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Regulation of Mammary Differentiation by the Extracellular Matrix

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In multicellular organisms cell growth and differentiation are influenced by soluble factors, cell-cell interactions and cell-extracellular matrix interactions. We have used the rat mammary gland as a model system to study the role of extracellular matrix components in the regulation of milk protein gene expression. Since mammary epithelial cells differentiate on a basement membrane *in vivo*, we investigated the effects of basement membrane components on the expression of the milk protein genes, α-casein, α-lactalbumin, and transferrin. We have demonstrated that a basement membrane gel, as well as its major basement membrane component, laminin, induced α-casein and α-lactalbumin expression as much as 160-fold compared to tissue culture plastic. We demonstrate that laminin affects mRNA stability as well as having an effect on protein stability and secretion.

Laminin interacts with mammary epithelial cells via an 68 kD cell surface receptor which is capable of interacting with the cellular cytoskeleton. In order to provide evidence that laminin affects on mammary differentiation are mediated through this receptor via the cytoskeleton, we examined the effects of cytoskeletal disrupting agents on milk protein gene expression. We demonstrate that cytochalasin D or colchicine selectively block laminin-mediated milk protein gene expression by affecting mRNA stability. Based on these experiments, we propose a model in which laminin affects mammary gene expression through interaction with cell surface receptors which interact with the cytoskeleton resulting in stabilization of mRNAs for milk protein genes.

Introduction

An understanding of the regulation of gene expression in multicellular organisms is a central issue of cellular and molecular biology. There is substantial evidence that these genes are regulated by different sets of external signals. These signals may be grouped into soluble factors; cell-cell interaction and cell-extracellular matrix (ECM) interaction. These regulatory factors are summarized in Figure 1. Normal cell growth and differentiation are regulated by complex interactions of these pathways. Malignant transformation characterized by unregulated growth and defective differentiation occurs through alterations in these pathways.

Our laboratory has used the rat mammary gland as a model system to explore the role of ECM components in the regulation of tissue specific gene expression. This is an excellent system to study differentiated function because, during development, mammary epithelial cells undergo growth and differentiation that is characterized by the expression of a defined set of milk protein genes. *In vitro* studies have indicated that tissue-specific gene expression is influenced by hormonal factors and cell-cell interactions, as well as by the ECM. In this paper we review the biochemistry of ECM components and recent data on ECM receptors. We then discuss the composition of the ECM in the mammary gland and the role of ECM in mammary differentiation. Based on this data we present a molecular model for regulation of gene expression by the ECM.

Structure and Composition of Basement Membranes

In vivo mammary epithelial cells rest on a basement membrane that forms a thin, continuous sheet between epithelia and underlying connective tissue stroma. The

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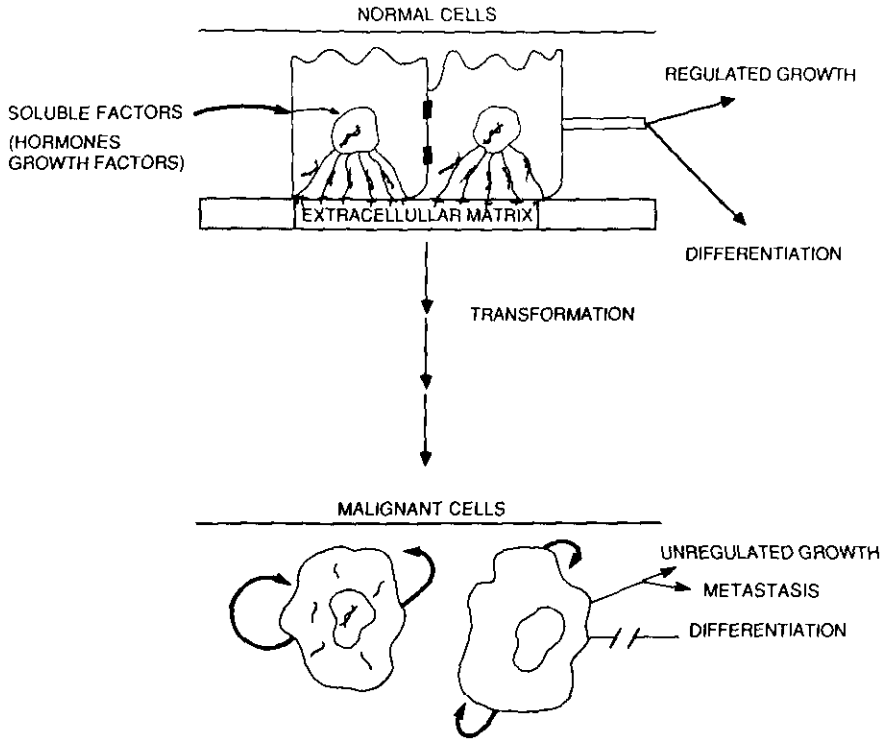

FIGURE 1. Regulation of cell growth and differentiation.

composition of the basement membrane was first approached by the biochemical analysis and fractionation of tissues such as the lens capsule (*1*) and the glomerulus (*2*) and by the analysis of basement membrane-producing tumors (*3–6*). The major components of the basement membrane include type IV collagen, proteoglycans, and glycoproteins. The major glycoproteins of the ECM include laminin and fibronectin, as well as the less-characterized basement membrane glycoproteins, entactin and nidogen. Laminin is the major basement membrane glycoprotein of basement membranes; fibronectin is the major glycoprotein of the ECM of stromal cells.

In addition to these basement membrane components that are widely distributed in adult tissues, there are ECM components that are expressed during development. A recently characterized class of proteins, the hexabrachions, are a representative of this type of ECM protein. The hexabrachions have been isolated from chick embryo and human fibroblast cultures and are expressed during embryogenesis (*7–9*). Tenascin, one of these hexabrachion proteins, is found in the mammary gland during embryogenesis and in mammary tumors but is lost during normal growth and development of the mammary gland (*7*).

Another example of a tissue-specific ECM component is hemonectin, a 60 kD protein that our laboratory has recently isolated from rabbit bone marrow matrix (*10*). This protein, which is found in bone marrow, promotes the attachment of granulocytic cells. It is anticipated that other tissue-specific or developmentally regulated ECM components will be isolated in the future.

Laminin

One of the major ECM components of the adult mammary gland is the basement membrane glycoprotein laminin. This large, complex glycoprotein of approximately 900 kD consists of three disulfide linked subunits, a 360 to 400 kD alpha chain subunit, a 225 kD beta 1 chain, and a 205 kD beta 2 chain (*11,12*). Laminin was first isolated as an intact molecule from the Engelbreth-Holm-Swarm (EHS) tumor. Using this tissue as a source, large amounts of laminin can be extracted by neutral salt solutions and then purified by DEAE-cellulose chromatography and agarose gel filtration (*5*), or alternately can be purified by lectin affinity chromatography (*13*).

As shown by rotary shadowing electron microscopy, laminin is a cross-shaped molecule with two globular domains at the ends of the short arms and one globular domain at the end of the long arm (*14*). Protease digestion has demonstrated that one cell binding domain is on a 260 to 300 kD fragment of the three short arms without the globular domains (P1 fragment) (*15*). A cell-binding domain of nine amino acids has been identified within the P1 region on the B1 chain (*16*). Unlike fibronectin and other adhesive matrix proteins, this cell-binding domain of laminin lacks the Arg-Gly-Asp (RGD) sequence (*16*). The lower 35 nm of the long arm, the E8 fragment, triggers neurite outgrowth (*17*) and contains a second cell-binding domain for several nonneural cells (*18*). Laminin molecules can form polymers and aggregates by binding to the globular domains (*19*). Laminin also binds to other ECM components, including heparan sulfate proteogly-

cans (*20*), type IV collagen, and nidogen (*21,22*). In summary, laminin is a large, complex molecule with multiple functional domains which enable the molecule to self-assemble and to interact with other ECM components and with cells. These characteristics may be important in cell-substrate interactions as well as in the assembly of basement membranes.

Laminin Receptor

Work from our laboratory as well as others have demonstrated that laminin interacts with the cell surface via a specific 68 kD cell surface receptor. This receptor has been purified by laminin affinity chromatography and binds to laminin with a K_d of approximately 2×10^{-9} M. It has been isolated from human breast cancer cells, mouse fibrosarcoma cells, myoblasts, murine melanoma cells, human neutrophils, and mouse macrophages (*23–28*). This receptor has characteristics of a membrane protein in that it is hydrophobic and can be incorporated into liposomes (*11*). Recently, cDNAs representing part of the receptor have been isolated (*29*). In our laboratory overlapping cDNA clones spanning the entire molecule have been isolated from a human endothelial library. We are using this cDNA to study the regulation of laminin receptor expression. In addition to the high affinity 68 kD laminin receptor, laminin binds to some cell types with lower affinity via cell surface glycolipids (*30*). The CSAT (cell substrate attachment antigen) protein has also been shown to bind to both laminin and fibronectin with a Kd of approximately 10^{-6} M (*31*).

Fibronectin

Fibronectin is a cell surface and plasma glycoprotein primarily synthesized by fibroblasts and endothelial cells and also by some epithelial cells. It is a multifunctional and well-characterized glycoprotein involved in cell attachment, spreading, and migration, cytoskeletal organization, hemostasis, differentiation, and metastasis (*32,33*). The subunits of fibronectin are approximately 220 to 250 kD and contain multiple functional domains capable of binding to cells, bacteria, collagens, fibrin, heparin, proteoglycans, and actin. One of the most important observations about the fibronectin molecule is the identification of the Arg-Gly-ASP (RGD) sequence in the cell binding domain (*34–36*). Through the use of the RGD tripeptide, a class of divalent cation-dependent cell surface receptors for a variety of adhesive proteins has been identified. The receptors for fibronectin, vitronectin, fibrinogen, collagen type I, and von Willebrand factor (*37–41*) all bind to RGD sequences in their ligands. The RGD tripeptide also binds to the platelet glycoproteins IIb/IIIa (*42*). Although these receptors are structurally and functionally related, specific ligand-receptor interactions must be mediated by important differences either in ligand availability or by specific regions or conformation of either the ligand or its receptor.

Other Glycoproteins

Entactin is a widely distributed, basement membrane, sulfated glycoprotein of approximately 150 kDa that was identified in murine endodermal cell lines and the EHS tumor and which has recently been partially cloned and sequenced (*43–45*). Entactin is localized in basement membranes of a variety of tissues including the mammary gland (*46*). Nidogen is a glycoprotein isolated from the EHS tumor that has been described in Reichert's membrane and is expressed during embryogenesis (*47,48*).

Collagens

A complete review of collagen biochemistry is beyond the scope of this chapter (*49–53*). The collagens are characterized by a triple helical domain with the repetitive sequence gly-X-Y with the X position amino acid often being proline and the Y position amino acid often being hydroxyproline or hydroxylysine. Types I, II, III, and V collagen are fibrillar interstitial collagens, and type IV collagen is a nonfibrillar protein found in basement membranes. The more recently described collagens types (VI-X) are also nonfibrillar.

Basement Membrane, Type IV Collagen

Type IV collagen, which is the major collagenous component of basement membranes, is a heterotrimer composed of pro-α1(IV), 185 kD, and pro-α2(IV), 170 kD chains (*54*). It has a 400-nm triple helical portion with a globular domain at the carboxy terminus (*55*). Aggregation of type IV collagen monomers occurs *in vitro* and appears to be mediated by covalent interactions between the noncollagenous domains of the molecule as described by Timpl et al. (*55*). Type IV collagen has also been shown to specifically interact with other ECM components. Kleinman et al. have shown that there is an increase in turbidity when type IV collagen and laminin are incubated at 35°C (*4*). Charonis et al. reported that laminin and type IV collagen form complexes that can be observed by rotary shadowing electron microscopy (*21*). Heparan sulfate proteoglycans have similarly been shown to associate with type IV collagen (*56*). Although functional interactions have not yet been proven *in vivo*, they may prove extremely important in the organization and synthesis of basement membranes.

Type V collagen is a hetero or homotrimer, variably composed of α1(IV), α2(V), or α3(V) chains. The exact relationship of type V collagen to the basement membrane is not well defined. However, type V collagen has been reported to act as an anchoring protein of the cell and its basement membrane to the interstitial collagen below (*57,58*).

Several molecules that specifically bind to collagen have been isolated. A 47 kD cell surface receptor for type IV collagen has been isolated by affinity chromatography

from membrane preparations (*59*). A group of collagen type 1 binding proteins of 250 kD, 70 kD, and 30 kD have been isolated by affinity chromatography using a matrix of nondenatured collagen type 1 and eluting with an RGD-containing peptide (*39*). An integral membrane proteoglycan that binds collagen types 1 and 111 has been isolated from mouse mammary epithelial cells (*60*). This cell surface proteoglycan is proposed to act as a receptor for interstitial fibrillar but not basement membrane collagens.

Proteoglycans

Proteoglycans form a diverse group of ECM components composed of core proteins with glycosaminoglycan chains. There is considerable heterogeneity in the core proteins and in the size, composition, and degree of sulfation of the side chains. This heterogeneity in both the protein core and in the glycosaminoglycans may affect the properties and functions of the proteoglycans and appears to be tissue specific (*61*). Proteoglycans can also bind to other ECM macromolecules such as laminin, fibronectin, and collagen type I (*62,63*). Additionally, proteoglycans play a role in tissue-specific gene expression. Fujita and colleagues have shown that proteoglycans from a number of tissue sources augment levels of liver specific mRNAs and reduce levels of housekeeping gene mRNAs *in vitro* (*64*).

Extracellular Matrix and Gene Expression

Although it is clear that ECM affects gene expression in a variety of systems, the molecular mechanisms responsible for this remain largely unknown. From totipotential stem cells of the early embryo arise all of the ectodermal, endodermal, and mesodermal lineages. These determined cells then differentiate to form the variety of cells of the embryo and the adult organism. Potential regulatory events in this process involve initiation of transcription, elongation, RNA polyadenylation, splicing, transport, mRNA stability, translation, and posttranslational events. Studies of gene expression in cultured cells have been primarily done in mesenchymal cells that can survive and differentiate on a plastic substratum, bathed in basal medium supplemented with serum. However, epithelial cells either fail to survive or quickly de-differentiate under these conditions. These observations suggest that epithelial cells require specific hormonal or growth factor signals not found in serum and that they require specific ECM signals that the cells themselves are not able to adequately provide on tissue culture plastic. The ability to study the regulation of differentiation under defined hormonal and substrate conditions has greatly enhanced our understanding of the mechanisms involved in these processes.

Role of the Extracellular Matrix in Mammary Differentiation

There is considerable evidence that both ECM and hormonal components are involved in mammary gland morphogenesis, growth, and differentiation. In this section, we review data regarding the composition of mammary gland ECM and review some of the results in our laboratory and others regarding the role of ECM in mammary differentiation. We then describe recent data concerning the molecular mechanisms involved in the regulation of mammary differentiation by ECM components.

Extracellular Matrix Composition of the Mammary Gland

Immunohistochemical studies have demonstrated that basement membrane composition and architecture change during mammary gland morphogenesis (*46,65,66*). In the virgin gland, myoepithelial cells form a continuous layer around the epithelial cells. These cells rest on a continuous basement membrane containing laminin, type IV collagen, and heparan sulfate proteoglycan. Fibronectin, type I and V collagen, and entactin are associated with the interstitial connective tissue and are not in the basement membrane in the virgin gland. With alveolar growth during pregnancy and lactation, the epithelial cells directly abut the basement membrane, which remains continuous. With involution, dissolution of the basement membrane occurs (*67*). In order to design an experimental model of involution *in vivo*, our laboratory has used *cis*-hydroxyproline to inhibit collagen deposition *in vivo* in rat mammary glands. In this model we provided evidence that the continued deposition of type IV collagen in the basement membrane was necessary for normal mammary growth and that dissolution of the basement membrane led to mammary involution (*68*). We also demonstrated that the administration of *cis*-hydroxyproline to rats bearing DMBA-induced mammary carcinomas led to tumor involution (*69*). Thus, an intact basement membrane is required for the maintenance of architecture in the normal mammary gland and also is required for the growth of well-differentiated mammary adenocarinomas. In addition, growth and attachment of mammary cells *in vitro* requires type IV collagen. We have shown that *cis*-hydroxyproline reduces the attachment and growth of mammary cells on type I but not type IV collagen substrata (*70*). These findings suggest that basement membrane components play an important role in the morphogenesis and growth of the mammary gland.

Several studies have indicated that the synthesis and deposition of ECM components by mammary epithelium is influenced by both soluble factors and other ECM components. Type IV collagen deposition by virgin rat mammary cells is hormone dependent (*69*). Glucocorticoids suppress type IV collagenolytic activity, leading to increased type IV collagen deposition (*71*). In addition, a variety of growth factors, including epidermal growth factor and mammary-derived growth factor-1 (*72*), modulate

type IV collagen synthesis. TGF-beta, which has been shown to regulate matrix synthesis, also acts as a negative growth control factor in the mammary gland (*73*). Thus, basement membrane deposition, which is required for mammary growth *in vitro* and *in vivo*, may be regulated by soluble factors. Additionally, the synthesis and deposition of ECM components by mammary epithelium is also influenced by ECM components themselves. Thus, it has been shown that the incorporation of SO_4^{-2} and glucosamine into basement membrane proteoglycans is enhanced when mammary cells are cultured on floating collagen gels *in vitro*. Pulse-chase studies have indicated that this is partially due to reduced degradation (*74,75*). Furthermore, Parry et al. have demonstrated that mammary cells on floating collagen gels incorporate glycosaminoglycans into an ECM whereas those on tissue culture plastic or attached gels secrete most of the synthesized glycosaminoglycans into the medium (*76*).

Earlier investigations into the regulation of milk protein gene expression used organ cultures or primary cultures of mammary cells on floating gels of stromal collagen. Organ cultures that maintain normal cell-cell and cell-ECM contacts allow the investigation of hormonal regulation of milk protein gene expression (*77–84*) but are not useful for the analysis of the role of isolated ECM components in milk protein gene expression. Primary cultures of mammary cells on top of or embedded in floating pads of types I and III collagen gels allowed the expression of some milk proteins (*85–93*). The ability of collagen gels to induce and maintain mammary differentiation is associated with contraction of the gels. If gel contraction is prevented either by glutaraldehyde fixation or by leaving the gels attached to the culture dish, the expression of differentiated function is greatly reduced (*88–91*). These observations led to the suggestion that cell shape changes influenced by gel contraction are necessary for the expression of differentiated function. Alternatively, we and others have proposed that the effects of floating collagen gels may be directly due to the deposition of basement membrane components by mammary cells on the gels (*94,95*). Recently, studies have shown that cell polarity is also critical in regulating mammary differentiation. This cell polarity may be influenced by the deposition of ECM components by mammary cells (*96*).

In order to determine if ECM components could induce milk protein gene expression, our laboratory has previously used an acellular biomatrix derived from pregnant rat mammary glands as a substratum for rat mammary epithelium (*95*). This biomatrix contains both stromal and basement membrane components and has been found to induce α-lactalbumin (α-LA) protein synthesis in primary rat mammary cultures. If the induction of milk protein gene expression on collagen gels and on biomatrix were due to basement membrane components, then one would predict that basement membrane components themselves would enhance the expression of milk protein genes. Moreover, as indicated above, *in vivo* mammary epithelial cells rest directly on a basement membrane composed of laminin, type IV collagen, entactin, heparan sulfate proteoglycans, and other glycoproteins. These observations led our laboratory to investigate the effect of basement membrane components on the expression of milk protein genes in the rat mammary gland (*97*). These studies are described in more detail below. The mammary gland is an excellent system to study the effect of these components on differentiation since markers of differentiation consist of a well-defined group of milk protein genes.

Milk Proteins

During lactation, most of the protein secretory machinery of mammary epithelial cells is dedicated to the synthesis of milk proteins. Approximately 80% of the milk proteins that are secreted are caseins, a family of calcium-binding phosphoproteins, which are secreted into milk as aggregates termed casein micelles. In the rat, α-casein is synthesized as a doublet of 43 kD and 41 kD, β-casein is 25 kD in size and γ-casein is a series of bands between 18 and 22 kD (*98*). The cDNA and genomic sequences of the caseins have been extensively characterized by Jeffrey Rosen and his colleagues (*99–102*). Their studies indicate that there has been considerable divergence in the sequence of these genes, with the exception of three highly conserved structural domains, the signal sequence, the casein kinase domain, and the 5′ noncoding sequences. In addition to this structural divergence, Rosen and colleagues demonstrated that hormonal control of the expression of these genes is kinetically distinct. These investigators also demonstrated in organ cultures studies that the lactogenic hormones prolactin, insulin, and hydrocortisone regulate casein gene expression at both the transcriptional and posttranscriptional levels (*79*). Other important milk proteins include α-lactalbumin (α-LA), which is a 20 to 21 kD protein in the rat which functions as a cofactor for galactosyltransferase in the synthesis of the major milk sugar lactose and transferrin, the iron-binding protein that also acts as a growth factor and is secreted into milk (*91*). In the studies outlined in the proceeding sections, we investigated the effects of basement membrane components on casein, α-lactalbumin, and transferrin gene expression at both the mRNA and protein levels.

Effects of Basement Membrane Components on Mammary Differentiation

Since *in vivo* mammary epithelial cells rest on the basement membrane, we examined the effects of basement membrane components on the expression of milk protein genes. We examined the effects of a gel of basement membrane components and the isolated basement membrane component laminin on the expression of milk protein genes in primary rat mammary cultures (*97*). Primary mammary cells were cultured under serum-free hormonally defined conditions in the presence of prolactin, hydrocortisone, and insulin. We examined the effects of these substrata on milk protein gene expression at both

the mRNA and protein levels. A basement membrane gel was prepared from the Engelbreth-Holm-Swarm tumor as described by Kleinman et al. (*103*). This gel has been shown to contain laminin, type IV collagen, entactin, nidogen, and heparan sulfate proteoglycan. Laminin, the major ECM component of this gel, comprises 85% of the total protein. This gel is now marketed commercially as Matrigel (Collaborative Research Inc.). Laminin was prepared as described by Timpl et al. (*5*) and its purity assayed by SDS-PAGE and silver staining. In these experiments α-LA was assessed by radioimmunoassays as we have previously described (*95*). Caseins were assayed by immunoprecipitation of ^{35}S-methionine-labeled cultures.

Total casein synthesis representing the sum of α-casein in the cell layer and the medium was induced up to 160-fold on basement membrane gel relative to plastic. Lesser effects of α-casein expression were seen for cells on floating collagen gel. The purified basement membrane component laminin increased total steady-state α-casein levels approximately 10-fold compared to tissue culture plastic. α-Lactalbumin was measured by radioimmunoassay. This protein was induced by 70-fold on basement membrane gel compared to tissue culture plastic. Laminin also had a significant although lesser effect on α-LA production reaching 7-fold greater than tissue culture plastic. On the basis of these experiments, we postulated that changes in steady-state levels of milk protein expression on ECM components could be due to changes in milk protein stability or steady-state accumulation of milk protein mRNAs. In order to examine the level of regulation, we performed pulse-chase analysis of metabolically labeled immunoprecipitated milk proteins, as well as Northern analysis to assay steady-state mRNAs levels. Pulse-chase analysis revealed that the ECM component laminin decreases the intracellular turnover of α-1 and α-2 caseins, as well as increasing the secretion of α-casein (*97*). The effect of ECM components on steady-state levels of mRNA was assessed by dot blot and Northern blot analysis. A representative Northern blot is shown in Figure 2. By this analysis, α-casein steady-state level of mRNA was induced approximately 5-fold on laminin relative to tissue culture plastic. This Northern blot analysis confirmed that the α-casein mRNA was intact (*97*).

Analysis using cDNAs for β and γ-casein indicated that various milk protein mRNAs are differentially regulated by ECM substrata. Steady-state levels of α and β casein mRNAs accumulated up to 5-fold more on basement membrane substrata than on tissue culture plastic, while α-LA mRNA levels under the same conditions were 2-fold greater than tissue culture plastic. In contrast, there was no consistent induction of γ-casein mRNA by ECM substrata (*97*). The induction of differentiated function by ECM substrata was not due to changes in cell types or cell growth since under these culture conditions there was no significant cell growth and cell type distribution was unaffected as determined using monoclonal antibody markers (*97*). From these studies, we concluded that milk protein genes are not coordinately regulated by ECM components. Furthermore, since the amount of induction of milk proteins exceeded the amount of induction of mRNA for these proteins, we concluded that ECM components including laminin act at the level of steady-state accumulation of mRNA in addition to acting at a translational or posttranslational level. A recent publication examining the effects of basement membrane components on mouse mammary gland expression has also found that ECM components can induce the expression of mouse casein genes in a noncoordinate manner (*104*).

In order to further examine molecular mechanisms involved in the induction of mammary milk protein gene expression by ECM components, we have concentrated on the effects of the purified basement membrane component laminin. There are a number of advantages to using this purified component, including the ability to perform labeling experiments without the trapping problem inherent in gelled substrata. Furthermore, the use of a purified component allows more detailed explorations of mechanisms of action. Since we have isolated laminin receptors from mammary epithelial cells, this enables us to examine signal transduction mechanisms involved in the induction of differentiated function.

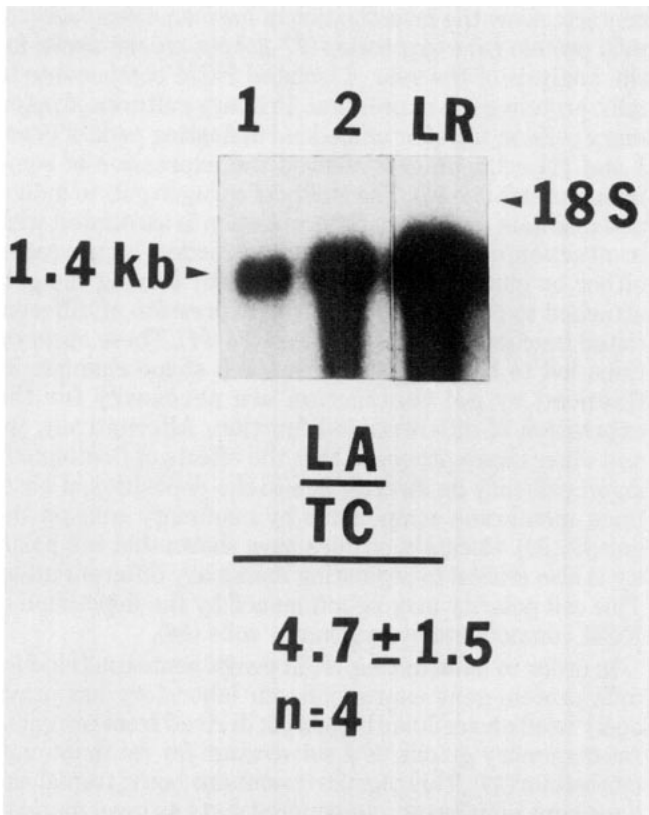

FIGURE 2. Northern blot of total cytoplasmic RNA. Total cytoplasmic RNA was isolated from 6-day primary rat mammary cells cultured on tissue culture plastic (TC) or laminin (LA) using detergent extraction. Equal amounts of total cytoplasmic RNA were loaded onto denaturing agarose gel, size fractionated, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled cDNA probe specific for α-casein gene. α-Casein transcript of 1.4 kb was detected. Lanes (1) TC, (2) LA, (LR) lactating RNA. The amount of α-casein RNA was quantitated by scintillation counting of nitrocellulose filter and the relative amount calculated as percent of lactating RNA. The ratio of α-casein RNA for LA versus TC is given and the standard deviation noted as calculated on the basis of four experiments.

Effects of Laminin on Casein mRNA

Northern blot analysis demonstrates that laminin induces α-casein mRNA (approximately 5-fold) compared to tissue culture plastic. This change in steady-state mRNA could be due to changes in transcription rate or mRNA stability. We have preliminary evidence that changes in mRNA levels are primarily due to changes in mRNA stability. This was determined by pulse-chase analysis, labeling RNA with ^{3}H-uridine as previously described (*80*). Cultures were pulsed for 1 hr with ^{3}H-uridine and then chased in the presence of 2 mM uridine and cytione. As shown in Figure 3, the turnover of casein mRNA is approximately 3- to 5-fold more rapid on tissue culture plastic than on laminin substrata. In contrast, total mRNA as indicated by poly A RNA turnover is unaffected by ECM. From these studies we conclude that differences in steady-state mRNA levels for the casein genes detected on laminin can be explained by alterations in mRNA half-life. Studies to determine whether laminin also effects mRNA transcription are in progress.

Laminin Receptor and the Cytoskeleton

Work in our laboratory and others has demonstrated that laminin binds to the surface of a variety of cells via a specific 68 kD cell surface receptor. It has been suggested that ECM may affect cell shape and the cytoskeleton. Sugrue and Hay demonstrated that when corneal epithelial are removed from their basement membrane, the basal cell surface forms numerous blebs that are associated with a disorganized cytoskeleton. Addition of soluble matrix molecules such as laminin or fibronectin causes actin to form filaments at the basal cell surface (*105*). On the basis of these studies, it was postulated that cells have transmembrane receptors capable of mediating the interaction of the ECM and the cytoskeleton. Work in our laboratory has provided evidence that the laminin receptor is such a molecule. The 68 kD laminin receptor protein purified by affinity chromatography is capable of bundling f-actin filaments *in vitro* (*106*). More recently, we have demonstrated that laminin can be clustered on the cell surface and that these clusters are resistant to detergent extraction with nonionic detergents, suggesting that they are linked to the cytoskeleton. Furthermore, when we treated these cells with cytochalasin D, the association of laminin with the detergent-resistant fraction was reduced (*107*). On the basis of these experiments, we have proposed a model in which laminin at the cell surface can cause rearrangement of the cellular cytoskeleton by the clustering of cell surface laminin receptors which, in turn, interact with actin in the cytoskeleton.

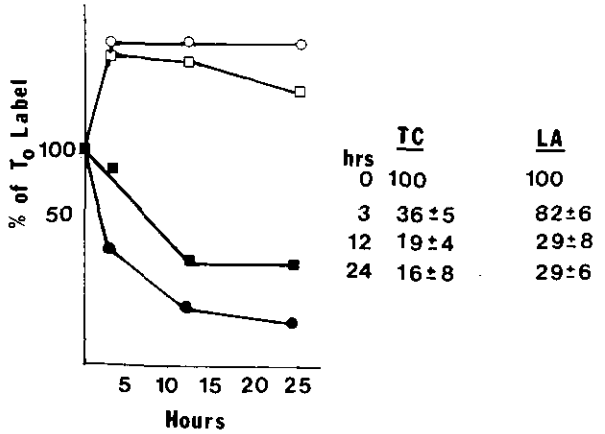

FIGURE 3. Pulse-chase analysis of α-casein mRNA. ^{3}H-labeled α-casein mRNA on laminin (■—■) and TC (●—●). ^{3}H-rRNA on laminin (□—□) and TC (○—○). Percent of T_0 ^{3}H-labeled α-casein mRNA at 3, 12, and 24 hr following the 1-hr pulse is shown and the standard deviation noted as calculated on the basis of two experiments.

Studies from other laboratories have demonstrated that cell surface receptors for other ECM components may also be capable of interacting with the cytoskeleton. The fibronectin receptor, which can be iodinated and incorporated into liposomes, also appears to colocalize with actin filaments at points of cell contact with the substratum (*37,108,109*). Additionally, fibronectin and actin appear to form a close transmembrane association termed the fibronexus (*110*). The CSAT antigen, which interacts with both laminin and fibronectin, also interacts with the actin-filament-associated protein talin (*111*). Rapraeger and Bernfeld have provided evidence that the integral membrane cell surface proteoglycan, which is a receptor for interstitial collagens, is also capable of interacting with the cytoskeleton (*112*). Hyaluronate, which is a principal glycosaminoglycan of the ECM, binds to 3T3 cells via an 85 kD integral membrane glycoprotein. This hyaluronate receptor has been shown to be associated with a cytoskeleton-rich detergent-resistant fraction, suggesting that the receptor is associated with actin or related proteins (*113*). Taken together, these studies demonstrate that cells have multiple ECM receptors that appear to be transmembrane proteins capable of interacting with the cellular cytoskeleton.

On the basis of evidence that mammary epithelial cells interact with laminin via a 68 kD receptor and that this receptor is capable of interacting with the cytoskeleton, we postulated that laminin effects on mammary differentiation might be mediated via the cytoskeleton. Therefore, we investigated the effects of the cytoskeletal disrupting agents, cytochalasin D and colchicine on laminin-induced milk protein mRNA, and protein expression in primary rat mammary cultures. Cytochalasin D inhibits actin polymerization, while colchicine prevents microtubule polymerization (*114–118*). In these experiments we measured the milk proteins α-casein and transferrin by immunoprecipitation, as well as the mRNAs for these proteins by Northern blot analysis. These experiments demonstrated that treatment of laminin-based cultures with either cytochalasin D or colchicine reduced the level of expression of α-casein and transferrin to levels below that detected in cultures on tissue culture plastic dishes in the absence of cytoskeletal inhibitors (Fig. 4*A,B*). We also assessed the effect of the cytoskeletal inhibitors on α-lactalbumin production. As shown in Figure 4*C*, cytochalasin D reduced the level of α-lactalbumin on laminin to below that detected on tissue culture plastic. The

effect of these inhibitors on the milk protein expression was not due to general effects on protein synthesis. This was determined by examining the effects of these compounds on TCA precipitable counts (Fig. 4*D*) and, furthermore, on total protein synthesis as assessed by one-dimensional and two-dimensional gel electrophoresis (data not shown). These experiments demonstrate that cytochalasin D and colchicine have only minor effects on total protein synthesis. Furthermore, SDS gel electrophoresis analysis showed that the majority of proteins were unaffected by these compounds. In contrast, the induction of milk protein genes induced by laminin was completely abrogated by cytoskeletal inhibitors.

Effects of Cytoskeletal Inhibitors on Milk Protein mRNA Accumulation

In order to determine whether the decrease in milk protein gene expression resulting from the addition of cytoskeletal inhibitors was due to decreased mRNA accumulation for these proteins, we performed Northern blot analysis. As we have previously described, the steady-state level of the milk protein mRNAs were increased on laminin substrata compared to tissue culture plastic. Both cytochalasin D and colchicine caused a marked reduction in the steady-state levels of mRNA for each of these milk protein genes. In the presence of these inhibitors, steady-state levels of mRNA on laminin substrata were considerably below those found on tissue culture plastic in the absence of cytoskeletal inhibitors (Fig. 5). These experiments suggest that the effects of cytoskeletal disrupting agents on laminin-induced milk protein gene expression occur predominantly at the level of accumulation of mRNAs for these proteins.

Effects of Cytoskeletal Inhibitors on mRNA Stability

Previous experiments have suggested that the increase in steady-state accumulation of mRNA for milk proteins on laminin substrata compared to tissue culture plastic could be accounted for by stabilization of these mRNA. Since treatment of cultures with cytoskeletal disrupting

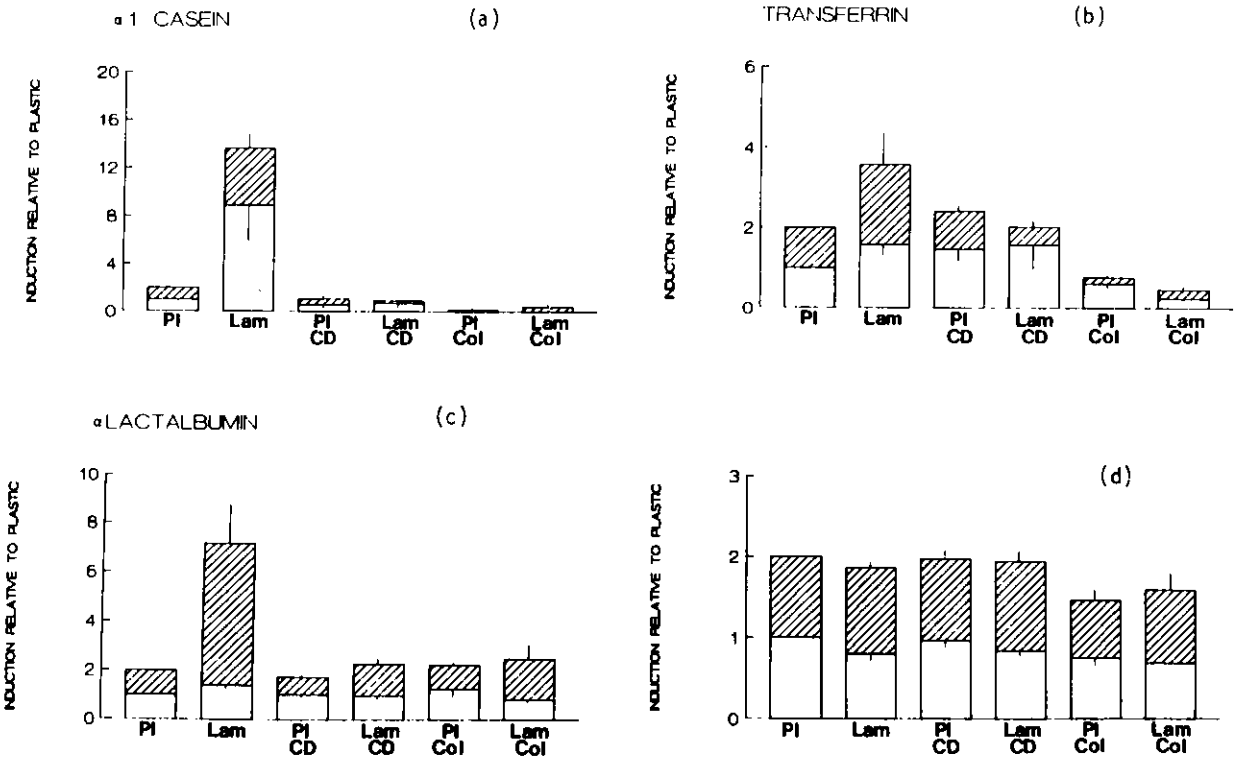

FIGURE 4. Effect of cytoskeletal inhibitors on milk protein and total protein synthesis. Mammary cells were maintained in culture for 6 days, then treated with cytochalasin D 2 μM or colchicine 1 μM for 24 hr. The cultures were metabolically labeled with ^{35}S-methionine for 1 hr, and the medium and cell lysates were harvested. Equivalent TCA precipitable counts of ^{35}S-methionine labeled medium (cross-hatched) and cell lysates (open) were immunoprecipitated, electrophoresed, dried, and fluorograms were obtained. α-Casein (*a*) and transferrin (*b*) bands were excised and counted. The mean amount of induction of (*a*) α-casein and (*b*) transferrin relative to that of cells on plastic dishes is shown. A unit of 1 is assigned to each of the medium and cell lysates on tissue culture plastic. Values are the mean of three to four experiments. Parallel cultures were assayed for (*c*) α-lactalbumin by a radioimmunoassay (*95*) and normalized for cell number (*97*). The mean amount of induction of α-LA synthesis relative to that of cells on plastic from four to nine experiments is shown. Duplicate aliquots from ^{35}S-methionine-labeled cultures described in (*a*) and (*b*) were used for TCA precipitation. The mean amount of induction of TCA precipitable counts relative to that of cells on plastic from three to nine separate experiments and normalized for cell number is shown in (*d*). Pl, tissue culture plastic; Lam, laminin; CD, cytochalasin D; Col, colchicine. Error bars indicate the standard error of the mean.

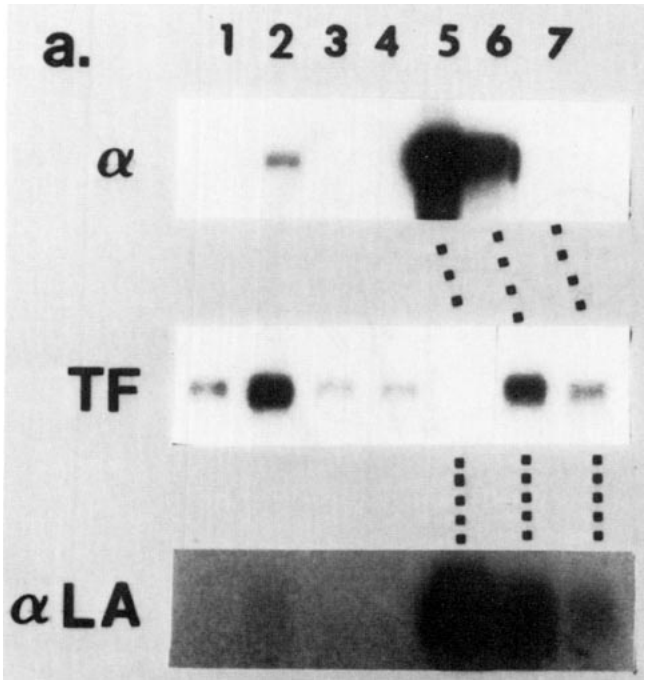

FIGURE 5. Effect of cytoskeletal inhibitors on the steady state accumulation of milk protein mRNAs. RNA isolated from primary rat mammary cell cultures on plastic or laminin in the presence or absence of cytoskeleton inhibitors was size fractionated on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P nick-translated cDNAs for rat α-casein, rat transferrin, and mouse α-lactalbumin. *Equal amounts (4 μg) of RNA were loaded in each lane.* Lanes (*a*) plastic (1) and laminin (2) controls; plastic (3) and laminin treated with cytochalasin D (4), lactating mammary gland RNA (5); perphenazine stimulated mammary gland RNA (6); virgin mammry gland RNA (7).

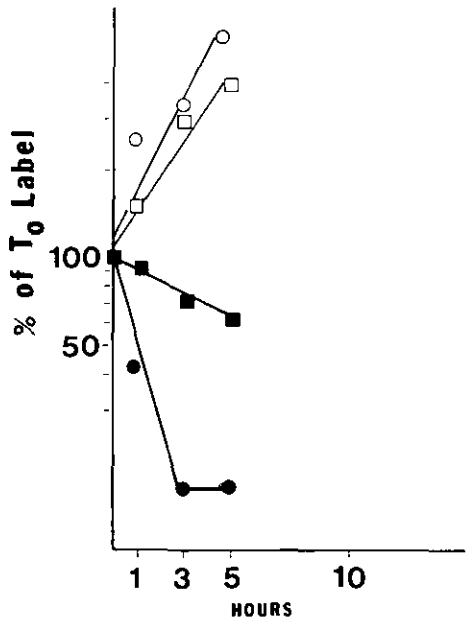

FIGURE 6. Turnover of α-casein mRNA in the presence of cytochalasin D. Pulse-chase analysis was performed as described in the legend to Fig. 2, except that the chase was performed in the presence of 2 mM cytochalasin D (CD) over a period of 5 hr using cells cultured on laminin-coated dishes. Levels of ^{3}H-labeled α-casein RNA were measured as described in Fig. 2 legend and plotted as percent of ^{3}H-labeled α-casein mRNA at time 0. α-Casein mRNA levels in the absence of CD (■–■) and in the presence of CD (●–●). Turnover of 28S rRNA is shown in the absence of CD (□–□) and in the presence of CD (○–○).

agents reduced the steady-state accumulation of mRNAs, we examined the effect of these inhibitors on mRNA turnover. mRNA was labeled by pulsing cells with ^{3}H-uridine and chasing with or without cytochalasin D. As can be seen in Figure 6, addition of cytochalasin D during the chase period increased the turnover of α-casein mRNA. These experiments suggest that the predominant effect of both laminin and cytoskeletal inhibitors on milk protein mRNA accumulation occur at the level of mRNA stability. Taken together, these data suggest that the cytoskeleton may serve to stabilize these mRNA species. Penman and colleagues have previously shown that mRNAs and polysomes are associated with the cytoskeleton. Furthermore, they demonstrated that treating cells with cytochalasin D releases mRNA from the cytoskeleton and reduces protein synthesis (*119–121*). Bonneau et al. have also provided evidence for association of specific mRNAs with the cytoskeleton (*122*). In our system, the increase in the steady-state mRNA level for α-casein may primarily reflect mRNA stabilization by the cytoskeleton. Indeed, preliminary experiments in our laboratory using detergent extraction to characterize mRNAs associated with the cytoskeleton indicate that in differentiating mammary cells *in vitro* over 90% of casein mRNA are associated with the cellular cytoskeleton. The observation that cytoskeletal inhibitors affect the expression of a small subset of total proteins suggests that there may be selective associations of specific mRNAs with the cytoskeleton. Alternately, the selective effects of cytoskeletal inhibitors may reflect intrinsic differences in the stability of mRNAs released upon depolymerization of the cellular cytoskeleton. Experiments to distinguish these possibilities are in progress.

Mechanism of Laminin-Induced Mammary Differentiation

On the basis of the experiments described in this chapter, we propose the following model to account for laminin-induced mammary differentiation (Fig. 7). We postulate that laminin interacts with specific transmembrane receptors on mammary epithelial cells that are capable of interacting with the cellular cytoskeleton. The interaction of these receptors with laminin on the basal cell surface establishes cell polarity. Furthermore, the orientation of laminin receptors on the cell surface may facilitate the organization of the cellular cytoskeleton. The cellular cytoskeleton in turn may influence gene expression at a variety of levels. The predominant effect in our system appears to be stabilization of mRNAs. Thus, the ECM may serve to stabilize the expression of genes whose transcription is regulated by soluble factors and hormones. The mammary system serves as a useful model to understand

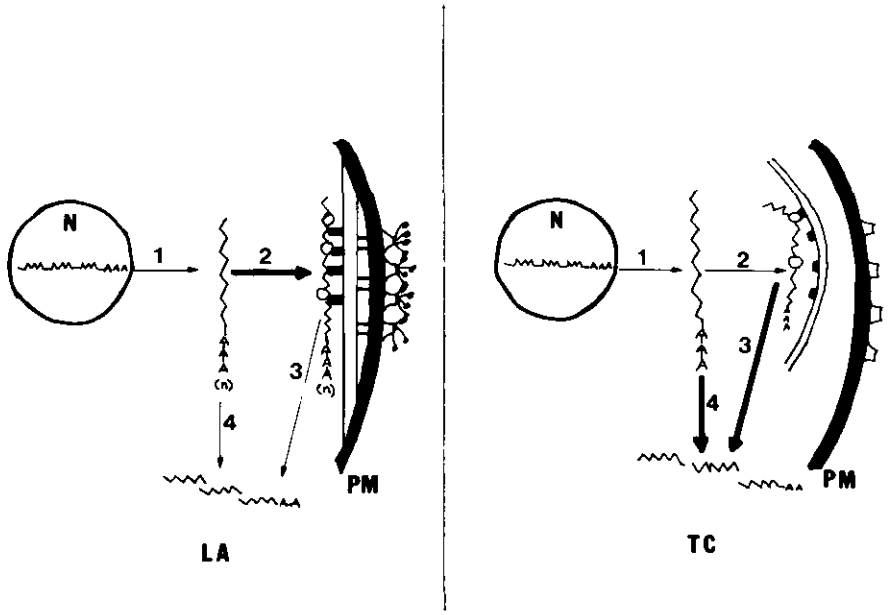

FIGURE 7. Schematic of pathways involved in the stabilization of α-casein mRNA by laminin. Pathways: (1) Maturation and transport of α-casein in mRNA to the cytoplasm; (2) Association of α-casein mRNA with ribosomes to form polysomes that are localized on cytoskeletal structures; (3), (4). Degradation of α-casein mRNA either prior to cytoskeletal attachment (4) or following association with the cytoskeleton (3).

the molecular mechanisms of regulation of gene expression by the ECM.

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