

# An Antiglycolipid Antibody Inhibits Madin-Darby Canine Kidney Cell Adhesion to Laminin and Interferes with Basolateral Polarization and Tight Junction Formation

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**Abstract.** Epithelial cells polarize not only in response to cell–cell contacts, but also to contacts with a substratum composed of extracellular matrix molecules. To probe the role of specific matrix constituents in epithelial cell polarization, we investigated the effects of an adhesion-blocking mAb, 12B12, on initial polarization of MDCK cells. The 12B12 antibody, raised against whole MDCK cells, blocks adhesion to laminin by 65% but has no effect on adhesion of cells to collagen type I. Taking advantage of this antibody's function-blocking activity, as well as the fact that MDCK cells secrete laminin, the role of endogenous laminin in polarization was examined by plating cells on collagen-coated substrata in the presence of the antibody. Under these conditions, cell spreading was reduced 1.5 h after plating,

and cells were flatter and had fewer microvilli after 24 h. Even though lateral cell membranes were closely apposed, transepithelial resistance in the presence of the antibody was significantly reduced relative to controls. When the polarization of specific apical and basolateral markers was examined both biochemically and immunocytochemically in the presence of the antibody, we observed that the apical marker polarized at normal rates while basolateral markers did not. Surprisingly, the 12B12 antibody was not directed against any known cell adhesion protein but reacted specifically with Forssman antigen, a glycosphingolipid. These results suggest that glycolipids may play a significant role in cell adhesion via laminin and in epithelial cell polarization.

COMPLEXITY of organisms is made possible in part by epithelia, which organize the body by partitioning it structurally and functionally. To accomplish their many roles, epithelia are morphologically, biochemically, and functionally polarized. Simple epithelial cells have three discrete membrane domains: apical, lateral, and basal, each of which has a specific protein and lipid composition. Apical surfaces interact with the organism's exterior, regulating access to the interior body compartments. Lateral domains communicate with and adhere to neighboring cells by forming cell–cell junctions. The tight junction (*zonula occludens*) divides the apical domain from the basal and lateral ("basolateral") membrane domains (Matlin and Caplan, 1992), prevents intermixing of lipids (Dragsten et al., 1981; van Meer and Simons, 1986; Gumbiner, 1993) and (most likely) proteins (Gumbiner, 1993), and unites cells to form a barricade to passive ex-

change via the paracellular pathway. Basal domains interact with the substratum, usually a basement membrane, which consists of extracellular matrix (ECM)<sup>1</sup> proteins such as laminin (LN), collagen IV, and heparan sulfate proteoglycans.

The biogenesis of the polarized phenotype is a complex multistep process that is contingent on many extracellular cues that exert specific effects on polarization (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989; Matlin and Caplan, 1992; Eaton and Simons, 1995). These extracellular cues include cell–substratum and cell–cell interactions. In the polarized MDCK cell line, cell–substratum interactions are necessary to establish the apical pole (Vega-Salas et al., 1987). Basolateral proteins localize only after cell–cell contacts are allowed to form, suggesting that cell–cell contacts are necessary for basolateral polarization

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1. *Abbreviations used in this paper:* CI, collagen type I; ECM, extracellular matrix; L-gln, L-glutamine; LN, laminin; PAP, peroxidase anti-peroxidase; PBS<sup>+</sup>, PBS containing 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>; PBS<sup>-</sup>, PBS without CaCl<sub>2</sub> or MgCl<sub>2</sub>; PLP, periodate-lysine-paraformaldehyde fixative.

(Vega-Salas et al., 1987). Further studies of MDCK cells cultured as cysts in suspension have also elucidated the importance of different cell contacts. Cell-cell contacts define "free" surfaces from those in physical contact with other cells, but cell polarity is believed to be consolidated upon ECM deposition into the cyst lumen (Wang et al., 1990b). Although each type of contact exerts specific effects, the sum of these signals culminates in the polarized phenotype.

Proteins that participate in cell-cell interactions, such as E-cadherin, have been intensively studied (McNeil et al., 1990; McNeil and Nelson, 1992; Hodiola and Watt, 1994). The molecules that mediate cell-substratum interactions, however, have been examined less thoroughly for their role in epithelial polarity. While epithelial cells in vivo principally interact with ECM in the form of a basement membrane, cells in vitro interact with artificial substrata that may be coated with serum factors and other medium constituents that may themselves affect cell growth and adhesion. In addition, the substratum itself may influence cellular characteristics because of its impermeability or electrical charge. For example, MDCK cells cultured on permeable supports are able to import nutrients from the basal surface, as they do in vivo, which results in a "more" polarized phenotype than when cultured on plastic (Simons and Fuller, 1985). The cells themselves may secrete components onto the substratum; for example, MDCK cells in culture synthesize and basally secrete components of the basement membrane, including LN, heparan sulfate proteoglycans, and possibly collagen type IV (Caplan et al., 1987; Wang et al., 1990b; Boll et al., 1991; Taub, 1991; Ecay and Valentich, 1992).

The response of MDCK cells to ECM can be dramatic, affecting cell polarity, morphology, and behavior. When apical membranes of MDCK cysts in suspension contact collagen type I (CI) gels, the cells "reverse" their polarity, forming apical domains that face the lumen and basal domains at the cell-CI interface (Wang et al., 1990a; Ojakian and Schwimmer, 1994). When MDCK cells, whether subconfluent and on plastic (Hall et al., 1982) or confluent and on permeable supports (Zuk and Matlin, 1996), are overlaid with CI, the cells respond first by reorganizing into a bilayer and then a tubulocyst. During bilayer development, apical proteins are absent from cell surfaces and basolateral proteins are randomly distributed on plasma membranes. After lumen formation, apical markers are again expressed on free surfaces, and basolateral proteins relocate to cell-cell and cell-substratum contacts (Zuk et al., 1996). Further evidence that the ECM modulates MDCK polarity, morphology, and behavior is provided by culturing explants of MDCK cells on CI gels. When cells contact the CI matrix, they break away from the explant, exchange their apical-basal polarity for front-end/back-end polarity, become fusiform in shape, and migrate over the matrix (Zuk et al., 1989).

Although CI can dramatically modify epithelial cell morphology, polarity, and behavior, even more fundamental cues for cell differentiation and polarization are provided by LN. LN-1 (Burgeson et al., 1994) is a large (1,000-kD) molecule with a cruciform structure (Timpl et al., 1979) that influences cell adhesion, spreading, growth, and migration (reviewed in Yamada, 1991; Tryggvason, 1993;

Timpl and Brown, 1994). LN is also necessary for the differentiation and maintenance of epithelia (Klein et al., 1988; Ekblom et al., 1990; Sorokin et al., 1990, 1992). If cell-LN interactions are perturbed during the mesenchymal-epithelial transformation in renal development, either by anti-LN or anti-LN receptor antibodies, the formation of an epithelium is inhibited (Klein et al., 1988; Sorokin et al., 1990, 1992).

While LN can affect differentiation, its specific role in MDCK cell polarity has not been directly addressed. We have been investigating the role of the ECM in the biogenesis of epithelial cell polarity using the MDCK cell line as a model system (Schoenenberger et al., 1994; Zuk et al., 1989; Zuk and Matlin, 1996). We hypothesize that LN, which is synthesized and secreted by MDCK cells, provides essential extracellular cues that polarize MDCK cells in culture. To test our hypothesis, we raised mAbs to whole MDCK cells and selected one that immunolabeled cell membranes and specifically inhibited MDCK cell adhesion to LN but not to CI. Cells treated with this antibody delay tight junction formation and the polarization of a basolateral marker but not of an apical marker. Although we have previously reported that MDCK cells adhere to ECM via the integrins (Schoenenberger et al., 1994), a superfamily of cell-surface receptors (Hynes, 1992), the antibody is directed against a neutral glycosphingolipid, the Forssman antigen.

## Materials and Methods

### Cell Culture

MDCK II HD cells were used (passages 7-33; Matlin and Simons, 1984). The suffix "HD" (Heidelberg) designates the place where this substrain was originally cloned (Louvard, 1980); this substrain has been extensively used in previous studies (see Matlin et al., 1981; Matlin and Simons, 1983; Schoenenberger et al., 1994; for review see Simons and Fuller, 1985). The clarification of the MDCK II substrain has recently become an important point since different substrains occasionally yield contradictory results (see Discussion and Mays et al., 1995). The cells were grown in Dulbecco's modified minimal essential media (DMEM; Mediatech, Herndon, VA) in 5% FBS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (L-gln), and 10 mM Hepes, pH 7.4, at 37°C/5% CO<sub>2</sub>. Confluent cultures were subcultured twice a week at a 1:5 dilution with trypsin-EDTA (GIBCO BRL, Gaithersburg, MD).

In most experiments, cells were cultured on Transwell polycarbonate permeable supports (0.4 μm pore size, 12 mm diameter; Costar, Cambridge, MA). Cells released by trypsin-EDTA were seeded onto prewetted permeable supports at a density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> and were fed every 2-3 d with growth medium (DMEM with 5% FBS, 10 mM Hepes, pH 7.4, 2 mM L-gln, and 100 U/ml penicillin-100 μg/ml ampicillin-0.25 μg/ml amphotericin B [antibiotics/antimycotic from GIBCO BRL]).

### Antibodies

**Production of Anti-MDCK Adhesion Antibodies.** To study LN's role in MDCK cell polarization, function-blocking rat mAbs were raised (Harlow and Lane, 1988) against whole MDCK cells that had been nonenzymatically harvested. The resulting hybridoma supernatants were screened for their ability to stain MDCK cell membranes by immunofluorescence and for their ability to interfere with MDCK cell adhesion to Matrigel, an LN-containing, basement membrane-like preparation from EHS sarcomas (Collaborative Research Inc., Bedford, MA).

MDCK cells were subcultured at a 1:5 dilution using trypsin-EDTA 24-30 h before use. For the initial immunization of 22-d-old CD (caesarian derived) rats (Charles River Laboratories, Wilmington, MA), cells were harvested from the subconfluent cultures by rinsing twice with PBS<sup>-</sup> (PBS

that does not contain  $\text{CaCl}_2$  or  $\text{MgCl}_2$ ), once with 4 mM EDTA/1 mM EGTA in  $\text{PBS}^-$ , and incubating for 40 min with EDTA/EGTA/ $\text{PBS}^-$  at  $37^\circ\text{C}/5\% \text{CO}_2$ . The cells were centrifuged for 6 min at 1,000 rpm at  $4^\circ\text{C}$  and resuspended in media modified for suspension cultures (S-MEM; GIBCO BRL) containing 2 mM L-gln, 10 mM Hepes, pH 7.4, and antibiotics/antimycotic. Harvested cells ( $5 \times 10^6$ ) were emulsified in complete Freund's adjuvant (GIBCO BRL) and injected intraperitoneally. A booster injection of  $1.5 \times 10^7$  cells emulsified in incomplete Freund's adjuvant (GIBCO BRL) was administered 21 d later. After 1 mo, a second booster injection was given. Sera from tail bleeds were tested for their ability to prevent MDCK cells from adhering to Matrigel when compared to preimmune bleeds (using the adhesion assay in Schoenenberger et al., 1994; see below) and by their reaction with MDCK cells by indirect immunofluorescence (according to Schoenenberger et al., 1991, and Zuk et al., 1989). Spleenocytes were fused to murine NS-1 myeloma cells (American Type Culture Collection, Rockville, MD). Hybridomas were selected with HAT (hypoxanthine, aminopterin, thymidine) media and were grown in DMEM supplemented with 20% FBS (hybridoma tested), 0.015% oxalacetate, 0.005% pyruvate, 0.00082% bovine insulin 2 mM L-gln, 10 mM Hepes, pH 7.4, and antibiotics/antimycotic. Hybridomas were cloned in soft agar. Culture supernatants were screened as for the sera (see above). Clone 12B12, which reduced MDCK cell adhesion to Matrigel and immunolabeled MDCK cell membranes by immunofluorescence, was selected for further study. The 12B12 mAb is an IgG based on SDS-PAGE analysis of radiolabeled hybridoma supernatants. For routine culture, the 12B12 hybridoma was grown in DMEM with 10% FBS (complement inactivated by incubating at  $56^\circ\text{C}$  for 30–45 min), 10 mM Hepes, pH 7.4, 2 mM L-gln, and antibiotics/antimycotic.

**Antibodies to Apical and Basolateral Antigens.** The antiapical gp135 mAb producing hybridoma (3F21D8) and supernatant (Ojakian and Schwimmer, 1988) were supplied by Dr. George Ojakian (SUNY, Brooklyn, NY). The antibasolateral p58 mAb, 6.23.3, has been described elsewhere (Balcarova-Ständer et al., 1984). The antibasolateral E-cadherin (uvomorulin) mAb (rr1, [Gumbiner and Simons, 1986]) was obtained from the Developmental Biology Hybridoma Bank (University of Iowa, Iowa City, IA). The anti-ZO-1 hybridoma (R40.76; Anderson et al., 1988) was supplied by Dr. Lynn Jesaitis (Harvard Medical School, Boston, MA). This rat mAb recognizes an intracellular epitope of the ZO-1 protein. The anti-Forsman antigen mAb, 33B12 (Sonnenberg et al., 1986), was from Dr. Arnold Sonnenberg (Netherlands Cancer Institute, Amsterdam, The Netherlands).

### Adhesion Assay

To identify antiadhesion mAbs, the same colorimetric adhesion assay described previously was used (Schoenenberger et al., 1994). In brief, MDCK cells in suspension were treated with hybridoma supernatants at a 1:1 dilution before plating onto ECM-coated 96-well plates (Immulon 2; Dynatech Laboratories, Chantilly, VA) at  $10^5$  cells/well. After 1.5 h incubation, nonadherent cells were washed away with  $\text{PBS}^+$  ( $\text{PBS}$  containing 1 mM  $\text{CaCl}_2$  and 0.5 mM  $\text{MgCl}_2$ ), and adherent cells were fixed, stained, and quantitated with a microplate reader (Molecular Devices, Menlo Park, CA). Adhesion was calculated by subtracting the background of coated wells to which no cells had been added from the values that were obtained from coated wells with cells.

To visualize the mAb's effects on cell spreading, the same procedure was followed, except that 16-well Nunc chambers on glass slides (Nunc Inc., Naperville, IL) replaced the 96-well plates. Adherent cells were washed with  $\text{PBS}^+$  and fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA)/75 mM lysine/10 mM sodium-*m*-periodate in 37.5 mM phosphate buffer (PLP; McLeane and Nakane, 1974) for 20–30 min at  $24^\circ\text{C}$  (room temperature). The cells were then mounted in 50% glycerol/ $\text{PBS}^-$  and viewed with Nomarski optics on an Axioplan microscope (Carl Zeiss, Inc., Thornwood, NY). Images were recorded on T-MAX or Technical Pan film at ASA 100 (Eastman Kodak Co., Rochester, NY).

### Immunocytochemistry

Immunoperoxidase staining (Zuk and Hay, 1994) was used to localize apical and basolateral markers during the polarization assay (see below). Cells cultured on permeable supports were fixed in PLP and permeabilized for 4 min with 0.1% Triton X-100/ $\text{PBS}^-$ . Endogenous peroxidase was quenched with 1%  $\text{H}_2\text{O}_2$  (Fisher Scientific, Fair Lawn, NJ)/ $\text{PBS}^-$  for 10–15 min. After blocking with 10% normal goat serum/ $\text{PBS}^-$ , the permeable supports were incubated with 25–35  $\mu\text{l}$  of antibody basally and 50–

100  $\mu\text{l}$  antibody apically for 1 h at room temperature or overnight at  $4^\circ\text{C}$  in a humidified chamber. After incubations with the appropriate link antibody (goat anti-mouse or goat anti-rat, both human and mouse IgG adsorbed; Cappel-Organon Teknika, Durham, NC) diluted 1:10 in blocking solution, followed by incubation with peroxidase-antiperoxidase (PAP) antibodies (mouse PAP [Sigma Immunochemicals, St. Louis, MO] at 1:100 or rat PAP [Calbiochem; San Diego, CA] at 1:300, both diluted in blocking buffer), the peroxidase was reacted with a fresh solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) dissolved in 0.01%  $\text{H}_2\text{O}_2$  in 50 mM Tris, pH 7.5. Some cultures were postfixed with 1%  $\text{OsO}_4$  (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer, pH 7.4. Cells were dehydrated in a series of ethanols (50% [vol/vol], 75%, and 95%) at  $-20^\circ\text{C}$ , rinsed three times in 100% room temperature ethanol, and incubated 20–30 min at room temperature ( $22\text{--}24^\circ\text{C}$ ) in fresh 100% ethanol. Polycarbonate membranes were excised from their plastic supports and infiltrated successively with 100% propylene oxide (Polysciences, Warrington, PA), 50% propylene oxide/50% plastic (dodecylsuccinic anhydride [DDSA]-Poly/Bed 812-Araldite 6005 [2.4:1:1] with 2% 2,4,6-Tri[*N,N*-dimethylaminomethyl] phenol [DMP-30] [Polysciences]), and then 100% plastic. The cultures were embedded by polymerizing the plastic overnight at  $60^\circ\text{C}$ . Sections (0.5–1  $\mu\text{m}$ ) were cut on an ultramicrotome (Reichert-Jung, Vienna, Austria) and stained with 0.05% toluidine blue in 0.1% sodium borate before viewing them on a Zeiss Axioplan microscope. Images were photographed with Technical Pan film at ASA 100.

### Polarization Assay

Polarization of MDCK cells plated onto ECM-coated permeable supports was assessed biochemically by domain-specific biotinylation (Gottardi and Caplan, 1992) of antigens that are normally found on the apical and basolateral domains in fully-polarized cells. Signal was detected by probing blots of immunoprecipitated proteins with streptavidin. Tight junction formation was monitored by recording electrical resistance.

**Preparation of ECM-coated Permeable Supports.** 12-mm diameter Costar Transwell polycarbonate permeable supports were coated with either 10  $\mu\text{g}/\text{cm}^2$  of CI (from rat tail,  $\geq 85\%$  pure; Upstate Biotechnologies, Inc., Lake Placid, NY) in  $\text{PBS}^-$  or 20  $\mu\text{g}/\text{cm}^2$  of LN (LN-1; purified from EHS tumors,  $\geq 90\%$  pure; Upstate Biotechnologies, Inc.) in 0.1 M sodium bicarbonate, pH 8.3, in 200  $\mu\text{l}$ /permeable support. After incubation at  $37^\circ\text{C}/5\% \text{CO}_2/100\%$  humidity for 2 h, nonspecific binding sites were blocked with 500  $\mu\text{l}$ /permeable support of 1% heat-treated BSA/ $\text{PBS}^-$  at  $4^\circ\text{C}$  for 2 h to overnight. The filters were then washed twice with cold  $\text{PBS}^+$ . For experiments without function-blocking antibodies, 250  $\mu\text{l}$  of 1% heat-treated BSA/serum-free growth medium was added to the apical compartment, and 1.5 ml of serum-free growth medium (without BSA) was added to the basal compartment. For experiments that used hybridoma supernatants, 250  $\mu\text{l}$  of 50% supernatant/1% BSA/49% serum-free growth medium was added to the apical compartment and 1.5 ml of 50% supernatant/50% serum-free growth medium was added to the basal compartment.

**Preparation and Plating of MDCK Cells.** MDCK cells that had been subcultured at a 1:5 dilution with trypsin-EDTA 24–30 h before were nonenzymatically detached as described in "Production of Anti-MDCK Adhesion Antibodies." A suspension of  $1.4 \times 10^6$  trypan-blue excluding cells/ml was diluted in S-MEM, and  $3.5 \times 10^5$  cells (250  $\mu\text{l}$ ) were plated into the apical compartments of the prepared permeable supports and incubated at  $37^\circ\text{C}/5\% \text{CO}_2/100\%$  humidity. Cultures were fed 2–3 h after plating with prewarmed growth medium. For experiments that examined antibody perturbations, cells were diluted to  $2.8 \times 10^6$  cells/ml in S-MEM and then diluted 1:1 with hybridoma supernatants and incubated 30 min on ice before plating  $3.5 \times 10^5$  cells (250  $\mu\text{l}$ )/permeable support. These cultures were not fed after plating.

**Measurements.** To ascertain functional cell-cell junction formation, we measured electrical resistance of the developing monolayers using an epithelial voltohmmeter (World Precision Instruments, Inc., Sarasota, FL). Cultures were equilibrated at room temperature for 10 min before measurements were taken; coated permeable supports without cells were used for background readings.

Cell-surface biotinylation (Gottardi and Caplan, 1992) of marker proteins was used to assess polarization of the developing monolayers. Cultures were washed three times with cold  $\text{PBS}^+$  on ice and once with 10 mM triethanolamine, pH 9.0/150 mM NaCl/2 mM  $\text{CaCl}_2/0.5$  mM  $\text{MgCl}_2$ . All subsequent steps were carried out at  $4^\circ\text{C}$ , unless otherwise stated. Surface proteins were selectively biotinylated with 0.5 mg/ml sulfo-succinimidobiotin (sulfo-NHS-biotin; Pierce Chemical Co., Rockford, IL) in triethanolamine buffer as described by Gottardi and Caplan (1992). After biotinylation, the cells were washed twice with cell culture medium

and then twice with PBS<sup>+</sup>. If the cultures had been treated with antifunctional antibodies, bound antibody was stripped from the cell surface by washing three times for 5 min with 50 mM sodium borate, pH 11/2 mM CaCl<sub>2</sub>/0.5 mM MgCl<sub>2</sub> on ice. The cultures were washed twice with PBS<sup>+</sup> before removing them from their supports and extracting them in 0.5% sodium deoxycholate (added fresh)/1% Triton X-100/0.5% SDS/20 mM Tris, pH 7.5/150 mM NaCl/2 mM EDTA/10 mM L-gly with the following protease inhibitors: 0.2 mg/ml iodoacetamide, 200 mM PMSF, 10 μg/ml aprotinin, 17.5 μg/ml benzamide, 1 μg/ml antipain, 1 μg/ml pepstatin. Extracts were filtered through Centrax 0.45-μm cellulose acetate filter units (Schleicher & Schuell, Inc., Keene, NH). After immunoprecipitating overnight, the samples were incubated with washed protein A–trisacryl beads (Pierce) for 1–2 h with rotation. Bound antibody–antigen complexes were washed three times with 0.5% sodium deoxycholate/1% Triton X-100/0.5% SDS/20 mM Tris, pH 7.5/500 mM NaCl/2 mM EDTA, and once with 10 mM Tris, pH 8.6. Samples were solubilized in 200 mM Tris, pH 8.8/5 mM EDTA/0.1% bromophenol blue/12% glycerol/20 mM DTT (Fisher Scientific) at 95°C for 3–5 min. After alkylation with 0.1 M iodoacetamide at 37°C for 15–30 min, the proteins were resolved by 6% SDS-PAGE at 200 V (Laemmli, 1970).

**Visualization of Biotinylated Proteins.** According to the method of Lisanti et al. (1989), gels were equilibrated in transfer buffer (20% methanol (Fisher Scientific), 39 mM glycine, 0.038% SDS, and 48 mM Tris base) for 10–15 min. A semidry blotting device (Bio Rad Laboratories, Hercules, CA) was used to blot proteins for 30 min at 90 mA to polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking nonspecific binding sites with 3% BSA/1% nonfat milk in TGG buffer (1 M D-glucose [Mallinckrodt, Paris, KY]/10% glycerol [J.T. Baker, Inc., Phillipsburg, NJ]/0.5% polyoxyethylene-sorbitan monolaurate [Tween 20]) for 1 h, the blots were washed with 0.5% Tween 20/PBS and then probed with 80 ng/ml streptavidin-HRP (Pierce) for 20 min. The membranes were washed four times for 5 min with 0.5% Tween 20/PBS<sup>-</sup> before reacting the HRP with Renaissance chemiluminescence reagents (DuPont, Wilmington, DE). The membranes were exposed to Reflection autoradiography film (DuPont) and developed in a Kodak X-OMAT apparatus.

### Blotting of MDCK Glycolipids

Lipids were extracted and blotted as previously described (van Genderen et al., 1991). Briefly, cellular lipids were extracted in a single phase (Rose and Oklander, 1965) and resolved by TLC on aluminum-backed silica sheets (SG 60; Merck, Darmstadt, Germany) that had been prerun in the resolving solvent (chloroform/methanol/0.22% CaCl<sub>2</sub>, 60:35:8 [vol/vol]). After developing the TLC sheets for 45 min, they were dried with a hair dryer for 30 min. The chromatograms were then incubated in 0.4% polyisobutyl methylacrylate (300,000 mol wt; Aldrich, Brussels, Belgium) in hexane for 1 min. After drying, nonspecific binding sites were blocked

with 5% nonfat milk in 50 mM Tris, pH 7.8/2 mM CaCl<sub>2</sub> for 30 min. The blots were incubated with hybridoma supernatants with agitation for 1 h and washed four times for 1 min with 5% nonfat milk in 50 mM Tris, pH 7.8/2 mM CaCl<sub>2</sub>. After incubating for 1 h with <sup>125</sup>I-protein A, blots were washed as before and then washed twice in ice-cold PBS<sup>-</sup>. The TLC sheets were air-dried and exposed to x-ray film at -70°C (Magnani et al., 1987).

Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO).

## Results

### The Kinetics of MDCK Cell Polarization Are Similar on CI and LN

As a first step toward understanding how the ECM can influence MDCK cell polarity, we assayed the kinetics of polarization in cells plated onto LN- and CI-coated permeable substrata. Cells were only able to interact with the coating ECM, since nonspecific binding sites were blocked with BSA. We plated MDCK cells at confluent density and used domain-specific biotinylation of an apical and basolateral marker protein to assess cell polarity. Because differential labeling of cell membrane domains depends on functional tight junctions to prevent the biotin reagent leaking from one compartment to the other, we also measured electrical resistance in the developing monolayers. Biotinylated proteins were detected by probing blots of immunoprecipitated markers with streptavidin-HRP and chemiluminescence.

The polarization kinetics of an apical marker, gp135, were determined first. Acquisition of electrical resistance is shown in Fig. 1 A. Between 1.5 and 6 h, the cells exhibited a resistance of 25 Ω·cm<sup>2</sup>; after 12 h, resistance increased sharply to 350 Ω·cm<sup>2</sup>. Regardless of matrix, trends of resistance in six trials were similar, although resistance tended to be slightly lower in cells on LN-coated permeable supports (data not shown).

Biochemical results are shown in Fig. 1 B. A band corresponding to gp135 was detected at lower levels in cells on

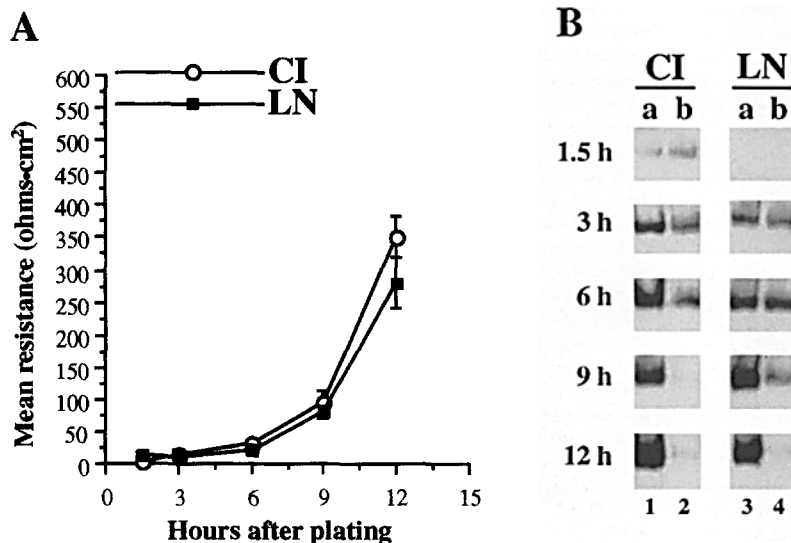


Figure 1. Polarization assay of an apical surface membrane marker, gp135. Restriction to the apical domain occurs within 12 h after plating MDCK cells onto CI or LN. Filter supports were coated with 10 μg/cm<sup>2</sup> of CI or 20 μg/cm<sup>2</sup> of LN before blocking nonspecific binding sites with heat-treated BSA. Cells were then plated at confluent density. (A) Resistance of cells plated onto CI (○) or LN (■) was recorded during the polarization assay represented in B to monitor tight junction formation. Little to no resistance is detected from 1.5 to 6 h; at 9 h, resistance increases to 100 Ω·cm<sup>2</sup>, and at 12 h, to 275 (LN) and to 350 (CI) Ω·cm<sup>2</sup>. Electrical resistance of cells on CI or LN are similar. Values represent mean ± SD. (B) At the indicated time points, a sulfo-NHS-biotin solution was applied to the apical (a) or basolateral (b) compartment of the filter chambers. Biotinylated cells were extracted, the gp135 protein was immunoprecipitated, resolved by SDS-PAGE, and transferred to membranes.

Biorelated proteins were detected with streptavidin-HRP complex and chemiluminescent substrates. The marker is differentially labeled at 3 h in cells plated on CI (lanes 1 a and 2 b) and at 9 h in cells plated on LN (lanes 3 a and 4 b). The marker appears fully polarized by 9 h on CI and by 12 h on LN.

LN-coated permeable supports (Fig. 1 *B*, lanes 3 and 4) when compared to CI-coated supports at 1.5 h (Fig. 1 *B*, lanes 1 and 2), but signal strength for the cells on LN quickly attained that of cells on CI at 3 h (Fig. 1 *B*, compare lanes 3 and 4 to lanes 1 and 2). At 3 h, more signal was seen apically than basally in cells on CI (Fig. 1 *B*, lanes 1 and 2), while the signal in cells on LN was approximately the same in both apically and basally biotinylated cultures (Fig. 1 *B*, lanes 3 and 4). Between 9 and 12 h, the signal in cultures on CI was detected almost exclusively apically (Fig. 1 *B*, lane 1). This observation indicated that the marker protein was localized to the apical surface at this time. In monolayers that initially interacted with LN-coated substrata, however, the marker signal was not detected primarily in apically derivatized cultures until 9 h (Fig. 1 *B*, lanes 3 and 4), and it was detected only in apically biotinylated cells at 12 h (Fig. 1 *B*, lanes 3 and 4), indicating an apical localization for this protein. Even though this particular experiment indicated that cells plated onto CI-coated substrata polarize this marker more quickly than cells on LN, the 9-h time point was not easily reproducible. Overall, the data indicate that gp135 polarizes within 9–12 h, regardless of substratum.

We also examined the polarization kinetics of a basolateral marker, E-cadherin. At 9 h, the first time point when polarization of basolateral markers begins (see below), the resistance of cells on CI reached  $150 \Omega \cdot \text{cm}^2$  (Fig. 2 *A*, open circles), while cells on LN lagged at  $50 \Omega \cdot \text{cm}^2$  (Fig. 2 *A*, closed squares). At 15 h, regardless of substratum, monolayer resistance peaked at  $\sim 525 \Omega \cdot \text{cm}^2$  and subsequently declined. Trends were similar in a total of three trials.

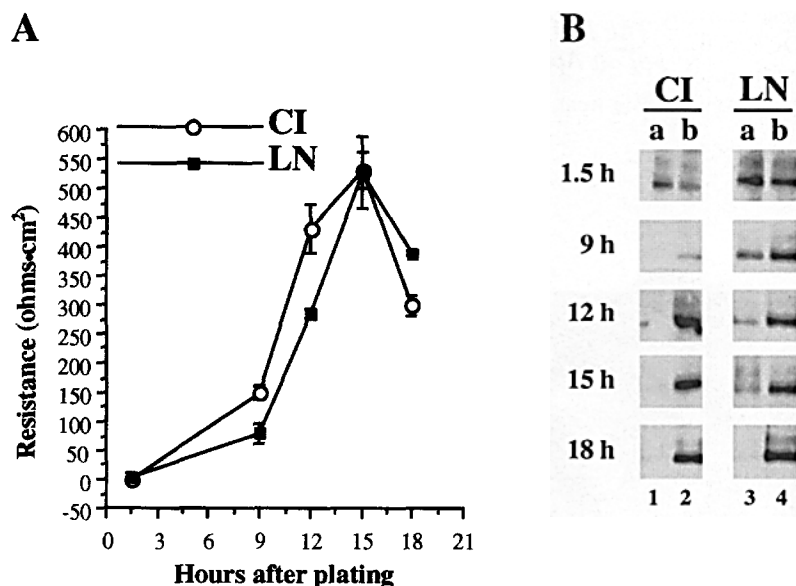
When the basolateral marker was assayed biochemically, little difference was observed between cells that initially interacted with LN or CI. Between 1.5 and 9 h, distribution of the marker was not polarized, regardless of coating matrix (data not shown). At 9 h, the cells on CI have mostly polarized the marker (Fig. 2 *B*, compare lanes 1 and 2). Cells plated onto LN began to restrict the basolateral marker at 9 h (Fig. 2 *B*, lanes 3 and 4); at 12 h, a more

asymmetric labeling was seen. Cells interacting with exogenous LN required more time than those on CI to maximally polarize the marker, between 15 and 18 h (Fig. 2 *B*, compare lanes 3 and 4 to lanes 1 and 2). Based on three trials, our data suggest that cells interacting with CI require 15 h to polarize the basolateral E-cadherin protein, while cells on LN require 15–18 h.

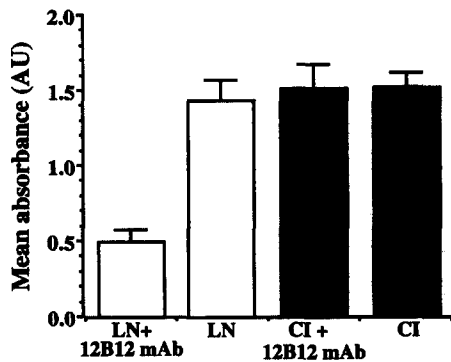
In summary, when MDCK cells were plated at confluent density onto LN- and CI-coated substrata, an apical marker polarized more rapidly than a basolateral marker, regardless of ECM coating. The apical marker, gp135, localized within 9–12 h, while the basolateral marker, E-cadherin, required 15–18 h. Electrical resistance, a measure of functional tight junctions, became measurable between 3 and 6 h after plating. These resistance profiles are similar to those observed by Cerejido et al. (1978) and Matlin and Simons (1984). Resistance of  $25\text{--}75 \Omega \cdot \text{cm}^2$  correlated with consistent asymmetric biotinylation seen between 6 and 9 h (Fig. 1 *B*) in all polarization assays of the apical marker, indicating that at this time, monolayers were impermeable to the labeling reagent.

### The 12B12 mAb Inhibits MDCK Cell Adhesion to Laminin but Not to Collagen Type I

To test the role of LN in MDCK cell polarization, a function-blocking mAb, 12B12, was generated by injecting whole MDCK cells into rats. In an adhesion assay, this antibody inhibited cell adhesion to Matrigel, a matrix resembling basement membranes (see Materials and Methods for details). Because Matrigel is rich in LN ( $\sim 60\%$ ; Collaborative Biomedical product literature), we wished to determine if 12B12 specifically inhibited MDCK cell adhesion to LN. MDCK cells were challenged to adhere to LN or CI in the presence of the antibody. After 1.5 h of incubation, MDCK cell adhesion to CI was unaffected by the antibody when compared to controls with no antibody (Fig. 3, solid bars). In contrast, the antibody reduced adhesion to LN to 34.6% of controls (Fig. 3, open bars), indicat-



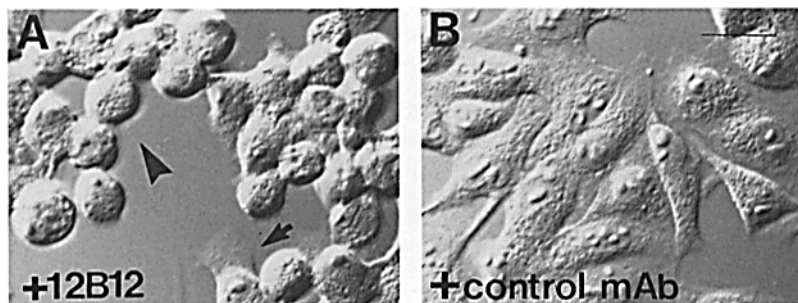
**Figure 2.** Polarization assay of a basolateral marker, E-cadherin. Restriction to the basolateral domain occurs within 18 h after plating MDCK cells onto CI or LN. MDCK cells were plated onto filter supports coated with  $10 \mu\text{g}/\text{cm}^2$  of CI or  $20 \mu\text{g}/\text{cm}^2$  of LN and processed as described in Fig. 1 and in Materials and Methods. (A) Electrical resistance of cells plated onto CI (○) and LN (■) was recorded during the polarization assay represented in *B* to monitor tight junction formation. At 1.5 h, resistance is nominal for both plating conditions. At 9 h, resistance has increased to  $150 \Omega \cdot \text{cm}^2$  (CI) and to  $75 \Omega \cdot \text{cm}^2$  (LN). Resistance peaks at 15 h for both matrices then declines. Resistance of cells on CI or LN are similar. Values represent mean  $\pm$  SD. (B) The polarization kinetics of basolateral E-cadherin. E-cadherin is differentially biotinylated by 9 h in cells on CI (lanes 1 and 2) and slightly so for cells on LN (lanes 3 and 4). At 15 h, the marker appears fully polarized in cells on CI whereas it requires 18 h to attain a similar distribution in cells that initially contact LN.



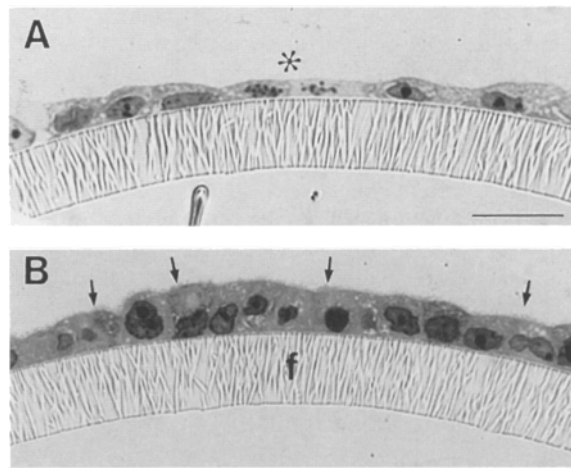
**Figure 3.** The 12B12 mAb specifically blocks the adhesion of MDCK cells to LN but not to CI. Plates (96 wells) were coated with 3  $\mu\text{g}/\text{well}$  LN or CI in the presence or absence of the 12B12 hybridoma supernatant diluted 1:1 with serum-free media. Cells were incubated for 30 min before plating with or without the 12B12 mAb (1 vol of cells to 1 vol of supernatant). At 1.5 h after plating, nonadherent cells were washed away. The remaining cells were fixed, stained with crystal violet, and quantitated with a microplate reader. In the presence of 12B12, cell adhesion to LN is reduced to 34.6% of controls (*open bars*), but adhesion to CI is unaffected (*solid bars*). Values represent mean  $\pm$  SE of three experiments. AU, arbitrary units.

ing that it specifically interfered with MDCK cell adhesion to this molecule.

MDCK cells synthesize and secrete their own LN (Caplan et al., 1987; Wang et al., 1990b; Boll et al., 1991; Taub, 1991; Ecay and Valentich, 1992; Zinkl, G.M. and K.S. Matlin, unpublished observation). Given that this antibody specifically inhibited adhesion to LN, we hypothesized that the 12B12 mAb would also interfere with the cells' ability to interact with endogenously secreted LN. To test if the 12B12 mAb interferes with MDCK cell–endogenous LN interactions, the morphology of cells plated at confluent density onto CI in the presence of the antibody was examined. MDCK cells treated with the 12B12 mAb or a control rat mAb, R40.76 (which recognizes an intracellular epitope of ZO-1, a tight junction protein), were plated onto CI-coated tissue culture chamber glass slides and allowed to adhere for 1.5 h. Cells adhered under these conditions, since the 12B12 mAb has no effect on MDCK cell adhesion to CI (see Fig. 3). Most cells treated with the 12B12 mAb (Fig. 4 A) exhibited a rounded morphology



**Figure 4.** The 12B12 mAb reduces spreading of MDCK cells on CI. Tissue culture chamber glass slides were coated with 3  $\mu\text{g}/\text{well}$  CI, and non-specific sites were blocked with BSA. After a 30-min incubation with antibody supernatants (1 vol cells to 1 vol supernatant), cells were plated into the wells with 12B12 mAb or R40.76 (anti-ZO-1), a control rat mAb, diluted 1:1 with serum-free media. Nonadherent cells were washed away 1.5 h after plating. The remaining cells were fixed and viewed using Nomarski optics. (A) MDCK cells incubated with the 12B12 mAb show a rounded morphology (*arrowhead*); some cells have begun to spread (*arrow*). (B) Cells treated with the control mAb are well spread and flatter. Bar, 10  $\mu\text{m}$ .

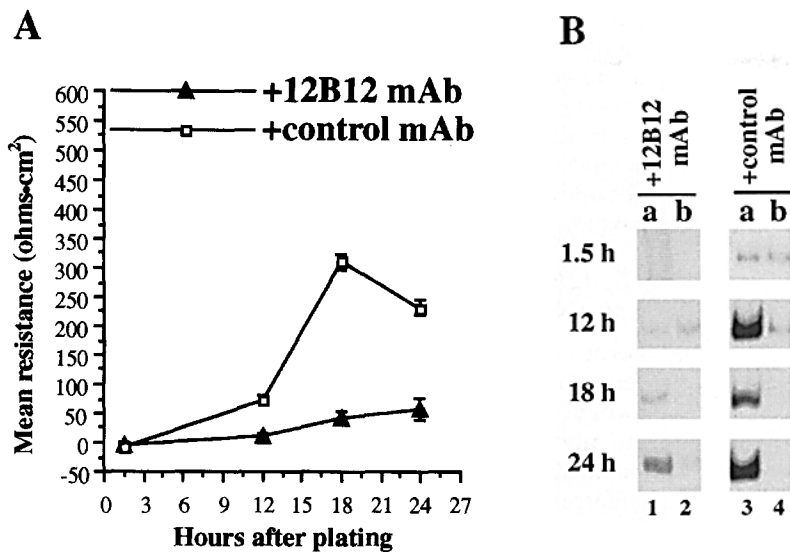


**Figure 5.** The 12B12 mAb alters cell morphology. Cells were prepared with 12B12 and R40.76 mAbs as described in Fig. 4, but were plated onto CI-coated filters at confluent density. Filters were embedded in plastic, sections (0.5  $\mu\text{m}$ ) were cut, and cells were stained with 1% toluidine blue. When the cells are grown for 24 h with 12B12 (A), cells are flat and have few microvilli. In contrast, cells treated with the control mAb (B) are cuboidal-shaped, and microvilli are well developed (*arrows*). Cell density is higher in control cultures (B) than those treated with 12B12 (A). Note the mitotic figures (\*) in A. f, filter. Bar, 20  $\mu\text{m}$ .

(*arrowhead*); only a few cells have begun to spread (*arrow*). In contrast, cells treated with a control rat mAb were flat and well spread (Fig. 4 B). Cells plated at confluent density onto CI-coated permeable supports and grown in the presence of the 12B12 mAb for 24 h formed a confluent monolayer, although some clumps of cells were observed (not shown) (Fig. 5 A). The cells were flatter, had fewer microvilli, and less well-developed lateral borders when compared to cells cultured in the presence of the control mAb (Fig. 5 B). Control cells were cuboidal shaped, had well-developed microvilli (Fig. 5 B, *arrows*), and well-defined lateral borders. Overall, the monolayer appeared more dense with the control antibody (compare  $\sim 16$  nuclei in Fig. 5 B to 8 nuclei in Fig. 5 A).

#### The 12B12 mAb Does Not Affect the Polarization of an Apical Marker

We next asked if disrupting cell–endogenous LN interac-



Control cells maintain the polarized distribution of the marker for the remainder of the experiment (lanes 3 and 4). At 12 h, the 12B12 mAb-treated cells polarize the marker, and at 24 h, signal strength increases; the cells have maintained an asymmetric distribution of the marker. Despite the low resistance of the 12B12 mAb-treated monolayers (A), differential labeling of the apical gp135 marker is observed at 18 and 24 h when resistance is 25 and 50  $\Omega\text{-cm}^2$ , respectively.

tions with this antibody also affected the biogenesis of MDCK cell polarity. MDCK cells were plated at confluent density onto CI-coated permeable supports in the presence of the 12B12 mAb or the control rat mAb R40.76 (anti-ZO-1). To verify that tight junctions formed in 12B12 mAb-treated cultures, transmonolayer electrical resistance was measured. As indicated in Fig. 6 A, resistance of 12B12 mAb-treated monolayers (*closed triangles*) did not parallel that of the control cells (*open squares*); resistance was reduced at all time points. Monolayers treated with the 12B12 mAb achieved a maximum resistance of 50  $\Omega\text{-cm}^2$  at 24 h, while control monolayers peaked and diminished to 250  $\Omega\text{-cm}^2$ . Similar trends were observed in three separate trials.

When polarization of apical gp135 was assayed under these conditions by biotinylation, a low signal was detected in 12B12 mAb-treated cultures at all time points when compared to controls (Fig. 6 B, compare lanes 1 and 2 with lanes 3 and 4). At 12 h, the control cells localized the marker to the apical surface as expected (Fig. 6 B, lanes 3 and 4; see also Fig. 1 B, lanes 1 and 2). In cells treated with the 12B12 mAb, however, the antigen was detected almost equally on the apical as well as the basal surfaces (Fig. 6 B, lanes 1 and 2) at 12 h. At 18 and 24 h, 12B12 mAb-treated cultures apically restricted the marker (Fig. 6 B, lanes 1 and 2), as did the control antibody cultures (Fig. 6 B, lanes 3 and 4).

Because the 12B12 mAb reduced the electrical resistance of the monolayer during these experiments, it was possible that the apparent lack of polarization of gp135 at 12 h (see Fig. 6 B, lanes 1 and 2) was caused by "leaky" monolayers. To verify that gp135 was randomly distributed on the cell surface at 12 h, cells were plated in the presence of 12B12 or control mAbs onto CI-coated permeable supports as done for the polarization assay, but were fixed at 12 h and the gp135 marker was immunolocalized by the PAP technique. When treated with the 12B12 mAb, the apical marker

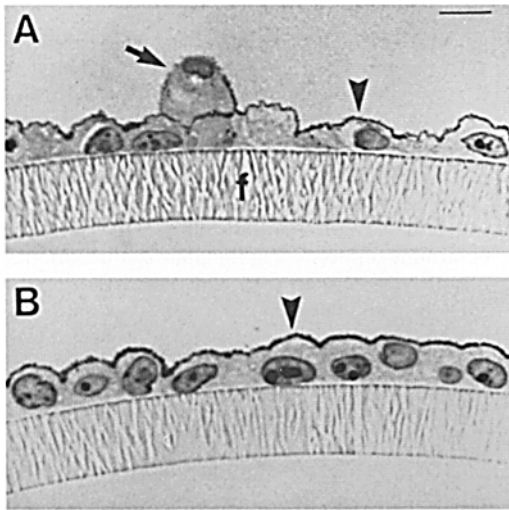
localized exclusively to the apical cell surface (Fig. 7 A, *arrowhead*) in a monolayer of cells, as did controls (Fig. 7 B, *arrowhead*). Based on these results, 12B12 does not inhibit polarization of gp135, even though it does perturb electrical resistance.

#### The 12B12 mAb Prevents Polarization of a Basolateral Marker

To examine the effects of the 12B12 mAb on localization of basolateral membrane proteins, we repeated the biochemical assay of cell polarization for the basolateral marker, E-cadherin. We assayed later time points (24 and 48 h) to assure that the cells had ample opportunity to polarize basolateral surface proteins. Electrical resistance of 12B12 mAb-treated monolayers exhibited a gradual increase that was much lower than controls until 48 h (Fig. 8 A, *closed triangles*). In control mAb-treated cells, resistance peaked at 24 h and decreased at 48 h (Fig. 8 A, *open squares*).

When examined biochemically, the basolateral marker protein was equally labeled in the apical and basal compartments in the presence of the 12B12 mAb at 1.5, 24, and 48 h after plating (Fig. 8 B, lanes 1 and 2). In control mAb-treated cells, the marker polarized by 24 h (Fig. 8 B, lanes 3 and 4), as expected (see Fig. 2 B, lanes 1 and 2); the cells maintained this distribution through 48 h. Unlike 12B12 mAb-treated cultures examined for the apical antigen, where signal strength was much less than controls (see Fig. 6), signal strength was comparable to that of controls for this marker.

To confirm that the 12B12 mAb disrupted basolateral polarity, we immunolocalized a basolateral antigen during the polarization assay. Cells treated with the 12B12 and control R40.76 mAbs were plated onto CI-coated permeable supports at confluent density. Because an anti-E-cadherin antibody that yielded consistent PAP staining was unavailable, another basolateral antigen, p58, was used.



**Figure 7.** The apical gp135 marker localizes to the apical cell surface at 12 h in MDCK cells treated with 12B12. Light micrograph of a 0.5- $\mu\text{m}$  section of cells that were immunostained by PAP for gp135 and counterstained with 0.1% toluidine blue to visualize nuclei. (A) To determine the localization of the apical gp135 membrane marker in cells treated with the 12B12 mAb, MDCK cells were plated onto filter supports coated with 10  $\mu\text{g}/\text{cm}^2$  of CI in the presence of the 12B12 mAb. Nonspecific binding sites were blocked with BSA. The marker was immunolocalized by PAP; localization is indicated by a dense precipitate, the product of the peroxidase reaction. The marker localizes to the apical cell surface (arrowhead). Some clumping of cells is observed where the signal is randomized (arrow). (B) Control cultures were treated with a control rat mAb (R40.76, anti-ZO-1) and then stained for gp135 with PAP. The apical marker localizes to the apical cell surface at 12 h after plating. *f*, filter; Bar, 10  $\mu\text{m}$ .

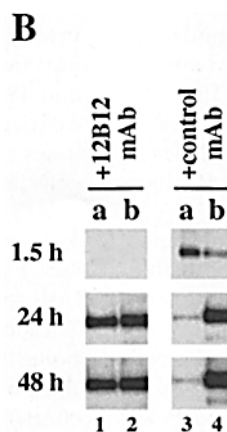
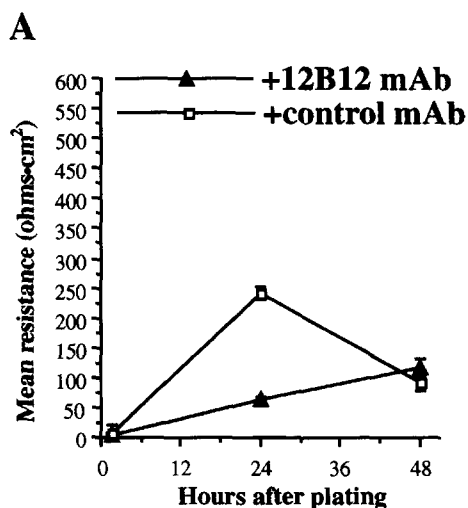
At 24 h, 12B12 mAb-treated cells (Fig. 9 A) were immunostained with the anti-p58 antibody. Clear staining of the apical (Fig. 9 A, arrowhead) and lateral surfaces (arrows) was observed, with some possible basal staining. Even though signal strength was low in these experiments, it was

much higher than background controls (Fig. 9 B), which omitted the anti-p58 antibody. In control mAb-treated monolayers, p58 was localized to the basolateral surface as expected at 24 h (Fig. 9 C).

Overall, these results demonstrate that if cell-LN interactions were perturbed by the 12B12 mAb, basolateral but not apical polarity was delayed. In addition, the antibody perturbed electrical resistance.

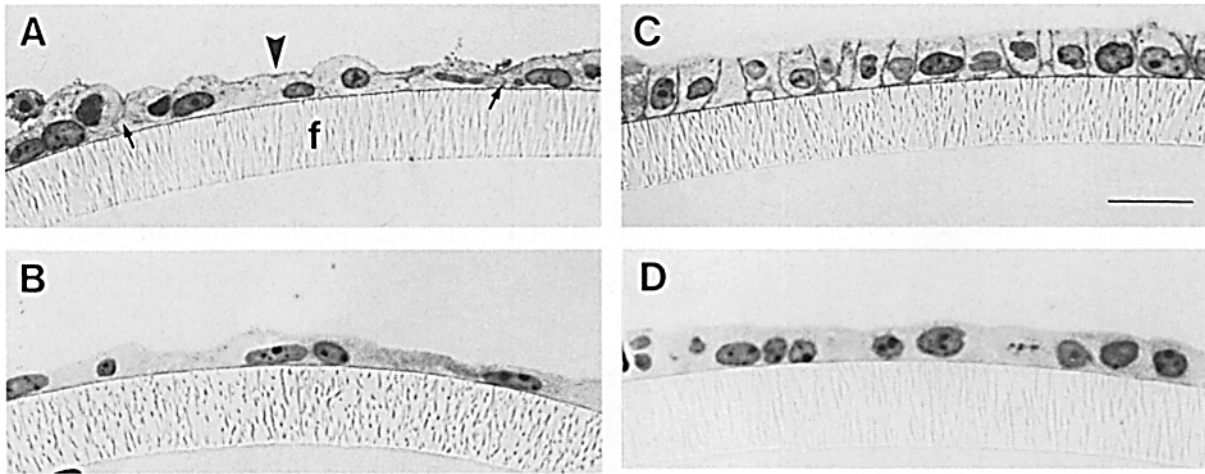
### The 12B12 mAb Recognizes a Glycolipid of the Apical Cell Surface

Because the 12B12 mAb specifically interfered with MDCK cell adhesion to LN, we hypothesized that the antibody recognized an integrin LN receptor. Previous work from our laboratory showed that MDCK cell attachment to LN was mediated by the  $\beta 1$  family of integrin receptors (Schoenberger et al., 1994). Nevertheless, multiple attempts to immunoprecipitate and immunoblot a protein antigen of 12B12 mAb under a variety of conditions were unsuccessful. When MDCK lipids were extracted and resolved on TLC plates and then reacted with the 12B12 mAb, however, a band was identified (Fig. 10, lane 2) that comigrated with and had a similar shape as a band that reacted with an authentic anti-Forsman antigen mAb (Fig. 10, lane 4, arrowhead). To confirm that 12B12 mAb recognized Forsman antigen, a strain of MDCK cells that does not express Forsman antigen, MDCK I (a high resistance clone), was used (Hansson et al., 1986; Nichols et al., 1986b; Hansson, 1988). MDCK I lipids were extracted as before and probed with the 12B12 mAb and the anti-Forsman antigen mAb. Consistent with previous reports, no MDCK I lipids reacted with the anti-Forsman antigen mAb (Fig. 10, lane 3); likewise, the 12B12 mAb also failed to yield a signal (lane 1). Comparable data were obtained in a separate TLC solvent system (chloroform/methanol/water 60:35:8 [vol/vol]), which separates Forsman glycolipid from sulfatide, a second lipid in MDCK II cells that is absent from MDCK I (Hansson et al., 1986). This supports the conclusion that the 12B12 mAb recognized Forsman



**Figure 8.** Polarization of E-cadherin in MDCK cells treated with the 12B12 mAb is perturbed. (A) Cells treated with 12B12 mAb gradually acquire electrical resistance ( $\blacktriangle$ ) whereas the control mAb has little effect ( $\square$ ). At 24 h, 12B12 mAb-treated cells acquire a resistance of  $\sim 50 \Omega\text{-cm}^2$ , while the resistance of control cells peaks and then declines to  $100 \Omega\text{-cm}^2$  at 48 h. The 12B12 mAb-treated monolayers reach a resistance of  $150 \Omega\text{-cm}^2$  at 48 h. Values represent mean  $\pm$  SD. (B) To determine differences in the polarization kinetics of E-cadherin in cells treated with the 12B12 mAb, MDCK cells were plated onto filter supports coated with 10  $\mu\text{g}/\text{cm}^2$  of CI as previously described. Whereas the marker is well polarized at 24 and 48 h after plating in controls (+control mAb, lanes 3 and 4), it is not basolaterally segregated in cells treated with the 12B12 mAb (+12B12 mAb, lanes 1 and 2). Note that despite the resistance of the 12B12 mAb-treated monolayers at 24 and 48 h, the E-cadherin marker is not differentially labeled (lanes 1 and 2).





**Figure 9.** Light micrographs of 0.5- $\mu\text{m}$  sections of MDCK cells that are stained for p58 with PAP and counterstained with 0.1% toluidine blue to visualize nuclei. The p58 basolateral marker is randomly expressed on cell surfaces 24 h in MDCK cells cultured with the 12B12 mAb. (A) To determine the localization of the basolateral p58 membrane marker in cells treated with the 12B12 mAb, MDCK cells were plated onto filter supports coated with 10  $\mu\text{g}/\text{cm}^2$  CI in the presence of the 12B12 mAb and processed as described in Fig. 7 and in Materials and Methods. Despite low expression of this marker, signal is found on the apical (arrowhead) and lateral (arrows) cell surfaces. Cells are in apposition, forming a monolayer. Apical surfaces appear “rounded.” (B) Background staining for A in which the primary antibody was omitted from 12B12 mAb-treated cultures. (C) In cultures treated with a control mAb, R40.76 (anti-ZO-1), the basolateral marker p58 is localized exclusively to the basolateral cell surface at 24 h after plating. The cells are cuboidal, with “even” apical membranes, and are taller than their 12B12 mAb-treated (A) counterparts. (D) Background staining for C in which the primary antibody was omitted from R40.76-treated cultures. *f*, filter; Bar, 20  $\mu\text{m}$ .

antigen and not another comigrating lipid; a different clone of MDCK II cells (Mays et al., 1995) also gave similar results (data not shown). In addition, the 12B12 mAb does not affect MDCK I adhesion to or cell spreading on CI or LN (data not shown).

Because previous immunofluorescence studies suggested that surface-expressed Forssman glycolipid in MDCK cells localizes principally to the apical membrane domain (Hansson et al., 1986; Butor et al., 1991), we immunostained for the 12B12 mAb antigen in polarized MDCK monolayers. The 12B12 mAb antigen was localized on nonpermeabilized cells by PAP immunostaining. A strong signal was detected apically (Fig. 11 A, arrowhead), while some signal was detected on lateral cell surfaces (arrow). We also localized the 12B12 mAb antigen at early times during the polarization assay when the antibody disrupts adhesion to LN. MDCK cells that were plated onto CI-coated permeable supports were immunostained 1.5 h later. A randomized plasma membrane distribution of the glycolipid was seen (Fig. 11 B); thus, the glycolipid has the potential to interact with the substratum soon after cell plating.

## Discussion

The results we report here indicate that an antibody that interferes with the adhesion of MDCK cells to LN is able to specifically disrupt the polarization of basolateral proteins and also perturbs the establishment of functional tight junctions. Surprisingly, this antibody reacts with a prominent glycolipid, the Forssman antigen.

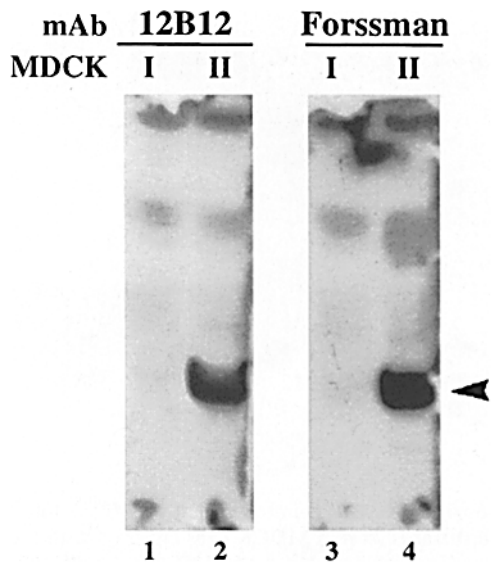
### *The 12B12 mAb Is Likely to Perturb Endogenous LN-MDCK Cell Interactions*

To study LN's contribution to MDCK cell polarization, we

took advantage of the cells' ability to synthesize their own ECM and to adhere to exogenous ECM. First, in culture, MDCK cells synthesize and secrete LN (Caplan et al., 1987; Wang et al., 1990b; Boll et al., 1991; Taub, 1991; Ecay and Valentich, 1992; Zinkl, G.M., and K.S. Matlin, unpublished observation). Second, while the cells may depend in part on LN for adhesion to a substratum, they are competent to adhere to other ECM molecules, such as CI (Schoenenberger et al., 1994). Thus, we are able to perturb MDCK cell-endogenous LN interactions in cells that are attached to a CI substratum. In this way, we can analyze the effects of inhibiting LN-cell interactions on MDCK cell polarization.

To interfere with MDCK cell-LN interactions, we used the 12B12 mAb, which prevents attachment to exogenous LN but not to CI. While our data do not directly demonstrate that 12B12 perturbs MDCK cell interactions with endogenous LN, two pieces of evidence support this contention. First, 12B12 specifically inhibits MDCK cell adhesion to exogenous LN. Second, the antibody reduces cell spreading early in culture. Because LN not only mediates adhesion, but also promotes cell spreading (Tryggvason, 1993; Timpl and Brown, 1994), these results suggest that the cells do not spread because they cannot interact with endogenous LN. It is unclear, however, whether the glycolipid exerts its effect by binding directly to LN or by some other mechanism.

By perturbing cell-LN interactions, the 12B12 mAb disrupts basolateral but not apical surface polarity. When MDCK cells are treated with 12B12 and plated at confluent density onto CI-coated supports, an apical antigen polarizes by 12 h, as did controls. While the results from the biochemical polarization assay suggested that gp135 is detected apically and basally in the presence of the 12B12 mAb, this observation was not confirmed by immunoper-

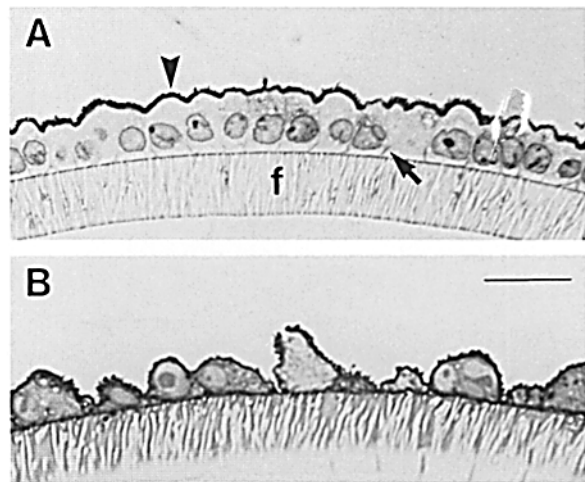


**Figure 10.** The 12B12 mAb recognizes a glycolipid on MDCK II cells that comigrates with and has a similar band shape as Forssman antigen. Lipids from MDCK strain I (I, lanes 1 and 3) or strain II (II, lanes 2 and 4), the strain used in this study, were extracted and resolved on TLC plates (resolving solvent, chloroform/methanol/0.22% CaCl<sub>2</sub> 60:35:8 [vol/vol]); origin, bottom; top, solvent front. Lipids were reacted with the 12B12 mAb (lanes 1 and 2) or an anti-Forssman antigen mAb (lanes 3 and 4) and protein A conjugated to <sup>125</sup>I. A band is seen in lane 2 which comigrates with the anti-Forssman antigen mAb reactive band seen in lane 4 (arrowhead). Because MDCK strain I does not express the Forssman antigen, strain I glycolipids fail to react with the anti-Forssman antigen mAb (lane 3); strain I lipids also fail to react with the 12B12 mAb (lane 1).

oxidase experiments. The contradictory biochemical results were most likely caused by leaky monolayers, an effect that the antibody exerts on tight junction formation, particularly at early time points. However, polarization of a basolateral antigen is significantly retarded when cells are cultured with the antibody, an observation confirmed both biochemically and immunocytochemically. Not only does 12B12 specifically inhibit basolateral polarization in MDCK cells, but it also deters functional tight junction formation. These results mimic those studies that find polarization of apical markers is established without tight junctions, which are, however, required for polarization of basolateral markers (Vega-Salas et al., 1987).

#### **Nature of the 12B12 mAb's Antigen and Its Effects on MDCK Cells**

The 12B12 mAb recognizes a glycosphingolipid of MDCK cells, the Forssman antigen (Hansson et al., 1986; Nichols et al., 1986a,b; van Meer et al., 1986). We base this conclusion on blots of MDCK cell lipids in which the 12B12 mAb recognizes a band that comigrates with a band that reacts with an authentic anti-Forssman antigen mAb. Previous work by Hansson et al. (1986) used mass spectrometric analysis to confirm that the band that reacted with the authentic anti-Forssman antigen is the Forssman glycolipid. Additionally, the 12B12 mAb neither reacts with lipids from the MDCK I cell line, which does not express the



**Figure 11.** The 12B12 mAb localizes its antigen to the apical cell surface in confluent MDCK monolayers (A) and is randomly distributed 1.5 h after plating (B). (A) To localize the 12B12 mAb antigen in confluent MDCK monolayers, cells were grown on uncoated filter supports for 1 wk. After fixation, they were stained with the 12B12 mAb using the PAP technique and the nuclei were counterstained with 0.1% toluidine blue. An intense dark precipitate is found on the apical cell surface (arrowhead), while very weak staining is seen on the lateral surface (arrow). (B) To understand how the 12B12 mAb could exert an effect on cell adhesion, its antigen was localized at 1.5 h after plating MDCK cells onto supports coated with 10 μg/cm<sup>2</sup> CI and nonspecific binding sites blocked with heat-treated BSA. The PAP signal is found over the entire cell surface, indicating a randomized distribution of the 12B12 mAb antigen. *f*, filter; Bar, 20 μm.

Forssman antigen (Hansson et al., 1986; Nichols et al., 1986b; Hansson, 1988) nor does this antibody affect adhesion or cell spreading on LN or CI. Although a Forssman epitope has been found on a few glycoproteins (Mori et al., 1986; Kijimoto-Ochiai et al., 1990), we do not believe that a glycoprotein with this epitope exists in MDCK II HD cells. We fail to immunoprecipitate and immunoblot proteins from MDCK II HD cells with 12B12. Although glycolipid polarity in epithelia is well documented (van Meer, 1989), it is unlikely that a particular sugar modification would be found only on apical proteins. The Forssman glycolipid has been used as a stage-specific marker for teratocarcinoma (Stern et al., 1978) and embryonic cells (Willison and Stern, 1978). The glycolipid consists of five sugar groups linked to the C-1 of ceramide (*N*-acetylgalactosaminyl-(α1-3)-*N*-acetylgalactosaminyl-(β1-4)-galactosyl-(α1-4)-galactosyl-(β1-4)-glucosyl-(β1-1)-ceramide) (Siddiqui and Hakomori, 1971). While some glycolipids have been suggested to bind ECM proteins (Cheresh et al., 1986; Hakomori, 1990; Kalb and Engel, 1991; Mecham, 1991; Jungalwala, 1994; Kobayashi et al., 1994), they usually are charged because of sulfate, phosphate, or other modifications to the head group, suggesting an electrostatic mechanism of binding. This cannot be the case for the neutral Forssman glycolipid and LN interactions in MDCK cells. Previous work using a different anti-Forssman antigen antibody that reacted with MDCK cells suggested that Forssman glycolipid did not play a role in cell-substratum adhesion per se, but was necessary for the "establishment" of cells in culture (Butor et al., 1991). When this antibody was added to cul-

tures at cell plating, the cells often died; if added at any other time point, cells adhered to the filter. However, these studies were all performed on bare polycarbonate filters, and no cell adhesion assays were used. The fact that cells died under these conditions and that cell adhesion was not directly measured does not prove or disprove a role for the Forssman glycolipid in MDCK cell adhesion.

Our data are not the first to suggest that lipids may play important roles in cell differentiation, although they are the first to suggest that a neutral glycosphingolipid mediates both MDCK cell adhesion to LN and basolateral polarization. Examining the anionic disialoganglioside  $G_{D3}$  (a ganglioside is a glycosphingolipid with one or more anionic sialic acids), Sariola et al. (1988) were able to perturb the conversion of metanephric mesenchyme to epithelia. They showed that the anti- $G_{D3}$  antibodies reacted only with a subpopulation of metanephric mesenchymal cells and not with the inducing ureteric bud epithelium. However, it cannot be argued that these antibodies exerted their effects by blocking cell-LN interactions, since LN is not expressed until just before epithelium formation (Klein et al., 1988; Ekblom et al., 1990). In fact, Sariola et al. (1988) suggest that cell-cell interactions were inhibited, thus suggesting that lipids may play significant roles not only in cell-substratum contacts, but also in cell-cell interactions. In addition, Cheresch et al. (1986) showed that two disialogangliosides,  $G_{D2}$  and  $G_{D3}$ , are involved in cell-substrate adhesion. When human melanoma (M21) and human neuroblastoma (SK-NAS) cells were plated onto fibronectin or LN in the presence of function-blocking anti- $G_{D2}$  or - $G_{D3}$  antibodies, adhesion was inhibited. This inhibition was detected as early as 5 min after plating, suggesting that these gangliosides play an early role in cell adhesion. Interestingly, Szulman (1975) indicated that an anti-Forssman antigen polyclonal sera was able to perturb tubule formation in a model of histotypic reaggregation of chicken mesonephric cells. He also suggested that Forssman antigen may play an important role in cell-cell interactions.

While studies have suggested important roles for lipids in cell adhesion, little is known about the underlying mechanisms. Do these lipids bind ECM molecules themselves, or do they modify the membrane microenvironment of surface protein receptors to enhance or discourage binding? There is some evidence supporting both possibilities. Cheresch and his colleagues in their  $G_{D2}/G_{D3}$  studies hypothesized that the anionic sialic acid groups bind  $Ca^{2+}$ , which is necessary for cell adhesion to ECM (Cheresch et al., 1986). In vitro, Kalb and Engel (1991) found that the phospholipids phosphatidylcholine (zwitterionic) and phosphatidylglycerol (anionic) bound LN in a  $Ca^{2+}$ -dependent manner, suggesting a role for lipids on the cell surface to aid in LN self-assembly into a matrix. Mohan et al. (1990) also found that some sulfated but not neutral glycolipids bound specifically to LN in vitro. In contrast, other studies suggest that the membrane microenvironment affects receptor binding to ECM. Using purified vitronectin receptor (an integrin) incorporated into liposomes containing various phospholipids and cholesterol, Conforti et al. (1990) found that the efficiency of binding to vitronectin correlated with lipid composition. They also found a conformational difference between receptors in liposomes that bound well to vitronectin and those in other liposomes

that did not, suggesting that the lipid microenvironment can affect protein structure. Others have observed similar cases of lipids affecting the activity of ECM receptors (Hermanowski-Vosatka et al., 1992; Zheng et al., 1993).

### *Models for 12B12 mAb Action*

Our observations suggest several models for the role of Forssman glycolipid in MDCK cell adhesion and polarity. These models take into account the observations that MDCK cells secrete LN and adhere to LN by  $\beta 1$ -integrins (Schoenenberger et al., 1990). Although we focus on integrins in our models, other proteins that bind LN, such as a 67-kD MDCK protein (Salas et al., 1992) or nonmuscle  $\alpha$ -dystroglycan (Durbeek et al., 1995), may also be involved. One model ("glycolipid zipper") suggests that the glycolipid facilitates LN adhesion directly. In culture, when suspended MDCK cells are plated onto a substratum, electrostatic interactions may initially allow the cells to attach. At this stage, they do not spread. As the cells begin to secrete LN at higher concentrations into the medium, some LN is deposited onto the substratum. The Forssman glycolipid (or related glycolipids in other epithelia, such as the blood group B-like glycolipid in MDCK strain I cells [Hansson, 1988]), which is randomly distributed on the cell surface, "scouts" for this LN, zippering the cell to the substratum, thereby aiding integrin-LN binding. This mechanism may be more efficient than allowing receptors alone to attach to the many sites on LN unaided, since lipids may be able to bind indiscriminately to the LN molecule whereas receptors bind to only one specific site; in addition, there are likely to be more molecules of the glycolipid than LN receptors. When cell-cell interactions develop, occupied integrin receptors would then initiate a signal cascade (Hynes, 1992; Clark and Brugge, 1995; Yamada and Miyamoto, 1995) to cue the cell to assemble the polarized phenotype. As integrins bind LN, the glycolipid localizes to the apical cell surface. By perturbing these early steps in cell-LN adhesion, the 12B12 mAb prevents the cascade, and the cells do not fully polarize.

Another model ("glycolipid context") suggests that interactions between the glycolipid and LN are not key; rather, the glycolipid helps to create a lipid microenvironment for integrin LN receptors such that ligand binding is optimized by stabilizing an LN-binding conformation. When the integrin receptor binds LN, the conformation is stabilized, and the glycolipid is no longer necessary and localizes apically. Since the 12B12 mAb interferes with this interaction, the LN integrin receptors are unable to bind LN efficiently, detrimentally affecting polarity. The lipid environment can affect integrin specificity and avidity, lending plausibility to this model (Conforti et al., 1990; Hermanowski-Vosatka et al., 1992; Zheng et al., 1993). Because our data do not directly demonstrate an LN-binding role for Forssman antigen, this model is also an attractive possibility.

Similarly, a "glycolipid organizer" model that incorporates the fact that glycolipids can bind LN (and other ECM proteins) (Hakomori, 1990; Kalb and Engel, 1991; Mecham, 1991) and thus may facilitate the organization of the ECM network would relegate integrins as "downstream" players. As the cells attach to the substratum, either by

electrostatic interactions or by serum factors, they begin to secrete LN. The glycolipid, which is randomly expressed on the cell surface, binds the secreted LN to help concentrate it at the cell surface–substratum interface. This action helps form a matrix “nucleus” that promotes polymerization of a LN/ECM network. The 12B12 mAb would then interfere with the three-dimensional LN network formation, to which cells would never optimally adhere, resulting in poorly polarized cells.

### ***Apical vs. Basolateral Polarity and Effects of ECM***

Under normal conditions, an apical marker is fully polarized by 12 h, while a basolateral marker requires 18 h, regardless of substratum. These differences in apical and basolateral kinetics were first observed by Vega-Salas et al. (1987), who observed that a 185-kD apical marker polarized more swiftly than a basolateral marker, p63. Using semiquantitative immunofluorescence measurements of frozen sections of MDCK cells that were plated at “high” density onto CI gels on impermeable substrata, they found that the apical marker required 24 h to fully polarize, while a basolateral marker required >72 h. Our data confirm this trend, but with a biochemical approach. The differences in the kinetics can be attributed to the particular antigens studied, the use of impermeable substrata, or the clone of MDCK cells used. Indeed, an issue that has recently become more important in the study of epithelial cell polarity is the clonal variations between different MDCK II cell lines. In a recent study, Mays et al. (1995) demonstrated the unexpected result that delivery of the Na<sup>+</sup>/K<sup>+</sup>-ATPase was not directly targeted to the basolateral domain in a clone of MDCK II cells. This difference in targeting was subsequently correlated with the missorting of glycosphingolipids. We have observed other variations between subclones of MDCK cells (Zuk and Matlin, 1996).

The observation that apical antigens polarize before basolateral ones suggests that one signaling and assembly pathway mediates the polarization of apical proteins and another pathway leads to the polarization of basolateral proteins. Not only is there likely to be cross-talk between these pathways, but each path may also have a hierarchy of signaling (Rodriguez-Boulant and Nelson, 1989; Mays et al., 1995). Full assembly of the polarized phenotype is therefore not realized when steps in the signaling hierarchy are perturbed. Previous observations suggest that signaling hierarchies are involved in MDCK cell polarization. MDCK cysts in suspension culture are partially polarized because apical markers are found on the cyst periphery, while basolateral antigens are at the sites of cell–cell contact. However, not until the deposition of ECM into the lumen does the protein ZO-1 localize to the tight junction (Wang et al., 1990b). Thus, not until the appearance of ECM do cells in cysts finally fully polarize, suggesting that ECM may play a later role in cyst cell polarization. Previous work from our laboratory also suggests that the pathways for apical and basal polarity are distinct and involve a hierarchy of signals. We developed and examined the polarity of Kirsten-*ras* transformed MDCK cells (Schoenenberger et al., 1991). Kirsten-*ras* is a constitutively “on” small GTP-binding protein; stably transfected MDCK cell

lines exhibit the characteristics of transformed cells, forming tumors in nude mice and multilayers in culture. These cells exhibit basolateral but not apical polarity. Thus, by perturbing a second messenger signaling pathway, a specific aspect of cell polarity, apical polarity, can be disrupted. Finally, our results with the 12B12 mAb suggest that by interfering with early adhesion to LN, polarization is selectively perturbed, also arguing that a single path in polarization (basolateral) is disrupted.

Kirsten-*ras* MDCK cells not only exhibit selective defects in polarity of apical proteins, but they also exhibit an altered pattern in ECM receptor expression. Further work demonstrated a correlation between *ras* expression and alterations in the integrin profile and expression levels (Schoenenberger et al., 1994). These results led to the hypothesis that integrins, in conjunction with their ECM molecule ligands, may act as morphoregulatory molecules (Schoenenberger et al., 1994). Our current results support this notion at the level of the ECM. Although we do not have direct evidence linking integrins to MDCK polarity, we do present evidence in this report that argues that LN (to which at least one MDCK integrin binds) mediates an aspect of MDCK cell polarity.

In summary, we have demonstrated that a glycolipid-binding mAb inhibits not only tight junction formation and adhesion to LN, but also inhibits basolateral but not apical polarity. These results suggest that cell–LN interactions may play an important role in the establishment of basolateral polarity. Future studies will concentrate specifically on the role of LN-binding integrins and MDCK cell polarity, and ultimately on the signal transduction cascade from integrin binding to assembly of the polarized phenotype.

We are very grateful to Drs. Lynn Jesaitis, George Ojakian, and Arnaud Sonnenberg for their gifts of antibodies. We thank Dr. Jim Casanova (Massachusetts General Hospital) for his idea of stripping monolayers of bound antibody, helpful discussions, and for critically reviewing the manuscript. We thank Drs. Iain Drummond and Bob Ezzell (Massachusetts General Hospital) for help with the figures. We also recognize the dedicated technical support of Susan Bell, Suzanne Miller, and Jessica Radin. We acknowledge Dr. Gary Pritchard (Tufts University) for his technical assistance and for his careful review of the manuscript.

This work was supported by the National Institutes of Health (R01 DK46768 and P01 DK38452).

Received for publication 8 January 1996 and in revised form 19 February 1996.

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