

**Division of Labor among the  $\alpha\beta4$  Integrin,  $\beta1$  Integrins, and an E3 Laminin Receptor to Signal Morphogenesis and  $\beta$ -Casein Expression in Mammary Epithelial Cells**

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LBNL/DOE funding & contract number: DE-AC02-05CH11231

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Contact of cultured mammary epithelial cells with the basement membrane protein laminin induces multiple responses, including cell shape changes, growth arrest, and, in the presence of prolactin, transcription of the milk protein  $\beta$ -casein. We sought to identify the specific laminin receptor(s) mediating the multiple cell responses to laminin. Using assays with clonal mammary epithelial cells, we reveal distinct functions for the  $\alpha 6\beta 4$  integrin,  $\beta 1$  integrins, and an E3 laminin receptor. Signals from laminin for  $\beta$ -casein expression were inhibited in the presence of function-blocking antibodies against both the  $\alpha 6$  and  $\beta 1$  integrin subunits and by the laminin E3 fragment. The  $\alpha 6$ -blocking antibody perturbed signals mediated by the  $\alpha 6\beta 4$  integrin, and the  $\beta 1$ -blocking antibody perturbed signals mediated by another integrin, the  $\alpha$  subunit(s) of which remains to be determined. Neither  $\alpha 6$ - nor  $\beta 1$ -blocking antibodies perturbed the cell shape changes resulting from cell exposure to laminin. However, the E3 laminin fragment and heparin both inhibited cell shape changes induced by laminin, thereby implicating an E3 laminin receptor in this function. These results elucidate the multiplicity of cell-extracellular matrix interactions required to integrate cell structure and signaling and ultimately permit normal cell function.

## Introduction

Cell contact with the extracellular matrix (ECM) serves as a dominant regulator of cellular structure and function (for reviews, see Roskelley *et al.*, 1995; Giancotti, 1997). The ECM functions both as a scaffold for cell attachment and cytoskeletal organization and as an array of signaling molecules. Cell surface receptors for ECM molecules integrate the three cellular responses of attachment, cytoskeletal organization, and signaling. Consequently, cellular structure and signaling events are coupled within these receptors, as shown by adhesion dependence of cell growth and cell shape dependence of some signaling pathways leading to cell survival and tissue-specific gene expression (Petersen *et al.*, 1992; Roskelley *et al.*, 1994; Boudreau *et al.*, 1996; Chen *et al.*, 1997a; Kheradmand *et al.*, 1998; Wang *et al.*, 1998). Although the multiple ECM receptors on the cell surface are presumed to play different roles in signaling and morphogenesis, their distinct functions are not well characterized in the same cell system.

Our laboratory has been dissecting the mechanism by which the ECM regulates epithelial cell behavior using assays of normal cell function in both primary mammary epithelial cells and cell lines. Cells of mammary epithelial origin comprise the myoepithelial and milk-secreting cells of the mammary gland. Like all epithelial cells, they contact the basement membrane, and signaling from the basement membrane is important in all stages of mammary gland development (for review, see Roskelley *et al.*, 1995). Removing mammary epithelial cells from contact with the basement membrane and placing them on tissue culture plastic leads to altered cellular structure and growth, increased apoptosis, and a loss of function, the latter being measured by the cell's inability to respond to lactogenic hormones by producing milk proteins (Emerman and Pitelka, 1977; Barcellos-Hoff *et al.*, 1989; Boudreau *et al.*, 1995; Lin *et al.*, 1995). However, many of these functions can be recovered by culturing cells in the presence of either a reconstituted basement membrane (Matrigel) or the purified basement membrane glycoprotein laminin. Primary mammary epithelial cells, and certain cell lines, cultured in the presence of laminin will arrest growth and reorganize to form rounded cell clusters that regain the ability to respond to lactogenic hormones and to express  $\beta$ -casein mRNA and protein (Roskelley *et al.*, 1994; Streuli *et al.*, 1995).

The specific receptors mediating the signaling responses to laminin in mammary epithelial cells have not been identified. Laminin, which exists in many isoforms, has in excess of 12 reported cell surface receptors. The best characterized laminin receptors belong to the integrin receptor family; these include the  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ,  $\alpha 7\beta 1$ ,  $\alpha 9\beta 1$ , and  $\alpha 6\beta 4$  integrins (Mercurio, 1995). In addition to the integrins, several other cell surface molecules have been implicated in cell-laminin interactions, including dystroglycan, the 67-kDa laminin receptor, an isoform of syndecan-1, and potentially others (Salmivirta *et al.*, 1994; Henry and Campbell, 1996; Hinek, 1996; Chen *et al.*, 1997b). Nearly all of the laminin receptors listed above have been implicated in linkages to the cytoskeleton and may transmit distinct signals via their unique cytoplasmic domains (Sastry and Horwitz, 1993; Henry and Campbell, 1996; Carey, 1997).

Previous studies from our laboratory showed that receptor binding to the E3 domain of laminin is required for  $\beta$ -casein expression and that antibodies blocking  $\beta 1$  integrins inhibit  $\beta$  casein production (Streuli *et al.*, 1995). These studies utilized primary cell cultures and the mammary epithelial cell line CID-9 (Schmidhauser *et al.*, 1990), both of which contain a mixture of epithelial and mesenchymal-like cells. Because primary and mixed cell cultures have the potential to produce an endogenous basement membrane, we have more recently employed a clonal mammary epithelial cell line, SCp2, which has lost the ability to assemble a functional basement membrane and, therefore, circumvents the interference from endogenous laminin deposition (Desprez *et al.*, 1993). This cell line nevertheless responds to reconstituted basement membrane or laminin by making  $\beta$ -casein. Using SCp2 cells, we previously reported two distinct signaling pathways for  $\beta$ -casein expression in response to ECM, a morphogenic and a biochemical pathway (Roskelley *et al.*, 1994). The morphogenic signal is the induction of a rounded morphology in cells exposed to laminin. This signal is a prerequisite for subsequent biochemical signals leading to transcription and translation of the  $\beta$ -casein gene.  $\beta$ -Casein expression was perturbed by a tyrosine kinase inhibitor, whereas the morphological changes were unaffected (Roskelley *et al.*, 1994; Roskelley and Bissell, 1995). Therefore, the morphogenic and biochemical signaling pathways induced by laminin were separated, yet the precise receptor(s) initiating these signals were still to be determined. In the present study, we have used reagents that block receptor-ligand interactions at the cell surface to dissect the function(s) of the laminin receptors operating in mammary epithelial cells. We demonstrate distinct but cooperative roles for the  $\alpha 6\beta 4$  integrin,  $\beta 1$  integrins, and an E3 laminin receptor in the functional differentiation of mammary epithelial cells. We also show that some of these signaling functions can be masked when the population in the cell culture is heterogeneous.

## Materials and Methods

### Antibodies and Reagents

The function-blocking integrin antibodies against  $\alpha 1$  (Ha31/8),  $\alpha 5$  (5H10-27),  $\alpha 6$  (GoH3),  $\alpha v$  (H9.2B8), and  $\beta 1$  (Ha2/5 and 9EG7) subunits were purchased as azide- and endotoxin-free reagents from PharMingen (San Diego, CA). The anti-integrin  $\beta 4$  subunit (clone 346-11A) was also purchased from PharMingen. Polyclonal anti- $\beta$ -casein antibody was generated against whole mouse milk in our laboratory as described (Lee *et al.*, 1984). The monoclonal antirat  $\beta$ -casein

antibody was a gift from Dr. C. Kaetzel (Kaetzel and Ray, 1984). The anti-E-cadherin antibody (C20820) was purchased from Transduction Laboratories (Lexington, KY). Laminin fragments were prepared as previously described (Schittny and Yurchenco, 1990; Sung *et al.*, 1993) and dialyzed against PBS. Heparin and heparan sulfate were purchased from Sigma Chemical (St. Louis, MO), product numbers H3393 and H9902, respectively.

### **Cell Culture and $\beta$ -Casein Assays**

The SCp2 cell line (Desprez *et al.*, 1993) is a functionally normal murine mammary epithelial line cloned from the heterogeneous cell strain CID-9 (Schmidhauser *et al.*, 1990). SCp2, NIH3T3, and primary mammary epithelial cells were cultured in DMEM/F12 medium (1:1) supplemented with insulin (5  $\mu\text{g}/\text{ml}$ ) (Sigma Chemical) and 2% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Primary mammary epithelial cells were isolated from midpregnant CD-1 mice, as described (Lee *et al.*, 1984).

To assay  $\beta$ -casein expression in mammary epithelial cells treated on tissue culture plastic, cells were plated at subconfluence in serum-free DMEM/F12 medium supplemented with insulin (5  $\mu\text{g}/\text{ml}$ ) and hydrocortisone (1  $\mu\text{g}/\text{ml}$ ) (Sigma Chemical) at a density of  $\sim 50,000$  cells/ $\text{cm}^2$ . Cells were allowed to attach and spread for 2 d before treatment. Once completely spread, they were treated with fresh serum-free medium, insulin, hydrocortisone, and prolactin (3  $\mu\text{g}/\text{ml}$ ) with or without laminin or Matrigel. Laminin or Matrigel diluted in the culture medium rapidly fall out of solution, forming a precipitate covering the cultured cells and thereby producing a high concentration of laminin at the cell surface. Cells were treated for 5 d, with one change of medium after 3 d, and then extracted for protein analysis. For extraction, cells were rinsed once with PBS, frozen and thawed in 100 ml of protein extraction buffer (50 mM Tris-HCl, pH 7.4, 30 mM NaCl, 1% [vol/vol] NP-40, 1% [wt/vol] deoxycholate, 0.1% [wt/vol] SDS, and protease inhibitor cocktail [Calbiochem, La Jolla, CA]), and cleared by centrifugation for 5 min at 12,000  $\times g$ . The resulting supernatant was added to reducing protein sample buffer and separated by SDS-PAGE as described below. Ovine prolactin-20 (AFP 10677C) was a gift from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Bethesda, MD). Purified Engelbreth-Holm-Swarm laminin was purchased from Sigma Chemical and included in the assays at 150  $\mu\text{g}/\text{ml}$ . Matrigel was purchased from Collaborative Biomedical Products (Bedford, MA) and used at a 1.5% dilution ( $\sim 150$ – $200$   $\mu\text{g}$  protein/ml).

For assays of  $\beta$ -casein expression and survival in prereduced cells, cells were first cultured in suspension by placing  $4.0 \times 10^6$  cells in a 10-cm culture dish coated with the nonadhesive substratum poly(2-hydroxyethyl methacrylate) (polyHEMA) (Sigma Chemical) in 10 ml of serum-free medium, plus insulin and hydrocortisone. Cells were allowed to aggregate in suspension for 2 d and then divided into either 48- or 96-well culture dishes coated with polyHEMA ( $2.0 \times 10^5$  or  $1.2 \times 10^5$  cells per well, respectively) in serum-free medium plus insulin, hydrocortisone, and prolactin, with or without laminin. Cells were incubated for 3 d before extraction for protein analysis. For extraction, cells were transferred to Eppendorf tubes, centrifuged at 3000  $\times g$  for 5 min, and lysed in protein extraction buffer, as described above. Viability of treated cells in suspension was assayed after 4 d using the Alamar Blue vital dye assay (Accumed International, Westlake, OH) according to the manufacturer's instructions. PolyHEMA-coated dishes were prepared using a solution of 6 mg/ml polyHEMA in 95% ethanol

added to culture plates at 0.05 ml/cm<sup>2</sup> and allowed to evaporate to dryness.

### **Immunoblotting and Immunoprecipitations**

SDS-PAGE was performed as previously described (Laemmli, 1970). For  $\beta$ -casein immunoblots, cell extracts equivalent to ~50,000 cells per sample were separated on 13% acrylamide gels and transferred to an Immobilon-P membrane (Amersham, Arlington Heights, IL). Filters were blocked with 5% (wt/vol) BSA in TBST (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% [vol/vol] Tween 20) and probed with either an anti-mouse milk polyclonal antisera or an anti-rat  $\beta$ -casein monoclonal antibody, diluted in TBST plus 1.0% (wt/vol) BSA. Antibody binding was detected by a horseradish peroxidaseconjugated secondary antibody and an ECL reagent (Amersham), according to the manufacturer's instructions.

For integrin immunoprecipitations, SCp2 cells were metabolically labeled for 16 h with 200  $\mu$ Ci of [<sup>35</sup>S]methionine (Amersham) per milliliter of culture medium. Labeled cells were washed several times with cold medium and extracted in NP-40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1.0% [vol/vol] NP-40). Antibodies were added to aliquots of the extract at a final concentration of 10  $\mu$ g/ml and incubated overnight at 4°C. Simultaneously, protein G-agarose (Sigma Chemical) was blocked by incubation overnight with a nonradioactive SCp2 cell extract at 4°C, then rinsed several times with NP-40 extraction buffer. Subsequently, the protein G-agarose was incubated with the antibody/extract mixture for 1 h at 4°C, washed three times with NP-40 extraction buffer, once with 1 M sucrose in NP-40 extraction buffer, and twice with 50 mM Tris-HCl, pH 7.5. The precipitated proteins were recovered from the beads in nonreducing SDS-PAGE sample buffer and separated on 7% SDS polyacrylamide gels. The gels were dried and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY).

## **Results**

### **Laminin-induced $\beta$ -Casein Expression Is Perturbed by Function-blocking Antibodies against the $\beta$ 1 and $\alpha$ 6 Integrins without Perturbing the Induction of Cell Shape Changes**

Signals induced by laminin in mammary epithelial cells include a two-step process leading to induction of tissue specific gene expression as measured by  $\beta$ -casein production (Figure 1A). To identify the laminin receptor(s) mediating these distinct signals, assays for both cell rounding and  $\beta$ -casein expression were performed in the presence of available function-perturbing antibodies against murine integrins. These included antibodies against the  $\beta$ 1,  $\alpha$ 1,  $\alpha$ 5,  $\alpha$ 6, and  $\alpha$ v subunits. Assays were performed using the cell line SCp2, a clonal murine mammary epithelial cell line that, like primary mammary epithelial cells, responds to contact with laminin by producing  $\beta$ -casein in the presence of lactogenic hormones (Desprez *et al.*, 1993).

The treated cells were tested for the ability to signal  $\beta$ -casein expression when exposed to laminin in the presence of function-perturbing anti-integrin antibodies. Assays for  $\beta$ -casein expression were performed on cells initially attached and spread on cell culture plastic. Spread cells were treated with serum-free medium containing soluble laminin, lactogenic hormones, and function-perturbing antibodies against integrin receptors. Both pure laminin and the laminin-

rich reconstituted basement membrane (Matrigel) were used in these studies, and both led to expression of  $\beta$ -casein, as previously demonstrated (Roskelley *et al.*, 1994; Streuli *et al.*, 1995). After 5 d of exposure to laminin, hormones, and antibodies, the treated cells were extracted and assayed for  $\beta$ -casein expression by immunoblotting. Treatment with the  $\alpha 1$ -,  $\alpha 5$ -, and  $\alpha v$ -blocking antibodies had no inhibitory effect (Figure 1B). In contrast, treatment with the function-blocking antibody against  $\beta 1$  integrins inhibited  $\beta$ -casein expression almost completely, as shown previously for primary cultures and CID-9 cells (Streuli *et al.*, 1995). Contrary to previous observations in primary cultures (Streuli *et al.*, 1991), the GoH3 antibody, directed against the integrin  $\alpha 6$  subunit, also blocked the expression of  $\beta$ -casein. Titration of the GoH3 antibody showed a significant blockage of  $\beta$ -casein expression at concentrations between 2 and 5  $\mu\text{g/ml}$  (Figure 1C).

The ability of laminin to induce the rounded cell morphology was not impaired by any of the function-blocking antiintegrin antibodies (Figure 2 and data not shown). The cells exposed to laminin in the presence of  $\beta 1$ - and  $\alpha 6$ -blocking antibodies were indistinguishable in morphology from those exposed to laminin alone, as were those exposed to laminin in the presence of both antibodies in combination (data not shown). Therefore, the inhibition of casein expression by the two integrin antibodies did not appear to occur by the inhibition of prerequisite cell shape changes. To confirm this,  $\beta$ -casein was assayed in cells forced to adopt a rounded conformation by culturing on a nonadhesive substratum (polyHEMA). Under these conditions, the cells were rounded and aggregated before laminin exposure and remained so throughout the assay (Figure 3A). Cells were assayed after just 3 d of laminin exposure because the induction of  $\beta$ -casein was more rapid in prerounded cells than in flat cells, permitting the correspondingly shorter assay duration (Roskelley *et al.*, 1994). In prerounded cells,  $\beta$ -casein expression was still inhibited by both the  $\beta 1$ - and  $\alpha 6$ -blocking antibodies (Figure 3B), demonstrating that the inhibition of  $\beta$ -casein expression by these antibodies was not caused by effects on cell shape. These results also indicate that yet another laminin receptor, distinct from the  $\beta 1$  and  $\alpha 6$  integrins, is required to mediate the cell shape changes induced by laminin.

Blocking of  $\beta 1$  integrin function has been demonstrated previously to initiate programmed cell death in mammary epithelial cells under specific conditions (Boudreau *et al.*, 1995; Pullan *et al.*, 1996), and enhanced cell death alone could have caused the observed loss of  $\beta$ -casein expression. However, no obvious signs of cell death were apparent under our culture conditions. This is likely due to the fact that rounded and clustered mammary epithelial cells are more resistant to apoptosis than single cells or cells spread on plastic (Boudreau *et al.*, 1996; Pullan *et al.*, 1996). To be certain that cell death was not enhanced significantly in the cell populations treated with  $\beta 1$ - and  $\alpha 6$ -blocking antibodies, we assayed the relative viability of each treated population using a vital dye. Cell viability was assayed in cultures of prerounded cells under conditions identical to those used for  $\beta$ -casein assays. Cells were exposed to laminin, hormones, and each of the function-blocking antibodies for 4 d, 1 d beyond the usual end point of the  $\beta$ -casein assay, to capture any cell death that might have been initiated when the  $\beta$ -casein was assayed. A slight reduction of cell viability was observed in the population treated with  $\beta 1$ -blocking antibodies, but this was no greater than the effects observed for  $\alpha 5$ - and  $\alpha v$ -treated cells, which showed no inhibition of  $\beta$ -casein expression (Figure 3C). Therefore, the inhibition of  $\beta$ -casein expression by  $\beta 1$ - and  $\alpha 6$ -blocking antibodies was not caused by enhanced cell death.

## **Both $\beta 1$ Integrin and $\alpha 6\beta 4$ Integrin Functions Are Required to Signal $\beta$ -Casein Expression**

The  $\alpha 6$  subunit is a component of two laminin receptors, the  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  integrins, both of which are reported to bind the laminin E8 fragment (Hall *et al.*, 1990; Lee *et al.*, 1992). Therefore, the  $\alpha 6$ -blocking antibody, GoH3, could target either the  $\alpha 6\beta 1$  or the  $\alpha 6\beta 4$  heterodimer, or both. The  $\beta 1$ -blocking antibody, HA2/5, would target all  $\beta 1$  integrin heterodimers (Mendrick and Kelly, 1993). Because both of these antibodies inhibited  $\beta$ -casein expression, one could conclude that the  $\alpha 6\beta 1$  integrin is the receptor responsible for the signaled  $\beta$ -casein expression. Alternatively, the two antibodies could perturb  $\beta$ -casein expression by distinct mechanisms, one through blocking the  $\alpha 6\beta 4$  integrin and the other through blocking one or more  $\beta 1$  integrins. The second possibility was found to be the case by immunoprecipitation of the  $\alpha 6$  integrins from the SCp2 cell line. Immunoprecipitations of the  $\alpha 6$  integrins using the GoH3 antibody revealed that the  $\beta 4$  subunit was the exclusive partner of the  $\alpha 6$  subunit in the SCp2 cells (Figure 4). The quantity of  $\alpha 6$  subunit immunoprecipitated was the same whether the  $\alpha 6$  or the  $\beta 4$  antibody was used, and the  $\beta 1$  subunit was undetectable in the  $\alpha 6$  subunit precipitations. The absence of the  $\alpha 6\beta 1$  heterodimer in cells expressing both the  $\beta 1$  and  $\beta 4$  subunits has been reported by several other laboratories and demonstrates a dominant preference of the  $\alpha 6$  subunit for dimerization with the  $\beta 4$  subunit (Lee *et al.*, 1992; Delcommenne and Streuli, 1995; Spinardi *et al.*, 1995; DiPersio *et al.*, 1997; Hodivala-Dilke *et al.*, 1998). In addition, the 9EG7 antibody, reported to alternately block or stimulate the function of a subset of  $\beta 1$  integrins, including the  $\alpha 6\beta 1$  integrin (Lenter *et al.*, 1993; Driessens *et al.*, 1995), did not inhibit or stimulate  $\beta$ -casein expression in our assays (our unpublished results). Therefore, the  $\alpha 6\beta 1$  integrin is not involved in signaling  $\beta$ -casein expression, but the  $\alpha 6\beta 4$  integrin is essential for transmitting signals for  $\beta$ -casein expression in mammary epithelial cells. The inhibition of  $\beta$ -casein expression by the  $\beta 1$ -blocking antibody occurs by a different mechanism, either through blocking yet another laminin receptor (e.g., the  $\alpha 3\beta 1$  integrin) or through events unrelated to signaling from laminin (e.g., disruption of other  $\beta 1$  integrin functions not related to binding laminin).

## **A Receptor for the E3 Domain of Laminin Mediates the Cell Shape Changes, Independent of $\beta 1$ and $\beta 4$ Integrins**

The results described above demonstrated that both the  $\alpha 6\beta 4$  integrin and  $\beta 1$  integrins are required for induction of  $\beta$ -casein expression, but neither are required to mediate the cell shape changes induced by laminin. Therefore, the receptor mediating the prerequisite cell shape change appeared not to be among the known integrin laminin receptors. Other receptors that might perform this function include those that bind the laminin E3 domain. Previous studies in our laboratory, with primary cell cultures and CID-9 cells, had identified a role for the E3 domain of laminin in signaling  $\beta$ -casein expression; purified E3 laminin fragment inhibited  $\beta$ -casein expression (Streuli *et al.*, 1995). Because neither the  $\alpha 6\beta 4$  integrin nor  $\beta 1$  integrins are thought to bind the laminin E3 domain (with the possible exception of  $\alpha 3\beta 1$  [Gehlsen *et al.*, 1992]), the mechanism by which the E3 fragment inhibited  $\beta$ -casein was not clear.

We hypothesized that the E3 laminin fragment may inhibit  $\beta$ -casein expression through inhibition of the receptor(s) mediating changes in cell shape. The E3 and E8 laminin fragments alone, or in combination, did not signal either the cell shape change or  $\beta$ -casein expression in SCp2 cells.

However, the ability of cells to round and cluster when exposed to laminin was strongly inhibited by the E3 fragment but not by the E8 fragment or the BSA control (Figure 5A, a–f). Titration of the E3 fragment showed strong inhibition of cell rounding at 100  $\mu\text{g/ml}$ , with diminishing effects at lower concentrations. The concentration of E3 fragment necessary to affect cell shape paralleled the concentrations needed to block  $\beta$ -casein expression in primary cell cultures (Streuli *et al.*, 1995). This indicated that the E3 laminin fragment perturbs a laminin receptor that mediates the cell shape change.

The E3 laminin fragment could inhibit  $\beta$ -casein expression solely through effects on cell shape, or it could perturb additional signaling functions required for  $\beta$ -casein expression. To distinguish between these two possibilities, the laminin fragments were tested in assays of  $\beta$ -casein expression in both flat and prerounded cells. Immunoblots for the resulting  $\beta$ -casein expression showed that the E3 fragment inhibited  $\beta$ -casein expression in both flat and rounded SCp2 cells (Figure 5B and data not shown). Therefore, the inhibition of  $\beta$ -casein expression by the E3 fragment occurs through both effects on cell shape and inhibition of other functions that are yet to be determined. The laminin E8 fragment and the BSA control did not inhibit  $\beta$ -casein expression at concentrations up to 100  $\mu\text{g/ml}$  (data not shown).

Receptors reported to bind the laminin E3 domain include syndecan-1 and dystroglycan, which are believed to bind, in part, through carbohydrate interactions with the heparinbinding region of laminin (Ervasti and Campbell, 1993; Salmivirta *et al.*, 1994). Consequently, their interactions with laminin are inhibited by heparin. We tested whether heparin also inhibited signals for the cell shape change and  $\beta$ -casein expression. Heparin strongly inhibited the cell shape change at a concentration of 400  $\mu\text{g/ml}$  (Figure 5Ai). Heparan sulfate and chondroitin sulfates A, B, and C were not effective inhibitors of cell rounding at the same concentration (Figure 5Ag, and data not shown). In assays of  $\beta$ -casein expression, heparin mimicked the activity of the laminin E3 fragment, whereas heparan sulfate did not. Heparin inhibited the induction of  $\beta$ -casein expression in assays of both flat and prerounded cells at the same concentrations that inhibit cell rounding (Figure 5C and data not shown). This indicates that the heparin-binding region within the laminin E3 domain participates in the interaction of laminin with the E3 laminin receptor. Heparan sulfate did not inhibit  $\beta$ -casein expression at concentrations up to 400  $\mu\text{g/ml}$ .

### **The Requirement of $\alpha 6\beta 4$ Integrin to Signal $\beta$ -Casein Expression Is Obscured in Primary Cell Cultures because of Paracrine Signaling Leading to Formation of Endogenous Basement Membrane**

The results described above, using the clonal mammary epithelial cell line SCp2, differed in part from our previously published results with primary mammary epithelial cell cultures and the CID-9 mammary epithelial cell line. In the previous studies, the GoH3 antibody was found not to inhibit the induction of  $\beta$ -casein expression (Streuli *et al.*, 1991). Therefore, either the SCp2 cell line had acquired a new signaling requirement for  $\beta$ -casein expression or some common aspect of the primary cultures and CID-9 cell line obscured or circumvented the requirement for the  $\alpha 6\beta 4$  integrin.

We hypothesized that endogenous basement membrane formation, occurring in primary cultures and the heterogeneous CID-9 cell line, may interfere with the detection of

signaling by the  $\alpha6\beta4$  integrin. It has been established previously that paracrine signaling between the mesenchymal and epithelial compartments results in the deposition of an endogenous basement membrane (Reichmann *et al.*, 1989; Cunha and Hom, 1996). The principal differences between the primary cultures, CID-9, and the SCp2 cell lines are that the latter is clonal and unable to form a functional basement membrane (Desprez *et al.*, 1993; Roskelley *et al.*, 1994). In mixed cultures, preformed  $\alpha6\beta4$ -laminin complexes might resist disruption by the GoH3 antibody.

This hypothesis was tested by two independent means. First, if primary cultures were able to form an endogenous basement membrane, then it would follow that  $\beta$ -casein expression could be induced in primary cultures by simply forcing a rounded cell conformation in the presence of lactogenic hormones but without the addition of exogenous laminin. The induction of  $\beta$ -casein expression was assayed in parallel cultures of primary murine mammary epithelial cells plated either onto tissue culture plastic, where they attached and spread, or in wells coated with polyHEMA, where they remained in suspension and maintained a clustered and rounded conformation. After 2 d, both cultures were treated with medium containing lactogenic hormones without the addition of laminin, and after 3 d of exposure to hormones, the cells were extracted and assayed for  $\beta$ -casein expression. As predicted, primary cells cultured on plastic (flat cells) did not produce significant  $\beta$ -casein; however, the same cells cultured on polyHEMA showed an induction of  $\beta$ -casein expression despite the absence of exogenously added laminin (Figure 6A). In contrast, the clonal SCp2 cell line expressed little or no  $\beta$ -casein, regardless of cell shape, if exogenous laminin was not present (Figures 1B, 3B, and 6A). Second, we tested whether the previous results obtained with primary cultures could be duplicated with the SCp2 cells if we added a mesenchymal component. Mesenchymal cells such as NIH3T3 fibroblasts do not express milk proteins. SCp2 cells were cocultured with NIH3T3 fibroblasts at a 10:1 ratio (epithelial cells: fibroblasts) and tested for  $\beta$ -casein expression in the absence of exogenous laminin. Coculture of the SCp2 cells and NIH3T3 fibroblasts resulted in expression of  $\beta$ -casein when cultured on polyHEMA but not when cultured on plastic, in which case they remained flat (Figure 6A). The resulting cell behavior of the cocultured epithelial cells and fibroblasts was identical to that of primary cell cultures, in which  $\beta$ -casein expression was induced by cell rounding without the addition of exogenous laminin.

Finally, the inhibition of the  $\beta$ -casein signal by the integrin-blocking antibodies was tested in cocultured SCp2 and NIH3T3 cells. The cocultured cells were exposed to the function-blocking antibodies in suspension without the addition of exogenous laminin. Under these conditions, the signaled  $\beta$ -casein in the coculture experiments was still inhibited by the  $\beta1$ -blocking antibody but was less efficiently inhibited by the GoH3 antibody (Figure 6B), consistent with results previously described for primary cell cultures (Streuli *et al.*, 1991). Therefore, the ability or inability to produce a functional basement membrane appears to be responsible for the different results obtained with the SCp2 cells as opposed to primary mammary epithelial and CID-9 cultures.

## Discussion

### Division of Labor

The division of labor among laminin receptors has been presumed on the basis of the unique structural properties of the different receptors and on the basis of a limited number of functional studies in vivo and in cell culture. Structurally, the cytoplasmic domains of different laminin receptors are distinct from each other, yet highly conserved, reflecting the selective conservation of unique functions within each different receptor (Sastry and Horwitz, 1993). Receptor knockout experiments, in which the function of a number of the known laminin receptors has been eliminated, lead to different phenotypes, most being lethal at various stages of development (Hynes, 1996; Williamson *et al.*, 1997). Among laminin receptors expressed in epithelial cells, the integrin  $\alpha 3$  and  $\alpha 6$  subunit knockouts displayed distinct alterations in the cell-basement membrane junctions (DiPersio *et al.*, 1997). In culture, cell binding to either the E3 or E8 domains of laminin had different effects in assays of kidney and salivary gland morphogenesis (Klein *et al.*, 1988; Sorokin *et al.*, 1992; Durbeej *et al.*, 1995; Kadoya *et al.*, 1995). In addition, ligation of different laminin receptors resulted in distinct downstream signaling events, including differences in protein phosphorylation and Shc activation (Kornberg *et al.*, 1991; Jewell *et al.*, 1995; Mainiero *et al.*, 1995; Wary *et al.*, 1996; Xia *et al.*, 1996). Finally, laminin receptors were found to localize to different membrane-cytoskeleton junctions in both muscle and epithelial cells (Bao *et al.*, 1993; Burgeson and Christiano, 1997). Therefore, the different functions of laminin receptors can be tied to their roles as mediators of unique membrane-cytoskeleton interactions in addition to their different signaling properties.

Despite the extensive characterization of different laminin receptors, little is known about their downstream influence on cell function beyond cell adhesion. We demonstrate here the distinct roles of at least two laminin receptors in signals leading to transcription of the milk protein  $\beta$ -casein in mammary epithelial cells. In addition to resolving the different signaling functions of laminin receptors, this work assigns clear downstream consequences of cell behavior to ligation of specific laminin receptors: morphogenic changes are induced by an E3 laminin receptor; and  $\beta$ -casein expression requires signaling by the  $\alpha 6\beta 4$  integrin,  $\beta 1$  integrins, and an E3 laminin receptor.

The key reagents used in this study, the  $\alpha 6$ - and  $\beta 1$ -blocking antibodies and the laminin E3 fragment, not only resolved the distinct functions of laminin receptors but also revealed their partial independence. The integrin-blocking antibodies did not perturb the cell shape changes mediated by the E3 laminin receptor. The independence of these receptors suggests that they do not associate at the cell surface to enact their functions but more likely segregate to distinct membrane-cytoskeleton junctions. Indeed, the  $\alpha 6\beta 4$  is a hemidesmosome component known to interact with the intermediate filament cytoskeleton, whereas all receptors so far reported to bind the E3 domain of laminin are thought to interact with the actin cytoskeleton (Gehlsen *et al.*, 1992; Henry and Campbell, 1996; Carey, 1997). Although these receptors function independently for the cell shape change, the integrins and E3 laminin receptor are codependent for signaling  $\beta$ -casein expression. Because the  $\alpha 6\beta 4$  integrin has been reported to bind the laminin E8 domain, it was surprising that the E8 fragment failed to inhibit  $\beta$ -casein expression.

One possible explanation is that the purified E8 fragment does not compete efficiently with intact laminin, but other interpretations may have to be considered. It should be noted also that the E8 fragment has a molecular mass four times greater than that of the E3 fragment. Therefore, much higher E8 protein concentrations may be required for inhibition to be observed in these assays.

## **Cell Shape**

The mechanism by which cell shape participates in the  $\beta$ -casein signaling pathway is unknown. Although shape dependence of signaling pathways has been demonstrated for many functions, the underlying molecular mechanisms are just beginning to be revealed (e.g., see Kheradmand *et al.*, 1998). The four known signaling pathways for  $\beta$ -casein expression emanate from the  $\alpha 6\beta 4$  integrin,  $\beta 1$  integrins, an E3 laminin receptor, and the prolactin receptor. Whether only one of these pathways is cell shape dependent, or whether all require a particular cell structure before they can signal, remains to be determined. Shape dependence implies a requirement for a particular cytoskeletal organization. Both the  $\alpha 6\beta 4$  integrin and  $\beta 1$  integrins are associated with the cytoskeleton, and this may be true also for the E3 laminin receptor. Therefore, signaling through one or all of these receptors may be altered by the organization of the cytoskeleton.

## **$\alpha 6\beta 4$ Integrin Function**

What is the role of  $\alpha 6\beta 4$  in signaling  $\beta$ -casein expression? Although biochemical signals have been shown to emanate from the  $\alpha 6\beta 4$  integrin (Giancotti, 1996; Mainiero *et al.*, 1997), this receptor is also a mediator of epithelial architecture. Ligation of the  $\alpha 6\beta 4$  integrin to laminin is considered to be the nucleating event in hemidesmosome formation (Giancotti, 1996), which in turn organizes components of the intermediate filament cytoskeleton. Therefore, the induction of  $\beta$ -casein expression by  $\alpha 6\beta 4$  ligation may operate, at least in part, through effects on cell architecture that in turn permit other pathways to function (e.g., those responding to lactogenic hormones). Previous results from our laboratory have shown that a program of normal epithelial morphogenesis in cultured human breast cells can be perturbed by blocking  $\alpha 6\beta 4$  integrin function, leading to disorganized and uncontrolled cell growth (Weaver *et al.*, 1997). The question of whether cell polarity per se is a requirement for  $\beta$ -casein expression has been addressed previously, and it was determined not to be essential because single cells (by definition apolar) embedded in a laminin-rich ECM produced  $\beta$ -casein (Streuli *et al.*, 1991). However, a much higher proportion of cells expressed  $\beta$ -casein when allowed to form multicellular aggregates. Furthermore, the time course for detection of signals for  $\beta$ -casein expression is uncharacteristically slow for simple biochemical signaling, requiring a minimum of 8 h for detection, even in prerounded cells (Roskelley *et al.*, 1994). Therefore, we propose that structural reorganization of the cell is one essential component of  $\beta$ -casein signaling, whether it is mediated by the  $\alpha 6\beta 4$  integrin,  $\beta 1$  integrins, the E3 laminin receptor, or all three.

## **E3 Laminin Receptor Function**

In addition to assigning a function to signaling from the  $\alpha 6\beta 4$  integrin, we now have revealed a clear consequence of cell interaction with the laminin E3 domain on cell morphology and function. The mechanism by which cell binding to the E3 domain induces cell rounding is

unknown. As described for  $\alpha 6\beta 4$ , the E3 laminin receptor could mediate its function through biochemical signaling or through direct effects on cytoskeletal organization, or both. This rounding function is insensitive to the tyrosine kinase inhibitor genistein (Roskelley *et al.*, 1994), so tyrosine phosphorylation events may not be required; however, the activity is inhibited by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate which affects the cytoskeleton. The fact that the E3 domain alone could not induce the rounding response, but was instead inhibitory, indicates that simple ligand binding is insufficient for this signaling event to occur and that a higher molecular organization of laminin is required.

The E3 laminin receptor responsible for inducing the cell shape change remains to be identified. However, dystroglycan is a strong candidate (Henry and Campbell, 1996). Dystroglycan is expressed in the SCp2 cells as well as in mammary epithelial cells *in vivo* (Durbeej *et al.*, 1998; our unpublished results). Dystroglycan is reported to bind the laminin E3 domain, and this binding is inhibited by heparin, but less effectively by heparan sulfate, and not at all by chondroitin sulfates (Pall *et al.*, 1996). Moreover, the high concentration of heparin required to inhibit cell rounding in our assays (200–400  $\mu\text{g/ml}$ ) corresponds to the concentration of heparin required to inhibit laminin binding to muscle  $\alpha$ -dystroglycan, which inhibits at a 50% inhibitory concentration of 250  $\mu\text{g/ml}$  (Pall *et al.*, 1996). Dystroglycan was shown recently to mediate the assembly of laminin at the cell surface (Henry and Campbell, 1998). Based on these results, it has been suggested that dystroglycan might act as a coreceptor for laminin and may thereby influence the function of other laminin receptors at the cell surface. Interpreting our results through this model, one can propose that basement membrane assembly by dystroglycan is required for correct signaling through the  $\alpha 6\beta 4$  or  $\beta 1$  integrins. This model offers an attractive explanation for why at least two laminin receptors are required to signal  $\beta$ -casein expression and why the laminin E3 domain function is required continuously. On the other hand, it is still possible that these receptors each contribute essential but entirely independent functions.

Other candidate E3 receptors include syndecan-1, whose binding to the G domain of laminin has been implicated in acinar formation in epithelial cells of the salivary gland (Hoffman *et al.*, 1998). Syndecan-1 is expressed in SCp2 cells (our unpublished results), but it is unknown whether the laminin-binding isoform (Salmivirta *et al.*, 1994) is present. Unlike dystroglycan, syndecan-1 binding to laminin-1 is not differentially inhibited by heparin, heparan sulfate, and chondroitin sulfate, although these interactions were not assayed in mammary epithelial cells (Salmivirta *et al.*, 1994; Hoffman *et al.*, 1998). In addition, the AG73 peptide, reported to compete with laminin for syndecan-1 binding (Hoffman *et al.*, 1998), did not induce or perturb significantly the cell shape change in our assays (our unpublished results). Aside from dystroglycan and syndecan-1, many cell surface proteoglycans have the potential to bind laminin through heparin-binding domains such as the E3 domain of laminin. It is possible that multiple cell surface molecules can perform this function; however, all redundancy must exist among E3 laminin receptors because the E3 fragment alone was able to inhibit cell rounding. Other cell surface molecules may also be required to effect the cell shape change, in cooperation with the E3 receptor, but so far we have found that only an E3 laminin receptor is essential.

## **$\beta$ 1 Integrin Function**

The mechanism of inhibition of  $\beta$ -casein expression by the  $\beta$ 1-blocking antibody remains to be deciphered. Inhibition might occur through the blocking of yet another required laminin receptor. The  $\alpha$ 3 $\beta$ 1 integrin is a logical candidate because it functions in epithelial interactions with laminin and is expressed in the SCp2 cells in culture (our unpublished results). Function-perturbing antibodies for the integrin  $\alpha$ 3 subunit, however, are still not available in the mouse system, but once available they will allow a resolution of this question. Alternatively, it is possible that the inhibition of  $\beta$ -casein expression could result from the blocking of other  $\beta$ 1 integrins, independent of effects on any laminin receptor. The  $\beta$ 1-blocking antibody might induce some form of transdominant inhibition of the  $\alpha$ 6 $\beta$ 4 integrin, E3 laminin receptor, or other molecules, as has been described previously for some integrins (Diaz-Gonzalez *et al.*, 1996; Hodivala-Dilke *et al.*, 1998). So far, we know that blocking of  $\alpha$ 1,  $\alpha$ 5, and  $\alpha$ v integrins had no observable effect on  $\beta$ -casein expression.

Finally, an absolute requirement for  $\beta$ 1 integrin,  $\alpha$ 6 $\beta$ 4 integrin, or E3 laminin receptor signaling in lactation remains to be demonstrated *in vivo*. Knockouts of the  $\alpha$ 6,  $\beta$ 4, and  $\beta$ 1 subunits have proven lethal at the neonatal and early embryonic stages (Fassler and Meyer, 1995; Stephens *et al.*, 1995; Dowling *et al.*, 1996; Georges-Labouesse *et al.*, 1996; van der Neut *et al.*, 1996), long before lactation could be assessed. One recent study, however, demonstrated that perturbation of  $\beta$ 1 integrin function, in transgenic mice expressing a chimeric  $\beta$ 1 integrin/CD4 molecule, led to decreased expression of milk proteins, including  $\beta$ -casein (Faraldo *et al.*, 1998).

## **The Interference of Endogenous Basement Membrane Deposition**

The current study was made possible by the use of a clonal epithelial cell line instead of mixed cultures containing both epithelial and mesenchymal cell types. Earlier studies from our laboratory had concluded that the E3 domain of laminin alone may be the only domain of laminin required for  $\beta$ -casein expression and that the GoH3 antibody was not inhibitory (Streuli *et al.*, 1991, 1995). However, these studies used either primary cell cultures or the CID-9 cell line, both of which contain some mesenchymal components. Paracrine signaling between mesenchymal and mammary epithelial cells results in the deposition of an endogenous basement membrane, which, in turn, can induce  $\beta$ -casein expression in the presence of lactogenic hormones (Reichmann *et al.*, 1989). In the present study, we concluded that the presence of an endogenous basement membrane in primary and CID-9 cultures had obscured the two-step signaling requirement; mechanical cell rounding was sufficient to induce  $\beta$ -casein expression in both primary and “mixed” (SCp2/NIH3T3) cultures without the addition of exogenous laminin. We propose that the GoH3 antibody was less effective at inhibiting  $\beta$ -casein expression in experiments in which mixed cell types were present because it does not efficiently disrupt the preformed complexes of  $\alpha$ 6 $\beta$ 4 integrins bound to endogenous laminin deposits. These results demonstrate the usefulness of homogeneous, but functional, epithelial cell lines for studies of extracellular matrix signaling from laminin. They also underscore the importance of defining the contribution of endogenously deposited ECM molecules when cultured cells are used for functional studies.

## Acknowledgments

The authors thank Dinah Levy for technical assistance and Marina Simian for assistance with primary cell cultures. We also thank Todd Mathis and Holly Colognato for assistance with laminin fragment preparation. We are grateful to Drs. Valerie Weaver, Michael Henry, and Zena Werb for helpful discussion. This work was sponsored by National Institutes of Health grant NIH-CA57621 and Department of Energy grant DE-AC03-76-SF00098. J.M. was supported by a National Institutes of Health Postdoctoral Fellowship and by a Department of Defense Breast Cancer Research Fellowship.

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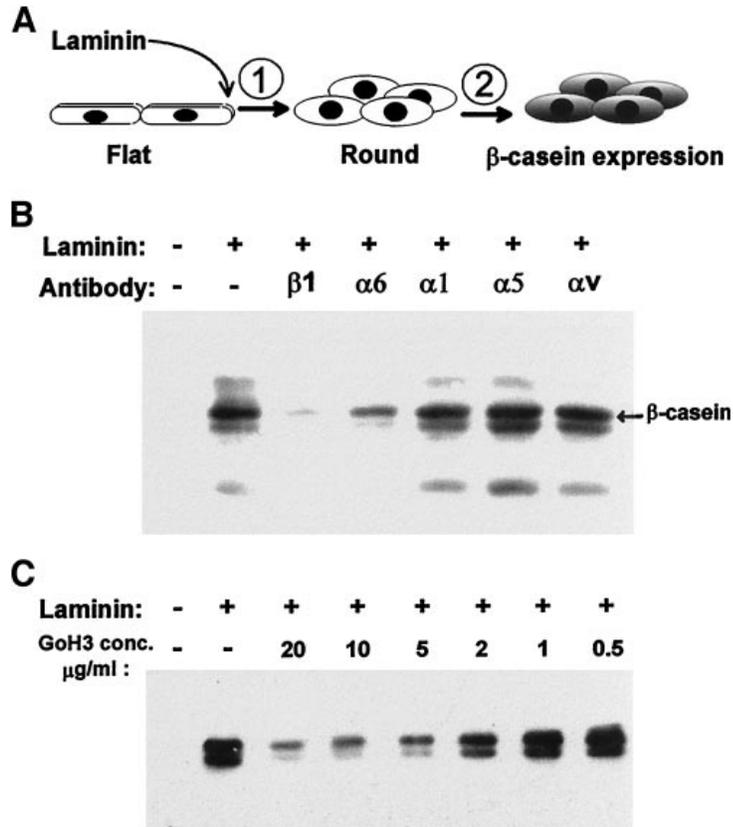
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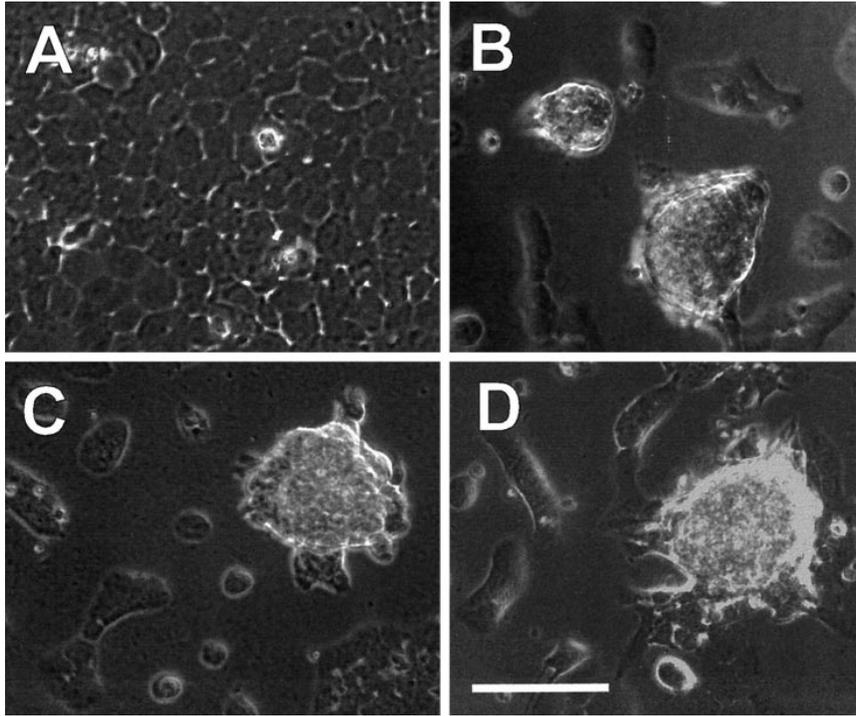
## Figures

FIGURE 1



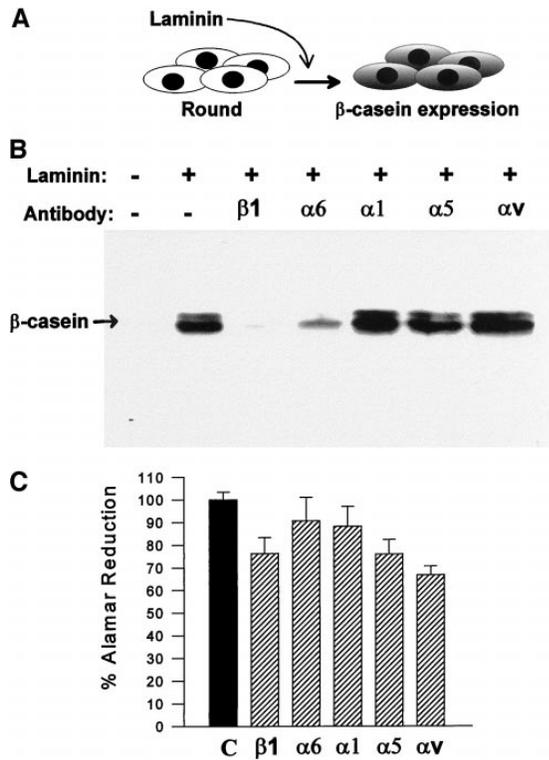
Inhibition of  $\beta$ -casein expression by function-blocking integrin antibodies. Assays for the induction of  $\beta$ -casein expression in SCp2 mammary epithelial cells were performed on cells initially spread on plastic and subsequently exposed to medium containing the lactogenic hormone prolactin, plus or minus laminin, and function-blocking antibodies against the  $\beta$ 1,  $\alpha$ 6,  $\alpha$ 1,  $\alpha$ 5, and  $\alpha$ v integrin subunits. (A) Schematic representation of the assay. A two-step signaling process leads to  $\beta$ -casein expression after cell contact with laminin, beginning with a prerequisite cell shape change (rounding), followed by subsequent biochemical signaling events. (B)  $\beta$ -Casein expression was assayed by immunoblots of cell extracts and appears as a doublet migrating at  $\sim$ 34 kDa. Laminin-induced  $\beta$ -casein expression was inhibited in the presence of  $\beta$ 1 and  $\alpha$ 6 integrin-blocking antibodies. (C) Titration of the  $\alpha$ 6-blocking (GoH3) antibody shows maximal inhibition in the range of 2–5  $\mu$ g/ml.

FIGURE 2



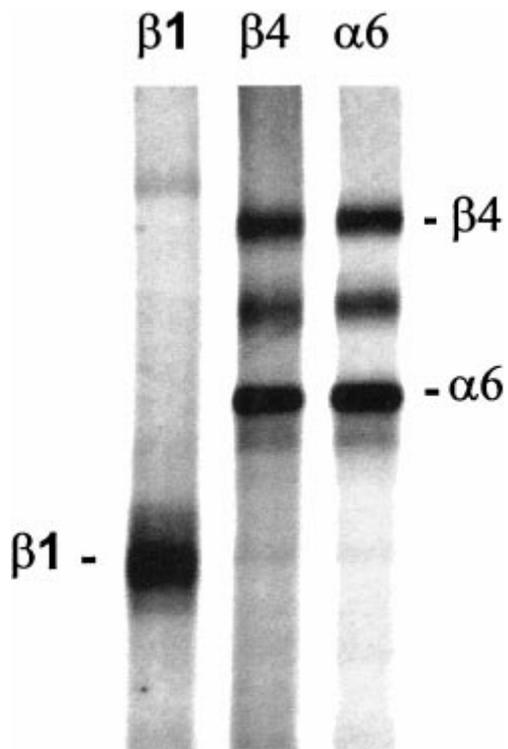
Morphogenic changes induced by laminin are unaltered by the presence of  $\alpha 6$ - and  $\beta 1$ -blocking antibodies. Assays for the induction of  $\beta$ -casein expression in SCp2 mammary epithelial cells were performed on cells initially spread on plastic and subsequently exposed to medium containing the lactogenic hormone prolactin, plus or minus laminin, and function-blocking antibodies against the  $\beta 1$  and  $\alpha 6$  integrin subunits. In the absence of added laminin, cells remained attached and spread on the plastic and continued to grow to confluence (A). In contrast, cells exposed to laminin underwent cell rounding, and those in contact with other cells clustered into multicellular aggregates, leaving much of the plastic culture dish exposed (B). Cells exposed to laminin in the presence of functionblocking antibodies against the  $\alpha 6$  (C) and  $\beta 1$  (D) integrin subunits continued to undergo the cell shape changes induced by laminin even though these same antibody treatments perturbed signals for  $\beta$ -casein expression.

FIGURE 3



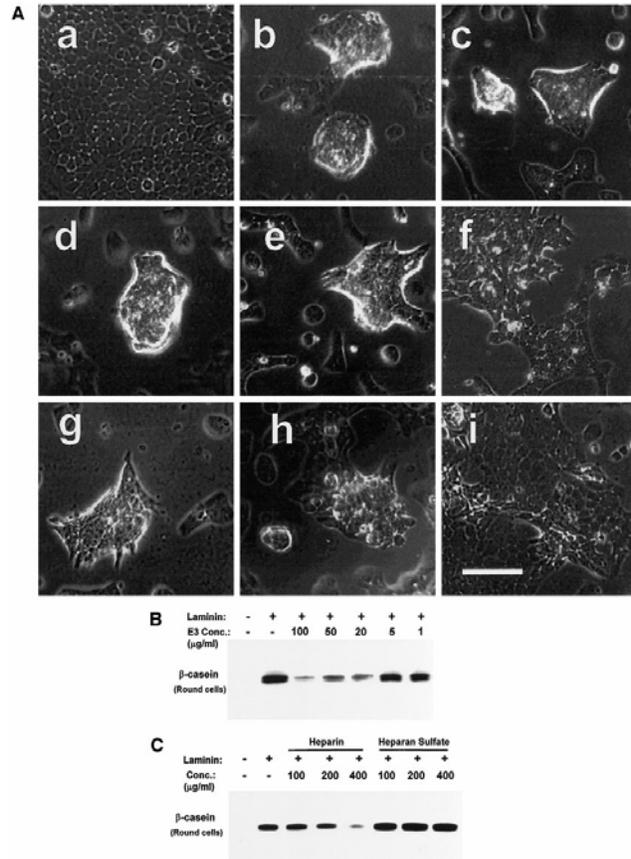
$\beta$ -Casein expression and cell survival in the presence of function-blocking antibodies against  $\beta 1$  and  $\alpha 6$  integrins in prerounded cells. SCp2 cells cultured in suspension were treated with prolactin, plus or minus laminin, and function-blocking antibodies against the  $\beta 1$ ,  $\alpha 6$ ,  $\alpha 1$ ,  $\alpha 5$ , and  $\alpha v$  integrin subunits. (A) Assays of  $\beta$ -casein expression in suspension cultures measure signaling events subsequent to, and independent of, the required cell shape changes. (B) Immunoblots of cell extracts after 3 d of laminin exposure show that laminin-induced  $\beta$ -casein expression in prerounded cells was still inhibited in the presence of  $\beta 1$  and  $\alpha 6$  integrinblocking antibodies. (C) Cell viability was assayed by Alamar Blue dye reduction in duplicate wells under  $\beta$ -casein assay conditions identical to those described for B, but culture was for 4 d (1 d longer) to measure any cell death that may have been initiated during the course of the  $\beta$ -casein assay.

FIGURE 4



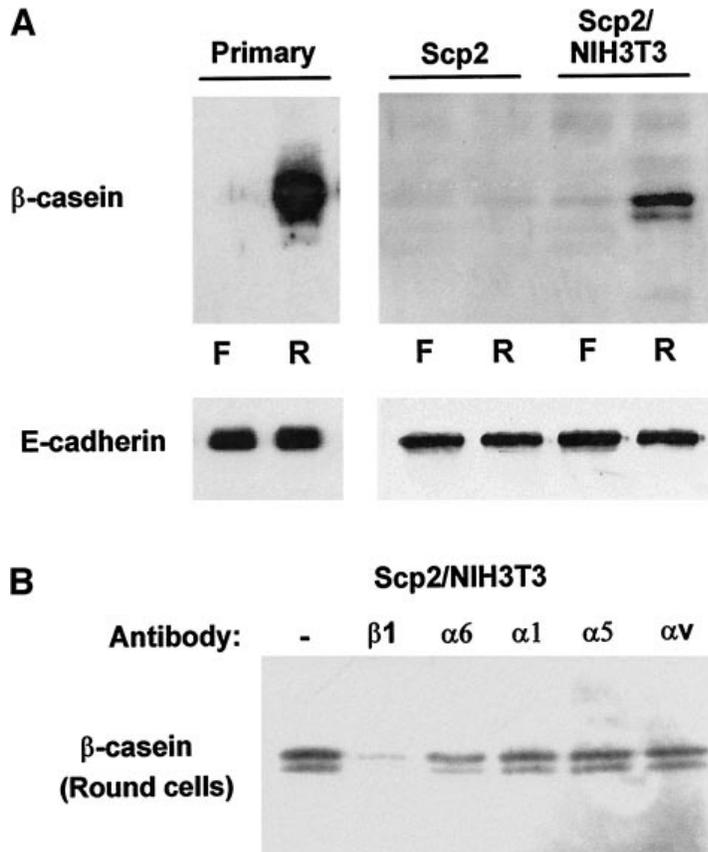
Immunoprecipitation of  $\alpha 6$  integrins from the SCp2 cells. The  $\beta 1$ ,  $\beta 4$ , and  $\alpha 6$  integrins were immunoprecipitated from [ $^{35}\text{S}$ ]methionine-labeled cell extracts and separated on a 7% SDSpolyacrylamide gel. Bands for both the  $\beta 4$  and  $\alpha 6$  subunits are evident in the immunoprecipitations using the  $\beta 4$  and  $\alpha 6$  subunit antibodies, demonstrating the presence of the  $\alpha 6\beta 4$  heterodimer. The  $\beta 1$  subunit was not detectable in precipitations of the  $\alpha 6$  integrins, demonstrating the absence of the  $\alpha 6\beta 1$  heterodimer in the SCp2 cells. The exclusive dimerization of  $\alpha 6$  with the  $\beta 4$  subunit is further supported by the fact that the yield of the  $\alpha 6$  and  $\beta 4$  subunits is equal between the  $\alpha 6$  and  $\beta 4$  immunoprecipitations, indicating that most or all of the  $\alpha 6$  subunit is dimerized with the  $\beta 4$  subunit in these cells.

FIGURE 5



Morphogenic changes and  $\beta$ -casein expression are blocked by the laminin E3 fragment and by heparin. (A) Assays for the cell shape changes induced by laminin in SCp2 mammary epithelial cells were performed on cells initially spread on plastic and subsequently exposed to medium containing laminin plus or minus the elastase-generated laminin fragments E3 and E8. In the absence of added laminin (a), cells remain attached and spread on the plastic and continue to grow to confluence. Cells exposed to laminin (b) undergo cell rounding, and cells sharing cell-cell contacts cluster into multicellular aggregates. Cells continue to undergo the cell shape changes when exposed to laminin in the presence of BSA at 100  $\mu\text{g/ml}$  (c), the laminin E8 fragment at 100  $\mu\text{g/ml}$  (d), the laminin E3 fragment at 50  $\mu\text{g/ml}$  (e), heparan sulfate at 400  $\mu\text{g/ml}$  (g), or heparin at 100  $\mu\text{g/ml}$  (h). However, cells are strongly inhibited from undergoing the cell rounding and clustering when exposed to laminin in the presence of the laminin E3 fragment at 100  $\mu\text{g/ml}$  (f) or heparin at 400  $\mu\text{g/ml}$  (i). (B) The E3 laminin fragment was tested as a competitive inhibitor in assays of  $\beta$ -casein expression in prerounded cells. The E3 fragment continues to inhibit  $\beta$ -casein expression even in assays of prerounded cells, with partial inhibition at concentrations as low as 20  $\mu\text{g/ml}$ . (C) Heparin and heparan sulfate were tested as competitive inhibitors in assays of  $\beta$ -casein expression in prerounded cells. Heparin inhibits  $\beta$ -casein expression in assays of prerounded cells at 400  $\mu\text{g/ml}$ , whereas heparan sulfate has no effect at the same concentration.

FIGURE 6



Assays of  $\beta$ -casein expression, in the absence of added laminin, in primary cell cultures, clonal epithelial cells, and cocultures of clonal epithelial cells and fibroblasts. (A) Primary murine mammary epithelial cell cultures, SCp2 clonal epithelial cells, and cocultures of SCp2 cells and NIH3T3 fibroblasts (10:1) were assayed for  $\beta$ -casein expression in both flat cells (F) and rounded cells (R) (suspension culture) exposed to prolactin in the absence of added laminin. Cell rounding in suspension cultures permitted the induction of  $\beta$ -casein in primary cultures but not in the clonal SCp2 cell line. Coculture of SCp2 cells with a mesenchymal component (NIH3T3 fibroblasts) permitted the induction of  $\beta$ -casein. The same immunoblot filters were also probed for E-cadherin to demonstrate normalization for equal cell number. (B) SCp2 cells and NIH3T3 fibroblasts were cocultured in suspension (prerounded) and exposed to prolactin in the presence of function-blocking antibodies against the  $\beta$ 1,  $\alpha$ 6,  $\alpha$ 1,  $\alpha$ 5, and  $\alpha$ v integrin subunits, without the addition of laminin.  $\beta$ -Casein expression induced by endogenous basement membrane formation was inhibited by the  $\beta$ 1 integrin blocking antibody but was not inhibited effectively by the  $\alpha$ 6 integrin-blocking antibody GoH3.