INTRODUCTION

The extracellular matrix (ECM) has been demonstrated to be an important factor in the regulation of developmental processes for a variety of tissues and organs, including the differentiation and organization of epithelia (Hay, 1983; Lin and Bissell, 1993). Collagen and laminin are ECM components that are found expressed in early kidney development (Ekblom, 1989) and changes in their expression patterns coincide with nephron maturation (Laurie et al., 1989; Ekblom et al., 1990), providing good evidence that ECM gene regulation is very important in the biogenesis of the renal nephron tubular epithelium (Saxen, 1987; Ekblom, 1989).

Epithelial cells attach to the ECM in order to establish cell polarity, with the epithelial basolateral membrane that adheres to the ECM being biochemically distinct from the apical membrane which borders the epithelial lumen (Nelson, 1992; Rodriguez-Boulan and Powell, 1992). Epithelial cell-substratum attachment is mediated by integrins, a family of ECM receptors which bind to a variety of ECM components including collagen, fibronectin and laminin (Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991; Hynes, 1992). Integrins are heterodimers consisting of α and β subunits with the ligand specificity residing within the α subunit (Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991; Hynes, 1992). There is now considerable evidence that these transmembrane receptors play an important role in the regulation of gene expression and signal transduction (Damsky and Werb, 1992; Juliano and Haskill, 1993; Schaller and Parsons, 1994).

SUMMARY

Previous studies have demonstrated that incubation of MDCK cell epithelial cysts in collagen gel induced a reversal in cell surface polarity that was regulated by β1 integrins. Further experiments were done to identify the specific collagen binding integrin involved by applying collagen gel overlays to the apical membrane of subconfluent MDCK monolayers. Cell surface levels of the apical membrane glycoprotein gp135 were monitored by ELISA to quantitate the extent of collagen-mediated membrane remodeling. After an 8 hour incubation with collagen, there was a 35% reduction in gp135 while the cell surface levels of the α2, α3 and β1 integrin subunits were not affected. Immunofluorescence microscopy confirmed the loss of gp135 from selected regions of the apical cell surface while the α2 and β1 integrin subunits were distributed in small clusters over the entire apical membrane in both control and collagen-treated monolayers. Collagen-mediated loss of gp135 was inhibited by monoclonal antibodies which recognize either the α2 or β1 integrin subunits but not by a monoclonal antibody against the α6β1 integrin. These results demonstrated that remodeling of the apical membrane had occurred, allowing the selective retention of β1 integrins but not gp135. They were supported by the observation that collagen-mediated loss of apical membrane microvilli was inhibited by the monoclonal antibody against the α2 integrin subunit. Incubation of confluent monolayers with collagen gel induced the formation of polarized epithelial tubules within 16 hours. Epithelial tubule biogenesis was completely inhibited by monoclonal antibodies against either the α2 or β1 integrin subunits, providing strong evidence that the α2β1 integrin is essential for collagen-mediated epithelial membrane remodeling and tubule formation.

Key words: α2β1 integrin, epithelial polarity, epithelial tubule formation
establishing apical cell surface polarity and are supported by the observation that apical polarity of the mammary membrane glycoprotein PAS-O appeared to require the interaction of a vitronectin binding integrin with the ECM (Parry et al., 1990). The concept that cell-ECM interactions are important in epithelial cell polarity development is strengthened by studies done on MDCK cysts. MDCK cells cultured in suspension form polarized epithelial cysts with the apical membrane glycoprotein gp135 (Ojakian and Schriemer, 1988) and microvilli on the outer membrane and the basolateral membrane with (Na+K+)ATPase lining the cyst lumen (Wang et al., 1990a). Resuspension of MDCK cysts in collagen gel induced extensive apical membrane remodeling accompanied by the loss of microvilli and endocytosis of gp135, the subsequent retargeting of gp135 to the cyst lumen and basolateral proteins to the outer membrane (Wang et al., 1990b, 1994). There results suggested that collagen-mediated epithelial remodeling and polarity reversal could be due to integrin-ECM interactions and this possibility has been studied in greater detail. Our laboratory and others have determined that MDCK cells express a variety of β1 integrins (Boll et al., 1991; Ojakian and Schriemer, 1994; Schoenerger et al., 1994) including α2β1 and α3β1, which have been shown to bind collagen in a variety of cell types (Staatz et al., 1989; Elices et al., 1989, 1991; Albeda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991). Subsequently, we demonstrated that the inclusion of function blocking monoclonal antibodies (mAbs) against the β1 integrin subunit in the collagen gel inhibited MDCK cyst epithelial membrane remodeling and cell polarity reversal (Ojakian and Schriemer, 1994). These results provide strong evidence that β1 integrins are important in the regulation of epithelial morphogenesis and the development of cell surface polarity.

In this paper, we extend these studies by identifying the specific β1 integrin involved in collagen-mediated epithelial polarity development. This was accomplished by utilizing MDCK cell monolayers rather than cysts so that quantitative measurements of several membrane proteins including β1 integrins could be done at the same time. We took advantage of previous observations demonstrating that application of a collagen gel overlay on the apical membrane of MDCK, thyroid, liver or mammary epithelial cell monolayers induced the formation of polarized tubular structures lined by microvilli (Chambard et al., 1981; Hall et al., 1982; Warren and Nelson, 1987; LeCluyse et al., 1994). Here we present evidence that mAbs against the α2β1 integrin subunits inhibit collagen-mediated MDCK membrane remodeling and tubule formation, and propose that α2β1 and collagen expression plays an important role in the modulation of renal nephron epithelial biogenesis during development.

MATERIALS AND METHODS

Cell culture

MDCK cells were grown in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (DMEM/FBS) and passaged as described previously (Herzlinger and Ojakian, 1984; Wang et al., 1990a). Data for the experiments using subconfluent cultures (Figs 2–7) were derived using MDCK clone 8 cells (Wang et al., 1990a, 1994; Wollner et al., 1992; Ojakian and Schriemer, 1994), although identical results were also obtained using MDCK strain II cells (Herz and Ojakian, 1989). Experiments using confluent monolayers were done with MDCK strain II cells (Figs 8–10). For subconfluent cultures, MDCK cells were plated at 2.5 × 10⁶ cells/cm² on type I collagen-coated (prepared from rat tail tendon; see Wang et al., 1990a) 96-well plate cultures for enzyme-linked immunoassay (ELISA), coverglasses for immunofluorescence microscopy, or micropore filters (Millipore Corporation; 0.45 μm pores) for electron microscopy, and cultured for 24 hours. For confluent cultures, cells were plated at 1 × 10⁵ cells/cm² on collagen-coated coverglasses or micropore filters and cultured for 72 hours. Epithelial membrane remodeling and tube formation was initiated by the addition of collagen gel overlays on the MDCK apical cell surface as described previously (Hall et al., 1982; Warren and Nelson, 1987; Ojakian and Schriemer, 1994). After culturing the cells for the appropriate time (4, 8, 16, 24 or 48 hours) at 37°C, collagen gels were removed by aspiration, the cells washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde/0.1% glutaraldehyde/PBS, washed with PBS containing 2% bovine serum albumin (BSA/PBS), and prepared for ELISA, immunofluorescence or electron microscopy. It was determined by both ELISA and phase-contrast microscopy that collagen gel removal did not detach cells from the substratum. For experiments using function blocking or control antibodies, mAbs 5E8 (50 μg/ml), AIIB2 or J1B5 (1:10 dilutions of hybridoma supernatants) were included in the collagen gels during gel formation. The 8 hour collagen gel incubations had no additional DMEM added while, in the 24 hour incubations, DMEM/FBS containing the appropriate mAb was added after collagen gel formation. Culture medium components were purchased from Gibco Inc. and plastic cultureware from Falcon.

Antibodies

Mouse mAbs 3F2 against gp135 and E1 against gp23 were produced by our laboratory (Ojakian et al., 1987; Ojakian and Schriemer, 1988); mouse mAb 5E8 against the α2 integrin subunit (Chen et al., 1991) was obtained from Drs Richard Bankert and Fang-An Chen (Roswell Park Cancer Institute); mouse mAb 6F1 against the α2 integrin subunit (Coller et al., 1989) from Dr Barry Coller (Mt Sinai College of Medicine); mouse mAb 12F1 against the α2 integrin subunit (Pischel et al., 1987) from Dr Virgil Woods (University of California, San Diego); mouse mAbs against the α3 (J143) and β1 (A2) integrin subunits (Kantor et al., 1987) from Drs Lloyd Old and Mary John (Memorial Sloan-Kettering Cancer Institute); and rat mAbs against the α6 (J1B5) and β1 (AIIB2) integrin subunits (Hall et al., 1990; Damsky et al., 1992) from Dr Caroline Damsky (University of California, San Francisco). All of the anti-integrin mAbs utilized in this study have been shown to recognize MDCK integrins by either immunofluorescence microscopy or SDS-PAGE (Ojakian and Schriemer, 1994). SDS-PAGE characterization of the mAbs used extensively in this study is presented in Fig 1. These data demonstrate that mAb 5E8 and 6F1 against the α2 integrin subunit and mAb J143 against the α3 integrin subunit immunoprecipitate 150 and 125 kDa polypeptides (see Ojakian and Schriemer, 1994, for biotinylation and immunoprecipitation procedures). It should be noted that mAb 12F1 has been demonstrated to be useful for immunofluorescence microscopy (Ojakian and Schriemer, 1994). However, by SDS-PAGE analysis we have not been able to consistently detect polypeptides and have concluded that this mAb is not efficient in immunoprecipitation of the MDCK α2β1 integrin.

ELISA

After collagen-mediated epithelial remodeling for 4 and 8 hours, the cells were aldehyde-fixed as described above. Cell surface levels of integrins, gp23 and gp135 were quantitated by sequential 1 hour incubations with the primary mAb (3F2, 1:200 dilution in BSA/PBS; 5E8, 20 μg/ml; AIJ2, 1:200; J1B5, 1:100; E1, 1:30), rabbit anti-mouse (5 μg/ml) or anti-rat IgG (10 μg/ml; Cappel, Durham, NC) and goat anti-rabbit IgG coupled to alkaline phosphatase (1:2000 dilution; Sigma).
Fig. 1. SDS-PAGE analysis of MDCK integrins. Biotinylated cell surface integrins were immunoprecipitated with the following antibodies: lane a, no antibody; lane b, mAb 5E8 against the α2 integrin subunit; lane c, mAb 6F1 against the α2 integrin subunit; lane d, mAb J143 against the α3 integrin subunit; lane e, polyclonal antibody (Chemicon Inc., #1920) against the α2 integrin subunit. All antibodies immunoprecipitate 150 kDa and 125 kDa polypeptides (arrowheads).

Chemical Co.). After incubation with the alkaline phosphatase substrate p-nitrophenyl phosphate (1 mg/ml) for 30 minutes, the relative levels of membrane proteins were determined spectrophotometrically on a microtiter ELISA reader at 405 nm. Since only subconfluent cells were used in this assay and immunofluorescence microscopy demonstrated that anti-integrin mAbs had access to both the apical and basolateral membranes (see Fig. 7), it is likely that membrane protein levels were quantitated for the entire cell surface.

**Immunofluorescence microscopy**

The distribution of membrane proteins on subconfluent cells incubated with collagen gel for 8 hours was done after aldehyde fixation. For confluent monolayers incubated with collagen gel overlays for 24 hours, the cells were aldehyde-fixed, then permeabilized with methanol (5 minutes at −20°C) to allow antibodies access to the entire cell surface. Membrane proteins were then localized by sequential 30 minute incubations with the primary mouse mAb (3F2, 1:2 dilution; 5E8, 50 μg/ml or A2J, 1:20), rabbit anti-mouse IgG (50 μg/ml) and goat anti-rabbit IgG coupled to rhodamine (50 μg/ml). Samples were mounted in 90% glycerol/10% PBS, viewed in a Zeiss microscope equipped with epifluorescence optics and photographed on Kodak Tri-X film. All photographs for an experiment had identical exposure and print times so that qualitative comparisons could be made.

**Immunogold electron microscopy**

Confluent monolayers incubated with collagen gel for 24 hours were fixed with aldehydes for 1 hour. The cells were then permeabilized by incubation in BSA/PBS containing 0.05% saponin (saponin/BSA/PBS) for 30 minutes at 4°C. All subsequent incubations were done in saponin/BSA/PBS to ensure that cell permeability was maintained and the antibody reagents had access to the entire cell surface. After sequential 1 hour incubations with mAb 3F2 (1:2 dilution), rabbit anti-mouse IgG (100 μg/ml) and goat anti-mouse IgG coupled to 1 nm diameter colloidal gold (1:50 dilution; Nanoprobes Inc., Stony Brook, NY), the immunogold particles were enlarged using silver (5 minutes at −24°C), the primary mAb 3F2 was not included. The cells were done in saponin/BSA/PBS to ensure that cell permeability was maintained and the antibody reagents had access to the entire cell surface. For light microscopy, 1 μm thick sections were mounted on glass slides, lightly stained with toluidine blue and photographed on Tri-X film. For electron microscopy, thin sections were stained and photographed as described above.

**Light and electron microscopy of epithelial tubule formation**

The extent of collagen-mediated epithelial remodeling was evaluated after fixation with 2.5% glutaraldehyde, embedded in Epon 812 and thin sections stained with uranyl acetate and lead citrate (Ojakian and Schwimmer, 1992) were viewed and photographed in a JEOL 100C electron microscope.

**RESULTS**

Previous work by our laboratory and others has demonstrated that incubation of MDCK cells with collagen gel overlays induced the formation of tubular structures (Chambard et al., 1981; Hall et al., 1982; Warren and Nelson, 1987; Ojakian and Schwimmer, 1994). To extend our studies on the role of β1 integrins in epithelial morphogenesis, we utilized collagen overlays applied to the apical membrane of subconfluent MDCK cells to induce membrane remodeling. After a 48 hour incubation, numerous lumens similar to those described previously (Hall et al., 1982) were observed by phase-contrast microscopy (Fig. 2A). Electron microscopy demonstrated that the lumens were lined by microvilli (Fig. 2B), indicating that this was the epithelial apical membrane. The membrane adjacent to the collagen lacked microvilli and represents the basolateral membrane (Fig. 2B).

**Analysis of gp135 and integrin cell surface levels**

To quantitate membrane remodeling, after application of collagen gel, the cell surface levels of gp135 were determined by ELISA. After incubation at 37°C, gp135 levels were significantly reduced after 4 hours (~19%) and 8 hours (~32%; n=15; P<0.001). Since the α2β1 and α3β1 integrins are present on MDCK cells (Ojakian and Schwimmer, 1994; Schoenberger et al., 1994) and are capable of binding collagen in other cell types (Albelda and Buck, 1990; Hemler, 1990; Hynes, 1992), we compared gp135 levels measured after an 8 hour collagen incubation with those obtained for the α2, α3 and β1 integrin subunits and gp23, another membrane glycoprotein (Herz and Ojakian, 1989). Despite considerable membrane remodeling, no changes in the integrin subunit or gp23 levels were observed (Fig. 3). These results provide strong evidence that the existing levels of the α2β1 and α3β1 integrins were sufficient for regulation of epithelial membrane remodeling. The observation that gp23 levels did not change are not surprising, since this glycoprotein has a non-polarized distribution on MDCK cells (Herz and Ojakian, 1989). The loss of cell surface gp135 was also observed by immunofluorescence microscopy. In control cells, gp135 was distributed across the apical membrane with the punctate staining representing microvillus staining (Fig. 4B; also see Ojakian and Schwimmer, 1988). After 8 hours with a collagen gel overlay, apical gp135 staining was only observed on selected areas of the monolayer and was absent from others (Fig. 4D). These results indicate that gp135 uptake by endocytosis does not occur synchronously over the entire apical membrane (Wang et al., 1990b). It should also be noted that the uneven staining observed in gp135 positive areas strongly suggests that apical membrane integrin-collagen interactions had occurred initiating membrane remodeling in these regions.

**Regulation of collagen-mediated membrane remodeling by the α2β1 integrin**

This monolayer system was used to identify specifically the β1 integrin involved in collagen-mediated epithelial remodeling. In preliminary experiments, we screened several mAbs against the collagen binding α2 and α3 integrin subunits to determine if they had anti-functional activity which would be useful for our studies. It was determined that the mAbs 12F1 and 6F1 against the α2 subunit (Pischel et al., 1987; Coller et al., 1989),
mAb J143 against the α3 subunit (Kantor et al., 1987) and mAb J1B5 against the α6 integrin subunit (Damsky et al., 1992) did not prevent either MDCK cell or cyst binding to collagen using the adhesion assays described previously (Ojakian and Schwimmer, 1994). However, mAb 5E8 against the α2 integrin subunit (Chen et al., 1991) inhibited cyst binding to type IV collagen and was used in subsequent studies. It should also be pointed out that all of the anti-integrin mAbs utilized in this study have previously been shown to bind to the MDCK cell surface (Ojakian and Schwimmer, 1994). These results demonstrate that mAb 5E8 inhibition of both cell binding and epithelial membrane remodeling (see below) is probably due to the recognition of a specific epitope on the α2 integrin subunit.

MDCK cells were incubated for 8 hours in the absence or presence of collagen gel overlays that either lacked or contained mAbs against different integrin subunits. In the absence of mAb, or the presence of the control mAb J1B5, a 35% decrease in gp135 was observed (Fig. 5). However, incubation with collagen gel containing either mAb 5E8 or mAb AIIB2 against the β1 integrin subunit (Hall et al., 1990) completely inhibited gp135 uptake (Fig. 5). These data provide strong evidence that the α2β1 integrin is the ECM receptor responsible for regulation of collagen-mediated epithelial remodeling.

In previous studies, we demonstrated that apical microvilli from MDCK cysts were lost after incubation in collagen gel (Wang et al., 1990b; Ojakian and Schwimmer, 1994) and that this epithelial membrane remodeling could be blocked by mAb AIIB2, which inhibited MDCK cell and cyst binding (Ojakian and Schwimmer, 1994). To determine if similar remodeling events were occurring in MDCK monolayers, after incubation with collagen gel overlays for 8 hours, the cells were examined by electron microscopy. Since apical membrane remodeling was incomplete at this time point (Figs 3, 4) apical microvilli were observed on some cells and not on others (Fig. 6A). However, if mAb 5E8 was present during the incubation, apical microvilli were observed on all cells (Fig. 6B), providing further evidence that the α2β1 integrin was involved in collagen-mediated epithelial remodeling. The cell surface distribution of the α2β1 integrin was examined by immuno-
fluorescence microscopy to determine if changes in cell surface distribution had occurred. After an 8 hour incubation either with or without a collagen gel overlay, the α2β1 integrin was localized on both the apical and basolateral cell surfaces (Fig. 7). Furthermore, α2β1 appeared to be in small clusters which were distributed evenly over the entire apical membrane (Fig. 7), suggesting that the collagen-mediated remodeling did not require integrin redistribution or the formation of focal adhesions. Identical observations were also made for the β1 integrin subunit and are not shown here.

The α2β1 integrin and formation of epithelial tubules
All of the experiments described above were done with subconfluent cells. The extent of collagen-mediated epithelial remodeling was also examined in confluent monolayers by immunofluorescence microscopy. After 24 hours with collagen gel overlays, numerous lumenal structures were observed by phase-contrast microscopy (Fig. 8A,C), many of which were more elongated than those described previously (Hall et al., 1982) or observed in subconfluent cells (Fig. 2A). Immunofluorescence microscopy of intact monolayers demonstrated that gp135 was not present on the apical membrane contacting the collagen (Fig. 8B), indicating that extensive collagen-mediated membrane remodeling had occurred. However, if the monolayers were permeabilized with methanol prior to immunofluorescence staining, then gp135 was localized to the lumenal structures (Fig. 8D), indicating that they are normally sealed. These data demonstrated that collagen gel overlays induced the formation of an anastomosing network of interconnected lumenal structures as well as some smaller, circular profiles (Fig. 8D).

The lumenal morphology was examined in detail by light, electron and immunogold electron microscopy. After a 16 hour incubation with collagen, the cells appeared to be arranged into
tubular structures organized around a central lumen with each tubule being separated by stratified cell columns (Fig. 9A). Ultrastructural examination of 24 hour cultures demonstrated that the tubule lumens were lined by apical membrane microvilli (Fig. 9B) identical in appearance to those observed on MDCK monolayers and cysts (Hall et al., 1982; Warren and Nelson, 1987; Ojakian and Schwimmer, 1988, 1994; Wang et al., 1990a,b; also see Fig. 2B). Immunogold electron microscopy localized gp135 only to the lumenal membrane (Fig. 9C), indicating that apical membrane remodeling was complete and that the tubules were polarized.

We used tubule formation to further study the role of β1 integrins in epithelial remodeling and polarity development. MDCK monolayers were incubated with collagen gel in either the absence or presence of function-blocking mAbs for 24 hours. After aldehyde fixation, gp135 was localized on methanol-permeabilized monolayers by immunofluorescence microscopy. Control monolayers had punctate apical staining (Fig. 10A) as demonstrated previously (Ojakian and Schwimmer, 1988, 1992), while in monolayers with collagen gel epithelial remodeling was complete as only gp135 positive-tubules were observed (Fig. 10B). Inclusion of anti-functional mAbs 5E8 and AIIB2 against the α2 and β1 integrin subunits, respectively, completely blocked collagen-mediated epithelial remodeling as demonstrated by the presence of apical gp135 staining and corresponding lack of lumen formation (Fig. 10B).

![Fig. 5](image1.png)

**Fig. 5.** Monoclonal antibodies against the α2β1 integrin inhibit epithelial membrane remodeling. Cells were incubated in either the absence (filled bars) or presence (hatched bars) of collagen gel overlays for 8 hours, either lacking (control) or containing mAbs J1B5 (anti-α6), 5E8 (anti-α2) or AIIB2 (anti-β1). Cell surface levels of gp135 were measured by ELISA and presented as absorbance at 405 nm (OD) ± s.e.m. It was determined that mAbs against subunits of the α2β1 integrin inhibited gp135 uptake while those against the α6 subunit did not.

![Fig. 6](image2.png)

**Fig. 6.** Ultrastructural observation of collagen-mediated membrane remodeling. After incubation with a collagen overlay for 8 hours (A), apical membrane microvilli were observed on some cells (arrowheads, left side) while on other cells, microvilli were not present (arrows, right side), indicating that remodeling had occurred. If mAb 5E8 was included in the collagen gel (B), then apical microvilli were observed on all cells, demonstrating that membrane remodeling was inhibited. Bar, 0.67 μm.
10C,D). In control experiments not shown here, we determined that mAbs 12F1 and J1B5 against the α2 and α6 subunits, respectively, did not inhibit membrane remodeling and lumen formation. These results support the data presented above for subconfluent MDCK cells and provide further evidence that the α2β1 integrin appears to play an important role in the regulation of collagen-mediated epithelial remodeling.

**DISCUSSION**

The role of β1 integrins in the development of epithelial tubules and cell surface polarity was studied in both subconfluent and confluent MDCK monolayers. Using the collagen gel overlay method (Chambard et al., 1981; Hall et al., 1982), we were able to do quantitative studies on ECM-mediated epithelial remodeling and utilize this model system to identify the specific β1 integrin involved in the regulation of this process. Previous work by our laboratory using function blocking mAbs demonstrated that β1 integrins appeared to be responsible for modulating collagen-mediated cell surface polarity reversal of epithelial cysts (Ojakian and Schwimmer, 1994). Here we demonstrate that mAb 5E8 against the α2 β1 integrin subunit completely inhibited collagen-mediated remodeling of the apical membrane in subconfluent cells and epithelial tubule formation in confluent cells. These data provide strong evidence that the α2β1 integrin is responsible for regulation of collagen-mediated epithelial tubule morphogenesis and cell surface polarity development in MDCK cells. This hypothesis is consistent with previous observations that
α2β1 is a collagen binding integrin in a variety of cell types (Staatz et al., 1989; Elices and Hemler, 1989; Albelda and Buck, 1990; Hemler, 1990; Ruoslahti, 1991; Hynes, 1992).

Since anti-functional mAbs against the α3β1 integrin in MDCK cells are not presently available, our studies cannot exclude the possibility that this receptor, which binds collagen in some cell types (Elices et al., 1991), is also involved in ECM-regulated cell polarity development. This possibility seems unlikely, since mAb 5E8 completely inhibited collagen-mediated epithelial remodeling, suggesting that other integrins were not involved. Using an in vitro model for kidney differentiation, other workers have proposed that the interaction of the α6β1 integrin with laminin is responsible for regulation of nephron development (Klein et al., 1988; Sorokin et al., 1990).

Although this is an interesting proposal, it should be pointed out that mAb GoH3 against the α6β1 integrin only produced a partial inhibition of epithelial tubule formation and no localization of membrane proteins was done to demonstrate that cell surface polarity had developed (Sorokin et al., 1990), suggesting that α6β1 is not the only integrin involved in renal tubule morphogenesis.

The possibility that collagen and collagen binding integrins could play an important role in renal tubule formation and nephron development is supported by observations that α2β1 localized on embryonic kidney distal and collecting tubule basolateral membranes (Korhonen et al., 1990). These results and those demonstrating that type IV collagen expression occurs at the same time as nephron formation (Laurie et al., 1989) suggest that distal and collecting tubule differentiation could be regulated by α2β1-collagen interactions. Although epithelial cells do not usually contact type I collagen, the experiments described here have physiological relevance, since the relative affinity of α2β1 for types I and IV collagen is almost equal (Staatz et al., 1989). Our observations that α2β1 appears to be the critical integrin for regulation of collagen-mediated MDCK epithelial morphogenesis does not exclude the possibility that other integrins can have a similar function in renal nephron development. For example, in human kidney, α2β1, α3β1 and α6β1 have distinct as well as overlapping nephron distributions (Korhonen et al., 1990). This suggests the interesting possibility that multiple integrins could influence the development of nephron segments or the maintenance of differentiated function, depending upon the local ECM microenvironment.

Subconfluent MDCK cells have non-polarized distributions of basolateral membrane proteins (Herzlinger and Ojakian, 1989). Subconfluent MDCK cells have non-polarized distributions of basolateral membrane proteins (Herzlinger and Ojakian, 1989).
1984; Vega-Salas et al., 1987), including the α2β1 integrin, and their interactions with collagen can probably be attributed to the abundant levels of α2β1 integrin found in the apical membrane (see Fig. 7). However, since α2β1, as well as other integrins, has a polarized cell surface distribution on confluent MDCK cells (Ojakian and Schwimmer, 1994; Schoenberger et al., 1994), this suggests that only low levels of α2β1 are necessary to initiate collagen-mediated epithelial remodeling. Furthermore, the observation that mAbs against either subunit of the α2β1 integrin inhibited membrane remodeling and epithelial tubule formation in confluent cells strongly suggests that functional levels of this integrin were present in the apical membrane even though they were not detected by the biochemical and morphological techniques used (Ojakian and Schwimmer, 1994; Schoenberger et al., 1994). Although β1 integrins appear to be vectorially targeted to the basolateral membrane of MDCK cells (Boll et al., 1991), it is likely that α2β1 that is mistargeted to the apical membrane is involved or that our cell culture conditions have delayed development of the membrane protein sorting pathways (Wollner et al., 1992).

Fig. 9. Morphological characterization of the lumenal structures demonstrates that they are polarized epithelial tubules. Confluent monolayers incubated for either 16 hours (A) or 24 hours (B-D) with collagen gel overlays were prepared for light (A) or electron microscopy (B-D). By light microscopy (A) tubular structures with a central lumen (arrows) were observed. Numerous microvilli (mv) were observed within these lumens by electron microscopy (B). Immunogold electron microscopy (C) demonstrated that gp135 was localized to the apical, or luminal, membrane (arrows), not the basolateral (BL) membrane. For comparison, samples were prepared for immunogold localization without the primary mAb and no gold particles were observed (D). Bars: 12 μm (A), 1 μm (B), 0.5 μm (C,D).
This suggestion is supported by observations that a variety of cell types, including MDCK, mammary and thyroid epithelial cells and endothelial cells, can bind apically applied collagen (Chambard et al., 1981; Kramer, 1985; Jackson and Jenkins, 1991; Berdichevsky et al., 1992). Furthermore, mAbs against subunits of the α2β1 integrin block interactions of the MTSV1-7 mammary cell line with collagen fibrils (Berdichevsky et al., 1992), a result consistent with those presented here for MDCK cells.

Of considerable importance is the observation that, after an 8 hour incubation with collagen, the levels of gp135 were reduced while the levels of the α2, α3 and β1 subunits remained constant. These results provide evidence that the α2β1 levels on the apical cell surface were sufficient for epithelial membrane remodeling and increased synthesis of this collagen receptor were not required. While it is appropriate that α2β1 was not affected due to its interactions with collagen, the observation that the β1 integrin subunit levels did not change suggests that a selective endocytosis of apical membrane proteins such as gp135 occurred while allowing the retention of basolateral membrane proteins. These results are supported by data demonstrating that non-polarized proteins such as the α3 integrin subunit (Ojakian and Schwimmer, 1994) and gp23 (Herz and Ojakian, 1989) were not removed from the cell surface.

In many cell types, integrins are found in focal adhesions on the membrane contacting the ECM and there is strong evidence that these discrete structures provide sites of increased cell-substratum adhesion (Burridge et al., 1988). Using a mAb against vinculin, we determined by immunofluorescence microscopy that MDCK cells have focal adhesions on their basal membrane contacting the type I collagen substratum (data not shown). Interestingly, the data presented here, and in previous studies on MDCK cells (Ojakian and Schwimmer, 1994; Schoenberger et al., 1994), demonstrate that the α2β1 integrin is not found in focal adhesions, suggesting that epithelial cell-ECM interactions can occur in the absence of these adhesive structures. Instead, the α2β1 integrin was localized to small, punctate structures randomly distributed over the apical membrane on subconfluent MDCK cells both prior to and after incubation with a collagen gel overlay. Since membrane remodeling occurred in the presence of collagen, this is strong evidence that these punctate structures can serve as the functional unit regulating MDCK cell-ECM interactions. Similar structures termed point contacts have been observed in a variety of cell types (see Tawil et al., 1993, and references therein) and there is evidence that the αvβ3 integrin was found in focal adhesions while the αvβ5 integrin on the same cell was localized to point contacts (Wayner et al., 1991) similar to those observed for the MDCK α2β1 integrin. Furthermore, it has been reported that fibronectin-coated microbeads added to the apical membrane of cultured endothelial cells can rapidly assemble focal adhesion proteins into structures that are morphologically distinct from both focal adhesions and point contacts (Plopper and Ingber, 1993). Although the mechanisms regulating collagen-mediated epithelial membrane remodeling are unknown, it is possible that α2β1 interactions with collagen could initiate signal transduction events, regulated through the focal adhesion kinase pp125FAK (Schaller et al., 1992; Juliano and Haskill, 1993; Schaller and Parsons, 1994), that allow the selective endocytosis of apical membrane proteins such as

---

**Fig. 10.** The α2β1 integrin regulates collagen-mediated epithelial tubule formation. In control monolayers (A), gp135 was localized to the apical membrane by immunofluorescence microscopy. After 24 hours with a collagen gel overlay (B), gp135 was localized only to the tubule lumens. If either mAb 5E8 (C) or AIIB2 (D) was included in the collagen gel, then gp135 was found localized only to the apical membrane and no tubule formation was observed, demonstrating that the α2β1 integrin plays a critical role in epithelial morphogenesis. Bar, 8 μm.
gp135 during membrane remodeling and the subsequent retargeting of proteins to the newly defined apical and basolateral membrane domains.

We thank Drs Eva Cramer, Robert Garofalo, John Lewis and Sam Santoro for helpful discussion and the following investigators for providing us with monoclonal antibodies: Drs Richard Bankert, Barry Coller, Fang-An Chen, Caroline Damsky, Mary John, Lloyd Old and Virgil Woods. We also thank Catherine Songster for typing the manuscript, Vincent Garofalo for photography and Alexander Fulop for assistance with the electron microscopy.

REFERENCES


(Received 30 November 1994 - Accepted 10 March 1995)