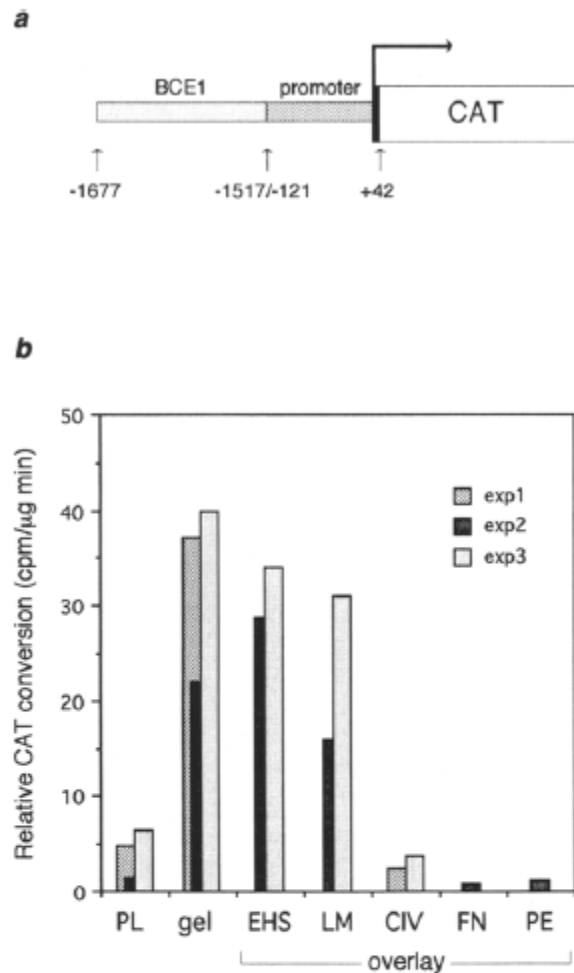


Figure 4. Transcription from the β -casein promoter can be activated by laminin. (a) The β -casein promoter contains a transcriptional enhancer (BCE1) normally located 1,517 bp upstream of the transcription initiation site. BCE1 can faithfully reproduce both ECM- and hormone-dependent transcriptional control when linked to a nonfunctional minimal casein promoter (Schmidhauser et al., 1992). This schematic diagram shows the CAT fusion gene that was transfected into CID-9 cells. The numbers indicate the positions in their natural context of the BCE1 enhancer (-1677 to -1517), minimal β -casein promoter (-121 to 0), and 42 bp of the 5' coding sequence (+1 to +42) of the β -casein gene relative to the transcription start site. (b) CID-9 cells stably expressing this construct were plated on top of an EHS gel (gel) or on tissue culture plastic (PL), and they were cultured for 3.5 d with lactogenic hormones. In the overlay experiments, cells were cultured on tissue culture plastic for 3.5 d in the presence of 200 μ g/ml EHS matrix (EHS), purified laminin (LM), collagen IV (CIV), or fibronectin (FN), or 20 μ g/ml perlecan (PE). Each type of bar (black, grey, and dashed) represents a separate experiment, although not every condition was used in each experiment. The relative levels of CAT indicate a dramatic substratum dependence of promoter activity; only purified laminin or the complete basement membrane matrix induced a significant increase in the activity of the casein promoter.

a

Schematic diagram of the $\alpha 1(2)$ casein gene construct. The construct is a fusion protein consisting of the N-terminal 12 amino acids of $\alpha 1$ casein (TG-1), followed by a flexible linker (F-12), and then the N-terminal 12 amino acids of $\beta 1$ casein (F-9). The construct is flanked by N and M termini. The construct is expressed in a cell line, and the resulting protein is purified. The protein is then subjected to immunoblotting using anti- $\alpha 1$ and anti- $\beta 1$ antibodies. The immunoblot shows a single band for $\alpha 1$ casein and a single band for $\beta 1$ casein, indicating that the construct is expressed as a single protein.

b

Immunoblotting results for the $\alpha 1(2)$ casein gene construct. The blot shows the expression of $\alpha 1$ casein (top row) and $\beta 1$ casein (bottom row) in the presence of various peptides. The peptides are: F-16, TG-1, GD-3, GD-8, GD-5, GD-1, and GD-2. The results show that the construct is expressed as a single protein, and the expression is not affected by the presence of the peptides.

c

Immunoblotting results for the $\alpha 1(2)$ casein gene construct. The blot shows the expression of $\alpha 1$ casein (top row) and $\beta 1$ casein (bottom row) in the presence of various peptides. The peptides are: GD-2 and GD-2s. The results show that the construct is expressed as a single protein, and the expression is not affected by the presence of the peptides.

Figure 5. Peptides pinpoint a domain of laminin required for differentiation. (a) Schematic diagram of laminin, indicating the location of peptides used in this study, and the E3 and E8 regions. (b) Milk proteins synthesized by mammary cells cultured with 100 $\mu\text{g}/\text{ml}$ laminin and lactogenic hormones. This immune precipitation shows the effect of including 100 $\mu\text{g}/\text{ml}$ peptide in the overlay assay. These results and data from a similar experiment where peptides F-9 and F-12 also had no effect on laminin-induced casein expression are summarized in Table I. (c) Titration of peptide GD-2 and the scrambled sequence GD-2s (0.5, 2.5, 10, and 50 $\mu\text{g}/\text{ml}$).

FIGURE 6

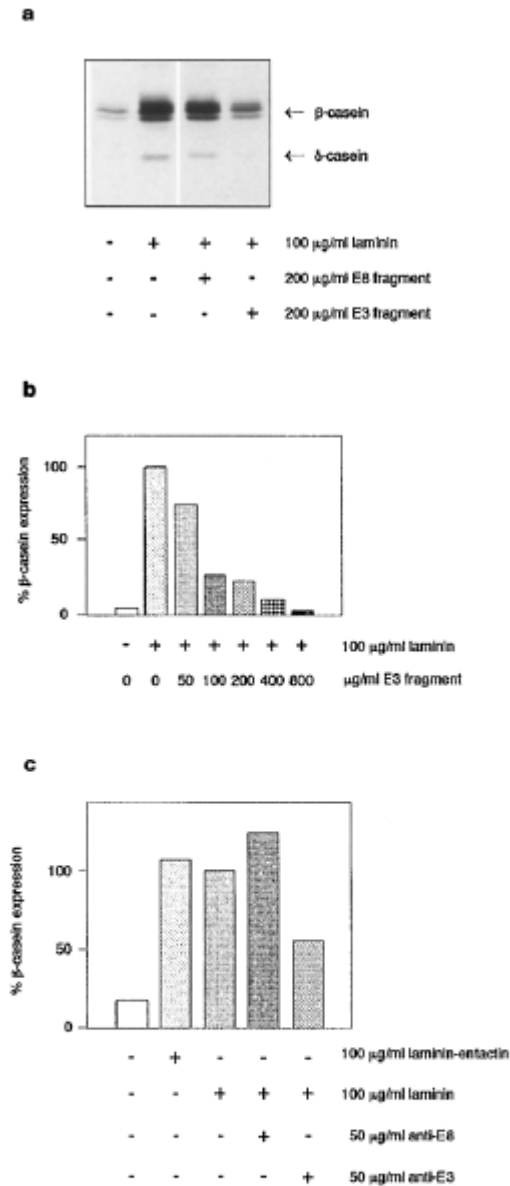


Figure 6. The laminin signaling domain is contained within the E3 region. (a) Mammary cells cultured in hormone-containing medium for 3.5 d synthesized β -casein in the presence of 100 μ g/ml laminin. This was competitively blocked by 200 μ g/ml E3 fragment, but not by 200 μ g/ml E8 fragment. (b) Dose response of the inhibition by E3 fragment of laminin-induced casein expression. The results in this experiment and in c were obtained by quantitative analysis of immune precipitates prepared as in Fig. 3, using a Fuji BAS 2000 Bio Imaging Analyzer. The amounts of β -casein present in each experimental condition are expressed as a percentage of the total β -casein induced by 100 μ g/ml laminin alone. (c) The induction of β -casein expression by laminin was blocked by antibodies raised against the E3 domain, but not against the E8 domain. These antibodies were raised against pure laminin domains, and IgG fractions were finally purified by affinity chromatography on a laminin column.

FIGURE 7

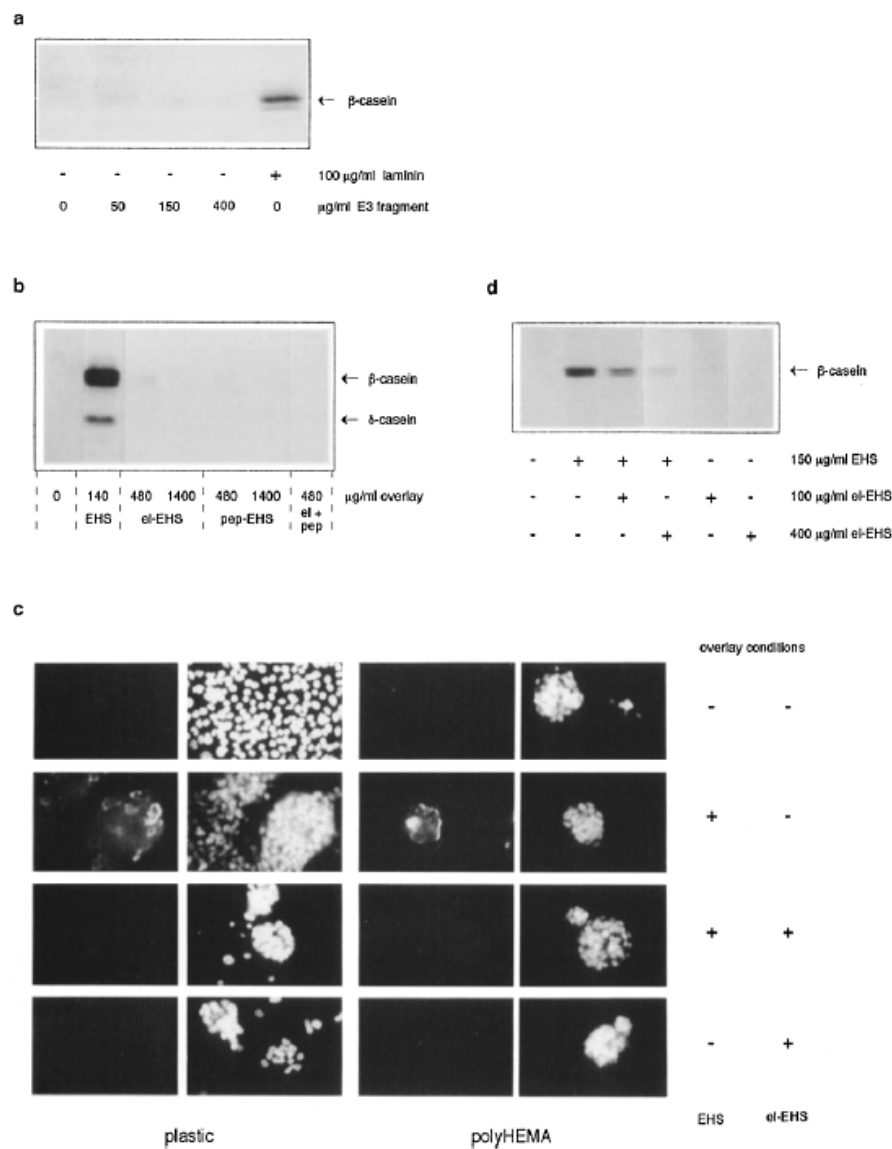


Figure 7. An intact laminin molecule is required for its signaling activity. (a) Purified E3 fragment was unable to induce casein expression in mammary cells cultured for 3.5 d with lactogenic hormones. 100 μ g/ml laminin was included as a positive control. (b) Caseins synthesized in mammary cells overlaid with EHS matrix (EHS), the matrix digested with either elastase (el-EHS) or pepsin (pep-EHS), or the different digests mixed together (el + pep). (c and d) Induction of β -casein expression in the SCp2 cell line. (c) SCp2 cells were either plated on tissue culture plastic (plastic) and then induced to differentiate by addition to 150 μ g/ml EHS matrix (EHS) to the culture medium for 2.5 d, or they were preclustered by culture on nonadhesive poly 2-hydroxyethylmethacrylate (polyHEMA) before addition of the matrix. β -casein expression was visualized by indirect immunofluorescence (left-hand panels), and cell distribution was determined by counterstaining the same cultures with DAPI (right-hand panels). 400 μ g/ml elastase-related EHS matrix (el-EHS) blocked differentiation induced by the intact matrix, but it was unable to direct casein expression itself. (d) SCp2 cells were clustered on poly 2-hydroxyethylmethacrylate, and differentiation was induced by the addition of EHS matrix for 2.5 d. Immune precipitates prepared from pulse-labeled cultures (as in Fig. 3) confirm that 100 and 400 μ g/ml elastase-treated EHS matrix blocked differentiation, but that this material was unable to induce casein expression itself.

TABLE 1

Table 1. Laminin Peptides Used in This Study

		Net charge	Hydropathy index	Blocks casein expression
F-9	RYVVLPRPVCFEKGMNYTVR	+3	-3.9	-
F-12	VEGVEGPRCDKCTRGY	0	-14.8	-
F-16	AQKTEESADARRKAEL	0	-24.8	-
TG-1	RPVRHAQCRVCDGNSTNPRERH	+5	-31.0	-
GD-1	KATPMLKMRTSFHGCIK	+5	-5.1	-
GD-1s	LGHMITFKKPSKTMKAC	+5	-5.0	-
GD-2	KEGYKVRLDLNITLFRITTSK	+2	-10.1	+
GD-2s	LGIKTKSRKDLEFTLVNTREY	+2	-10.1	-
GD-3	KNLEISRSTFDLLRNSYGVRK	+3	-8.6	-
GD-5	TSLRKALLHAPTGSYSYGQ	+2	-11.8	-
GD-8	GRLHFMFDLGKGRKVSHP	+5	-8.2	-

Net charge and hydropathy index are from Charonis et al., 1988; Skubitz et al., 1991; Wilke and Skubitz, 1991. GD-1s and GD-2s are randomly scrambled sequences with the same amino acid content as GD-1 and GD-2.