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Regulation of epithelial cell surface polarity reversal by β_1 integrins

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SUMMARY

The role of extracellular matrix in the regulation of epithelial cell surface polarity development was studied using MDCK cells. Previous work has demonstrated that MDCK cells cultured in suspension form epithelial cysts having polarized cell surface distributions of several membrane proteins. When MDCK suspension cysts are incubated within collagen gel, a dynamic epithelial membrane remodeling occurs that is accompanied by the reversal of cell surface polarity (Wang et al., 1990b, *J. Cell Sci.* 95, 153-165), suggesting that extracellular matrix is important in the modulation of epithelial polarity development. To determine if members of the integrin receptor family were involved, MDCK cyst binding studies were done utilizing antifunctional monoclonal antibodies (AIIB2 and AJ2) against the β_1 integrin subunit. These antibodies inhibited cyst binding to type I collagen, type IV collagen and laminin, providing evidence that functional β_1 integrin heterodimers were present on the cyst outer membrane. Integrin localization on suspension cysts demonstrated that the α_2, α_3 and α_6 integrin subunits had a non-polarized cell surface distribution and were localized to both the apical and basolateral membranes. Interestingly, immunofluorescence microscopy determined that the β_1 subunit had a polarized, basolateral membrane distribution although cyst binding studies using inhibitory monoclonal antibodies suggested that functional β_1 subunits were present on the cyst outer membrane. After incubation of suspension cysts in collagen gel for 8 hours, the β_1 integrin subunit was detected on the outer membrane, suggesting that the formation of additional integrin α/β heterodimers could be involved in epithelial remodeling. To establish the role of β_1 integrins in polarity reversal, experiments were done on cysts incubated in collagen gel. After 6 hours in collagen gel, considerable membrane remodeling had occurred as determined by a reduction in outer membrane microvilli. However, the presence of monoclonal antibody AIIB2 inhibited membrane remodeling by preventing both microvillar loss and the endocytosis of the apical membrane glycoprotein gp135. These results provide strong evidence that members of the β_1 integrin family are involved in the regulation of epithelial polarity reversal, and demonstrate that MDCK cysts constitute an excellent model system for studying the role of cell-extracellular matrix interactions in the regulation of epithelial plasticity and cell surface polarity development.

Key words: extracellular matrix, integrin, epithelial polarity

INTRODUCTION

Transporting epithelial cells are organized into sheets and tubules that function by vectorially transporting amino acids, ions and sugars against transepithelial concentration gradients (Berridge and Oschman, 1972). To accomplish these vectorial functions, the epithelial plasma membrane is divided into two domains: the apical membrane that borders the tubule lumen and the basolateral membrane that contacts the basal lamina. These membrane domains are morphologically and biochemically polarized with each being composed of unique enzymes, receptors and transport systems that are separated by tight junctions located at the boundary between the apical and basolateral membranes (reviewed by Nelson, 1992; Rodriguez-Boulan and Powell, 1992). Cell surface polarity is required for proper physiological function (Nelson, 1992; Rodriguez-Boulan and Powell, 1992) and there is evidence that dynamic changes in cell polarity (termed epithelial plasticity) contribute to these processes.

The cellular mechanisms that modulate biogenesis of polarity are only poorly understood and the regulatory processes involved are currently under intense investigation. There is evidence that interactions between epithelial cells and the extracellular matrix (ECM) are responsible for regulation of gene expression, organization of the cytoskeleton and the maintenance of differentiated function in epithelia (Hay, 1983; Lee et al., 1985; Barcellos-Hoff et al., 1989; Streuli et al., 1991). The ECM has been shown to be extremely important in kidney development and there are multiple ECM components expressed in the embryonic kidney (Saxen, 1987; Ekblom, 1989; Laurie et al., 1989), which could be involved. For example, the appearance of type IV collagen and laminin adjacent to forming S-shaped bodies suggests that these ECM components are critical for nephron development (Ekblom, 1981, 1989; Ekblom et al., 1981, 1990).

Epithelial cell attachment to the ECM occurs through the interaction of the integrin superfamily of cell surface receptors

(reviewed by Albelda and Buck, 1990; Hemler, 1990; Ruoslathi, 1991; Hynes, 1992). Integrins are heterodimers composed of α and β subunits (Wayner and Carter, 1987) and, to date, 14 α and 8 β subunits have been identified (Hynes, 1992). Unique combinations of α/β subunits bind to different ECM components (Wayner and Carter, 1987; Elices and Hemler, 1989; Elices et al., 1991; Hynes, 1992) with the ECM specificity residing within the α subunit (Albelda and Buck, 1990; Hemler, 1990; Hynes, 1992). Epithelial cells typically have a variety of integrins, giving them the capability of binding to different ECM components (Wayner and Carter, 1987; Hynes, 1992) and there is evidence that gene expression can be regulated by integrin-ECM interactions (Werb et al., 1989; Hynes, 1992). There is some evidence that integrins are involved in epithelial polarity development. Using an in vitro kidney model system, it was demonstrated that nephron formation could be inhibited by the presence of a monoclonal antibody (mAb) against the α_6 integrin subunit (Sorokin et al., 1990). Furthermore, Parry et al. (1990) have presented data suggesting that the vitronectin receptor may be responsible for establishing the polarized cell surface distribution of a mammary epithelial membrane glycoprotein.

Cell lines have been widely utilized for investigating epithelial cell polarity development. Many of these studies have been done on Madin-Darby canine kidney (MDCK) cells, an epithelial cell line that exhibits differentiated properties of renal tubular epithelium and has polarized distributions of plasma membrane lipids and proteins (Nelson, 1992; Rodriguez-Boulan and Powell, 1992). There is evidence that attachment of MDCK cells to ECM requires specific receptors (Salas et al., 1987) and subsequent work has demonstrated that MDCK cells express a variety of β_1 integrins (Boll et al., 1991), which could be involved in cell-substratum interactions.

We have utilized MDCK cells as a model system in which to study the role of ECM in epithelial polarity development. When MDCK cells are grown within collagen gel, they develop into polarized epithelial cysts with the basolateral membrane in contact with the collagen and the apical surface lining an internal lumen (Hall et al., 1982; McAteer et al., 1987). However, when grown in suspension culture, MDCK cells form epithelial cysts with the opposite orientation, having apical microvilli on the outer membrane, while the basolateral membrane lines the lumen (Wang et al., 1990a). Suspension cysts have polarized cell surface distributions of gp135, an apical cell surface glycoprotein (Ojakian and Schwimmer, 1988, 1992), and the basolateral membrane proteins Na^+,K^+-ATPase and uvomorulin (Wang et al., 1990a). After incubation of suspension cysts in collagen gel, a dynamic reversal of cell polarity occurs that is accompanied by extensive remodeling of the apical and basolateral membranes (Wang et al., 1990b). Apical surface gp135 is removed from the cyst outer membrane by endocytosis and newly synthesized gp135 is targeted to the luminal cell surface. Since concomitant changes were also observed in the redistribution of the basolateral proteins Na^+,K^+-ATPase and uvomorulin (Wang et al., 1990b), it appears that reversal of cell surface polarity in MDCK cysts constitutes an excellent model system for studying the cellular dynamics of epithelial plasticity.

In this paper, we present a more complete characterization of MDCK β_1 integrins as well as evidence that β_1 integrins on the MDCK cell surface are utilized for both MDCK cell and cyst adhesion to a variety of ECM components. Since incubation of suspension cysts in collagen gel induces polarity reversal (Wang et al., 1990b), it is likely that integrins are involved in the initiation of epithelial membrane remodeling and subsequent polarity reversal. Evidence for this proposal is provided by demonstrating that a antifunctional monoclonal antibody mAb to the integrin β_1 subunit inhibited MDCK cyst polarity reversal within collagen gel, suggesting that β_1 integrins are important modulators of epithelial polarity development.

MATERIALS AND METHODS

Cell culture

MDCK cells were cultured in Dulbecco's minimal essential medium (DME) containing 10% fetal bovine serum (DME-FBS) in a 5% CO_2 atmosphere and passaged weekly as described previously (Herzlinger and Ojakian, 1984; Wang et al., 1990a). The majority of these studies were done with MDCK clone 8 cells (Wang et al., 1990a; Wollner et al., 1992); however, the MDCK cells used in our laboratory previously (Herzlinger and Ojakian, 1984; Ojakian and Schwimmer, 1988; Herz and Ojakian, 1989) gave identical results. Formation of MDCK cysts was accomplished by plating 10^5 cells into 100 mm tissue culture dishes (Falcon) coated with 1% agar and culturing them in suspension for 7-10 days. At 2 day intervals, additional DME-FBS (2 ml) was added to the culture dishes without medium removal. When the majority of cysts were determined by phase-contrast microscopy to have developed a hollow lumen (usually by 7 days), they were utilized for experiments. For studies on epithelial polarity reversal, MDCK cysts were gently harvested by low-speed centrifugation (500 ***g*** for 3 minutes), washed with DME, then pelleted. Type I collagen extracted from rat tail tendon in acetic acid (from Dr Kathryn Miles) was used to form collagen gels by mixing 9 vol. collagen, 1 vol. 10× DME and 0.1 vol. 0.34 M NaOH at 4°C. The cysts were resuspended in this collagen solution by gentle pipetting. 250 μl (containing ~10^5 cysts) were added to 24-well culture plates and allowed to gel at 37°C (Wang et al., 1990a). For experiments requiring the antifunctional mAb A11B2 (diluted 1:5), mAb was incorporated into the collagen gels during their formation. These gels were overlaid with DME-FBS containing additional mAb AIIB2, or control mAb J1B5, and the cysts incubated for 6 hours at 37°C in a 5% CO_2 atmosphere. For the formation of inverted cysts with microvilli lining the internal lumen (McAteer et al., 1987; Wang et al., 1990a), subconfluent cells grown on type I collagen-coated micropore filters (0.45 μm pores; Millipore Corp.) were covered with a collagen gel overlay and cultured in DME-FBS for 48 hours (Hall et al., 1982; Warren and Nelson, 1987).

Antibodies and extracellular matrix components

Mouse mAb 3F2 against gp135 was produced by our laboratory (Ojakian and Schwimmer, 1988); mouse mAb 3A3 against the α_1 integrin subunit was obtained from Dr David Turner (SUNY Health Science Center at Syracuse); mouse mAb 6F1 against the α_2 integrin subunit (Coller et al., 1989) from Dr Barry Coller (SUNY Health Science Center at Stony Brook); mouse mAb 5E8 against the α_2 integrin subunit (Chen et al., 1991) from Drs Richard Bankert and Fang-An Chen (Roswell Park Cancer Institute); mouse mAb 12F1 against the α_2 integrin subunit (Pischel et al., 1987) from Dr Virgil Woods (University of California, San Diego); mouse mAb TS2/7 against the α_1 integrin subunit and a rabbit antiserum against the α_2 integrin subunit (Elices and Hemler, 1989) from Dr Martin Hemler (Harvard Medical School); mouse mAbs against the α_3 (J143) and β_1 (AJ2) integrin subunits (Kantor et al., 1987) from Drs Lloyd Old and Mary John (Memorial Sloan-Kettering Cancer Institute); rabbit

antiserum against the α_3 integrin subunit was purchased from Chemicon Inc. (catalog #1920); rat mAbs against the α_5 (BIIG2), α_6 (J1B5) and β_1 (A11B2) integrin subunits (Werb et al., 1989; Hall et al., 1990) from Dr Caroline Damsky (University of California, San Francisco); rat mAb GoH3 against the α_6 integrin subunit (Sonnenberg et al., 1988) from Dr Arnond Sonnenberg (The Netherlands Red Cross Laboratory); and mouse mAb LM609 against the $\alpha_v\beta_3$ integrin (Cheresh and Spiro, 1989) from Dr David Cheresh (Scripps Research Institute). All of the mAbs were utilized as dilutions of ascites fluid with the exception of mAbs AIIB2, BIIG2, GoH3 and J1B5, which were dilutions of hybridoma supernatants. In some experiments, AIIB2 ascites fluid was also utilized and gave results similar to those described for the hybridoma supernatent. The antibodies utilized in this study were characterized and it was determined that, with the exception of mAbs LM609, BIIG2, 3A3 and TS2/7, they all immunoprecipitated polypeptides of 150 kDa and 125 kDa from MDCK cells. In comparison, mAb LM609 recognized polypeptides of 150 kDa and 100 kDa, while mAbs BIIG2, 3A3 and TS2/7 did not immunoprecipitate any polypeptides. Type I collagen for binding assays was purchased (Sigma Chemical Co.), mouse type IV collagen and laminin were gifts from Dr Roy Ogle (University of Virginia School of Medicine) or were purchased (Collaborative Research), human fibronectin and the adhesion peptides GRGDSP and GRGESP were purchased (Telios Pharmaceuticals Inc.).

Cell and cyst binding assays

Cell binding

Non-tissue culture, 96-well polystyrene plates (Nunc) were coated with either ECM components or 2% bovine serum albumin (BSA; as a control) for 18 hours at 4˚C, then rinsed with PBS and any remaining protein-binding sites blocked with 2% BSA-PBS for 1 hour at 4˚C. For cell binding, subconfluent, 2-day-old MDCK cells were removed from culture dishes by incubation in 0.2% trypsin-2 mM EDTA for 10 minutes at 37˚C, incubated in 0.2% soybean trypsin inhibitor, washed with PBS, then resuspended in DME (total time ~30 minutes). ECM-coated wells were incubated with 100 μl cell suspensions (10^5 cells/well) for 1 hour at 37˚C in DME, or DME containing either mAbs or RGD adhesion peptides (100 μg/ml), then rinsed 3 times with PBS and fixed in 3% paraformaldehyde-PBS (pH 7.4) for 15 minutes at room temperature. Quantitation of cell binding was determined on quadruplicate samples using toluidine blue dye absorbance recorded at 600 nm on a microtiter ELISA reader (Hall et al., 1987). The absorbance of cells bound to the BSA-coated wells was determined to be minimal (A<0.005) and, therefore, was not subtracted from absorbances obtained on cells bound to ECM-coated wells. The maximum number of cells binding to wells coated with the highest ECM concentrations utilized were counted by phase-contrast microscopy and determined to be 80% of the cells added. Since cell binding to poly-L-lysine-coated plates was determined to be equivalent to that observed for the higher ECM concentrations, it was not considered in our calculations. After determining cell binding over a relatively large ECM concentration range (see Fig. 1, below), subsequent cell and cyst binding studies were done using the following ECM concentrations: type IV collagen (10 μg/ml), fibronectin (10 μg/ml) and laminin (25 μg/ml).

Cyst binding

Quadruplicate samples containing 5×10^4 suspension cysts in 100 μl DME-BSA (1 mg/ml) in either the presence or absence of mAbs or RGD adhesion peptides were incubated in ECM-coated (see concentrations above) 96-well plates for 4 hours at 37˚C. These concentrations allowed the levels of cyst binding necessary for quantitation using the toluidine blue dye absorbance assay. The maximum number of cysts bound were determined by microscopy to be 30% of those added for the ECM concentrations utilized. Incubation times of 4 hours or 6 hours were utilized so that comparisons to our previous studies (Wang et al., 1990a,b) could be made.

Immunoprecipitation and SDS-PAGE characterization of integrins

Biochemical characterization of MDCK integrins was done to complement data obtained by immunofluorescence and immunogold electron microscopy. Subconfluent cells were incubated for 1 hour at 4˚C in sulfo-NHS-biotin (500 μg/ml; Pierce Chemical Co.) a charged, water-soluble reagent that has been utilized to label plasma membrane proteins of MDCK and other epithelial cells (Sargiacomo et al., 1989; Le Bivic et al., 1990; Wollner et al., 1992). Cells were scraped from the plates, solubilized in 1% Triton X-100, PBS, 1 mM PMSF for 1 hour at 4˚C and pelleted in a microcentrifuge. The supernatant was removed and analyzed for biotinylated cell surface integrins by immunoprecipitation and SDS-PAGE using a procedure for MDCK membrane proteins (Herz and Ojakian, 1989). Briefly, supernatant samples were incubated with mAbs (1:100 dilution) for 18 hours at 4˚C, then in either goat anti-mouse IgG-Sepharose 4B for 2 hours, or sequentially in rabbit anti-rat IgG and Protein A-Sepharose 4B for 1 hour each. Immunoprecipitated integrins were eluted from the Sepharose beads, mixed with SDS sample buffer, separated by SDS-PAGE on 8% non-reducing gels and electrophoretically transferred to Immobilon-P membranes (Millipore Corp.) at 100 milliamps for 2 hours (Herz and Ojakian, 1989). These Immobilon-P membranes were then incubated in Streptavidin-horseradish peroxidase (0.5 μg/ml; Sigma Chemical Co.) for 1 hour, and the biotinylated integrins detected by chemiluminescence (ECL kit; Amersham) and exposure to X-ray film.

Immunofluorescence microscopy

Confluent MDCK monolayers on micropore filters, suspension-grown cysts, or cysts formed within collagen gel were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde for 1 hour at 4˚C. They were infiltrated with 30% sucrose-PBS for ~18 hours at 4˚C, then frozen in liquid nitrogen and 5 μm frozen sections were cut on a Leitz cryostat and mounted on 0.1% poly-L-lysine-coated glass slides. Integrin subunits were localized by sequential 1 hour incubations at room temperature in primary antibody (diluted 1:100 in BSA-PBS), either rabbit anti-mouse or anti-rat IgG (100 μg/ml), and goat anti-rabbit IgG coupled to rhodamine (1:10 dilution). Sectioned monolayers and cysts were observed in a Zeiss microscope equipped with epifluorescence optics and photographed on Kodak Tri-X film. All commercial secondary and tertiary antibodies used for both immunofluorescence and immunogold electron microscopy were purchased from Cappel Laboratories.

Immunogold electron microscopy

Suspension cysts were fixed in paraformaldehyde-glutaraldehyde as described above and β_1 integrins localized on the outer membrane prior to embedding. Briefly, the cysts were sequentially incubated for 1 hour each at room temperature with mAbs against integrin subunits (1:10 dilution), either rabbit anti-mouse or anti-rat IgG (100 μg/ml), and Protein A-10 nm colloidal gold (1:25 dilution; Sigma Chemical Co.). Control experiments were done using rabbit anti-rat IgG and Protein A-gold only. After washing with PBS, the stained cysts were post-fixed for 15 minutes with 2.5% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.3) prior to embedding in Epon 812. Thin sections were cut on a diamond knife, stained with uranyl acetate and lead citrate, and photographed in a JEOL 100C electron microscope.

Electron microscopic observation of membrane remodeling

For these experiments, suspension cysts were incubated in DME/FBS in either the presence or absence of mAbs A11B2 or J1B5 (1:5 dilutions) for 15 minutes at 37˚C. These cysts were then resuspended in collagen gel and incubated in DME-FBS in the presence or absence of these mAbs for 6 hours at 37˚C. For observation by conventional electron microscopy, the cysts were fixed in 2.5% glutaraldehyde-0.1

M sodium phosphate followed by postfixation in 1% OsO_4 for 1 hour each at room temperature. For the immunolocalization of gp135 during collagen-induced epithelial polarity reversal, the cysts were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde for 1 hour at 4˚C. To determine if membrane remodeling had occurred, collagen gels containing fixed cysts were loosened from the culture wells and incubated in mAb 3F2 (1:2 dilution) for 18 hours at 4˚C. After washing in BSA-PBS for 1 hour, the cysts were incubated in biotinylated goat anti-mouse IgG (100 μg/ml) for 8 hours followed by Streptavidin-10 nm colloidal gold (100 μg/ml) for 18 hours. The biotin and Streptavidin reagents were purchased from the Sigma Chemical Co. After postfixation in 2.5% glutaraldehyde, 0.1 M sodium phosphate, the cysts were embedded in Epon 812 for thin sectioning. Quantitation of microvillar number was done from electron micrographs for each experimental condition by measuring cyst outer membrane length, counting connected microvillar projections and expressing these data as microvilli/μm.

RESULTS

Characterization of MDCK cell and cyst binding to ECM

MDCK cell binding done in plastic wells coated with ECM components demonstrated that MDCK cells exhibited ECM concentration-dependent binding to types I and IV collagen, fibronectin and laminin which leveled off at ~25 μg/ml (Fig. 1). The cells preferentially bound to type I collagen at lower concentrations than the other ECM components; however, these differences were not observed at higher ECM concentrations. Although these results were not completely consistent with previous MDCK binding data (Salas et al., 1987), a different cell binding assay was utilized and direct comparisons were not possible.

To determine if β_1 integrins were involved in the binding of MDCK cells to ECM, further binding studies were done in the presence of an antifunctional monoclonal antibody. Using the mAb A11B2 against the integrin β_1 subunit (Hall et al., 1990), it was determined that cell binding to type IV collagen was inhibited by >95% while binding to fibronectin was only inhibited 25% (Fig. 2). Although not shown here, mAb A11B2 inhibited cell binding to laminin by ~85%. In control experiments using hybridoma supernatants containing mAbs B11G2 or J1B5 (1:5 dilutions) obtained from the same laboratory (Werb et al.; Hall et al., 1990), we observed no inhibition of cell binding to either type IV collagen, fibronectin or laminin.

The possibility of integrin involvement in MDCK cell binding was also tested by using the hexapeptide GRGDSP, which can block the interaction of some integrins with the ECM (Ruoslathi and Pierschbacher, 1987; Albelda and Buck, 1990; Hemler, 1990). GRGDSP produced an ~50% inhibition of cell binding to fibronectin but did not inhibit binding to type IV collagen while the control hexapeptide GRGESP did not prevent cell binding to either ECM component (Fig. 2).

Since previous studies by us (Wang et al., 1990b) and others (Hall et al., 1982; Warren and Nelson, 1987) indicated that the MDCK apical cell surface appeared capable of interacting with type I collagen, MDCK cyst binding studies were also done. They demonstrated that cysts, as well as cells, were capable of binding to types I and IV collagen, fibronectin and laminin (see Figs 3, 4 for cyst binding data). The GRGDSP peptide

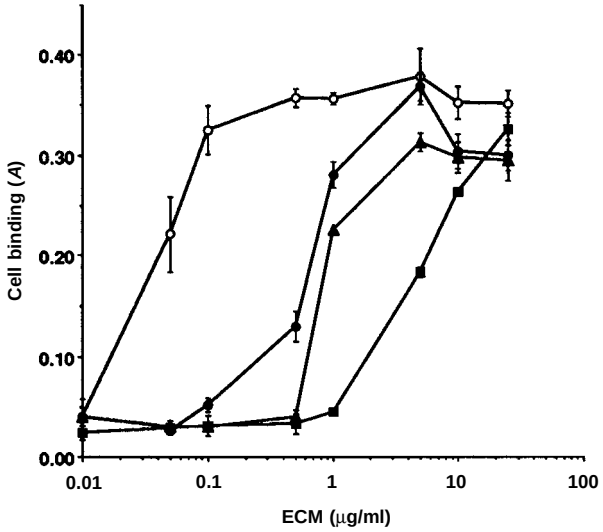


Fig. 1. MDCK cell binding to extracellular matrix components. Cell suspensions were incubated for 1 hour at 37°C on plastic wells coated with either type I collagen (○), type IV collagen (●), laminin (■) or fibronectin (▲) and the levels of binding were measured. This is a representative experiment (n=3) with each point (mean ± s.e.m.) being done in quadruplicate.

inhibited cyst binding to collagen IV by ~50% and to fibronectin by ~90% but did not inhibit binding to laminin (Fig. 3). The unexpected observation that cyst, but not cell, binding to type IV collagen was inhibited by the GRGDSP peptide suggests the interesting possibility that cyst collagen binding integrins were in a conformational state capable of recognizing the RGD sequence.

To study further the involvement of β_1 integrins, cyst binding to ECM was done in the presence of the antifunctional mAb AIIB2. These data demonstrate that mAb AIIB2 inhibited cyst binding to type IV collagen and laminin by >90% and to fibronectin by 25% (Fig. 4). In experiments not presented here, cyst binding to ECM components could also be inhibited by >90% using either mAb AIIB2 or mAb AJ2 ascites fluid. Since subsequent studies on epithelial polarity reversal were done in type I collagen gels, cyst binding to collagen gel was also done. However, due to excessive collagen gel dye staining, the colorimetric binding assay could not be used here and quantitation of cyst binding was done microscopically. These data demonstrate that cysts bind to collagen gel (121.5±5.0 cysts/mm^2) and this binding was reduced by ~80% (23.9±1.2 cysts/mm^2) in the presence of mAb AIIB2. These data, and those using the GRGDSP hexapeptide, provide strong evidence that functional β_1 integrins are present on the MDCK cyst outer membrane and are potentially involved in epithelial polarity reversal. The observations that mAb AIIB2 did not effectively block both cell and cyst binding to fibronectin demonstrates that its antifunctional activity is not due to steric hinderance and probably involves specific inhibition of β_1 integrin function.

Biochemical characterization of integrins

To further characterize MDCK integrins, subconfluent cells were incubated with biotin, allowing this protein-labeling

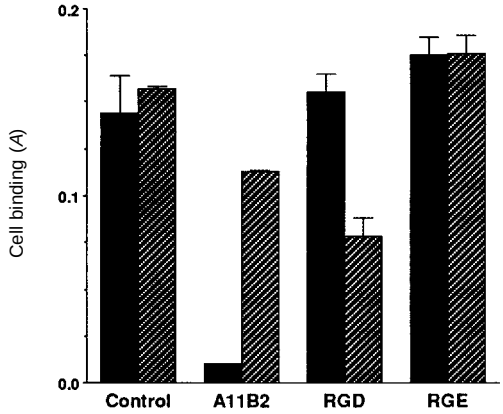


Fig. 2. Inhibition of cell binding to ECM by antifunctional mAb AIIB2 or GRGDSP hexapeptide. Cells were added to plastic wells coated with either type IV collagen (filled bars) or fibronectin (hatched bars). They were incubated in DME (Control), or DME containing either mAb AIIB2 (1:10 dilution), GRGDSP peptide (100 μg/ml) or GRGESP peptide (100 μg/ml) for 1 hour at 37°C prior to measuring cell levels bound (mean ± s.e.m.). *n*=3.

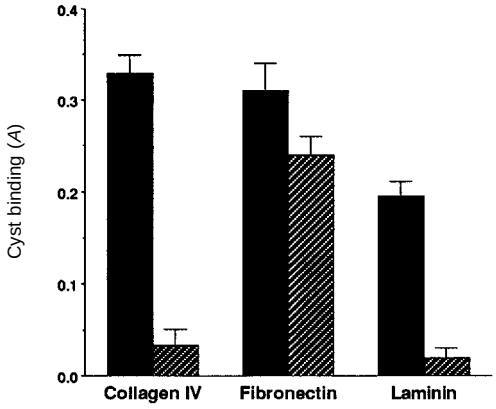


Fig. 4. Inhibition of cyst binding to ECM by the antifunctional mAb AIIB2. Suspension cysts were incubated in plastic wells coated with either type IV collagen, fibronectin or laminin in either DME-BSA (filled bars) or DME-BSA containing a 1:10 dilution of mAb AIIB2 (hatched bars) for 4 hours at 37°C prior to measuring cyst levels bound (mean ± s.e.m.). *n*=3.

reagent (Sargiacomo et al., 1989) access to the entire cell surface. The biotinylated cell surface integrins were identified by immunoprecipitation and SDS-PAGE using a panel of monoclonal and polyclonal antibodies against integrin subunits. Our data demonstrate that MDCK cells express a variety of integrins including: $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ and, possibly, $\alpha_6\beta_4$ (Fig. 5). All of the antibodies against the α subunits immunoprecipitated polypeptides with an apparent molecular mass of 150 kDa. In the samples that had been incubated with antibodies against α_2, α_3 and α_6 integrin subunits (Fig. 5, lanes b-d), polypeptides of 125 kDa were co-immunoprecipitated and it is likely that they represent the β_1 integrin subunit (Wayner and Carter, 1987; Albelda and Buck, 1990; Hemler, 1990; Hynes, 1992). This assumption is supported by data demonstrating that the anti-β_1 mAbs A11B2 (Hall et al., 1990) and AJ2 (Kantor et al., 1987) also immunoprecipitated 150 kDa and 125 kDa polypeptides (Fig. 5, lanes f and g). Although we have no direct evidence for the presence of the $\alpha_6\beta_4$ integrin, the co-immunoprecipitation of a 200 kDa polypeptide (Fig. 5, lane d) by mAb GoH3 against the α_6 integrin subunit (Sonnenberg et al., 1988) suggests that this laminin-binding receptor (Lee et al., 1992) is present. The 150 kDa and 100 kDa polypeptides immunoprecipitated by mAb

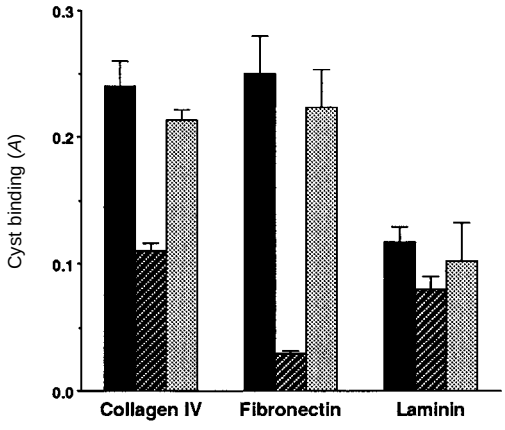


Fig. 3. Inhibition of cyst binding to ECM by the GRGDSP hexapeptide. Suspension cysts were incubated in plastic wells coated with type IV collagen, fibronectin or laminin in DME-BSA (filled bars), or DME-BSA containing either 100 μg/ml GRGDSP (hatched bars) or GRGESP (stippled bars) peptides for 4 hours at 37°C prior to measuring cyst levels bound (mean ± s.e.m.). *n*=3.

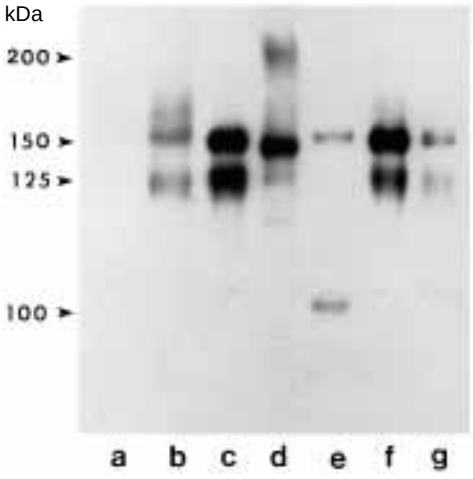


Fig. 5. Biochemical characterization of MDCK cell surface integrins. Biotinylated integrins were immunoprecipitated with the following antibodies: lane a, mAb TS2/7 against the α_1 subunit; lane b, polyclonal antiserum against the α_2 subunit; lane c, polyclonal antiserum against the α_3 subunit; lane d, mAb GoH3 against the α_6 subunit; lane e, mAb LM609 against $\alpha_v\beta_3$; lane f, mAb AIIB2 against the β_1 subunit; and lane g, mAb AJ2 against the β_1 subunit. After SDS-PAGE, apparent molecular masses were determined by comparison to Bio-Rad prestained standards: myosin (205 kDa), β-galactosidase (116.5 kDa), BSA (80 kDa), ovalbumin (49.5 kDa).

LM609 (Fig. 5, lane e) represent the $\alpha_v\beta_3$ integrin subunits of the vitronectin receptor (Cheresh and Spiro, 1987). Our observations that these anti-human mAbs were able to recognize canine integrins is consistent with previous molecular cloning data demonstrating a high degree of interspecies homology within this ECM receptor family (Takada and Hemler, 1989; Hemler, 1990; Takada et al., 1991; Hynes, 1992). However, the observation that mAbs TS2/7 (Fig. 5, lane a) and 3A3 (data not shown) against the α_1 integrin subunit did not immunoprecipitate any polypeptides does not exclude the possibility that the $\alpha_1\beta_1$ integrin was also present and not detected due to species differences in the epitopes that these mAbs recognize.

Cell surface distribution of integrins on suspension cysts

The observations that β_1 integrins appear to be involved in the binding of MDCK cells and cysts to collagen (Figs 2-4) and the demonstration that a variety of integrins are present on the MDCK cell surface (Fig. 5) provides strong supporting evidence that members of this ECM receptor superfamily play an important role in mediating epithelial polarity reversal within collagen gel. However, for such a regulation to be feasible, integrins such as $\alpha_2\beta_1$ and $\alpha_3\beta_1$, which bind collagen (Elices and Hemler, 1989; Elices et al., 1991), must be present on the apical membrane of suspension-grown cysts. This has been confirmed by immunofluorescence microscopy on frozen sections of suspension cysts, which demonstrated that the α_2, α_3 and α_6 integrin subunits all had a non-polarized distribution and were localized to both the apical (outer surface) and basolateral (luminal) membranes (Fig. 6A-C). Localization of the β_1 integrin subunit with mAb A11B2 gave quite a different result as β_1 had a polarized cell surface distribution and was localized to the basolateral membrane (Fig. 6D). An identical β_1 distribution was also observed after staining with mAb AJ2 and is not presented here. The β_1 integrin subunit cell surface distribution was quite surprising in view of the antifunctional studies using mAb AIIB2 (Fig. 4) that provided evidence for the presence of β_1 on the outer membrane. However, it should be pointed out that the immunofluorescence data do not exclude the possibility that low levels of the β_1 subunit were present on the suspension cyst outer membrane and their detection is below the resolution limits of this procedure.

This possibility was tested by utilizing immunogold electron microscopy to localize β_1 integrins on the suspension cyst outer membrane prior to embedding. These data demonstrate that the α_2 and α_3 subunits were expressed on the majority of cells and distributed primarily on the apical microvilli with some staining also being observed on the intermicrovillar membrane (Fig. 7A,B). They also support our previous data showing that cysts are sealed structures (Wang et al., 1990b), since no staining of integrin subunits was observed on the cyst lateral membranes. In addition, low levels of immunogold staining were observed when mAb AIIB2 was utilized as the primary antibody (Fig. 7C), suggesting the presence of the β_1 integrin subunit on the outer membrane. To be certain that this staining did not represent Protein A-gold non-specific binding, quantitative measurements of mAb AIIB2 immunogold staining were compared with those obtained lacking primary

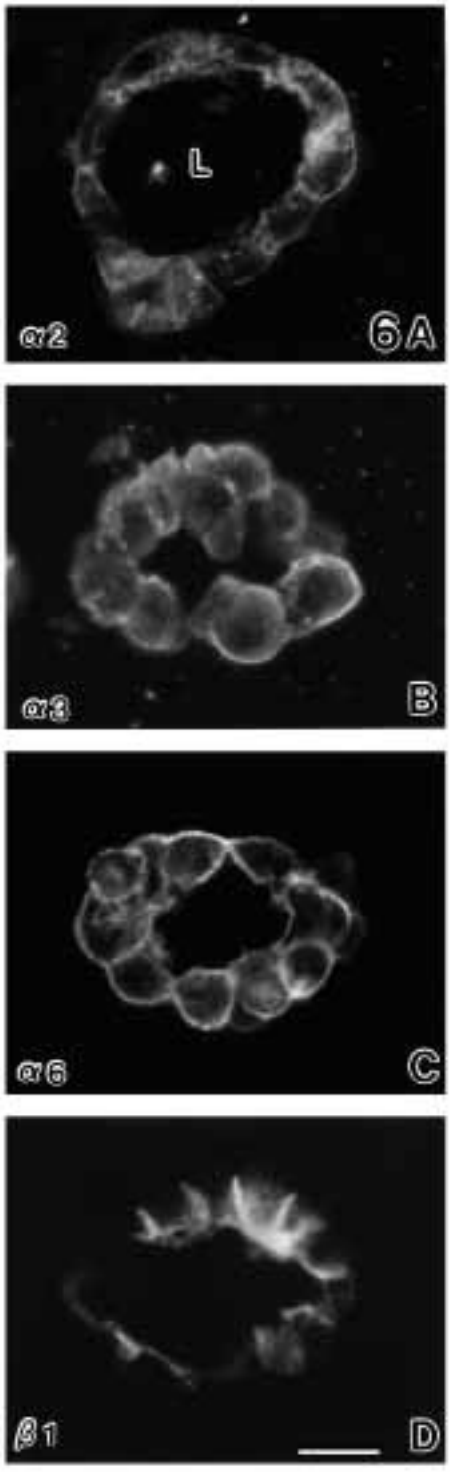


Fig. 6. The cell surface distribution of β_1 integrins on suspension cysts. Integrin subunits were localized on frozen sections by immunofluorescence microscopy using the following antibodies: (A) mAb 12F1 against the α_2 subunit, (B) polyclonal antiserum against the α_3 subunit, (C) mAb J1B5 against the α_6 subunit and (D) mAb AIIB2 against the β_1 subunit. These data demonstrated that the α subunits all had a non-polarized cell surface distribution and were localized to both the luminal (L) and outer membranes, while the β_1 subunit had a polarized distribution and was localized only to the basolateral membrane adjacent to the cyst lumen. Bar, 8 μm.

mAb. They demonstrate that the levels of membrane-bound Protein A-gold particles were 3-fold higher when mAb AIIB2 was present (Table 1). Since statistical analysis indicated that these differences were significant ($P<0.006$), it is possible that low levels of the β_1 integrin subunit were present on the cyst outer membrane. These data also provided evidence that the

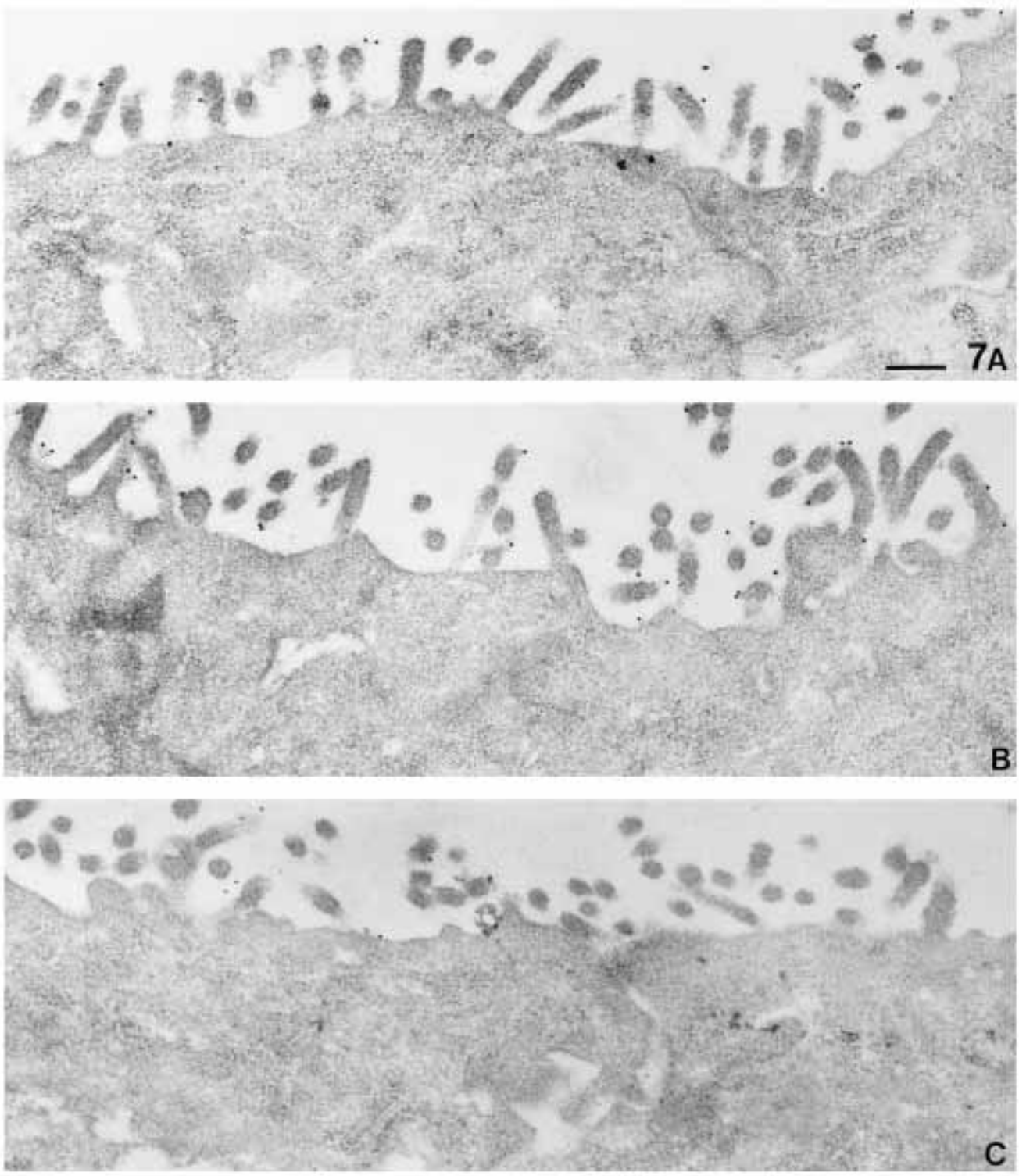


Fig. 7. Ultrastructural localization of β_1 integrins on suspension cysts. Integrin subunits were localized on the cyst outer membrane by immunogold electron microscopy using the following antibodies: (A) mAb 6F1 against the α_2 subunit; (B) mAb J143 against the α_3 subunit; and (C) mAb AIIB2 against the β_1 subunit. The majority of immunogold label was on apical microvilli although some staining was also observed on the intermicrovillar membrane. Bar, 0.26 μm.

α_2, α_3 and β_1 integrin subunits were in the proper transmembrane orientation, since the mAbs utilized recognize epitopes on the protein ectodomain (Kantor et al., 1987; Coller et al., 1989; Hall et al., 1990).

Cell surface distribution of integrins on monolayers and cysts formed in collagen

The non-polarized cell surface distribution of the α_2, α_3 and α_6 integrin subunits on suspension-grown cysts is interesting in view of our observations that other cyst membrane proteins have a polarized distribution (Wang et al., 1990a). Since the majority of MDCK epithelial polarity studies have been done on monolayers, as a frame of reference, the cell surface distribution of β_1 integrins was also determined for confluent cell monolayers grown on collagen-coated micropore filters. These data were obtained on MDCK monolayers having transepithelial electrical resistances of >200 ohms·cm^2, indicating the presence of tight junctions (Misfeldt et al., 1976; Cereijido et al., 1978) capable of maintaining epithelial polarity (Herzlinger and Ojakian, 1984; Vega-Salas et al., 1987). Immunofluorescence microscopy of frozen sections demonstrated that: gp135 had an apical distribution (Fig. 8A) as described (Ojakian and Schwimmer, 1988, 1992); the α_2 and β_1 integrin subunits a basolateral distribution (Fig. 8B,E); the α_3 integrin subunit a non-polarized distribution with some intracellular staining and the lateral membrane staining being much lower than the apical and basal (Fig. 8C); and the α_6 integrin subunit primarily a basal distribution (Fig. 8D). Since the cell surface distribution of the α_2 and α_6 subunits was different on suspension-grown cysts (Fig. 7) and monolayers (Fig. 8), immunofluorescence localization of integrins was also done on inverted MDCK cysts formed within collagen gel. Under these conditions, gp135 was localized to the apical, or luminal, cell surface (Fig. 9A,B) as described (Wang et al., 1990a,b). The α_2 and β_1 integrin subunits had a basolateral distribution and were now present on the cyst outer membrane (Fig. 9C,F). The α_3 subunit had a nonpolarized cell surface distribution including the presence of some intracellular staining (Fig. 9D), and the α_6 subunit was localized primarily to the basal membrane (Fig. 9E). It is important to point out that the cell surface distributions of integrins on MDCK cysts formed within collagen gel and monolayers grown on micropore filters were identical. These data suggest that attachment to an immobilized ECM may be involved in integrin targeting and this possibility is discussed below.

Table 1. Quantitative measurement of the β_1 integrin subunit on suspension cysts

	Membrane length (μm/cell)	Protein A-gold (particles/cell)	Protein A-gold (particles/μm)
No mAb	16.3±2.22	2.71±0.52	0.16±0.02
mAb AIIB2	16.5±2.24	9.10±1.02	0.55±0.13

MDCK suspension-grown cysts were fixed and the β_1 integrin subunit was localized on the outer membrane by immunogold electron microscopy. After staining in either the presence or absence of mAb AIIB2, Protein A-gold densities (mean ± s.e.m.) were determined for a total of 228.6 μm (no mAb) or 231.3 μm (mAb AIIB2) of cyst outer membrane. Statistical analysis by the 2-tailed *t*-test demonstrated that the differences between cysts treated with either no mAb or mAb AIIB2 were significant (P<0.006).

Since the majority of the β_1 integrin subunit is primarily on the basolateral membrane of suspension-grown cysts and α/β heterodimers are required for functional integrin-ECM interactions (Hynes, 1992), cysts were incubated in collagen gel to determine if changes in β_1 subunit distribution could be detected after membrane remodeling had occurred. The

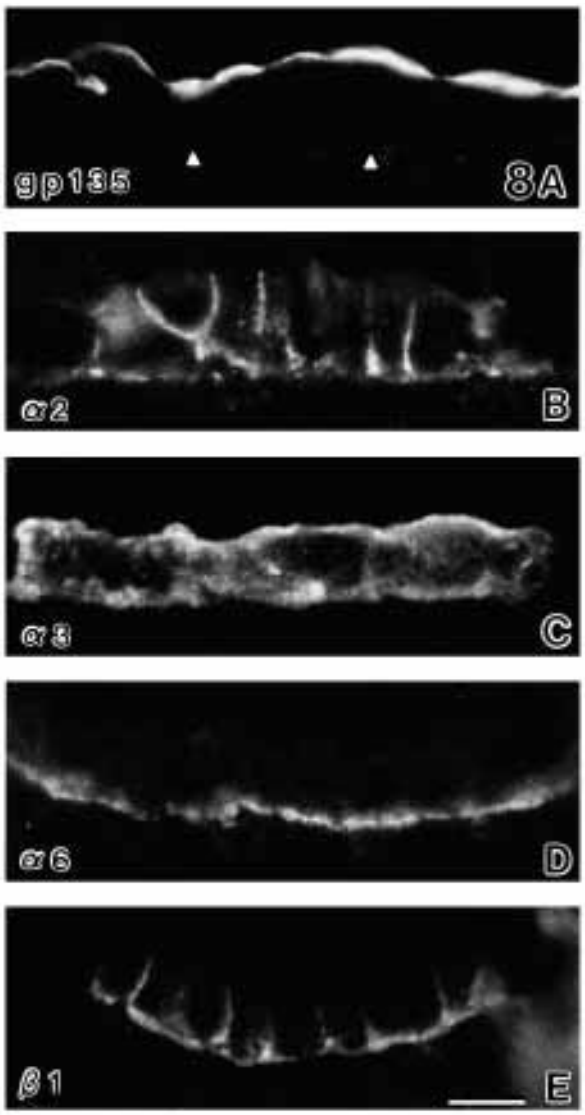
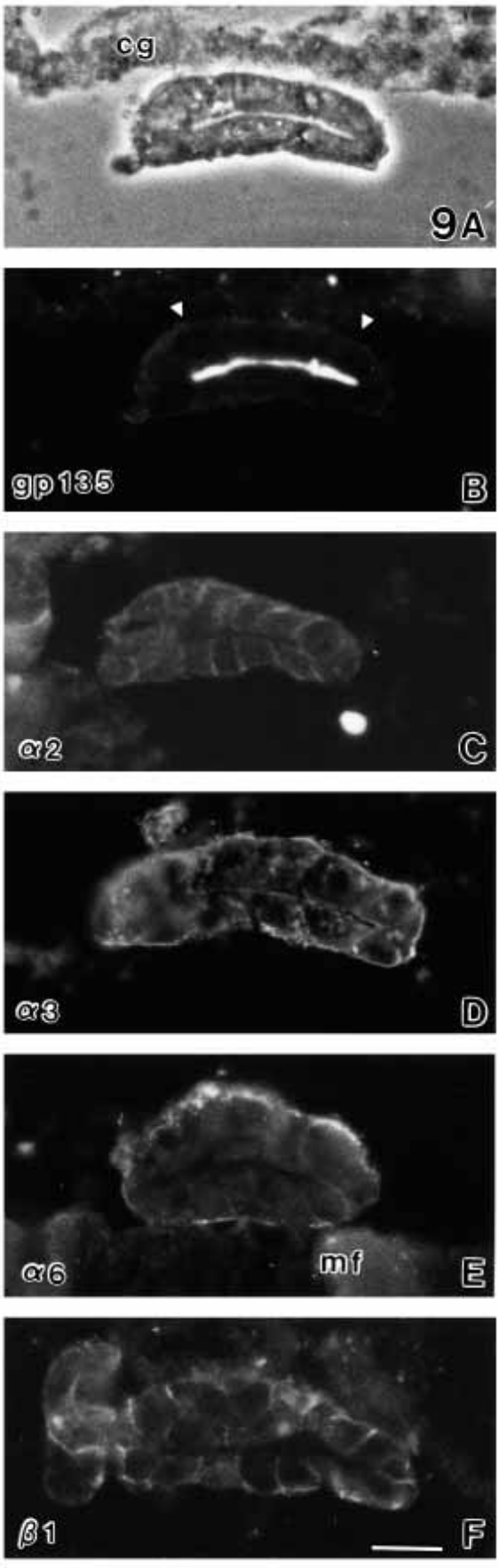


Fig. 8. Immunofluorescence localization of β_1 integrins on MDCK monolayers. Cell surface distribution of integrin subunits was determined on frozen sections by immunofluorescence microscopy using the following antibodies: (A) mAb 3F2 against gp135; (B) mAb 5E8 against the α_2 subunit; (C) polyclonal antiserum against the α_3 subunit; (D) mAb GoH3 against the α_6 subunit; and (E) mAb AJ2 against the β_1 subunit. Immunofluorescence staining was done on adjacent sections from the same sample. All monolayers shown were oriented with the apical membrane on top and the basolateral membrane contacting the collagen-coated micropore filter substratum. Note that gp135 (A) had an apical distribution and was not found on the basolateral membrane (arrowheads), while, in contrast, the α_6 subunit (D) had a basal membrane distribution. The α_2 and β_1 subunits (B,D) were localized to the entire basolateral membrane and the α_3 subunit (C) had a non-polarized cell surface distribution. Bar, 6.25 μm.



β_1 integrin subunit was observed on the cyst outer membrane by immunofluorescence microscopy after 8 hours in collagen gel (Fig. 10), suggesting that retargeting of newly synthesized β_1 subunit had occurred. These results indicate that collagen-mediated membrane remodeling was not completed at this time (also see Wang et al., 1990b), since the β_1 subunit was localized to the luminal membrane of some cells (Fig. 10B).

Monoclonal antibody inhibition of epithelial reversal

The observations that β_1 integrins appeared to be involved in cyst binding to ECM components (Figs 3, 4) and were found on the cyst outer membrane (Figs 6, 7) suggested that members of this ECM receptor family could be involved in epithelial polarity reversal. This proposal was tested by doing polarity reversal experiments in the presence of the antifunctional mAb A11B2 and analyzing epithelial membrane remodeling at the ultrastructural level. After incubation of suspension cysts in collagen gel for 6 hours, polarity reversal occurred as determined by the loss of apical microvilli on the cyst outer membrane and the appearance of microvilli on the luminal membrane (Fig. 11). This representative electron micrograph illustrating collagen-induced outer membrane remodeling is shown at higher magnification (Fig. 12B) and was compared with that of suspension-grown cysts, which were characterized by numerous outer membrane apical microvilli (Fig. 12A; also see Wang et al., 1990). After 6 hours in collagen gel, extensive membrane remodeling had occurred and few outer membrane microvilli were observed (Fig. 12B). However, if cysts were incubated in collagen gel containing mAb A11B2, an inhibition of epithelial polarity reversal was observed. The presence of this antifunctional mAb prevented the loss of outer membrane microvilli (Fig. 12C) and apical microvilli were not observed on the luminal membrane, indicating that polarity reversal had not occurred. This membrane remodeling was quantitated by counting the number of outer membrane microvilli under the different experimental conditions. When cysts were incubated for 6 hours in collagen gel containing mAb AIIB2, 3.11±0.26 microvilli/μm were measured on the outer membrane compared to 3.95±0.32 microvilli/μm on suspension cysts (Table 2). However, after incubation in collagen gel either lacking mAb, or containing the control mAb J1B5, which recognized the α_6 integrin subunit on suspension cysts

Fig. 9. Immunofluorescence localization of β_1 integrins on MDCK cysts formed within collagen gel. The distribution of gp135 was determined on frozen sections in corresponding phase-contrast (A) and immunofluorescence (B) micrographs using mAb 3F2. The remaining immunofluorescence micrographs were obtained after staining frozen sections with: (C) mAb 5E8 against the α_2 subunit; (D) polyclonal antiserum against the α_3 subunit; (E) mAb GoH3 against the α_6 subunit; and (F) mAb AIIB2 against the β_1 subunit. For orientation, portions of the collagen gel (cg) overlay (A) and the micropore filter (mf) support (E) are presented. Localization of gp135 (B) was confined to the slit-like luminal, or apical, membrane while the outer, or basolateral, membrane (arrowheads) was unstained. In contrast, the α_2 (C) and β_1 (F) subunits had a basolateral distribution, the α_6 subunit (E) primarily a basal distribution, and the α_3 subunit (D) a nonpolarized distribution including some intracellular staining. Bar, 8 μm.

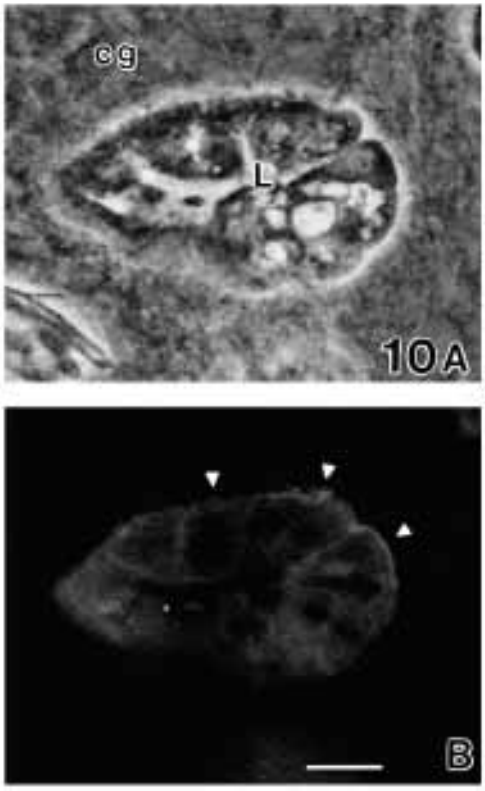


Fig. 10. Incubation of suspension cysts in collagen gel affects the distribution of the β_1 integrin subunit. Cysts were incubated in collagen gel (cg) for 8 hours at 37°C to initiate reversal of epithelial polarity. Corresponding phase-contrast (A) and immunofluorescence (B) micrographs of a frozen section demonstrated that mAb AIIB2 localized the β_1 subunit on the cyst outer membrane (arrowheads); however, staining of the luminal membrane (L) was observed on some cells. Bar, 8 μm.

(Fig. 6C), there was a considerable loss (~50%) in microvilli (Table 2). When these data were compared with those from cysts incubated in mAb AIIB2 (Table 2) these reductions in microvilli were determined to be statistically significant (P<0.0001). Therefore, these results demonstrate that mAb AIIB2 had an inhibitory effect on membrane remodeling and subsequent polarity reversal, suggesting that β_1 integrins play an important role in the regulation of epithelial polarity development.

Although loss of outer membrane apical microvilli appeared to be a good indicator of collagen-induced membrane remodeling, these experiments did not provide any information on changes in membrane composition. In our previous studies, the cell surface distribution of apical gp135 was utilized to qualitatively follow the temporal changes in epithelial polarity reversal (Wang et. al., 1990b). Here we utilized immunogold electron microscopy to quantitate gp135 levels on the outer membrane of cysts that had been incubated in collagen gel in either the presence or absence of mAb A11B2 for 6 hours. In control cysts, extensive membrane remodeling had occurred and gp135 could not be localized over large areas of outer membrane on most cysts (Fig. 13A). Also present in these samples are cysts in which the remodeling process was incomplete and small regions containing microvilli were present. In a typical example, gp135 was observed only on the microvilli and not on the adjacent membrane region of the same cell where remodeling had already occurred (Fig. 13B). In cysts that had been incubated in collagen gel containing mAb A11B2, gp135 was localized on outer membrane microvilli (Fig. 13C) demonstrating that AIIB2 prevented membrane remodeling. Quantitative immunogold electron microscopy demonstrated that there was a statistically (P<0.004) significant loss (~55%) in outer membrane gp135 on cysts incubated in collagen gel without mAb AIIB2 (Table 3). It is important to note that the reduction in outer membrane gp135 was consistent with that observed for microvilli loss (~50%) under equivalent incubation conditions (Table 2).

DISCUSSION

In a previous study, we demonstrated that incubation of suspension-grown MDCK cysts in collagen gel induced remodeling of apical and basolateral membranes, resulting in polarity reversal (Wang et al., 1990b). Although the specific nature of the membrane-collagen interactions was not analyzed, these results suggested the possibility that members of the integrin ECM receptor family were involved in the regulation of these events. In this report we present strong evidence that the interaction of β_1 integrins with collagen appears to be required for MDCK epithelial membrane remodeling and polarity reversal.

Immunoprecipitation and SDS-PAGE demonstrates that MDCK cells have the $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ and, probably, $\alpha_6\beta_4$ integrins on their cell surface. Although we have not determined the ligand specificity for these integrins, data from other workers has demonstrated that the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are capable of binding to multiple ECM components including collagen and laminin (Elices and Hemler, 1989; Albelda and Buck, 1990; Elices et al., 1991; Ruoslathi, 1991; Hynes, 1992) while $\alpha_6\beta_1$ and $\alpha_6\beta_4$ bind only laminin (Sonnenberg et al., 1988; Lee et al., 1992; Hynes, 1992). Although the $\alpha_6\beta_4$ integrin has been localized to keratinocyte hemidesmosomes (Stepp et al., 1990), these membrane specializations are not present in MDCK cells, leaving open the possibility that $\alpha_6\beta_4$ can also function as a non-junctional basal membrane laminin receptor in some epithelial cells. This suggestion is consistent with the observation that the α_6 integrin subunit in confluent MDCK monolayers and inverted cysts formed within collagen gel was localized primarily on the basal membrane contacting the substratum. However, such a proposal would require that basally secreted laminin (Boll et al., 1991) be incorporated into the collagen-coated micropore filter and collagen gel utilized in these experiments. The high level of cell and cyst binding to fibronectin in the presence of mAb A11B2 suggests that the $\alpha_v\beta_3$ integrin, which binds to both fibronectin and vitronectin (Cheresh and Spiro, 1987), is primarily responsible for MDCK-fibronectin interactions. Our data on the MDCK integrin profile are consistent with those presented in a preliminary report (Matlin et al., 1992) and suggest that the MDCK cell binding to ECM previously reported (Salas et al., 1987) was probably due to β_1 integrins. Finally, it

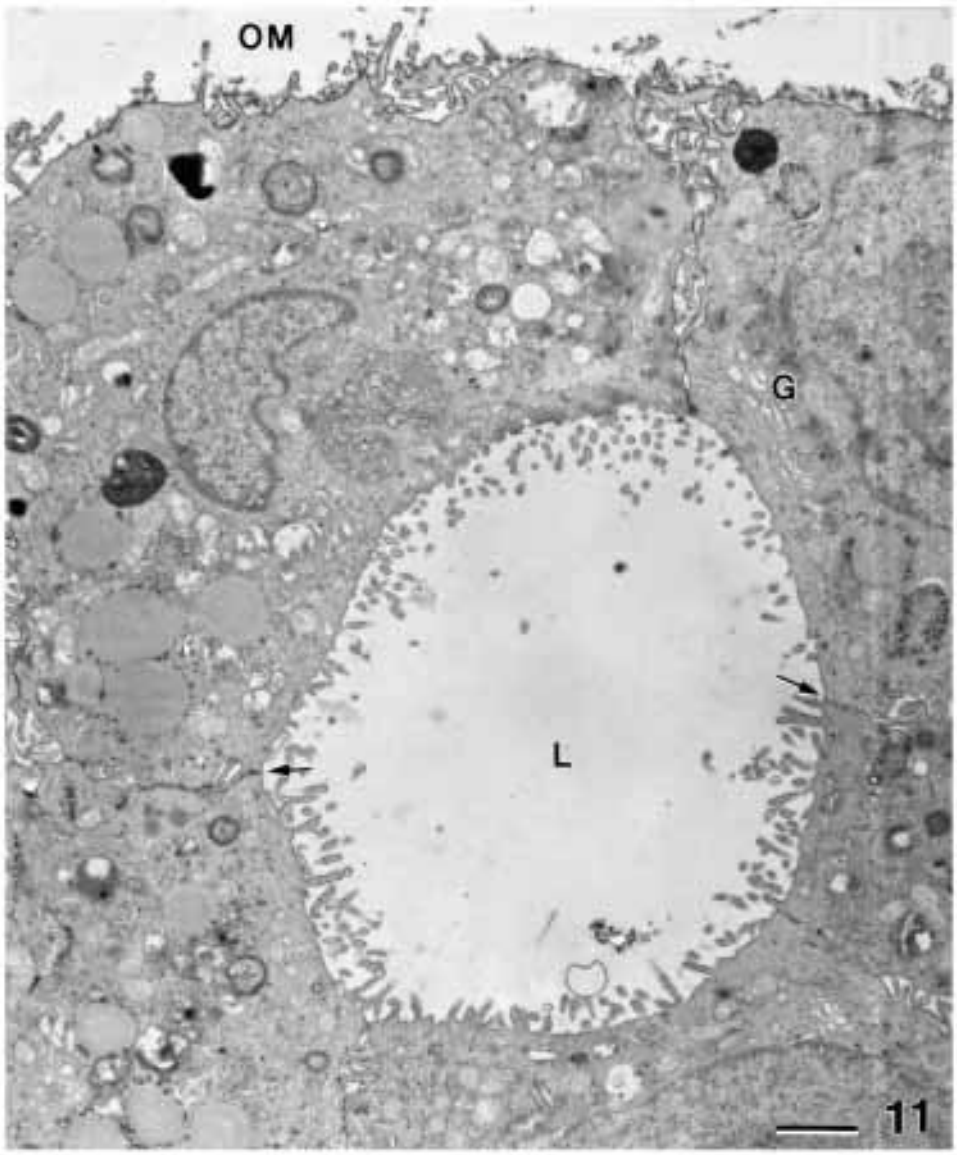


Fig. 11. The ultrastructure of collagen-mediated epithelial polarity reversal. Suspension cysts were incubated in collagen gel for 6 hours at 37°C, then prepared for thin sections. Remodeling of the outer membrane (OM) in contact with the collagen had occurred as few microvilli were observed. Microvilli were present on the cyst inner membrane lining the lumen (L) and tight junctions (arrows) appeared to be present. Also note that the Golgi apparatus (G) was oriented in a position between the nucleus and the luminal membrane. Bar, 1.17 μm.

should be noted that MDCK cells also have a non-integrin laminin receptor (Salas et al., 1992), which could contribute to the low level of laminin binding that still remains in the presence of mAb AIIB2.

Cell binding studies indicate that MDCK cells and cysts have receptors for types I and IV collagen, laminin and fibronectin. We have confirmed that these cell-substratum interactions are due to β_1 integrins by two different criteria. The antifunctional mAb A11B2 inhibited cell and cyst binding to both type IV collagen and laminin, providing conclusive evidence that β_1 integrins play an important role. These experiments are supported by data demonstrating that the GRGDSP peptide inhibited cell and cyst binding to ECM. Our morphological data demonstrating that mAb A11B2 can inhibit collagen-mediated remodeling of the MDCK cyst outer membrane and subsequent appearance of microvilli on the luminal membrane strongly suggest that β_1 integrins are involved in the regulation of epithelial polarity reversal. Although we have not identified which β_1 integrin(s) are involved, our observations that the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ collagen binding integrins (Elices and Hemler, 1989; Elices et al., 1991) are present on the cyst outer membrane make them excellent candidates. Further characterization of integrin-ECM interactions on MDCK cells will require the availability of integrin α subunit antifunctional mAbs, which recognize and inhibit the binding of canine integrins to ECM.

In studies on renal nephron formation, there is evidence that laminin A chain expression (Klein et al., 1988) and α_6 subunit interactions with laminin (Sorokin et al., 1990) are responsible for epithelial cell surface polarity development. However, it is important to point out that mAb GoH3 against the α_6 integrin subunit (Sonnenberg et al., 1988) only produced a partial inhibition of S-shaped body formation (Sorokin et al., 1990) and these workers did not demonstrate that polarized distributions of cell surface proteins were present. These data, those presented in this paper and the observation that type IV collagen expression coincides closely with nephron formation (Ekblom, 1989; Laurie et al., 1989), strongly suggest that a

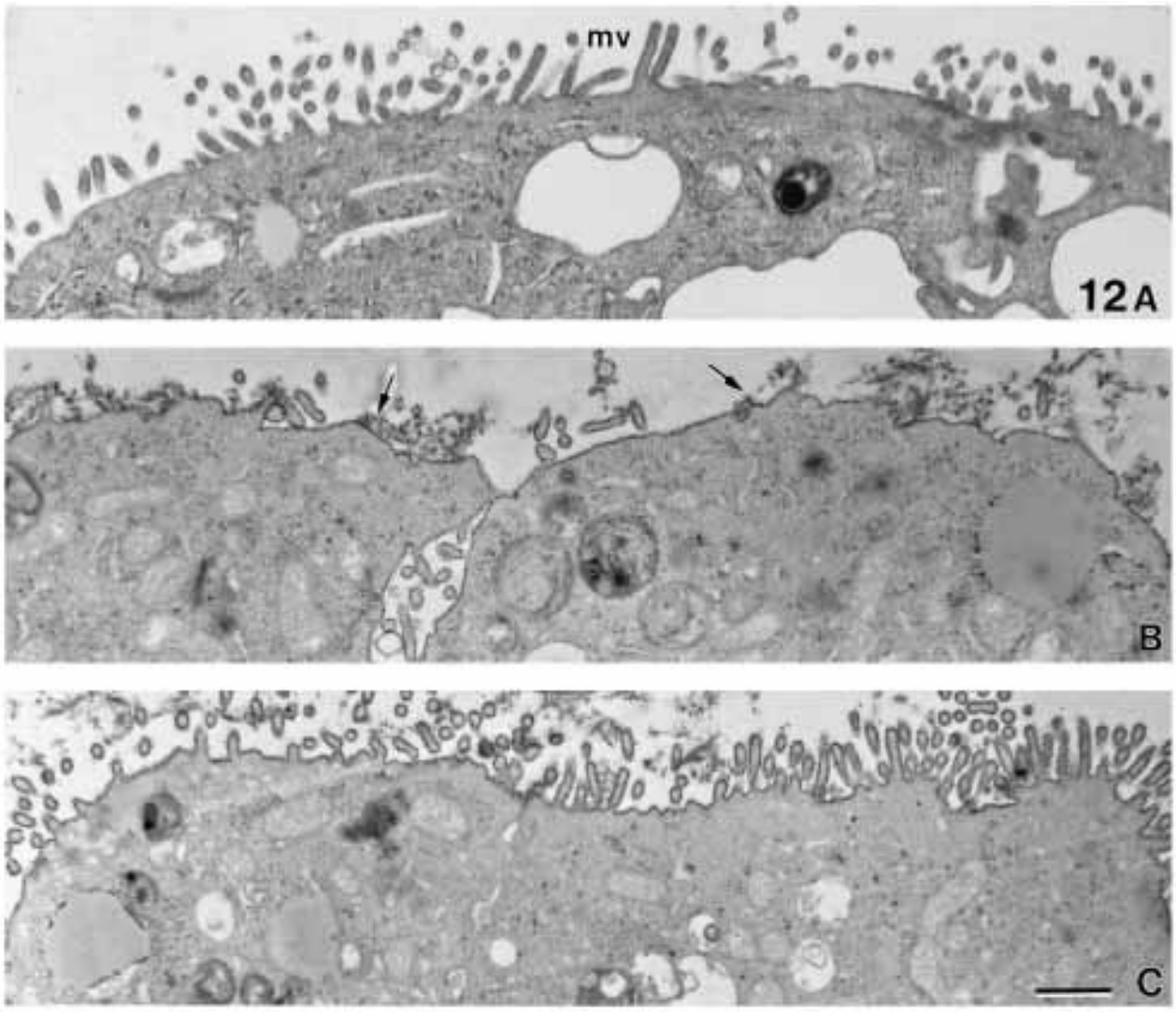


Fig. 12. Inhibition of epithelial remodeling by the antifunctional mAb AIIB2. Suspension cysts were either fixed (A) or incubated in collagen gel in the absence (B) or presence (C) of mAb AIIB2 for 6 hours at 37°C, then prepared for thin section electron microscopy. Numerous microvilli (mv) were observed on the outer membrane of control cysts (A) while cysts in collagen gel (arrows) had undergone outer membrane remodeling and had few microvilli (B). If mAb AIIB2 was included during the incubation (C), epithelial remodeling was inhibited and outer membrane microvilli appeared to be as numerous as in control samples (see Table 2 for quantitative data). Bar, 0.76 μm.

variety of ECM components and integrins, not just laminin and the α_6 integrin subunit, could be important in the development of epithelial cell surface polarity.

Since mAb A11B2 prevents MDCK cell and cyst binding and inhibits epithelial polarity reversal in collagen gels, it is reasonable to propose that this inhibition is due to prevention of β_1 integrin-collagen interactions. The mechanism by which this occurs has not been conclusively determined, since, although there appear to be considerable levels of the α_2 and α_3 integrin subunits on the cyst outer membrane, the β_1 integrin subunit is localized primarily to the basolateral membrane. Despite this, there appear to be sufficient levels of functional β_1 integrin heterodimers on the outer membrane available for cyst-collagen binding and initiation of polarity reversal. Although we do not at present know if formation of additional integrin α/β heterodimers is required for subsequent epithelial remodeling, this possibility seems likely, based on the observation that the β_1 integrin subunit was detected by immunofluorescence microscopy on the cyst outer membrane after incubation in collagen gel for 8 hours. This suggestion is supported by immunofluorescence data demonstrating that the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins are present on the outer membrane of collagen-grown inverted cysts. Since integrin α/β heterodimers are required for both binding ECM (Hemler, 1990; Hynes, 1992) and the formation of functional linkages to the actin cytoskeleton (Buck and Horwitz, 1987; Burridge et al., 1988; Otey et al., 1990), it seems likely that targeting of additional β_1 integrin subunits to the cyst outer membrane must occur after cyst-substratum interactions have been established. This proposal presents an interesting mechanistic consideration, since the β_1 integrin subunit is vectorially targeted to the basolateral membrane of MDCK mono-

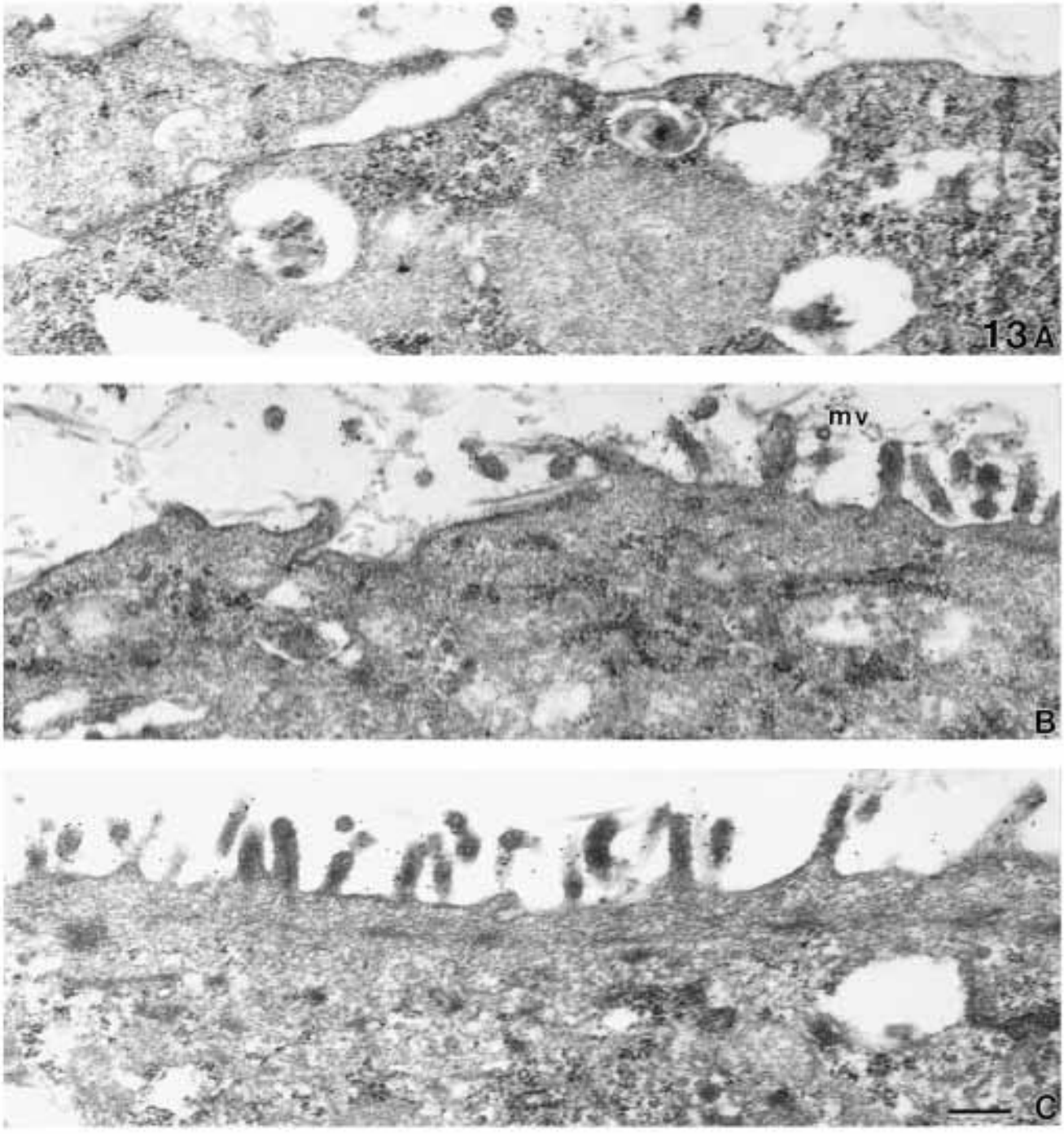


Fig. 13. Inhibition of collagen-mediated gp135 endocytosis by mAb AIIB2. Suspension cysts were incubated in collagen gel for 6 hours at 37°C in either the absence (A,B) or presence (C) of mAb AIIB2. After fixation, outer membrane gp135 was localized by immunogold electron microscopy. In membrane regions that have undergone extensive remodeling (A,B), immunogold labeling was absent, indicating that gp135 had been removed from the cell surface by endocytosis. In some cysts, membrane remodeling was incomplete (B) and adjacent regions of the same cell retaining microvilli (mv) stained positive for gp135. If mAb AIIB2 was present (C), then membrane remodeling was inhibited and numerous gp135-positive microvilli were present (see Table 3 for quantitative data). Bar, 0.26 μm.

layers grown on permeable filters (Boll et al., 1991). If it is assumed that identical vectorial targeting mechanisms are also operating in suspension cysts, then what factors are involved in establishing the retargeting of the β_1 integrin subunit to the cyst outer membrane? It is tempting to speculate that the formation of integrin-cytoskeletal linkages initiates a reorientation of intracellular targeting information, thereby providing new docking sites on the cyst outer membrane for vesicles that are responsible for transporting membrane proteins to the cell surface. This could be accomplished by a remodeling of the

Table 2. Quantitative measurement of cyst outer membrane microvilli levels after membrane remodeling in collagen gel

Treatment	Membrane length (μm/cell)	Microvilli/cell	Microvilli/μm
Suspension	6.59±0.82	26.0±3.32	3.95±0.32
mAb AIIB2	9.23±0.49	28.7±2.78	3.11±0.26
No mAb	9.29±0.99	15.2±2.10	1.64±0.13
mAb J1B5	11.1±0.80	18.1±2.28	1.63±0.14

Suspension-grown cysts were incubated within collagen gel in the absence (no mAb) or presence of either mAb AIIB2 or J1B5 for 6 hours at 37˚C. After processing for electron microscopy, the levels (mean±s.e.m.) of apical microvilli extending directly from the outer membrane (470.5 μm, 464.3 μm and 673.8 μm of membrane were measured for AIIB2, no mAb and J1B5 treatments, respectively) were counted directly from electron micrographs. For comparison, the microvilli of outer membrane (125.3 μm) on suspension cysts was measured. The differences in microvillar levels when either mAb J1B5-treated or untreated cysts were compared with mAb AIIB2-treated cysts were determined to be statistically significant by the 2-tailed *t*-test (P<0.00001 for mAb and mAb J1B5-treated and untreated cycts).

Table 3. Quantitative measurement of gp135 levels on suspension cysts after outer membrane remodeling

	Membrane length (μm/cell)	Streptavidin-gold (particles/cell)	Streptavidin-gold (particles/μm)
Control	7.37±0.86	15.7±4.73	2.13±0.56
mAb AIIB2	9.12±0.73	43.6±6.55	4.79±0.90

Suspension-grown cysts were incubated in collagen gel for 6 hours in either the presence or absence (Control) of mAb AIIB2. After fixation, gp135 was localized on the outer membrane (235.8 μm for Control, 200.6 μm for mAb AIIB2) by immunogold electron microscopy and Streptavidin-gold densities (mean±s.e.m.) were determined. Statistical analysis by the 2-tailed *t*-test determined that the differences in gp135 density on Control and mAb AIIB2 cysts were significant (P<0.004).

outer membrane cytoskeleton converting it from an apical cytoskeleton capable of interacting with membrane proteins such as gp135 (Ojakian and Schwimmer, 1988, 1992) to a basolateral cytoskeleton that forms complexes with Na^+,K^+-ATPase, fodrin and ankyrin (Nelson and Veshnock, 1987; Nelson, 1992).

The demonstration that the α_2 and α_6 integrin subunits have polarized, basolateral distributions on confluent MDCK monolayers and collagen-grown inverted cysts, but non-polarized cell surface distributions on suspension cysts suggests that ECM may be influencing the targeting of these epithelial membrane proteins. Based on immunofluorescence microscopy, it is clear that suspension-grown cysts are capable of generating polarized distributions of gp135, Na^+,K^+-ATPase and uvomorulin (Wang et al., 1990a) as well as the β_1 integrin subunit, demonstrating that the intracellular protein sorting and targeting mechanisms are functioning properly within these three-dimensional multicellular structures. One possible explanation for the basolateral distribution of the α_2 and α_6 integrin subunits on both cell monolayers and cysts within collagen gels is that the targeting of these subunits is influenced by the presence of an intact, immobilized ECM substratum, which serves as the functional equivalent of a basal lamina. This proposal is supported by recent data demonstrating that unstirred platelets in suspension become activated only after stirring allows integrin binding to an immobilized ECM (Fox et al., 1993). Although MDCK suspension cysts accumulate type IV collagen and laminin within the lumen (Wang et al., 1990a), there is no evidence that these ECM components can assemble into a functional basal lamina and allow normal cell attachment. Finally, we also have data demonstrating that suspension cysts incubated in agarose gels containing type I collagen, type IV collagen, fibronectin or laminin do not undergo epithelial polarity reversal (Ojakian and Schwimmer, unpublished results). Taken together, these observations strongly suggest that the presence of an immobilized ECM substratum appears to be essential for cyst attachment, membrane remodeling and epithelial polarity reversal.

There is biochemical data suggesting that integrin α subunits will accumulate within intracellular compartments and not reach the cell surface unless they form heterodimers with the β_1 subunit (Heino et al., 1989; Rosa and McEver, 1991). These data are not consistent with those demonstrating that non-polarized distributions were present for the α_2, α_3 and α_6 integrin subunits on MDCK suspension cysts and for the α_3 integrin subunit on monolayers and inverted cysts. Since the β_1 subunit distribution is primarily basolateral, it appears that the α_2, α_3 and α_6 subunits can have a plasma membrane localization even in the absence of heterodimer formation with the β_1 subunit. Our data showing that integrin α subunits have non-polarized cell surface distributions are consistent with those observed for the α_2 integrin subunit in both developing and adult human kidney (Korhonen et al., 1990), for the α_3 integrin subunit during human kidney development (Korhonen et al., 1990), and for the α_6 integrin subunit in mouse embryonic kidney (Sorokin et al., 1990). Since the α_3 integrin subunit has a non-polarized distribution on monolayers and inverted cysts, it is possible that our MDCK cell lines are deficient in components required for polarized sorting of this integrin or that polarity development occurs at a different rate than is observed for other membrane proteins (Wollner et al., 1992). In this regard, it is important to point out that the cell surface glycoprotein gp23 also has a non-polarized cell surface distribution on a variety of MDCK sublines (Ojakian et al., 1987; Herz and Ojakian, 1989). These observations suggest that MDCK cells may express a non-targeted form of the α_3 integrin subunit that is similar to that found in embryonic kidney (Korhonen et al., 1990). The localization of some α_3, but not β_1, subunit within the MDCK cytoplasm also supports the possibility that this accumulation constitutes an intracellular reserve that could be utilized during rapid tissue remodeling.

In addition to their well established role in cell-substratum adhesion, there is also evidence that β_1 integrins are involved in cell-cell adhesion as well. The $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins have been localized to the lateral membranes between adjacent keratinocytes (Larjava et al., 1990; Carter et al., 1990) and $\alpha_2\beta_1$ and $\alpha_5\beta_1$ to sites of endothelial cell-cell contact (Lampugnani et al., 1991). These data, and those demonstrating that either an RGD peptide or monoclonal antibodies against the α_2, α_3 or β_1 integrin subunits disrupted cell-cell contacts, have suggested that integrins also play an important role in epithelial cell interactions (Larjava et al., 1990; Carter et al., 1990; Lampugnani et al., 1991). Our observations that the $\alpha_2\beta_1$ and,

to a lesser extent, $\alpha_3\beta_1$ integrins are also localized to the lateral membranes between confluent MDCK monolayers is consistent with these data and suggest that β_1 integrins along with other cell adhesion molecules (Nelson, 1992; Rodriguez-Boulan and Powell, 1992) may be important in maintaining cell-cell contacts during epithelial polarity development.

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