INTRODUCTION

Epithelial cells form a selective permeability barrier separating the outside world from internal compartments. To perform this function, epithelial cells are polarized into discrete apical, lateral and basal domains. The apical plasma membrane contains unique proteins and lipids not found on basolateral plasma membranes (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). Specialized structures such as microvilli and cilia extend from the apical pole; the Golgi complex is positioned in the apical cytoplasm. Tight junctions, zonula adherens and desmosomes are found on lateral borders that also contain cell adhesion molecules, such as E-cadherin, that mediate interactions with neighboring cells. Basal plasma membranes contain integrin receptors (Ruoslahti, 1991) that interact with extracellular matrix (ECM) in the underlying basement membrane. Some integrins may also mediate cell-cell adhesion (Larjava et al., 1990; Carter et al., 1990).

Studies on the biogenesis of epithelial polarity have focused on the role of cell-cell and cell-substratum adhesion in establishing the polarized epithelial phenotype (Nelson and Veshnock, 1986, 1987a,b; Ekblom, 1989; Nelson and Hammerton, 1989; McNeil et al., 1990; Sorokin et al., 1990). In the polarized Madin-Darby canine kidney (MDCK) cell line, in vitro studies have demonstrated that interactions with the substratum are sufficient for establishing apical polarity (Rodriguez-Boulan et al., 1983; Vega-Salas et al., 1987). In the absence of cell-cell contacts, MDCK cells polarize apical plasma membrane glycoproteins in response to cell-substratum adhesion. Basolateral membrane proteins remain randomly distributed on plasma membranes until cell-cell contacts form (Nelson and Veshnock, 1986; 1987a,b; Nelson and Hammerton, 1989; McNeil et al., 1990). Studies on early

SUMMARY

A number of epithelia form tubulocysts in vitro when overlaid with type I collagen gel. Because collagen receptors are generally believed to be expressed on the basolateral domain, the mechanism by which collagen elicits this morphogenetic response from the apical surface is unclear. To investigate the role of β1 integrins, the major receptor family for collagen, in this process, we overlaid polarized monolayers of MDCK II cells grown on permeable supports with type I collagen gel and correlated integrin polarity with the polarity of other apical and basolateral membrane markers during tubulocyst formation. Polarized monolayers of one clone of MDCK II cells, referred to as Heidelberg MDCK, initially respond to collagen overlay by stratifying; within 48 hours, lumena develop between the cell layers giving rise to tubulocysts. Tight junctions remain intact during tubulocyst formation because transepithelial electrical resistance does not significantly change. Major alterations are observed, however, in the expression and localization of apical and basolateral membrane markers. β1 integrins are necessary for tubulocyst morphogenesis because a function-blocking antibody administered to the apical pole of the cells completely inhibits the formation of these structures. To determine how apical-cell collagen interactions elicit tubulocyst formation, we examined whether β1 integrins are mobilized to apical plasma membranes in response to collagen overlay. We found that in the absence of collagen, polarized monolayers of Heidelberg MDCK cells endogenously express on apical plasma membranes a small pool of the β1 family, including α2β1 and α3β1. Collagen overlay does not mobilize additional β1 integrins to apical domains. If β1 integrins are not already apically expressed, as in the C6 MDCK cell line (Schoenenberger et al. (1994) J. Cell Biol. 107, 527-541), β1 integrins are not directed apically and tubulocysts do not develop in response to collagen. Thus, interaction of β1 integrins pre-existing on apical plasma membranes of polarized epithelia with type I collagen gel is the mechanism by which apical application of collagen elicits the formation of tubulocysts. Depolarized integrins on apical plasma membranes of polarized epithelia may be relevant to the pathogenesis of disease and injury.

Key words: β1 Integrin, Epithelial polarity, Tubulocyst, MDCK
kidney development also indicate that polarization requires cell-cell and cell-substratum adhesion. In kidney tubulogenesis, the ureteric bud induces the metanephric mesenchyme to condense by forming extensive cell-cell contacts. Conversion of the condensates into polarized tubular epithelia is later mediated by cell-substratum interactions with ECM (Sorokin et al., 1990).

A number of studies have addressed the role of cell-cell contacts mediated by E-cadherin in epithelial polarization (Nelson and Vescnocket, 1986, 1987a,b; Nelson and Hammerton, 1989; McNeil et al., 1990); however, equivalent studies on cell-substratum adhesion, particularly the role of the ECM and its receptors, have been few. Cell-ECM interactions initiated by suspending MDCK cysts in type I collagen gel lead to membrane remodeling and polarity reversal (Wang et al., 1990; Ojakian and Schwimmer, 1994). A new basal surface develops at the site of cell-collagen interaction and an apical surface forms at the opposite end lining an internal lumen. Further evidence that the ECM is critical in establishing the polarized epithelial phenotype comes from studies on kidney development. Deposition of laminin A in the basement membrane surrounding the condensed mesenchyme stimulates the conversion of the aggregated cells into polarized epithelia (Sorokin et al., 1990).

Epithelial interactions with ECM are mediated by the integrins, a superfamily of cell surface receptors found on every eucaryotic cell except erythrocytes (for reviews, see Hynes, 1992; Hemler et al., 1995). Integrins are transmembrane glycoproteins that consist of unrelated α and β subunits. Their association into noncovalent heterodimers is necessary for cell surface expression and ligand binding. Most epithelia express integrins belonging to the β1, β3, β4 or β5 families. The β chains form complexes with a variety of different α subunits, yielding receptors for collagen, laminin, fibronectin and vitronectin. In addition, some αβ combinations recognize more than one ECM ligand. For example, the α1β1 and α2β1 are receptors for collagen or laminin whereas the α3β1 can recognize collagen, laminin, or fibronectin (Hynes, 1992; Hemler et al., 1995). Receptor-ligand binding activates transmembrane signaling, influencing cell differentiation, behavior and gene expression (for reviews, see Damsky and Werb, 1992; Juliano and Haskell, 1993; Clark and Brugge, 1995).

By mediating cell-substratum interactions, the integrins have been implicated in the pathogenesis of disease and injury. In carcinogenesis, novel integrin-substratum interactions influencing epithelial differentiation and polarization (Schoenenberger et al., 1994) have been suggested to contribute to metastasis (Plantefarber and Hynes, 1989; Schoenenberger and Matlin, 1991; Matlin and Caplan, 1992). New integrin-ECM interactions are established after epithelial injury and facilitate wound healing and repair (for reviews, see Gailit and Clark, 1994; Clark, 1995). In vitro models of acute renal failure, integrin-substratum interactions are altered (Gailit et al., 1993; Goligorsky et al., 1993); the α3β1 integrin redistributes from basal to apical cell surfaces and some integrins are lost from epithelial plasma membranes (Gailit et al., 1993).

We have been investigating integrin-mediated cell-substratum interactions in epithelial cell polarization using the MDCK cell line as a model system. We have been particularly interested in the role of the ECM in generating and maintaining discrete apical and basolateral domains. MDCK cells express the β1, β3, and β4 integrin families, including the α2β1, α3β1, an unidentified αβ1, αβ6β4 and αβ4β3 (Schoenenberger et al., 1994; Ojakian and Schwimmer, 1994). The β1, β3, and β4 families are present on basolateral plasma membranes. Only the β1 family mediates cell attachment to collagen types I and IV and laminin (Schoenenberger et al., 1994; Ojakian and Schwimmer, 1994). To further study the role of β1 integrins in epithelial polarization, we took advantage of a classic observation in which epithelia, including thyroid, mammary gland, intestine, liver and vascular endothelia, form tubulocysts when overlaid with type I collagen gel (Chambard et al., 1981; Hall et al., 1982; Montesano, 1983; Montgomery, 1986; LeCluyse et al., 1994). Because β1 integrins are the major receptor family for collagens (Hynes, 1992; Hemler et al., 1995) found on basolateral cell surfaces (Ruoslathi, 1991), the mechanism of how collagen at apical plasma membranes elicits this morphogenetic response was unclear. We hypothesized that apical cell-collagen interactions would mobilize β1 integrins to apical plasma membranes to mediate the formation of tubulocysts. Using the polarized MDCK cell line, we found to our surprise that in the absence of collagen overlay, β1 integrins were endogenously expressed at low levels on apical plasma membranes in one clone of MDCK, even though other apical and basolateral membrane markers localized exclusively to their respective plasma membrane domains. Moreover, apical application of collagen did not mobilize additional β1 integrins to apical cell surfaces. Pre-existing apical β1 integrins mediated tubulocyst formation because if receptor-ligand binding was inhibited or if β1 integrins were absent from this surface tubulocysts did not develop in response to collagen.

MATERIALS AND METHODS

Cell culture

MDCK II cells were originally cloned by Louvard (1980) for high growth rate and dome formation; this clone is referred to as Heidelberg (HD) MDCK cells to identify its place of origin. HD MDCK cells have been used extensively for studies on epithelial cell polarity (Matlin et al., 1981; Matlin and Simons, 1983; for review, Simons and Fuller, 1985). The C6 subclone of HD MDCK (MDCK pMV7 6.2.1) was used for some experiments. C6 cells are stably transfected with the empty pMV7 expression vector (Schoenenberger et al., 1991). They are identical to the parental line with regard to ultrastructure, growth rate and transepithelial electrical resistance.

For routine culture, HD (passages 7-33) and C6 (passages 7-15) MDCK cells were grown in 75 cm² flasks (Falcon; Becton Dickinson & Co., Boston, MA) in minimal essential medium (MEM) or Dulbecco’s MEM (DME; Mediatech, Herndon, VA) containing 5% fetal bovine serum (FBS; Sigma Chemical Co., St Louis, MO) or hyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine (Sigma) and 10 mM Hepes (Sigma), pH 7.4, at 37°C in 5% CO₂. Confluent cells were harvested with trypsin-EDTA (Gibco BRL, Life Technologies, Inc., Grand Island, NY) and subcultured at a 1:5 dilution twice weekly.

For culture of cells on permeable polycarbonate supports, cells were plated at 7.8 × 10⁶ cells on Transwells filters (24 mm, 0.4 μm pore size; Corning CoStar, Cambridge, MA) pre-wetted with medium. Cultures were fed every other day for 6-8 days as well as the day before collagen overlay. Antibiotic-antimycotic (Gibco) was included in the medium at a final concentration of 100 units/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B.
Antibodies

Anti-integrin antibodies

Rabbit anti-human \(\beta_1\) integrin antibody was obtained from Dr. E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). This antibody was generated against a 38mer peptide corresponding to the carboxy-terminal domain (Giancotti and Ruoslahti, 1990) of the human \(\beta_1\) integrin. Under non-denaturing conditions, this antibody and other polyclonal \(\beta_1\) integrin antibodies (Schoenenberger et al., 1994) immunoprecipitate \(\alpha_2\beta_1\), \(\alpha_3\beta_1\), an unidentified \(\alpha\beta_1\), and precursor \(\beta_1\) from MDCK cell extracts (Schoenenberger et al., 1994). Under denaturing conditions, this antibody and other polyclonal \(\beta_1\) integrin antibodies immunoprecipitate only two polypeptides corresponding to the \(\beta_1\) integrin subunit and its precursor (Schoenenberger et al., 1994).

The function-blocking rat monoclonal anti-human \(\beta_1\) integrin antibody, A1IB2 (Hall et al., 1990), was obtained from Dr. C. Damsky (University of California at San Francisco, San Francisco, CA). This antibody immunoprecipitates only mature \(\beta_1\) and its associated \(\alpha\) subunits in MDCK cells; it does not recognize the \(\beta_1\) precursor of MDCK (A. Zak, unpublished observation).

Antibodies to apical and basolateral membrane markers

Hybridomas secreting mouse monoclonal antibody 3F2ID8 recognizing the apical membrane glycoprotein, gp135, were obtained from Dr. G. Ojakian (SUNY, Brooklyn, NY; Ojakian and Schwimmer, 1988). Mouse monoclonal antibody 6.23.3 was generated against dog intestinal mucosa (Balcaraova-Ständer et al., 1984). This antibody recognizes a basolateral membrane protein of 58 kDa, referred to as p58. Mouse monoclonal antibody rr1, generated against strain 1 MDCK cells, recognizes the basolateral membrane protein E-cadherin (Gumbiner and Simons, 1986). Culture supernatant and the antibody-secreting hybridoma were obtained from Dr. B. Gumbiner ( Sloan-Kettering, New York, NY).

Collagen overlay

Rat tail tendon type 1 collagen (Upstate Biotechnology Inc., Lake Placid, NY) in 0.02 N acetic acid was dialyzed against 1/10th Ham’s F-12 (Gibco), pH 4.0, at 0°C. Hydrated collagen gels were cast as previously described (Zuk et al., 1989) by mixing on ice 7 ml of dialyzed collagen (3 mg/ml), 1 ml 10x F-12 (101 mg/ml), 1 ml 10x sodium bicarbonate (11.76 mg/ml; Gibco) and 1 ml FBS.

In preparation for collagen overlay, MDCK cells grown to confluency on permeable supports were rinsed once with growth medium prepared as described earlier. Medium (1.5 ml) was added to the basal compartment; the apical compartment was overlaid with 400 \(\mu\)l collagen solution after rinsing once with the same volume of collagen mixture. Within 20 minutes at 37°C, the collagen polymerized into a hydrated gel covering MDCK apical surfaces. In order to facilitate contact of apical cell surfaces with the polymerized type I collagen gel and to avoid detachment of the collagen overlay from this cell surface, additional medium was not added to the apical compartment of permeable supports overlaid with collagen. Control filters that were not overlaid with collagen had 400 \(\mu\)l medium added to the apical compartment. Cultures were incubated for up to 5 days. The volume of collagen and/or medium added to the apical or basal compartments was the minimal amount needed for adequate incubation of Transwell supports for the duration of the experiment.

For some experiments, antibodies or reagents were mixed with collagen at the time of overlay and also added basally. Cultures were fed from the basal compartment with fresh antibody or reagent 24 hours later. Antibodies included A1IB2; reagents included mitomycin C (Sigma), an inhibitor of cell proliferation. Heat inactivated FBS was used for antibody inhibition studies.

Metabolic labeling and immunoprecipitation

Integrins were radiolabeled to steady-state prior to collagen overlay. Polarized monolayers of HD MDCK cells grown on Transwell filters for 6-8 days were incubated from the basal compartment with 100 \(\mu\)Ci/ml \([^{35}S]\)methionine \([^{35}S]\)cysteine (EXPRE \(^{35}S^{35}S\), New England Nuclear, Boston, MA) in labeling medium (1/10 methionine-free MEM or DME containing FBS, glutamine, Hepes, and antibiotic/antimycotic) for 16-24 hours. Apical compartments contained labeling medium without isotope. After steady-state labeling, filters were prepared for collagen overlay; medium was removed only from the apical compartment and apical cell surfaces rinsed once with labeling medium and once with collagen solution; basal medium containing radiolabel was not removed. Apical cell surfaces were then overlaid with 400 \(\mu\)l collagen solution. Filters were further incubated for up to 12 hours before processing for differential surface biotinylation (Schoenenberger et al., 1994) and immunoprecipitation of integrins.

To examine the effects of collagen overlay on newly synthesized integrins, cells were pulse-labeled after collagen overlay. Pulse-chase experiments were done 12 hours after collagen overlay when cell layering and lumen formation are negligible (Fig. 2B). MDCK cells were rinsed three times, 10 minutes each at 37°C with methionine-free medium containing Hepes, glutamine, and 0.35g/l sodium bicarbonate (pulse-medium). MDCK cells were then pulsed for 10 minutes at 37°C from the basal cell surface with 100 \(\mu\)l of 100 \(\mu\)Ci \([^{35}S]\)methionine \([^{35}S]\)cycteine; 500 \(\mu\)l pulse-labeling medium without isotope was added apically. Radiolabeled proteins were then chased for up to 90 minutes at 37°C in MEM containing 10x the normal concentration of unlabeled methionine. MDCK integrins reach the cell surface within 60 minutes chase (G. M. Zinkl and K. S. Matlin, unpublished observation). After 90 minutes chase, integrins were differentially surface biotinylated and immunoprecipitated.

For surface biotinylation and immunoprecipitation, MDCK cells on ice were rinsed three times with PBS containing 1 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\) (PBS+) without disturbing the collagen overlay. MDCK cells were then differentially biotinylated twice, 15 minutes each, from the apical or basal compartment or both with 0.5 mg/ml sulfo-NHS-biotin (Pierce Chemical Co., Rockford, IL) dissolved in TEA buffer (10 mM triethanolamine, 125 mM NaCl, 2 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), pH 9.0; Gottardi and Caplan, 1992). Compartments that were not biotinylated received only TEA buffer. Filters were then rinsed once in serum-free medium without mixing of apical and basal washes; filters were then extensively rinsed with PBS+ before extraction.

Transwell filters were removed from their supports, cut in half and extracted in microfuge tubes (1.5 ml) containing extraction buffer (1% NP-40, 1% sodium deoxycholate (added fresh), 0.5% SDS, 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 10 mM glycine, 10 mM Tris-HCl, pH 9.0; Gottardi and Caplan, 1992). Compartments that were not biotinylated received only TEA buffer. Filters were then rinsed once in serum-free medium without mixing of apical and basal washes; filters were then extensively rinsed with PBS+ before extraction.

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Apical \(\beta_1\) integrin in polarized MDCK cells

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Cultures were labeled with $[3\text{H}]$thymidine (New England Nuclear) in accordance with the manufacturer’s directions. Values were calculated and basal (1 ml) compartments. Filters were allowed to equilibrate in $\text{H}_{2}\text{O}$, and $[3\text{H}]$thymidine incorporation was assayed. Filters were rinsed on ice three times with PBS+, 4 minutes each, without disturbing the collagen overlay. Filters were cut away from their supports with collagen intact and proteins precipitated on ice for a minimum of 2 hours in 1 ml 10% trichloroacetic acid (TCA). TCA was removed by aspiration, and samples rinsed twice with 1 ml fresh ice-cold 5% TCA. Samples were solubilized in 500 µl 0.2 M NaOH, 0.1% SDS for 1 hour at 37°C and then neutralized with an equal volume of 2 M Tris, pH 6.8. Radioactivity was measured by liquid scintillation counting from equal aliquots of samples. Counts per minute/sample were calculated by subtracting background counts (filter, with or without collagen). Significance ($P < 0.05$) was determined by the one-way analysis of variance and Student’s $t$-test.

**Light microscopy**

Cultures were fixed in half-strength Karnovsky fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.4) for 1 hour, osmicated (1% OsO₄), and stained en bloc with 1% uranyl acetate, as previously described (Zuk et al., 1989). Filters were dehydrated in a graded series of ethanols, cut from the filter holders leaving the collagen overlay intact, and infiltrated and embedded in Epon-Araldite containing 2% DMP-30.

Transverse sections (0.5-1 µm) were cut using a Reichert-Jung Ultracat microtome. Sections were stained with 1% toluidine blue (for light microscopy) or 0.1% toluidine blue (for immunoperoxidase stained filters) in 1% sodium borate and photographed with T-MAX ASA 100 film (light microscopy; Kodak) or Technical Pan Film ASA 100 (peroxidase immunohistochemistry; Kodak).

**Immunohistochemistry**

Apical and basolateral membrane markers were immunolocalized in whole-mount collagen overlaid cultures using a modification of a peroxidase-antiperoxidase technique previously described (Zuk and Hay, 1994). This procedure has the advantage of localizing with high resolution the stable peroxidase product in plastic-embedded samples. For peroxidase immunohistochemistry, an entire filter of MDCK cells overlaid with collagen was rinsed in PBS+ and fixed in 2% paraformaldehyde, 75 mM L-lysine, 10 mM sodium m-periodate (PLP; McLean and Nakane, 1974) for 1 hour. After further rinsing, endogenous peroxidase activity was quenched with 1% H₂O₂/PBS or 0.1% phenylhydrazine/PBS for 10 minutes; cells were rinsed with PBS and then permeabilized with 0.1% Triton X-100/PBS for 4 minutes. Non-specific binding sites were blocked by incubating in 10% normal goat serum (NGS) for 45 minutes-1 hour. For incubation in antibodies, filter holders were removed from 6-well plates and positioned basal side down on 150 µl of antibody pipetted on Parafilm in a humidified chamber; the apical compartment was covered with an additional 150 µl antibody. Filters were incubated overnight at 4°C in primary antibody. Filter holders were returned to 6-well plates, rinsed and then incubated as described above in link antibody (goat anti-mouse; Sigma) diluted 1:20 in 10% NGS for 1 hour at room temperature. After further rinsing, filters were incubated in peroxidase-antiperoxidase (PAP) mouse antibody (Sigma) diluted 1:100 in 10% NGS for 1 hour at room temperature. Filters were washed extensively and PAP complexes immunolocalized by reacting with a freshly prepared solution of 0.05% diaminobenzidine (Sigma) dissolved in 0.01% H₂O₂ in 50 mM Tris-HCl, pH 7.5, for 10-15 minutes at room temperature. The immunostained filters were then washed and processed for light microscopy as described earlier except that osmication and en bloc staining were omitted.

**Transepithelial resistance**

Transepithelial electrical resistance was measured in HD MDCK cells grown on permeable supports with or without collagen overlay using an Epithelial Voltohmmeter (World Precision Instruments, New Haven, CT). Warm (37°C) medium was added to the apical (1.5 ml) and basal (1 ml) compartments. Filters were allowed to equilibrate to room temperature for 20 minutes. Electrical resistance was measured according to the manufacturer’s directions. Values were calculated after subtracting the background contributed by blank filters plus or minus collagen overlay, as appropriate.

**Thymidine incorporation**

Cultures were labeled with $[3\text{H}]$thymidine (New England Nuclear) in order to measure cellular proliferation. For these experiments, HD MDCK cells either at the time of overlay (0 hour) or at subsequent 12 hour intervals up to 48 hours were labeled from the basal compartment with 1 µCi/ml $[3\text{H}]$thymidine in DME containing FBS, Hepes, glutamine and antibiotic-antimycotic. Twelve hours later, $[3\text{H}]$thymidine incorporation was assayed. Filters were rinsed on ice three times with PBS+, 4 minutes each, without disturbing the collagen overlay. Filters were cut away from their supports with collagen intact and proteins precipitated on ice for a minimum of 2 hours in 1 ml 10% trichloroacetic acid (TCA). TCA was removed by aspiration, and samples rinsed twice with 1 ml fresh ice-cold 5% TCA. Samples were solubilized in 500 µl 0.2 M NaOH, 0.1% SDS for 1 hour at 37°C and then neutralized with an equal volume of 2 M Tris, pH 6.8. Radioactivity was measured by liquid scintillation counting from equal aliquots of samples. Counts per minute/sample were calculated by subtracting background counts (filter, with or without collagen). Significance ($P < 0.05$) was determined by the one-way analysis of variance and Student’s $t$-test.

**RESULTS**

HD MDCK cells overlaid with type I collagen gel form tubulocytes

HD MDCK cells were cultured to confluency on Transwell filters and then metabolically labeled to steady-state with $[35\text{S}]$methionine $[35\text{S}]$cysteine. Apical and/or basolateral compartments were differentially cell surface biontylated and cell lysates immunoprecipitated with antibody recognizing the apical membrane glycoprotein, gp135 (mouse monoclonal antibody 3F21D8; Ojakian and Schwimmer, 1988) or the basolateral membrane marker, E-cadherin (mouse monoclonal antibody r11; Gumbiner and Simons, 1986). Autoradiograms of SDS-PAGE gels reveal that HD MDCK cells polarize apical and basolateral membrane markers to their respective plasma membrane domains. GP135 localizes exclusively to apical plasma membranes (lane 1, Fig. 1) and is not expressed baso-
laterally (lane 2, Fig. 1), whereas E-cadherin is found only on basolateral cell surfaces (lane 4, Fig. 1) and is not detected apically (lane 3, Fig. 1).

To induce formation of tubulocysts, polarized monolayers of HD MDCK cells on Transwell filters were overlaid from the apical surface with a solution of type I collagen that polymerizes into a hydrated gel with incubation. At the time of overlay (0 hours, Fig. 2A), HD MDCK cells are cuboidal shaped and morphologically polarized along an apical to basal axis (also Figs 4A, 5A). Monolayer morphology is uniform and organized at this time. Collagen fibrils within the polymerized gel contact apical epithelial surfaces (Fig. 2B). Twelve hours later (Fig. 2B), monolayer morphology is relatively unchanged; in a few areas, however, HD MDCK cells begin to layer (data not shown). Cell layering is more extensive at 24 hours (Fig. 2C), frequently becoming 2-4 cells thick. Monolayer morphology is disrupted in these areas as the epithelium reorganizes. Changes in cell shape are noted, ranging from cuboidal to squamous. As a second cell layer forms that adheres to the overlaid collagen (Fig. 2C), cell-cell contacts are retained within the original monolayer, the forming bilayer and regions undergoing epithelial rearrangement. The forming bilayer subsequently attaches to the collagen gel and populates this surface (Fig. 2C,D,E). Lumena then develop between cells attached to the filter and those attached to collagen. Morphogenesis of HD MDCK cells into a bilayer of cells separated by a lumen, i.e. tubulocyst, is complete at 48 hours (Fig. 2D,E) and extends across the filter. HD MDCK cells populating the gel surface and forming tubules reacquire cuboidal cell shape (Fig. 2D).

Cell proliferation accompanies tubulocyst formation (Fig. 3). Measurement of $[^3]$H]thymidine incorporation in HD MDCK cells at 12 hour intervals beginning at the time of overlay and extending up to 48 hours reveals no significant change in $[^3]$H]thymidine incorporation within the first 12 hours of culture in the absence or presence of collagen. Proliferation increases at subsequent time points in collagen overlaid cultures. The most significant increase ($P \leq 0.05$) is measured 24-36 and 36-48 hours after overlay, when MDCK cells populate the gel surface and form lumens. Mitomycin C (1-2 $\mu$g/ml), an inhibitor of cell proliferation, prevents formation of a complete bilayer; lumena are not well developed and tubulocyst formation is incomplete (data not shown). This suggests that cell division is not required for rearrangement of the

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**Fig. 2.** Light micrographs of transverse sections (0.5 $\mu$m) of HD MDCK cells overlaid from the apical surface with rat tail tendon type I collagen gel. Sections were stained with 1% toluidine blue. At 0 hours (A), confluent HD MDCK cells grown on Transwell filters are overlaid from the apical surface with type I collagen gel (see Materials and Methods). At the time of overlay, HD MDCK cells are cuboidal shaped and apically-basally polarized (also, Fig. 1). Twelve hours (B) later, monolayer morphology remains relatively unchanged. Collagen fibrils within the gel contact apical epithelial surfaces. At 24 hours (C), cell layering is extensive and monolayer morphology is altered in these areas. Note the heterogeneous cell shapes found throughout the re-organizing epithelium. Cells that appear between the monolayer and collagen gel are beginning to attach to, and layer against, the collagen; free surfaces are developing between the cell layers. At 48 hours (D,E), a bilayer of cells separated by a lumen extends across the filter; lumena are well developed in some areas. Bar, 10 $\mu$m.
monolayer into a bilayer but that proliferation of cells in the incipient bilayer is required for complete formation of tubulocytes. 

Transepithelial electrical resistance does not change during tubulocyst formation (Table 1). Transepithelial electrical resistance was measured at 12 hour intervals beginning at the time of overlay (0 hour) and ending 48 hours later. No loss of electrical resistance was detected during tubulocyst morphogenesis when values were compared to non-collagen overlaid controls.

Apical and basolateral membrane proteins redistribute in tubulocyst formation

From the dramatic reorganization of the MDCK cell epithelium during tubulocyst formation, it seemed likely that apical and basolateral plasma membrane proteins would also undergo parallel rearrangements. To examine this, an immunoperoxidase technique was used to follow the localization of gp135, an apical membrane glycoprotein (Ojakian and Schwimmer, 1988), and p58, a basolateral membrane marker (Balcaraova-Ständer et al., 1984). Although we detected E-cadherin on basolateral cell surfaces both biochemically (Fig. 1) and by immunofluorescence microscopy (data not shown), it was difficult to consistently detect this antigen with peroxidase immunohistochemistry using either monoclonal or polyclonal antibodies. GP135 and p58 were thus localized in collagen overlaid cultures fixed at 12 hour intervals starting at the time of overlay.

Light microscopy of PAP immunostained sections reveals that gp135 localizes exclusively to apical plasma membranes of confluent HD MDCK cells at the time of overlay (Fig. 4A), confirming our biochemical results (Fig. 1, lane 1). However, during tubulocyst formation, gp135 expression and localization changes. During early stages of cell layering, gp135 is lost from plasma membranes. It is frequently absent from regions of cell-collagen interaction (arrows, Fig. 4B,C) as attachments develop to the overlaid collagen gel. In a few areas (open arrow, Fig. 4B), however, gp135 persists at the site of cell-collagen contacts. When lumena develop (*, Fig. 4C,D) gp135 immediately localizes to apical plasma membranes of the new cell layer attached to collagen and to apical cell surfaces of MDCK cells attached to the filter. We did not detect internal vacuolar staining in tubular morphogenesis at any time point we examined.

Distribution of p58, a basolateral membrane marker, was examined next. Initially, p58 localizes only to basalateral and not to apical cell surfaces of polarized HD MDCK cells (Fig. 5A), confirming our biochemical results with the basolateral marker, E-cadherin (Fig. 1, lane 4). During cell layering, when expression of the apical marker gp135 is reduced at points of cell-collagen interaction, p58 is expressed over the entire plasma membrane at regions of cell-cell, cell-substratum, and cell-collagen interactions (Fig. 5B,C). As the epithelium populates the collagen gel surface and lumena develop between the cell layers, p58 reacquires a basolateral distribution and is lost from forming apical (i.e. free) surfaces (Fig. 5C,D).

Interestingly, we found that localization of apical and basolateral membrane markers do not change during mitosis. Whatever their distribution is prior to cell division, the same distribution is maintained during mitosis, confirming previously published results (Reinsch and Karsenti, 1994). When gp135 expression is downregulated during cell layering, it remains absent from plasma membranes during cell division (data not shown). When gp135 localizes to apical plasma membranes with the formation of free luminal surfaces, it retains this apical location during subsequent mitoses that are responsible for populating the gel surface (data not shown). The basolateral protein, p58, which is randomly distributed on plasma membranes during cell layering, remains randomized during cell division. When basolaterally segregated in the presence of a free surface, it persists on basolateral plasma membranes as cells within the bilayer divide (Fig. 5D).

Overall, these observations indicate that collagen contact with the apical surface of polarized HD MDCK cells leads to tubulocyst formation within 48 hours. This reorganization is accomplished without significant changes in transepithelial electrical resistance but with major changes in the distribution and expression of apical and basolateral membrane proteins.

The β1 integrin family on apical surfaces of HD MDCK cells mediates tubulocyst formation

Because β1 integrins are the major receptor family for collagen (Hynes, 1992; Hemler et al., 1995), we next determined whether they initiate tubulocyst formation by polarized HD MDCK cells overlaid with collagen gel. The function-blocking anti-β1 integrin antibody, AIB2, which inhibits
Fig. 4. Transverse sections (0.5 μm) of HD MDCK cells cultured on Transwell filters and overlaid from the apical surface with type I collagen gel (coll). Sections were photographed after PAP immunolocalization of gp135 with monoclonal antibody 3F21D8. Sections were stained with 0.1% toluidine blue. At 0 hour (A), confluent monolayers of HD MDCK cells grown on permeable supports are overlaid from the apical surface with type I collagen gel. Only the apical surface exhibits strong staining of gp135 at this time. After 12 hours of overlay (B) when the epithelium begins to layer, surface staining of gp135 decreases at regions of cell-collagen interaction. In some areas, gp135 is lost (filled arrow) whereas in others it persists (open arrow). Some regions of the monolayer continue to localize gp135 to apical plasma membranes. As the forming bilayer attaches to collagen (B,C), gp135 remains absent in regions of cell-collagen interactions (arrows). With the formation of free luminal surfaces (+ C,D), gp135 localizes to apical plasma membranes of HD MDCK cells attached to the filter or collagen gel. (C,D) Represent 36 hours after overlay. Bar, 10 μm.

Fig. 5. Transverse sections (0.5 μm) of HD MDCK cells cultured on Transwell filters and overlaid from the apical surface with type I collagen gel (coll). Sections were photographed after PAP immunolocalization of p58, a basolateral membrane marker. Sections were stained with 0.1% toluidine blue. A control section (A) shows that p58 localizes to basolateral cell surfaces of polarized HD MDCK cells. As the epithelium layers in response to collagen overlay and attaches to it (B,C), p58 randomizes to all cell surfaces. It localizes to surfaces in contact with collagen, as well to regions of cell-cell and/or cell-filter interactions. In the presence of a free surface (i.e. lumen, C,D), p58 localizes basolaterally. Note the basolateral location of p58 in the mitotic cell (D) of the bilayer populating the gel surface. (B) Represents 12 hours after overlay; (C,D) 36 hours after overlay. Bar, 10 μm.
MDCK cell attachment to collagens I and IV and laminin (Schoenenberger et al., 1994), was mixed with collagen prior to overlay of apical cell surfaces. We found that anti-β1 integrin antibody significantly inhibits layering and lumen formation. Polarized HD MDCK cells do not respond to apical collagen by reorganizing into tubulocysts. In fact, monolayer morphology does not change (Fig. 6A). We frequently observed during processing for light microscopy that collagen gels are not attached to apical plasma membranes. In contrast, the control antibody, AD7, which recognizes a Golgi-associated protein (Narula et al., 1992) has no effect on tubulocyst formation (Fig. 6B).

Previous results indicated that in polarized MDCK cells β1 integrins localize only to basolateral and not to apical cell surfaces (Schoenenberger et al., 1994) and that they are targeted directly to basolateral plasma membranes (Boll et al., 1991; G. M. Zinkl and K. S. Matlin, unpublished observation). Thus, the observation that an anti-β1 integrin antibody added to collagen at the time of overlay inhibits tubulocyst formation raised the question of how β1 integrins that are normally basolateral can exert an effect at apical plasma membranes. We hypothesized that apical application of type I collagen gel mobilizes β1 integrins to apical plasma membranes to mediate the formation of tubulocysts. In response to collagen overlay, β1 integrins would be redistributed from basolateral to apical cell surfaces and/or newly synthesized integrins would be delivered to apical plasma membranes. Even though we did not detect the β1 family on apical surfaces of polarized MDCK cells (Schoenenberger et al., 1994), we also considered the possibility that low levels are endogenously expressed on apical cell surfaces that were previously undetected.

The redistribution of β1 integrins from basolateral to apical plasma membranes in response to collagen overlay was first examined. For these experiments, HD MDCK cells were metabolically labeled to steady-state with [35S]methionine [35S]cysteine prior to collagen overlay. The apical compartment was then washed, overlaid with collagen and incubated further for up to 12 hours prior to differential surface biotinylation of apical and/or basolateral compartments and immunoprecipitation of β1 integrins. Under these conditions, any integrin detected on cell surfaces would be synthesized prior to collagen overlay and any redistribution from basolateral to apical plasma membranes would be due to apical cell-collagen interactions. The cultures were processed up to the 12 hour timepoint when layering and lumen formation are the sparse (see Fig. 2B).

We found that in the absence of collagen overlay, polyclonal anti-β1 integrin antibody (Giancotti and Ruoslahti, 1990) immunoprecipitates α2β1 and α3β1 integrins from basolateral plasma membranes at the 0, 6 and 12 hour timepoints under non-denaturing conditions (lanes 2, 6, 10, Fig. 7). Surprisingly, the same matrix receptors are also detected apically (lanes 1, 5, 9, 11). Identification of integrin subunits was tentatively identified by SDS-PAGE and based on mobility differences observed by SDS-PAGE after immunoprecipitation with subunit specific antibodies (Schoenenberger et al., 1994). The band indicated by the arrowhead identifies the α1 subunit.
11, Fig. 7) when levels are compared to non-overlaid apical controls (lanes 5, 9, Fig. 7). Levels of β1 integrins on basolateral cell surfaces are the same with (lanes 4, 8, 12, Fig. 7) or without (lanes 2, 6, 10, Fig. 7) collagen overlay. Thus, it appears that a small pool of the β1 integrin family is endogenously expressed on apical plasma membranes of HD MDCK cells and that the steady-state levels of β1 integrins on apical surfaces do not increase within 12 hours of collagen overlay. Because no major differences in levels of β1 integrins are detected on apical and basolateral plasma membranes in response to collagen, the results further indicate that β1 integrins do not redistribute from basolateral to apical cell surfaces after collagen overlay.

These observations did not rule out the possibility that apical application of collagen would induce increased targeting of newly synthesized integrins to apical cell surfaces. To test this, we pulse labeled HD MDCK cells 12 hours after collagen overlay and differentially surface biotinylated and immunoprecipitated β1 integrins under non-denaturing conditions after chase incubations. At 0 minutes chase, integrin is not detected on HD MDCK cell surfaces regardless of collagen overlay (lanes 2, 3, Fig. 8A). Only the precursor to β1 integrin is synthesized at this time (lanes 1-3, Fig. 8B). After 90 minutes chase, all members of the β1 family, including α2β1, α3β1 and the β1 precursor are made (lanes 4-9, Fig. 8B). As previously reported (Schoenenberger et al., 1994), it was difficult to resolve the ααβ1. The α2β1 and α3β1 are detected on basal plasma membranes in the absence or presence of collagen (lanes 7, 9, respectively; Fig. 8A) after 90 minutes chase. In agreement with our conclusion from steady-state labeling experiments, we found that in the absence of collagen overlay (lane 6, Fig. 8A), the β1 family, primarily α2β1, is detected on apical cell surfaces in amounts equivalent to that seen after steady-state labeling. Moreover, collagen overlay does not stimulate delivery of additional newly synthesized integrin to apical plasma membranes (lane 8, Fig. 8A).

The results indicate that confluent HD MDCK cells grown in transfilter culture and polarized with respect to apical and basolateral membrane markers endogenously express low levels of β1 integrins on apical plasma membranes and that these mediate tubulocyst formation in response to collagen overlay. β1 integrins are not mobilized to apical plasma membranes either by redistribution from basolateral to apical cell surfaces or by new synthesis and delivery to apical plasma membranes.

The C6 MDCK cell line does not form tubulocysts

We previously reported that β1 integrins are not detected on apical cell surfaces in another clonal MDCK cell line known as C6 (Schoenenberger et al., 1994). C6 MDCK cells contain the empty retroviral vector pMV7 and are identical to HD MDCK cells without additional receptor mobilization to the apical domain. To complement these observations, we used the C6 MDCK cell line which does not express apical β1 to determine whether the absence of this receptor family, cell-collagen interactions membranes of HD MDCK (lane 3, Fig. 9). The profile of β1 integrins expressed on basolateral plasma membranes are identical for both cell lines (lanes 2 and 4, Fig. 9).

The β1 integrins found on apical plasma membranes of HD MDCK cells are sufficient to mediate tubulocyst formation without additional receptor mobilization to the apical domain. Fig. 8. Autoradiograms of SDS-PAGE (6%, reduced) gels analyzing pulse-chase labeling of β1 integrins in HD MDCK cells twelve hours after collagen overlay. Cells were pulsed for 10 minutes and chased for 90 minutes. HD MDCK cells were then differentially surface biotinylated from either the apical (a), basolateral (b) or both (ab) cell surfaces and immunoprecipitated with polyclonal anti-β1 antibody under non-denaturing conditions. (A) Surface expression of β1 integrins. At 0 minutes chase, integrins are not detected on MDCK cell surfaces with (lane 3) or without (lane 2) collagen overlay. After 90 minutes chase, the β1 integrin family, including the α2β1 and α3β1, are detected on apical cell surfaces in amounts equivalent to that seen after steady-state labeling. (B) Immunoprecipitable antigen of the respective samples shown in (A). Aliquots (5 μl) were taken after immunoprecipitation with β1 integrin and Protein A Trisacryl but prior to precipitation of biotinylated integrins with streptavidin agarose. The band indicated by the arrowhead has been tentatively identified as the α3 integrin subunit. The α3 integrin subunit is difficult to resolve from the precursor to β1 (pβ1).
depolarize $\beta_1$ integrins to apical plasma membranes. Twelve hours after overlay, C6 cells were pulse-chased, differentially surface biotinylated and immunoprecipitated with polyclonal anti-$\beta_1$ integrin antibody. Confirming our steady-state labeling data (Fig. 9, lanes 1, 2), we found that the $\beta_1$ integrin family is expressed only on basolateral cell surfaces after a 90 minute chase (lane 8, Fig. 10A) and is not detected at appreciable levels on apical plasma membranes (lane 7, Fig. 10A). Low levels are detected with prolonged exposure of autoradiograms; however, the signal is significantly reduced when compared to the apical signal on HD MDCK cells (lanes 2 and 4, respectively). The profile of $\beta_1$ expression on basolateral surfaces are identical between the two cell types. The band indicated by the arrowhead has been tentatively identified as the $\alpha_1$ subunit.

Light microscopy of C6 cells overlaid with type I collagen gel further indicates that in the absence of apical $\beta_1$ integrins, this cell line does not form tubulocysts. Forty-eight hours after overlay (Fig. 11B), tubulocysts do not develop across the entire extent of the filter as they do in overlaid HD cells (compare to Fig. 2D and E); cell layering is negligible and lumena are absent. During processing for light microscopy, we observed that collagen gels were not attached to apical plasma membranes of C6 cells.

These data indicate that collagen overlay does not stimulate new synthesis or delivery of $\beta_1$ integrins to apical plasma membranes, confirming our observation using the HD MDCK cell line. Complementing the data which establishes a role for apical $\beta_1$ integrins in tubulocyst formation, experiments with C6 cells suggest that in the absence of apical $\beta_1$ tubulocysts do not develop in response to collagen.

**DISCUSSION**

We demonstrate that overlay of HD MDCK cells with type I collagen gel initiates changes in organization and cell polarity leading to the formation of tubulocysts. In particular, we show that $\beta_1$ integrins pre-existing on apical plasma membranes are necessary for tubulocyst formation. In the polarized HD
MDCK cell line, β1 integrins are partially depolarized to apical and basolateral cell surfaces even though other endogenous apical and basolateral membrane markers are segregated to their respective plasma membrane domains. If β1 integrins are not apically expressed, as in C6 MDCK cells, tubulocysts do not develop. Moreover, collagen overlay does not mobilize additional β1 integrins to apical plasma membranes either by redistribution from basolateral to apical cell surfaces or by new synthesis and delivery to apical plasma membranes. β1 integrins pre-existing at low levels on apical plasma membranes of HD MDCK cells actively bind type I collagen gel. Presumably, a cascade of intracellular signaling events is activated leading to the formation of tubulocysts.

**Tubulocyst formation by HD MDCK**

When polarized monolayers of MDCK cells in transfilter culture are overlaid with type I collagen gel, only apical epithelial surfaces contact collagen. Subsequent cell-collagen interactions initiate epithelial rearrangement resulting in tubulocyst formation, confirming previously published results (Hall et al., 1982; Schwimmer and Ojakian, 1995). In the former study, tubulocyst formation was observed only when subconfluent MDCK cells were overlaid, but not when confluent cultures were used. Based on our results, we suspect that this may have been due to the lack of apically expressed integrins once the cells polarized. In the work of Schwimmer and Ojakian (1995), subconfluent MDCK cells, which expose basolateral matrix receptors to the apical cell surface, were primarily used. In some experiments, confluent MDCK monolayers were employed but the issue of overall basolateral polarity was not rigorously addressed (Schwimmer and Ojakian, 1995).

For our studies we cultured MDCK cells to confluency on permeable supports and then overlaid polarized monolayers with a solution of type I collagen which polymerizes into a hydrated gel with incubation. Growth of cells on permeable supports allows studies on various aspects of epithelial cell polarization, including localization of apical and basolateral membrane markers, measurement of transepithelial electrical resistance and selective biochemical analyses of apical and/or basolateral plasma membranes. The growth of MDCK cells on permeable supports thus provided a technical advantage for studies addressing the mechanism(s) of tubulocyst formation in response to apical overlay with ECM. Moreover, polarization of apical and basolateral membrane markers and transepithelial electrical resistance could be monitored during the process.

The first step in tubulogenesis in response to apical cell-collagen interactions is cell layering. In the absence of cell proliferation, epithelial cells within the monolayer ‘migrate’ or ‘slide’ over one another, resulting in cell layering. As cells within the stratified epithelium adhere to the overlaid collagen, the epithelium undergoes cell division and populates the surface of the collagen gel. This interpretation is supported by our observation of cell division. We frequently observed mitotic images in the cell layer attached to collagen. Additionally, [3H]thymidine incorporation was greatest at the time coinciding with tubule formation. Lumena then develop between regions of epithelia undergoing stratification and are lined by cells from the original monolayer and the newly formed cell layer attached to collagen.

Cell-cell contacts are not lost during tubulogenesis. Although a recently published report demonstrates a loss of transepithelial electrical resistance within 2 hours after collagen overlay of thyroid cell monolayers (Garbi et al., 1996), we in this study did not measure any significant change in transepithelial resistance at the later time points we examined. The functional integrity of the tight junction thus remained intact. Cell-cell contacts were not disrupted in regions undergoing cell layering, in the original monolayer or in the forming bilayer. Maintenance of cell-cell contacts has also been reported during epithelial polarity reversal upon transfer of MDCK cysts to collagen gel (Wang et al., 1990). Because the tight junction functions as a fence separating apical from basolateral plasma membrane domains (van Meer and Simons, 1986), the observation that tight junction integrity persists in tubulogenesis argues against the possibility that changes in polarization of apical and basolateral membrane proteins are a consequence of loss of cell-cell contact and diffusion of plasma membrane proteins between different domains.

While cell-cell contacts remain continuously intact, the epithelium reorganizes, having significant effects on the polarity and expression of plasma membrane proteins. During cell layering, gp135, an apical membrane glycoprotein (Ojakian and Schwimmer, 1988), disappears from plasma membranes. It is absent from regions of cell-collagen interactions, from points of cell-cell contact in the stratified epithelium, and from cell-substratum (i.e. permeable support) contacts. This observation agrees with previously published reports that find gp135 absent from cell surfaces of MDCK cysts during polarity reversal in collagen gels (Wang et al., 1990) and in MDCK cells incubated with collagen overlays (Schwimmer and Ojakian, 1995). Unlike these studies, however, we did not detect any cytoplasmic vesicular staining of gp135 at any time point. This finding is puzzling considering that internalization of gp135 was readily observed by fluorescence microscopy in MDCK cysts twelve hours after suspension in collagen gels (Wang et al., 1990), a time point that we also examined.
In contrast to gp135, the basolateral membrane protein, p58, randomizes during cell layering. In the absence of a free surface, p58 distributes over the entire plasma membrane at points of cell-cell, cell-collagen and cell-substratum contacts. Randomization of other basolateral membrane proteins, including the Na\(^+\)K\(^+\)ATPase and E-cadherin, has been observed during epithelial polarity reversal of MDCK cysts cultured in collagen gel (Wang et al., 1990).

Thus, it appears that apical cell-collagen interaction initiates a reorientation of the apicobasal axis that leads to changes in polarization. The cells do not reverse polarity as do suspension cysts of MDCK cells cultured in collagen gel (Wang et al., 1990; Ojakian and Schwimmer, 1994); rather, polarization is altered as the epithelium undergoes morphogenesis. In the presence of both cell-cell and cell-substratum contacts, regardless of whether the substrate is the filter or overlaid collagen, the apical membrane marker gp135 decreases in surface expression during cell layering whereas the basolateral membrane marker p58 randomizes on plasma membranes. In order for apical and basolateral markers to re-polarize to specific plasma membrane domains, cell-cell and cell-substratum interactions alone are not sufficient; gp135 and p58 localize to apical and basolateral cell surfaces, respectively, only with the creation of free luminal surfaces coinciding with the immediate onset of tubule formation. Thus the key in re-polarization of apical and basolateral membrane glycoproteins in our cell culture system is the ‘asymmetry’ of contacts which re-establishes the apicobasal axis. Because we did not observe an intracellular pool, the apical appearance of gp135 is presumably due to synthesis of new protein delivered to apical plasma membranes. Whether p58 is internalized from incipient luminal surfaces and is then delivered to basolateral plasma membranes or whether it is newly synthesized and targeted directly basolaterally is a subject for further investigation.

**Role of apical integrins in tubulocyst formation**

The observation that epithelia from various tissues including cell lines form tubulocytes when overlaid with type I collagen gel raised the question of how collagen at apical plasma membranes of confluent epithelia (Chambard et al., 1981; Kramer, 1985; Jackson and Jenkins, 1991; Schwimmer and Ojakian, 1995) elicits this morphogenetic response. We hypothesized that apical-ECM interactions would mobilize \(\beta 1\) integrins to apical plasma membranes. Using radiolabeled HD MDCK cells that are confluent and polarized with respect to apical and basolateral membrane markers, we demonstrated biochemically that \(\beta 1\) integrins are predominantly expressed on the basolateral domain, confirming our previously published results (Schoenenberger et al., 1994). In this particular strain of MDCK cells, however, a small amount of \(\beta 1\) integrins were also detected on the apical domain.

Thus, we provide the first biochemical evidence that \(\beta 1\) integrins do indeed exist on apical plasma membranes of otherwise polarized MDCK cells. While previous studies have detected apical integrins on MDCK cells by immunocytochemical techniques (Ojakian and Schwimmer, 1994), in the absence of biochemical data the authors of this work were unable to conclusively determine if just \(\alpha\) subunits or \(\alpha\beta 1\) heterodimers were expressed apically. As these authors state, the former possibility would be unlikely given the biochemical evidence that only integrin heterodimers and not monomers are transported to the plasma membrane (Heino et al., 1989; Rosa and McEver, 1989). Indeed, their observation that polarity reversal of MDCK cell cysts was inhibited by an anti-\(\beta 1\) antibody strongly suggests that functional \(\alpha\beta 1\) integrins were present apically (Ojakian and Schwimmer, 1994). Apical \(\beta 1\) integrins have also been detected by immunocytochemistry on a subpopulation of confluent thyroid epithelia where they are believed to initiate reorganization of the cell layer in response to collagen overlay (Garbi et al., 1996).

We also show that \(\beta 1\) integrins expressed apically are necessary for tubulocyst formation. Two pieces of evidence support this conclusion. First, antibody perturbation experiments indicate that inhibition of receptor-ligand binding at apical plasma membranes prevents the formation of tubulocyst. In particular, the function-blocking anti-\(\beta 1\) integrin antibody, AIIB2, which we have shown inhibits MDCK cell adhesion to type I and IV collagen and laminin (Schoenenberger et al., 1994) prevents not only tubulocyst formation but physical attachment of collagen gels to apical plasma membranes. Second, experiments with C6 MDCK cells, demonstrate that this cell line expresses little or no \(\beta 1\) integrins on apical plasma membranes either before or after collagen overlay (Schoenenberger et al., 1994, and this study), and are unable to form tubulocytes. While it can be argued that the absence of apical \(\beta 1\) integrins is not the only trait that has been altered in the C6 MDCK cell clone, these results and the antibody perturbation experiments with the HD MDCK cells strongly suggest that without apical \(\beta 1\) integrins, tubulocytes do not form.

Contrary to our original hypothesis, our biochemical data clearly indicate that collagen overlay of the apical surface does not mobilize the appearance of \(\beta 1\) integrins to the apical plasma membrane domain. Through both pulse-chase experiments and steady-state labeling we showed that the level of apical integrins in HD MDCK cells was not increased through new integrin synthesis or redistribution of pre-existing integrins upon collagen overlay. In the C6 clone, collagen overlay did not lead to the expression of any significant amounts of \(\beta 1\) integrins on the apical surface. Thus, it seems unlikely that small amounts of integrins or other collagen receptors that exist on apical surfaces of polarized epithelia act as ‘sensors’ of ectopic ECM proteins and signal the cell to move additional integrins to the apical domain.

As this work was in progress, a study by Schwimmer and Ojakian (1995) appeared which also examined the role of specific integrins in tubulocyst formation. While we agree in general terms with their conclusions, we were unable to confirm or deny their finding that \(\alpha 2\beta 1\) in particular was responsible for tubulocyst formation because the antibody cited in their study gave inconsistent results in our hands. More importantly, we believe that our work extends their experiments by providing biochemical evidence for apical integrins, by demonstrating that these integrins are pre-existing and are not newly synthesized or redistributed from other nonapical pools upon collagen overlay, and by showing that the lack of apical integrins and the inability to form tubulocytes are correlated in the C6 MDCK cell clone.

The \(\beta 1\) integrins that pre-exist on apical plasma membranes actively bind fibrils within the polymerized type I collagen gel. We observed that within 12 hours of overlay, collagen gels are attached to apical plasma membranes of HD cells. This is an
interesting observation considering that apical plasma membranes of polarized epithelia are not specialized to form cell contacts. β1 integrins are typically found on basolateral cell surfaces and an apical location is not considered to be their normal context. Binding of receptor to its ligand at apical surfaces presumably induces assembly of the signal transduction machinery leading to the formation of tubulocysts. In a process known as outside-in signaling, receptor-ligand binding causes conformational changes in integrin cytoplasmic domains that in turn activate a cascade of cytoplasmic and nuclear signals resulting in changes in gene expression, cellular differentiation and behavior (for review, see Hynes, 1992; Clark and Brugge, 1995; Hemler et al., 1995; Yamada and Miyamoto, 1995). Although the nature of the transduction mechanism in integrin signaling is not well understood, it is likely to include activation of kinases, small molecular mass GTPases, phospholipid mediators and/or changes in cytoskeletal linkages. Preliminary observations suggest that phosphorylation of integrins or focal adhesion kinase is not involved (A. Zuk and K. S. Matlin, unpublished observation). In addition, preliminary studies using a number of reagents that affect intracellular signaling, including activators and inhibitors of protein kinase C, protein kinase A, and heterotrimeric G proteins, suggest that the signal transduction mechanism(s) mediating tubulocyst morphogenesis is complex, perhaps involving multiple pathways (A. Zuk and K. S. Matlin, unpublished observation).

**Possible roles of apical integrins in vivo**

Our findings raise the issue of the functional significance of depolarized β1 integrins on apical plasma membranes of polarized epithelia. In renal ischemia, redistribution of integrin receptors from basolateral to apical surfaces of kidney tubular epithelial cells has been suggested to contribute to acute renal failure (Goligorsky et al., 1993). Damaged cells exfoliated into the tubule lumen after injury are hypothesized to lose integrin polarity and attach to apically localized integrins on epithelia remaining attached to basement membranes, resulting in tubular obstruction (Goligorsky et al., 1993). Indeed in in vitro models of ischemic insult the α3 integrin subunit redistributes from a basal to apical location (Gailit et al., 1993). RGD containing peptides alleviate tubular obstruction indicating a possible role for RGD sensitive integrins in cell-cell attachments that may contribute to obstruction of the tubular lumen (Goligorsky and DiBona, 1993; Noiri et al., 1994). Although it has been reported that a basolateral membrane marker, the Na\(^+\)K\(^+\)ATPase, depolarizes after ischemia (Molitoris et al., 1988, 1992), it is unclear whether those same cells also depolarize integrins from basolateral to apical plasma membranes or whether apical proteins also redistribute. These questions are currently being pursued in our laboratory using an in vivo model of ischemic insult.

Apically directed integrins in polarized simple epithelia may also play a role in the pathogenesis of infection. The bacterial protein invasin is a component of the cell wall of *Yersinia* that directly binds plasma membranes of mammalian cells prior to entry (Iserberg and Leong, 1990). Invasin binds to members of the β1 family, including the α2β1 which our study shows is expressed at low levels on apical plasma membranes. In addition, the α2β1 which is detected on the apical domain of polarized HD cells, has recently been shown to function as a virus receptor, mediating surface attachment and infection by echovirus 1 (Bergelson et al., 1993), a human pathogen responsible for febrile illnesses and aseptic meningitis. It is unclear how bacteria and viruses in luminal compartments gain entry into the cellular interior of simple polarized epithelia such as those that line the gastrointestinal, kidney and respiratory systems. Although apically localized integrins are not normally detected in in situ tissue sections (Ruoslalti, 1991), our observation that a polarized epithelial cell line that segregates apical and basolateral membrane proteins to specific plasma membrane domains yet expresses partially depolarized integrin on apical plasma membranes raises the possibility that integrin receptors may also be expressed at low levels on apical surfaces of simple polarized epithelia in vivo. These in turn may mediate bacterial and viral entry and subsequent infection.

Apical integrins may also play an important role in epithelial oncogenesis. If integrins are depolarized at an early stage of carcinogenesis, then our results indicate that exposure of apical surfaces in the incipient neoplasm to ECM proteins would then initiate cell layering, ultimately resulting in the formation of a tumor. Indeed changes in integrin distribution and expression have been reported in carcinomas in situ and in malignant cells (Pignatelli et al., 1990, 1991; Koretz et al., 1991; Koukoulis et al., 1991; Weinel et al., 1992). Our previous results in fact suggest that oncogenic transformation and loss of integrin polarity may go hand-in-hand. When polarized MDCK cells are transformed by expression of the Kirsten ras oncogene, apical antigens and basolateral β1 integrins lose polarity as the monolayer reorganizes into a multilayer (Schoenenberger et al., 1991, 1994). Other basolateral antigens do not redistribute from areas of cell-cell contact and tight junctions remain functional and located at the border between the free surface and the lateral plasma membranes (Schoenenberger et al., 1991). How disruption of the ras signaling pathway leading to oncogenic transformation and the alteration in cell polarity and integrin expression are interrelated remain unanswered but important questions for the future.

In conclusion, we have in this report addressed the mechanism by which apical application of collagen on polarized epithelia results in the formation of tubulocysts. We show that the asymmetry of contact which develops with the creation of free luminal surfaces during tubulocyst formation re-establishes the apicobasal axis leading to re-polarization of apical and basolateral membrane markers. We emphasize the point that in one clone of MDCK cells apical and basolateral membrane markers are polarized to their respective plasma membrane domains yet β1 integrins which are considered to be basolateral matrix receptors are expressed on apical cell surfaces where they mediate the formation of tubulocysts. If apical β1 integrins are absent, tubulocytes do not form. Moreover, additional β1 integrins are not mobilized to apical plasma membranes in response to collagen. The β1 integrins expressed apically actively bind ECM despite an apical location not being their normal place of function. We suggest that previous studies documenting the ability of epithelia from various tissue sources to form tubulocysts in response to type I collagen overlay is likely due to β1 integrins pre-existing on apical plasma membranes.

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