INTRODUCTION

In adult females, the mammary epithelium is organized in two layers, a luminal epithelium and a basal layer containing myoepithelial cells and undifferentiated precursors. Basal cells express specific protein markers, such as keratin 14 (K14) and P-cadherin. To study the factors that regulate the basal mammary epithelial cell phenotype, we have established two clonal derivatives of the mouse HC11 cell line, BC20 and BC44, expressing high levels of K14 and P-cadherin. Unlike the parental HC11 cells, these basal cells did not produce β-casein in response to lactogenic hormone treatment; however their phenotype appeared to be plastic. Cultured in EGF-free medium, they exhibited enhanced cell-extracellular matrix adhesions and deficient cell-cell junctions, whereas long-term treatment with EGF induced a decrease of focal contact number and establishment of cell-cell junctions, resulting in downregulation of K14 and P-cadherin expression at the protein and mRNA levels. To determine whether cell-extracellular matrix interactions mediated by integrins have a role in the regulation of the expression of K14 and P-cadherin, the amounts of transcripts for the two proteins were analysed in the basal cells, which were plated on the function-blocking antibodies against β1 and α6 integrin chains, on fibronectin and on laminin 5. The amount of P-cadherin transcript was 2- to 4-fold higher in cells plated on the function-blocking anti-integrin antibodies and on the extracellular matrix proteins, as compared to cells plated on poly-L-lysine, whereas the K14 transcript levels were not significantly modified in response to adhesion. The data demonstrate that integrin-mediated cell interaction with extracellular matrix is directly implicated in the control of P-cadherin expression, and that EGF and cell-extracellular matrix adhesion events are important regulators of the basal mammary epithelial cell phenotype.

SUMMARY

The mammary epithelium is composed of a luminal epithelium and a basal layer containing myoepithelial cells and undifferentiated precursors. Basal cells express specific protein markers, such as keratin 14 (K14) and P-cadherin. To study the factors that regulate the basal mammary epithelial cell phenotype, we have established two clonal derivatives of the mouse HC11 cell line, BC20 and BC44, expressing high levels of K14 and P-cadherin. Unlike the parental HC11 cells, these basal cells did not produce β-casein in response to lactogenic hormone treatment; however their phenotype appeared to be plastic. Cultured in EGF-free medium, they exhibited enhanced cell-extracellular matrix adhesions and deficient cell-cell junctions, whereas long-term treatment with EGF induced a decrease of focal contact number and establishment of cell-cell junctions, resulting in downregulation of K14 and P-cadherin expression at the protein and mRNA levels. To determine whether cell-extracellular matrix interactions mediated by integrins have a role in the regulation of the expression of K14 and P-cadherin, the amounts of transcripts for the two proteins were analysed in the basal cells, which were plated on the function-blocking antibodies against β1 and α6 integrin chains, on fibronectin and on laminin 5. The amount of P-cadherin transcript was 2- to 4-fold higher in cells plated on the function-blocking anti-integrin antibodies and on the extracellular matrix proteins, as compared to cells plated on poly-L-lysine, whereas the K14 transcript levels were not significantly modified in response to adhesion. The data demonstrate that integrin-mediated cell interaction with extracellular matrix is directly implicated in the control of P-cadherin expression, and that EGF and cell-extracellular matrix adhesion events are important regulators of the basal mammary epithelial cell phenotype.

Key words: Mammary epithelial cell, Extracellular matrix, Integrin, Keratin 14, P-cadherin

INTRODUCTION

In adult females, the mammary epithelium is organized in two layers, a luminal epithelium and a basal layer, consisting of myoepithelial cells. Undifferentiated precursors of both luminal and myoepithelial cells are also believed to be basally localized. The mammary epithelium bilayer is surrounded by a basement membrane embedded in connective tissue. In the fully differentiated mammary gland, that is during the lactation period, luminal epithelial cells are specialized in the synthesis and secretion of milk proteins while basal myoepithelial cells display contractile properties (for reviews, see Daniel and Silberstein, 1987; Sakakura, 1991).

Basal cells in the mammary epithelial bilayer differ from luminal cells in that they express a set of smooth muscle differentiation markers (Lazard et al., 1993; Deugnier et al., 1995) and in addition, similar to basal keratinocytes, specific basal cell markers such as the keratin 5 and keratin 14 (K14) pair and P-cadherin (Taylor-Papadimitriou and Lane, 1987; Daniel et al., 1995). Keratin 5/14 filaments have an important role in the cytoarchitecture of basal epithelial cells, as they are connected to hemidesmosomes, which are multicomponent adhesion complexes attaching basal cells to the underlying basement membrane (Burgeson and Christiano, 1997). The absence of K14 in null mutant mice gives rise to blistering skin disorders involving cytolysis of the basal layer of epidermis (Lloyd et al., 1995). The second basal cell marker, P-cadherin, is a component of cell-cell adherens junctions, which are essential for the establishment and maintenance of epithelia (Nose and Takeichi, 1986; Takeichi, 1995). The adhesive function of cadherins depends on their association with a family of cytoplasmic regulatory molecules, the catenins, which link cadherins to the actin cytoskeleton (Aberle et al., 1996). Interestingly, inactivation of the P-cadherin gene leads to precocious development of the mammary gland, and therefore P-cadherin is believed to be involved in the control of growth and differentiation of mammary epithelium (Radice et al., 1997).

The mammary myoepithelial cells, similar to basal cells in epidermis, are enriched in integrins, the major extracellular matrix receptors. In the basal layer of the mammary epithelium, histological studies have revealed the integrin subunits α1, α2, α3 and α6, β1 and β4 (Sonnenberg et al.,
1986; Koukoulis et al., 1991; Glukhova et al., 1995). α1, α2 and α3 can form heterodimers with the β1 chain, whereas α6 can complex additionally with the β4 chain.

In vitro, β1-integrins are mostly concentrated at the cellular surface, in cell-extracellular matrix (ECM) focal adhesions. α6β4 integrin is a specific component of the hemidesmosomes (for recent reviews, see Borradori and Sonnenberg, 1996; Yamada and Geiger, 1997). The cytoplasmic parts of β1- and β4-integrin chains interact with a number of regulatory and signal transducing proteins, therefore integrins are capable of activating different intracellular signaling pathways and thus participate in the control of cell growth and cell type-specific gene expression in various tissues (reviewed in Laffenre and Yamada, 1996; Roskelley et al., 1995; Meredith et al., 1996; Giancotti, 1997). In particular in the mammary epithelium, integrin-mediated cell-ECM interactions have been shown to play an important role in the control of luminal epithelial cell differentiation. In vitro, the induction of β-casein in mammary epithelial cells and the activity of STAT5 transcription factor, an essential regulator of milk protein gene expression, have been reported to depend on cell adhesion to laminin (Streuli et al., 1995a,b). In vivo, perturbation of β1-integrin function resulted in reduced expression of WAP and β-casein (Faraldo et al., 1998).

To study the role played by cell-ECM interactions and integrins in the regulation of basal cell-specific gene expression in mammary epithelium, we have established and characterized mammary epithelial cell lines expressing high levels of the basal cell markers, K14 and P-cadherin, in the absence of EGF in culture medium. These cells exhibit enhanced cell-ECM adhesions and deficient cell-cell junctions. We report here that cell-ECM interactions mediated by integrins are important regulators of the basal mammary epithelial cell phenotype, and, in particular, of P-cadherin expression.

MATERIALS AND METHODS

Cell culture

HC11 mammary epithelial cells, derived from COMMA-1D mouse mammary gland cell line (Ball et al., 1988) (kindly provided by Dr N. Nynes, Friedrich Miecher-Institute, Basel, Switzerland), were routinely grown in RPMI 1640 (Gibco BRL, Life Technologies SA, France) containing 10% FCS (Seromed, Biochorm KG, Germany), 2 mM L-glutamine (Gibco BRL), 5 μg/ml bovine insulin (Sigma, MO, USA), 10 ng/ml murine EGF (Sigma) and penicillin-streptomycin (Gibco BRL). For cell cloning, the cells were plated into 96-well tissue culture plates (Falcon, Becton Dickinson Europe, France) at a concentration of 1, 2 and 5 cells per well and cultured in the EGF-free medium conditioned by parental HC11 cells. 45 single-cell colonies were obtained and screened for expression of K14, using immunofluorescence microscopy and quantitative flow cytometry. For further studies, two cell lines, termed as BC20 and BC44, were selected and routinely grown in EGF-free medium. Mouse mammary epithelial cells, EphH4 (Reichman et al., 1992), kindly provided by Dr H. Beug (Research Institute for Molecular Pathology, Vienna, Austria) were grown in DMEM (Gibco BRL) containing 10% FCS (Seromed), 2 mM L-glutamine (Gibco BRL), 25 mM Hepes buffer (Sigma) and penicillin-streptomycin (Gibco BRL).

Induction of β-casein

To induce β-casein, 2-day confluent cultures of HC11 and BC20 cells were treated for 4 days with lactogenic hormone-containing medium, i.e. RPMI 1640 supplemented with 10% FCS, 1 μM dexamethasone (Sigma), 5 μg/ml insulin and 5 μg/ml ovine prolactin (Sigma), as described previously (Ball et al., 1988; Chammas et al., 1994). Cells treated with medium containing dexamethasone and insulin served as controls.

Antibodies

The following primary antibodies were used: CKB1, mouse anti-cytokeratin peptide 14 (Sigma); LL001 and LE61, mouse anti-cytokeratin 8/18, respectively (gifts from Prof. B. Lane, University of Dundee, UK; Taylor-Papadimitriou and Lane, 1987); DECMA-1, rat anti-E cadherin (Sigma); PC-10, rat anti-P cadherin (a gift of Dr M. Takeichi; Nose and Takeichi, 1986); α18, rat anti-α-casein (a gift from Dr Hirohashi; Shimoyama et al., 1992); mouse anti-β-catenin and anti-γ-catenin (Transduction Laboratories, KY, USA); VIIF9, mouse anti-vinculin (Glukhova et al., 1990); mouse anti-desmplakins 1 and 2 (2 gifts from Prof. W. Franke, German Cancer Research Center, Heidelberg, Germany); hamster anti-β1-integrin subunit (Hmβ1-1), anti-α1-integrin subunit (Haα1/8), anti-α2-integrin subunit (Haα2/9); rat anti-α5-integrin subunit (5H10-27) and anti-β4-integrin subunit (346-11A) (Pharmingen, San Diego, CA, USA); rabbit polyclonal anti α3-integrin subunit (a gift from Dr F. Watt; ICRF, London, UK); rabbit polyclonal anti-αv-integrin subunit (Chemicon, Temecula, CA, USA); GoH3, rat anti-α6-integrin subunit (a gift from Dr A. Sonnenberg; Sonnenberg et al., 1986).

The following secondary antibodies were used: sheep anti-mouse Ig conjugated to FITC or Texas Red and donkey anti-rabbit Ig conjugated to FITC (Amersham, UK); donkey anti-rat IgG conjugated to FITC or Texas Red, goat anti-hamster IgG conjugated to FITC and goat anti-mouse IgM conjugated to phycoerythrin (Jackson ImmunoResearch, PA, USA). Phalloidin-FITC (Sigma) was used to reveal actin filaments.

Flow cytometry

Cells to be analysed for K14, α3- or αv-integrin subunit expression were harvested using trypsin/EDTA, washed with RPMI 1640 containing 1% FCS and fixed in a mixture of 4% formaldehyde (Merck, Germany) and 0.2% Triton X-100 (ICN Biochemicals, USA) in PBS for 15 minutes at room temperature. Fixed cells were washed with PBS containing 1% FCS, and incubated with primary antibody for 1.5 hours and then with secondary antibody for 1 hour at room temperature. Cells to be analysed for surface expression of α1-, α2-, α5-, α6-, β1- and β4-integrin subunits were harvested using trypsin/EDTA, washed, incubated with primary antibody for 1 hour at 4°C and then with secondary antibody for 45 minutes at 4°C. They were fixed overnight in 4% formaldehyde before being analysed. In control samples, primary antibody was omitted from the incubation medium. 5-10×10^3 cells per sample were analysed in a FACScan analyser (Becton Dickinson, CA, USA) using Cell Quest software.

Immunofluorescence microscopy

Cells were plated at high density onto glass coverslips in 24-well tissue culture plates (Falcon, Becton Dickinson Europe, France). To stain for K14, K8/18, catenins, desmoplakins and cadherins, cells were routinely fixed with methanol at −20°C for 10 minutes. To evaluate the detergent solubility of cadherins, cells were gently permeabilized with 0.5% Triton X-100 and then fixed with 4% formaldehyde for 10 minutes. To stain for actin and vinculin, cells were fixed with 4% formaldehyde in PBS for 10 minutes and permeabilized with 0.5% Triton X-100 for 5 minutes at room temperature. Cells were incubated with primary antibodies for 1 hour at 37°C and then with appropriate secondary antibodies for 45 minutes, at 37°C. Cells were mounted in Moviol (Hoechst, Germany), observed with an Axiphot microscope (Carl Zeiss, Germany) and photographed on Kodak Tmax 3200 ASA film or Kodak Ektachrome Panther 1600 ASA film.
Immunoblotting
Confluent monolayers of BC20 and BC44 cells were rinsed with PBS and extracted in electrophoresis sample buffer (Laemmli, 1970). Samples were electrophoresed in a 10% SDS-polyacrylamide gel and transferred to PVDF membrane (Immobilon-P, Millipore, MA, USA). The blots were blocked in PBS-T (PBS with 0.1% Tween 20) containing 5% non-fat milk, incubated with anti-α-, β- or γ-catenin antibodies, washed in PBS-T, and treated with peroxidase-conjugated secondary antibodies (Amersham, UK). Labelled proteins were detected by enhanced chemiluminescence as recommended by the manufacturer (Amersham, UK) on high performance autoradiography film (Amersham, UK).

Cell attachment to anti-integrin antibodies and to ECM proteins
To coat with anti-β1-integrin antibody, 10-cm tissue culture Petri dishes were incubated with 10 μg/ml rabbit anti-hamster IgG (Sigma) in PBS for 6 hours, washed twice with PBS, treated with 1% bovine serum albumin (Sigma) for 1 hour, washed and incubated with 50 μg/ml hamster anti-β1-integrin in PBS overnight. Similarly, to coat with anti-tα6 antibody, Petri dishes were incubated with 10 μg/ml donkey anti-rat IgG (Jackson Immunoresearch, USA) followed by anti-tα6 antibody (GoH3) used as tissue culture supernatant. Coating with 20 μg/ml fibronectin (Sigma), 10 μg/ml laminin-5 (Rouselle et al., 1995) or 100 μg/ml poly-L-lysine (Sigma) was performed overnight. All the incubations were performed at 4°C.

Confluent BC20 cells were harvested by trypsin/EDTA, rinsed with growth medium, resuspended in RPMI 1640 containing 0.2% fetal calf serum, 2 mM L-glutamine, 0.5% GMS-A supplement (Gibco BRL), plated on the antibody-, ECM protein- or poly-L-lysine-coated plastic at a concentration of 2.5×10^5 cells per 10 cm Petri dish and incubated for 7 hours at 37°C before RNA extraction.

RNA preparation and northern blot analysis
Total RNA was extracted from cell cultures using RNA-plus reagent (Bioprobe Systems, France) according to the manufacturer's instructions. 20 μg of total RNA were separated on 1% agarose/formaldehyde gels, transferred to nylon membranes (Hybond-N, Amersham, UK) and hybridized with γ-32PdCTP random-primed labeled cDNA probes. Probes for mouse P-cadherin (Nose et al., 1987), mouse K14 (Knapp et al., 1987) and mouse β-casein (Gupta et al., 1982) were provided by Drs A. Cano (Instituto de Investigaciones Biomédicas, Madrid, Spain), M. Quintanilla (Instituto de Investigaciones Biomédicas, Madrid, Spain) and J. Rosen (Baylor College of Medicine, Baylor, TX, USA). The blots were exposed to high performance autoradiography film (Amersham, UK). Quantitative analysis of the results was performed by phosphorimager (Molecular Dynamics).

RESULTS
HC11 cells and their clonal derivatives cultured in the absence of EGF upregulate the expression of basal cell markers, K14 and P-cadherin
Confluent cultures of HC11 mammary epithelial cells grown in the absence or presence of EGF for 1 week were examined for expression of the basal cell markers, K14 and P-cadherin (Fig. 1). Double immunofluorescence labeling revealed that, in the absence of EGF, cultures consisted of numerous cell islets containing a dense network of K14-positive filaments and expressed P-cadherin at sites of cell-cell contacts (Fig. 1A). On the contrary, in the presence of EGF, the majority of cells were negative for K14 and P-cadherin (Fig. 1B). To estimate the percentage and the relative fluorescence intensity of the K14-positive cells, we used immunostaining and flow cytometry. Representative histograms of fluorescence distribution are shown in Fig. 1C,D. After 1 week in culture in the absence of EGF, 67% of the cells were positive for K14 while cultures grown in the presence of EGF contained 31% positive cells (Fig. 1C,D). The K14-positive cell population was heterogeneous, with cells exhibiting medium and bright levels of fluorescence intensity.

To obtain a homogeneous cell population with basal cell characteristics, that is, expressing K14 and P-cadherin, the parental HC11 cells were subcloned by limiting dilution in the absence of EGF, and the obtained clones were screened for K14 and P-cadherin expression. For further studies, clones 20 and 44 were selected and are referred to hereafter as BC20 and BC44. They comprised approximately 80% of K14-positive cells, with a prevalence of brightly stained cells (Fig. 1E,F). Fig. 2 shows the expression of K14 and P-cadherin in BC20 and BC44 cells analysed by northern blot. The parental HC11 cells contain much lower levels of K14 transcript and almost no P-cadherin mRNA (Fig. 2). For comparison, we probed RNA isolated from the mammary epithelial cell line EpH4 for expression of the basal markers. As shown in Fig. 2, EpH4 cells contained extremely low amounts of K14 transcript and did not express any P-cadherin. The expression of K14 and P-cadherin in the cell lines was stable for at least 60 passages. BC20 and BC44 did not express keratins 8/18, characteristic of luminal mammary epithelial cells (Taylor-Papadimitriou and Lane, 1987), while the parental HC11 cells contained a keratin 8/18-positive cell population (data not shown).

The phenotype of the obtained clones appeared to be somewhat plastic, as after 2-3 passages in the presence of EGF, downregulation of K14 and P-cadherin expression was observed at the protein (Fig. 3A-D) and the mRNA levels (Fig. 4). However, unlike the parental HC11 cells that were reported to produce a milk protein β-casein if primed with EGF and treated with lactogenic hormones (Ball et al., 1988), the basal cells, regardless of EGF treatment, did not express β-casein in response to lactogenic stimuli (Fig. 5).

The basal cell clones exhibit well-developed cell-ECM adhesions and deficient cell-cell junctions
Analysis of actin and vinculin distribution in the basal cells has revealed the patterns that are characteristic of fibroblastoid rather than epithelial cells. Actin filaments were organized in stress fibers, while vinculin was found in cell-ECM focal adhesions. Neither actin nor vinculin were localized at the cell-cell boundaries. The basal cells did not form desmosomes, as indicated by the absence of intercellular staining with an anti-desmoplakin antibody (Fig. 3I).

In addition to downregulation of K14 and P-cadherin, treatment with EGF induced a reorganization of the cytoskeleton towards that characteristic of an epithelial phenotype. In the EGF-treated cultures, both actin and vinculin were concentrated at the sites of cell-cell contact, whereas actin stress fibers and vinculin-containing focal adhesions were significantly diminished (Fig. 3F,H). Consistent with an epithelial phenotype, the basal cells cultured in the presence of EGF formed desmosomes (Fig. 3J). After removal of EGF, the cells reverted back to their ‘mesenchymal’ morphological characteristics and upregulated P-cadherin and K14 expression (not shown).
In confluent basal cell monolayers, E- and P-cadherins were localized at the cell-cell boundaries (Fig. 6A,C). The absence of actin and vinculin from sites of the cell-cell contacts (Fig. 3E,G), however, indicated a deficiency of adherens junctions. Triton X-100 is known to solubilize the majority of cytosolic proteins, i.e. non-associated with the cytoskeleton. We therefore treated the monolayers of the basal cells with 0.5% Triton X-100 prior to staining with anti-cadherin antibodies. As expected, both E- and P-cadherins appeared to be Triton X-100-soluble and were not revealed at cell-cell boundaries after extraction (Fig. 6B,D). On the contrary, in the basal cells cultured in the presence of EGF (Fig. 6E,F), as well as in the mammary epithelial cell line EpH4 (Fig. 6G,H), E-cadherin remained in the cell-cell junctions regardless of Triton X-100 extraction.

As catenins are known to mediate the linkages of cadherins to the actin cytoskeleton, the expression of α-, β- and γ-catenins in basal cell lines was analysed. Immunoblotting experiments revealed that all three catenin molecules were expressed in the basal cell lines, but their expression levels varied depending on the presence or absence of EGF. In EGF-free conditions, the expression of α-catenin was significantly increased compared to EGF-containing conditions. The expression of β- and γ-catenins followed a similar pattern, with higher levels detected in the absence of EGF.

In summary, the results suggest that EGF plays a crucial role in the regulation of cadherin and catenin expression in basal cell lines, potentially influencing cell-cell adhesion and actin cytoskeleton organization.

Fig. 1. Expression of basal cell markers in parental HC11 cells and subclones. (A,B) Double immunofluorescence staining of parental HC11 cells with anti-K14 (in red) and anti-P-cadherin (in green) antibodies. HC11 cells were grown in EGF-free medium (A) or in EGF-containing medium (B). Bar, 35 mm. (C-F) Flow cytometry of parental HC11 cells and subclones labeled with anti-K14 antibody (grey histogram). Analyses were performed on parental HC11 cells grown in the absence (C) or in the presence of EGF (D), and on BC20 (E) and BC44 (F) cells cultured in EGF-free medium. The percentage of K14-positive cells is indicated. White histograms show negative controls where primary antibody was omitted. HC11 cells upregulate P-cadherin and K14 when EGF is omitted from the standard growth medium. Similarly the derivatives, BC20 and BC44, obtained in the absence of EGF, express high levels of K14.

Fig. 2. Autoradiogram showing the expression of K14 and P-cadherin in parental HC11 cells and in basal clones BC20 and BC44 analysed by northern blot. BC20 and BC44 express high levels of K14 and P-cadherin if compared to the parental HC11 cells cultured in the absence of EGF. For comparison, the mammary epithelial EpH4 cells express extremely low amounts of the two basal cell markers, if any. GAPDH was used to estimate the loading.
Integrins and basal cell marker expression

expressed in BC20 and BC44 cells (Fig. 7A). The distribution of the catenins was then examined in BC20 cells by immunofluorescence microscopy. Similar to E- and P-cadherin, β- and γ-catenins were localized at the sites of cell-cell contact (Fig. 7D,E), whereas α-catenin displayed an irregular, discontinuous distribution pattern (Fig. 7B). In parallel control assays performed with the mouse mammary epithelial cell line, EpH4, a regular staining of the cell-cell contacts was obtained using the same anti-α-catenin antibody (Fig. 7C).

The absence of α-catenin from sites of cell-cell contacts, together with the Triton X-100 solubility of E- and P-cadherins, strongly supported the notion that the established basal cells lacked organized adherens junctions.
Ligation of integrins and adhesion to ECM proteins result in increase of P-cadherin transcript level

Cellular attachment to ECM results in clustering of integrins, assembly of multimolecular focal complexes associated with the actin cytoskeleton and activation of intracellular signalling pathways leading to changes in gene expression (Yamada and Geiger, 1997).

Using flow cytometry, we have determined the integrin repertoire of the basal cells. As shown in Fig. 8, the basal cells expressed high levels of β1, α3, α6 and β4 integrin chains, and low to moderate levels of α5 and αv, whereas neither α1 nor α2 chains were revealed. Cell attachment assays have demonstrated that the basal cells adhered well to purified fibronectin and laminin 5 and practically did not attach to laminin 1 and collagens I and IV (data not shown). To determine whether cell-ECM interactions mediated by integrins have a role in the regulation of the expression of K14 and P-cadherin, BC20 cells were plated onto poly-L-lysine, the function-blocking antibodies against α6 and β1 integrin chains, fibronectin and laminin 5 in serum-free medium. After 7 hours, total RNA was extracted and probed for K14 and P-cadherin expression using northern blot analysis. The data presented in Fig. 9A show that the level of K14 transcript was not modified significantly in response to integrin ligation by the function-blocking antibodies against α6 and β1 integrin chains, fibronectin and laminin 5 in serum-free medium.
Integrins and basal cell marker expression

DISCUSSION

The basal myoepithelial cells play an important role in mammary gland bilayered parenchyma organization, as they are involved in both cell-cell and cell-ECM interactions. In adult mammary gland, the expression of K14 and P-cadherin is largely confined to the basal layer of myoepithelial cells. K14-containing intermediate filaments penetrate into desmosomes and hemidesmosomes and thus, being involved in intercellular interactions as well as in contacts with basement membrane, they appear to be essential for the maintenance of the basal cell layer integrity (Fuchs and Cleveland, 1998). The functions of P-cadherin, as well as the precise mechanisms that regulate its expression, remain uncertain (Faraldo and Cano, 1993; Faraldo et al., 1997). Recent studies by Radice et al. (1997) have demonstrated that P-cadherin-deficient virgin females displayed precocious differentiation of the mammary epithelium similar to early pregnant animals, and exhibited mammary hyperplasia with age. Therefore P-cadherin was suggested to play a role in the negative control of growth and differentiation of mammary epithelium (Radice et al., 1997).

HC11 is a mammary epithelial cell line derived from spontaneously immortalized COMMA-1D epithelial cells that were isolated from the mammary gland of mid-pregnant mice (Ball et al., 1988). During pregnancy, the mammary tree consists of branching ducts and alveolar buds that will further develop and give rise to secretory tissue. COMMA-1D and HC11 cells retain important characteristics of normal mammary cells, including morphogenetic properties in vivo and the ability for functional differentiation in vitro. In vivo, after transplantation into the cleared fat pad of BALB/c mice, both lines have been reported to generate outgrowths reminiscent of ductal and alveolar structures (Danielson et al., 1984; Humphreys and Rosen, 1997). In culture, a population of HC11 cells, similar to alveolar secretory cells, is able to synthesize the milk protein β-casein, in response to lactogenic hormone treatment (prolactin, dexamethasone and insulin) (Ball et al., 1988; Chammas et al., 1994). In addition, the HC11 line comprises cells expressing the basal cytokeratin, K14 (Cella et al., 1996). Thus the line appears to be heterogeneous, consisting of cells that display the characteristics of both luminal and basal mammary epithelial cells.

We report here the establishment of HC11 derivatives, BC20 and BC44, which exhibit high levels of expression of the basal cell markers, K14 and P-cadherin. These lines do not express keratins 8/18, which are characteristic of simple epithelia and luminal mammary epithelial cells (Taylor-Papadimitriou and Lane, 1987) and, unlike the parental HC11 cells, are not competent to produce β-casein in response to lactogenic blocking antibodies or adhesion to ECM proteins. On the contrary, the amount of P-cadherin transcript was 2-, 2.4-, 3.9- and 3.5-fold higher in basal cells plated onto anti-α6, anti-β1, fibronectin and laminin 5, respectively, compared to that in cells attached to poly-L-lysine (Fig. 9B).
hormones. Under standard culture conditions, neither BC20 nor BC44 cells contained detectable amounts of α-smooth muscle actin (data not shown). Conceivably, these two derivatives might behave as undifferentiated, P-cadherin-positive myoepithelial cell progenitors that have been identified in the basal layer of growing buds in the developing mouse mammary gland (Daniel et al., 1995). We are currently investigating possibilities of inducing the expression of smooth muscle-specific proteins in these cells.

In parental HC11 cells, expression of the basal cell markers K14 and P-cadherin significantly increased following removal of EGF from the growth medium. Therefore, the basal clones were established and cultured in the absence of EGF. The two basal clones displayed prominent cell-ECM focal adhesions and deficient cell-cell junctions, as revealed by (1) the absence of actin and vinculin from cell-cell boundaries, (2) the irregular distribution pattern of α-catenin and (3) the localization of E- and P-cadherin in the Triton X-100-soluble fraction. Consistently, no desmosomes were detected in the basal cells cultured under standard conditions in the absence of EGF. However, upon long-term exposure to EGF, cells acquired a more epithelial morphology. In particular, actin and vinculin were redistributed to cell-cell junctions, and α-catenin as well as desmoplakins were found at sites of cell-cell contacts, whereas focal adhesions and stress fibers almost disappeared. These alterations of cell-ECM and cell-cell interactions were accompanied by downregulation of P-cadherin and K14 expression. Similar changes are observed in epidermis, when keratinocytes proceed in their differentiation as stratification occurs. Indeed, keratinocytes of suprabasal layers shut off expression of K14 and P-cadherin, downregulate integrins, and establish stronger cell-cell contacts that include adherens junctions and desmosomes (Fuchs and Cleveland, 1998; Jones et al., 1995).

We have found that EGF acts as a negative regulator of the basal mammary epithelial cell phenotype. However, the long-term effects of EGF on basal cells remain to be further investigated. In vitro, EGF has pleiotropic effects on mammary epithelial cell phenotype. On the one hand, it is a mitogen and scattering agent. For instance, it was reported to induce a transient transition towards fibroblastoid phenotype in 184A1 human mammary epithelial cells (Matthey et al., 1993). On the other hand, it appears to be a differentiation factor, since it is required for promoting competency for β-casein production in HC11 cells (Ball et al., 1988; Wirl et al., 1995). In addition, EGF may alter cell-ECM relationships modifying ECM composition (Chammus et al., 1994; Wirl et al., 1995).

Several papers have reported the results of inactivation of EGF receptor gene or of targeted expression of its dominant negative mutant in vivo, in the epidermis and in the mammary luminal epithelium. On the basis of these studies, EGF (as well as other EGF receptor ligands) was suggested to regulate proliferation of the epidermis and orientation and maturation of hair follicles (Murillas et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995) and ductal development in the mammary gland (Xie et al., 1997). So far, no data have been reported on the mammary gland phenotype of the EGF receptor-null animals. If delivered in mammary gland in situ, EGF was shown to stimulate ductal growth in the resting state and to inhibit it in the developing gland (Coleman and Daniel, 1990). Thus, cellular responses to EGF may vary depending on the growth and differentiation state and on the cellular environment, i.e. established contacts with neighbouring cells and ECM. Whereas in vivo, cells are exposed to several growth factors and hormones at the same time, the in vitro model system that is being used in this study might allow us to dissect particular functions of EGF in the differentiation of mammary epithelium.

As revealed by flow cytometry analysis, BC20 and BC44 cells, similar to basal mammary epithelial cells in vivo, expressed high levels of α3, α6, β1 and β4 integrin chains (Berdichevsky et al., 1994). Expression of α3β1 and α6β4 integrins provides the basal cells with the ability to adhere to laminin-5, a component of epithelial basement membrane (Rousselle et al., 1991; Burgeson and Christiano, 1997). Expression of α5 and αv integrin chains in the basal cells is not surprising, as they are known to be upregulated under the conditions of culture. Potentially, α5β1 and αvβ1 integrins may serve as receptors for fibronectin (Hynes, 1992). The absence of α1-integrin chain from BC20 and BC44 cells correlates with the lack of smooth muscle marker expression. We have shown previously that, in vivo, expression of α1 integrin chain in myoepithelial cells is posterior to the expression of smooth muscle-specific actin and myosin isoforms (Deugnier et al., 1995). No significant amount of α2 integrin chain was revealed in the basal cells; however, its expression in mammary epithelial cells was reported to be susceptible to modulation by various stimuli (D’Souza et al., 1993; Lu et al., 1995).

To study directly the involvement of β1- and α6β4 integrins in the regulation of basal cell marker expression, BC20 cells were plated on function-blocking antibodies against β1- and α6-integrin chains and on ECM proteins, fibronectin and laminin 5. We have observed that cells plated onto function-blocking anti-β1- or anti-α6-antibodies as well as onto fibronectin and laminin 5 significantly upregulated P-cadherin expression. In contrast, K14 transcript levels were not significantly altered in response to integrin ligation or adhesion to ECM proteins. These data are in agreement with those reported by Bagutti et al. (1996), showing that expression of K14 was not affected in β1-deficient ES cells grown subcutaneously in vivo. Thus the results reported here suggest that EGF regulates both K14 and P-cadherin expression, whereas cell-matrix interactions are directly implicated in P-cadherin expression control.

Under standard culture conditions, basal cells exhibit well-developed cell-ECM adhesions and deficient cell-cell junctions, while long-term EGF treatment leads to disappearance of focal contacts and stress fibers and establishment of cell-cell junctions. Recent studies performed in different model systems have demonstrated the functional connection between cell-ECM and cell-cell adherens junctions. In general, it was found that enhancement of one adhesion system results in the weakening of the other (Finneman et al., 1995; Monier-Gavelle and Duband, 1997; Levenberg et al., 1998). A possible explanation of such cross-talk may be that cellular pools of cytoskeletal proteins such as actin, α-actinin and vinculin are shared by the two types of multimolecular adhesion complexes (Yamada and Geiger, 1997; Hazan et al., 1997). On the other hand, certain regulatory molecules, in particular members of the Rho-family of small GTPases and integrin-linked kinase, were suggested to be involved in the


