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Integrin expression and localization in normal MDCK cells and transformed MDCK cells lacking apical polarity

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SUMMARY

Epithelial cells polarize in response to contacts with the extracellular matrix and with neighboring cells. Interactions of cells with the extracellular matrix are mediated mainly by the integrin family of receptors. To begin to understand the role of integrins in polarization, we have investigated the expression and localization of three integrin families in the polarized Madin-Darby canine kidney (MDCK) epithelial cell line and in transformed MDCK cells lacking apical polarity. We find that MDCK cells express several β_1 integrins, including $\alpha_2\beta_1$, $\alpha_3\beta_1$, and an unidentified integrin designated $\alpha_x\beta_1$. The β_1 integrins are the major receptors for collagens I and IV and laminin in MDCK cells, since a blocking anti-β_1 antibody almost totally abolishes adhesion to these proteins. They also express a vitronectin receptor tentatively identified as $\alpha_v\beta_3$, and the epithelial-specific integrin $\alpha_6\beta_4$. The latter is not a laminin receptor in MDCK cells because a function blocking anti-α_6 antibody has no effect on cell adhesion to laminin. All three integrin families are expressed exclusively on both the basal and lateral surfaces, as determined by immunofluorescence microscopy and surface biotinylation. Transformed MDCK cells express β_1 integrins as well as $\alpha_v\beta_3$ and $\alpha_6\beta_4$, but show alterations in the β_1 family. Expression of α_x is lacking, and the relative amount of the β_1 subunit is diminished, resulting in the accumulation of Endo-H-sensitive α_3. In addition, surface biotinylation and immunofluorescence indicate that significant amounts of both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ appear on not only the basolateral but also the apical plasma membrane. These results indicate that integrins are the major receptors for the extracellular matrix in MDCK cells, and that they may affect epithelial cell polarization by mediating not only cell-substratum but also cell-cell contacts.

Key words: epithelial polarity, integrin, MDCK

INTRODUCTION

Epithelial cells form the interface between compartments in complex organisms and, by their secretory and absorptive functions, alter the compartments they separate (Matlin and Caplan, 1992). In kidney tubules, for example, the epithelium divides the urinary space from the interstitium while selective transporters act to concentrate the urine and recover essential minerals and metabolites. To play these roles, epithelial cells must be polarized (Matlin and Caplan, 1992; Rodriguez-Boulan and Nelson, 1989; Rodriguez-Boulan and Powell, 1992). Their plasma membranes are divided into three domains with distinct protein and lipid compositions. The apical or free surface faces what is topographically the outside world and is often characterized by specialized structures such as microvilli and cilia involved in the absorption or movement of luminal fluid. The lateral surface interacts with neighboring cells and is distinguished by the presence of a variety of complex intercellular junctions, such as the tight junction, and other proteins involved in cell-cell adhesion. The basal surface abutting the substratum participates in specific interactions with proteins of the basal lamina such as laminin, collagen IV and heparan sulfate proteoglycans (Boll et al., 1991; Matlin and Caplan, 1992; Rodriguez-Boulan and Nelson, 1989; Rodriguez-Boulan and Powell, 1992).

Studies of the biogenesis of polarity suggest that polarization requires both cell-cell and cell-substratum adhesion (Matlin and Caplan, 1992; Parry et al., 1990; Rodriguez-Boulan and Nelson, 1989; Rodriguez-Boulan and Powell, 1992). During early development of the kidney, for example, differentiation of the tubular epithelium occurs in two stages (Ekblom, 1989, 1992). In the first, induced cells of the metanephric mesenchyme become tightly adherent, forming cell aggregates called condensates. Next, a basal lamina is deposited along the periphery of the condensates causing its conversion from a solid mass of cells to a hollow ball composed of an inwardly facing, polarized epithelium (Ekblom, 1989, 1992). Experiments mainly utilizing the polarized cell line MDCK (Madin-Darby canine kidney) have permitted the dissection of the contributions of cell-cell

contacts and cell-substratum contacts to the polarization process. When cultured on artificial substrata under conditions in which cell-cell contacts do not form, MDCK cells are still able to polarize both endogenous and exogenous membrane proteins to the apical surface, suggesting that interaction with the substratum is adequate for the establishment of at least a rudimentary apical domain (Ojakian et al., 1990; Rodriguez-Boulan et al., 1983; Vega-Salas et al., 1987). Polarization of proteins to the basolateral pole, on the other hand, is largely dependent on the formation of cell-cell contacts (McNeil et al., 1990; Nelson and Hammerton, 1989; Nelson and Veshnock, 1986, 1987a,b; Rodriguez-Boulan and Nelson, 1989).

Although the functions of cell-cell adhesion molecules such as E-cadherin in the polarization process are under intense investigation, the roles of equivalent cell-substratum adhesion proteins in polarization have not been explored in detail (Boll et al., 1991; Parry et al., 1990). In particular, it is not known how the integrins, a large family of receptors for the extracellular matrix, influence the morphogenesis of the polarized phenotype. Integrins are heterodimeric transmembrane glycoproteins found in every eucaryotic cell type except erythrocytes (Hemler, 1991; Hynes, 1992; Ruoslahti, 1991). Most epithelial integrins are composed of complexes between the β_1, β_3 or β_4 subunits and a variety of different α subunits, yielding receptors for collagens, laminins, vitronectin and fibronectin (Hemler, 1991; Hynes, 1992; Ruoslahti, 1991). While most α subunits associate exclusively with a single β subunit, there are notable exceptions. The α_6 subunit, for example, interacts not only with β_1, where it forms a receptor for laminin, but also with β_4, creating a distinct receptor for laminin or components of the anchoring filaments of hemidesmosomes (Hemler, 1991; Hynes, 1992; Lee et al., 1992; Quaranta and Jones, 1991; Sonnenberg et al., 1991, 1990a; Stepp et al., 1990). Individual heterodimers may also display multiple ligand specificities. For example, $\alpha_2\beta_1$ can act as a receptor for either collagen or laminin, and $\alpha_3\beta_1$ can act as a receptor for either laminin, collagen or fibronectin (Hemler, 1991; Hynes, 1992; Ruoslahti, 1991).

Integrins are thought to play important roles in a number of pathological processes, which may involve the breakdown of epithelial polarity. During carcinogenesis, changes in integrin expression may lead to altered differentiation or induction of invasive behaviour (Giancotti and Ruoslahti, 1990; Pignatelli and Bodmer, 1990; Plantefaber and Hynes, 1989; Ruoslahti, 1991). Loss of integrin function following ischemic damage in kidney tubular cells might contribute to the sloughing off of cells into the lumen, thus obstructing urine production (Gailit et al., 1993; Goligorsky et al., 1993; Racusen, 1992). Following injury, expression of new integrins may lead to infiltration of inflammatory cells (Arnout, 1990; Springer, 1990), or facilitate cell migration to close wounds (Clark, 1988; Humphries et al., 1991).

As part of the long-term interests of our laboratory in understanding the biogenesis of epithelial polarity, we have begun to examine the functions of integrins in the polarized epithelial cell line MDCK. Here, we report an analysis of the integrins expressed not only by normal MDCK cells but also by oncogenically transformed MDCK cells that lack apical polarity and grow in multilayers. What we find is that MDCK cells utilize a complex set of integrins to interact with not only the substratum but possibly also with neighboring cells, and that the expression and localization of these integrins is perturbed by transformation. Our results lay the groundwork for future studies of the role of integrins in epithelial polarization.

MATERIALS AND METHODS

Cell culture

For the present study, MDCK II cells and MDCK cells transformed with the viral Kirsten-*ras* oncogene were used (Matlin and Simons, 1984; Schoenenberger et al., 1991). As a control, some experiments also utilize the MDCK cell line C6, which was derived from normal MDCK II cells by transfection with the same pMV7 vector used to introduce K-*ras* (Schoenenberger et al., 1991). C6 cells are identical to the parental cell line with regard to ultrastructure, distribution of plasma membrane proteins, and transepithelial resistance. The transformed MDCK cell lines designated R2, R3 and R5, express increasing levels of *ras* p21. In particular, the R5 line, which expresses the highest level, forms multilayers and exhibits basolateral but not apical polarity (Schoenenberger et al., 1991).

For routine culture, MDCK II (passages 7-33) and transformed (R2, R3, R5; passages 7-15) and non-transformed (C6; passages 7-15) cell lines were grown in MEM (GIBCO Laboratories, Grand Island, NY) supplemented with Earle's salts, 5% fetal bovine serum (FBS, Sigma Chemical Co., St Louis MO), 2 mM L-glutamine (Sigma) and 10 mM HEPES (Sigma), pH 7.4, at 37°C/5% CO_2. Confluent cells were split 1:5 with trypsin-EDTA (GIBCO) every 3-4 days.

For culture of cells on permeable polycarbonate filter supports (Millipore PCF, 0.4 μm pore size, 30 mm diameter; Millipore Corp., Bedford, MA), normal and transformed cell lines were seeded at a density of 7×10^5 cells per pre-wetted filter and cultured for 4-7 days. Cultures were fed every other day as well as the day before experimentation.

Antibodies

The monoclonal and polyclonal anti-integrin antibodies used in the present study are listed in Table 1.

The rabbit polyclonal antibody raised against the human fibronectin receptor ($\alpha_5\beta_1$) obtained from Telios Pharmaceuticals (La Jolla, CA) (Argraves et al., 1987) reacts under denaturing conditions with two polypeptides corresponding to β_1 and its precursor in MDCK cells (see Results). The same two polypeptides are also detected by two

Table 1. Anti-integrin antibodies

Specificity*	Type†	Source	Reference
$\alpha_5\beta_1$ (hu)	Rb p	Telios Pharm.‡	Telios data sheet
β_1 peptide (ch)	Rb p	Hynes	(Marcantonio and Hynes, 1988)
β_1 peptide (hu)	Rb p	Ruoslahti	(Giancotti and Ruoslahti, 1990)
β_1, AIIB2 (mo)§	Rt m	Damsky	(Hall et al., 1990)
β_3/β_5 (hu)	Rb p	Telios Pharm.	Telios data sheet
β_4 (hu)	Rb p	Hemler	Unpublished¶
α_2 ,peptide (hu)	Rb p	Hemler	(Takada and Hemler, 1989)
α_3, peptide (ch)	Rb p	Hynes	(Hynes et al., 1989)
α_4, peptide (hu)	Rb p	Hemler	(Takada et al., 1989)
α_5, peptide (ch)	Rb p	Hynes	(Hynes et al., 1989)
α_6, GoH3(hu)§	Rt m	Sonnenberg	(Sonnenberg et al., 1987)
α_6, J1B5 (mo)	Rt m	Damsky	Unpublished
α_6, peptide (ch)	Rb p	Reichardt	(Tamura et al., 1990)

*Antibodies made against proteins or peptides based on sequences of human (hu), chicken (ch), or mouse (mo) integrins.

†Antibodies made in either rabbit (Rb) or rat (Rt) and either polyclonal (p) or monoclonal (m).

‡Telios Pharmaceuticals, La Jolla, CA, now a division of Gibco/BRL.

§Adhesion-blocking antibody.

¶Raised against the SDS-gel purified β4 subunit derived from affinity-purified α6β4 (M. Hemler, personal communication).

different rabbit polyclonal antisera directed against peptides derived from the cytoplasmic tails of chicken and human β_1 obtained from Richard Hynes (Massachusetts Institute of Technology) (Marcantonio and Hynes, 1988) and Erkki Ruoslahti (La Jolla Cancer Research Center) (Giancotti and Ruoslahti, 1990) (data not shown). The rat monoclonal antibody AIIB2 against mouse β_1 obtained from Caroline Damsky (University of California, San Francisco) (Hall et al., 1990) immunoprecipitates only mature β_1 and not its precursor (data not shown).

The rabbit polyclonal antiserum raised against the original vitronectin receptor ($\alpha_v\beta_3$; Telios) (Suzuki, 1986) may also cross-react with $\alpha_v\beta5$ (Telios Technical Literature). This antiserum detects a single polypeptide under denaturing conditions in MDCK cells with a mobility corresponding to either the β_3 or $\beta5$ subunit (Fig. 3).

Polypeptides precipitated from MDCK cells under nondenaturing conditions with the rat monoclonal antibody GoH3 against mouse α_6 (obtained from Arnout Sonnenberg, Netherlands Blood Center, Amsterdam) (Sonnenberg et al., 1987) were identical to those precipitated with another rat monoclonal anti-α_6 (J1B5) obtained from Caroline Damsky, and a rabbit polyclonal anti-α_6 cytoplasmic tail antiserum obtained from Lou Reichardt (University of California, San Francisco) (Tamura et al., 1990; data not shown). On the basis of these and other data described in Results, one of the polypeptides has been identified as α_6 and the other as β_4 (see Results).

GoH3, J1B5 and AIIB2 do not react with MDCK cell polypeptides under denaturing conditions.

Both the anti-β_1 antibody AIIB2 and the anti-α_6 antibody GoH3 have been reported to block cell adhesion to their respective ligands (Hall et al., 1990; Sonnenberg et al., 1988).

Semi-thin frozen sections

Filter-grown transformed and non-transformed MDCK cells were processed for 0.5 μm cryosections as described by Kendall et al. (1992). Briefly, cells on filters were rinsed with phosphate buffered saline containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS+), fixed in PLP (2% paraformaldehyde, 75 mM L-lysine, 10 mM $NaIO_4$) (McLean and Nakane, 1974) for 1 hour and rinsed again. Filters were cut out of the filter holders, trimmed into 1 mm squares and embedded in gelatin. When the gelatin solidified, the filters were re-fixed, infiltrated in sucrose/PBS, and frozen onto specimen holders in freon precooled with liquid nitrogen, followed by immersion in liquid nitrogen. Semi-thin frozen sections (0.5-1.0 μm) were cut on an Ultracut microtome with a cryosectioning attachment (Reichert-Jung, Vienna, Austria) at −80°C. Sections were collected in sucrose, and transferred to microscope slides (Superfrost Plus, Fischer Scientific, Boston, MA) or coverslips previously coated with gelatin and chromium potassium sulfate.

Immunofluorescence

Indirect immunofluorescence was performed either on cryosections of filter-grown cells or on cells grown on filters that were fixed in PLP. For cryosections, sucrose was removed by rinsing three times in PBS lacking Ca^{2+} and Mg^{2+} (PBS−), and aldehyde groups blocked by quenching in 50 mM NH_4Cl/PBS(−) for 20-45 minutes. After further rinsing, non-specific binding sites were blocked with 0.2% gelatin/PBS(−) or 10% normal goat serum for 30-60 minutes (Kendall et al., 1992). For immunostaining, antibodies against the β_1 (1:150, Telios), β_3 (1:150, Telios) and α_6 subunits (GoH3) were used. Sections were incubated in the primary antibody for 45 minutes, rinsed, and incubated with fluorescein-conjugated secondary antibody directed against rabbit or rat (TAGO, Inc.; Burlingame, CA). They were then mounted in 90% glycerol/PBS(−) containing 0.l% *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine to reduce photobleaching, and viewed on a Zeiss Axiophot or Axioskop Microscope equipped with epifluorescence illumination. All images were photographed with T-MAX-400 film (Eastman Kodak Co., Rochester, NY).

Immunofluorescence of cells on filters was as described above, except that cells were quenched for 15 minutes with 1 mg/ml $NaBH_4$/PBS(−) followed by permeabilization for 4 minutes in 0.1% Triton X-100/PBS(−) (Bacallao et al., 1990). Filters were then cut into 5 mm squares and incubated cell side down on 50 μl drops of 10% normal goat serum, followed by primary and secondary antibodies. Filters were mounted onto glass slides for confocal microscopy using nail-polish spacers as described by Bacallao (1990). A Bio-Rad MRC 600 confocal system in conjunction with a Zeiss Axiovert and PlanApo ×63 objective was used to image the cells in the *xy* plane at 1.0 μm intervals (*z*-series). The raw images were transferred to a Macintosh format and contrast-adjusted using Adobe Photoshop (Adobe Systems, Mountain View, CA). All three images depicted for each antibody were contrast-adjusted and printed identically.

Immunoprecipitation

Filter-grown cells were metabolically labeled from the basal compartment for 16-24 hours with 100 μCi of [^{35}S]methionine (EXPRE^{35}S^{35}S, New England Nuclear, Boston, MA) per filter chamber in MEM containing one-tenth the normal methionine concentration (Sigma, St. Louis, MO) supplemented with 5% FBS and 10 mM HEPES. An equal amount of medium lacking isotope was added to the apical compartment. Radiolabeled cells were then washed three times on ice with cold PBS(+). Filters were cut in half, removed from the filter holder, and placed in microfuge tubes. Cells were then extracted under non-denaturing or denaturing conditions. Extraction under non-denaturing conditions, which preserves αβ heterodimeric complexes, was carried out by adding 1 ml of extraction buffer (1% NP-40, 1% sodium deoxycholate, 0.5% SDS, 0.15 M NaCl, 10 mM Tris, pH 7.5, or 1% Triton X-100, 0.1% SDS, 0.15 M NaCl, 20 mM Tris, pH 8.6) containing protease inhibitors (10 μg/ml aprotinin, 17.5 μg/ml benzamidine, 1 μg/ml antipain, 1 μg pepstatin, and 0.2 mg/ml iodoacetamide) to microfuge tubes, and vortexing the samples briefly before inverting at 0°C for 30 minutes. For extraction of integrins under conditions that denature αβ heterodimers into individual α and β subunits, filters were transferred to microfuge tubes and heated (95°C, 3 minutes) in 0.5% SDS, 0.15 M NaCl, 20 mM Tris, pH 8.6, containing 0.2 mg/ml iodoacetamide. Samples were vortexed, mechanically sheared, and diluted fivefold with 1% Triton X-100, 0.15 M NaCl, 20 mM Tris, pH 8.6, containing 10 μg/ml aprotinin, 17.5 μg/ml benzamidine, 1 μg/ml antipain and 1 μg/ml pepstatin. Filters were removed by centrifugation (5 minutes, 15,000 ***g***) and cell lysates transferred to new tubes and centrifuged again (20 minutes, 0°C). In some cases, lysates were filtered through 0.45 μm filter units (Ultrafree-MC Filters, Millipore Corporation, Bedford, MA).

For immunoprecipitation, extracts were incubated overnight with the primary antibody at 4°C. For rat monoclonal antibodies, rabbit anti-rat IgG (Sigma) was added at the same time as the primary antibody. Antigen-antibody complexes were recovered by adding Protein A-Trisacryl (Pierce Chemical Co., Rockford IL) and inverting for 2-4 hours at 4°C. Immunoprecipitates were then pelleted (15,000 ***g***, 15 seconds) and washed thrice in extraction buffer without protease inhibitors and once with 10 mM Tris, pH 8.6, to remove salts and detergent.

To analyze the precipitated polypeptides, Trisacryl beads were resuspended in electrophoresis sample buffer (200 mM Tris-HCl, pH 8.8, 5 mM EDTA, 15% sucrose, 0.1% bromophenol blue, 20 mM dithiothreitol, and 2% SDS) and heated at 95°C for 3-5 minutes. The samples were then alkylated with iodoacetamide for 15 minutes at 37°C and fractionated by SDS-gel electrophoresis according to the method of Laemmli (1970). Gels were fixed, impregnated with EN^{3}HANCE (E.I. DuPont deNemours & Co., Boston, MA), dried and exposed to preflashed KODAK X-OMAT film (Eastman Kodak Co., Rochester, NY).

Endoglycosidase H (Endo H; Boehringer Mannheim Biochemicals, Indianapolis, IN) digestion of integrins was performed as described

previously (Matlin, 1986). After immunoprecipitation of integrins under denaturing conditions, antigen was released from Triasacryl Protein A beads by heating (95°C, 3 minutes) in 100 μl 5 mM TES-NaOH, pH 7.4, 1% SDS followed by diluting tenfold with 0.2 M sodium citrate, pH 5.5, containing protease inhibitors. Protein A beads were then pelleted, the supernatant containing the released integrin was divided into two equal portions, and Endo H (25 milliunits/ml) was added to one portion. Both portions were then incubated at 37°C for 16 hour and the digested protein recovered by precipitation with an equal volume of 40% trichloroacetic acid for a minimum of 3 hours on ice. After centrifuging (30 minutes, 15,000 ***g***), pelleted samples were processed for SDS-gel electrophoresis as described above.

Surface labeling

For cell surface labeling with biotin, the high pH method of Gottardi and Caplan (1992) was used. After rinsing in PBS+, filters were rinsed once with TEA buffer (10 mM triethanolamine, 125 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 9.0). Freshly prepared sulfo-*N*-hydroxy-succinimide-biotin (sulfo-NHS-biotin, 0.5 mg/ml, Pierce) dissolved in TEA was added to each compartment to be biotinylated. Unlabeled compartments received TEA buffer. After incubating for 30 minutes on ice, monolayers were washed twice in ice-cold serum-free MEM, avoiding mixing of apical and basal washes. Filters were rinsed twice more with PBS(+), flooding the wells. Cells were extracted under non-denaturing conditions as described above with the exception that 10 mM glycine was added to the extraction buffer. After immunoprecipitation, antigen was released from the Trisacryl Protein A pellet by heating (95°C, 3 minutes) in 30 μl of 2% SDS and 20 mM Tris-HCl, pH 8.6. As a positive control, 5 μl was removed and prepared for SDS-gel electrophoresis. The remaining 25 μl were diluted with 250 μl of 1% Triton X-100, 0.15 M NaCl, and 20 mM Tris-HCl, pH 8.6. Biotinylated integrins were then precipitated with streptavidin-agarose (Pierce). Samples were inverted at 4°C overnight, washed and processed for SDS-gel electrophoresis.

Transepithelial resistance

Transepithelial resistance was measured with a Millicell ERS (Millipore Corp., Bedford, MA).

Adhesion assay

The adhesion assay was based upon the procedure of Ruoslahti and Hayman (1982).

Extracellular matrix proteins, bovine serum albumin (BSA), and poly-D-lysine for coating wells, were prepared as follows. Laminin (Upstate Biotechnologies, Inc. (UBI), Lake Placid, NY) and collagen IV (Collaborative Research, Inc., Bedford, MA), purified from the Engelbreth-Holm-Swarm mouse sarcoma, were thawed on ice before diluting in cold 0.1 M sodium bicarbonate, pH 8.3. Lyophilized human fibronectin and human vitronectin (Collaborative) were dissolved in PBS(−) to a concentration of 1 mg/ml, divided into aliquots, and stored at −20°C for up to one week. Rat-tail collagen I (UBI) was diluted in PBS(−) according to the manufacturer's directions. Bovine serum albumin (Sigma, Fraction V, no. A7906) was dissolved in PBS(−) to a concentration of 5% (w/v) and denatured at 70°C for 5 minutes with constant stirring in a water bath. The solution was filtered through a 0.45 μm syringe filter (Millipore) and aliquots stored at −20°C. Lyophilized, high molecular weight poly-D-lysine (Collaborative) was dissolved in 0.1 M sodium bicarbonate, pH 8.3, to 100 μg/ml.

On the day of the experiment, Dynatech (Chantilly, VA) Immulon 2 plates (96 well) were coated with 100 μl of extracellular matrix proteins diluted to various concentrations in 0.1 M sodium bicarbonate, pH 8.3. Standard controls for each assay included wells coated with 100 μl of 1% BSA/PBS(−), and 200 μl of 50 μg/ml poly-D-lysine/0.1 M sodium bicarbonate, pH 8.3. Wells were coated for 2 hours at 37°C/5% CO_2 during which time the plates were kept covered with Parafilm to prevent evaporation. Solutions were then aspirated and the covered plates incubated further (30 minutes). Non-specific binding sites were blocked with 200 μl/well of 1% BSA/PBS(+) at 4°C for 2 hours. The plates were washed twice with 200 μl/well of PBS(+) before adding 50 μl/well of 1% BSA/serum-free MEM (with 10 mM HEPES, pH 7.4). For antibody perturbation experiments, the wells to be plated with cells received antibody containing BSA/serum-free MEM. In each assay, eight wells were used for each experimental condition. Four wells received cells (see below), while the other four were used for background measurements.

Subconfluent MDCK cells that had been passaged on the previous day were washed twice with PBS(−) and once with 4 mM EDTA-1 mM EGTA/PBS(−), incubated with 5 ml of the EDTA/EGTA solution for 40 minutes at 37°C/5% CO_2 to dissociate them into single cells, and collected by centrifugation. Cells were resuspended in 2-5 ml of serum-free S-MEM (Suspension MEM; GIBCO) containing 15 mM Hepes, pH 7.4. A suspension of 1×10^5 viable cells/50 μl was plated onto the coated plates. In antibody perturbation experiments, cells were incubated on ice for 30 minutes in the presence of the antibody before plating.

The plated cells were then incubated covered at 37°C/5% CO_2 for 90 minutes, the time necessary for MDCK to maximally adhere to laminin (data not shown). Plates were then gently washed twice with 200 μl/well of cold 0.5% BSA in PBS(+), twice with 200 μl/well of cold PBS(+), and then fixed with 100 μl/well of 100% methanol for 10 minutes. The methanol was removed and the plates allowed to air dry.

The plates were stained for 5 minutes with 100 μl/well 0.1% crystal violet filtered through a 0.22 μm filter unit (Millipore), washed four times with 200 μl/well of distilled water, and air-dried before dissolving the dye in 1% sodium deoxycholate (100 μl/well) for 10-20 minutes. The amount of staining, which is proportional to the number of attached cells, was measured with a Molecular Devices plate reader at 590 nm and the background reading for each well at 405 nm subtracted. In addition, the average absorbance of four wells without cells was measured and subtracted from the average of four wells with cells to yield the absorbance due to the adherent cells only. Each graph represents the data from three independent experiments.

RESULTS

MDCK cells express β_1, β_3 and β_4 integrins

When extracts of MDCK cells labeled overnight with [^{35}S]methionine were immunoprecipitated with a polyclonal antibody directed against the β_1 integrin subunit (Table 1) under conditions that do not dissociate the α and β chains, and the immunoprecipitates analyzed by SDS-gel electrophoresis and fluorography, five polypeptides with relative molecular masses between 110,000 and 170,000 were detected (Fig. 1, lane 1). One of these, designated α_x, was often poorly resolved from the other polypeptides (Fig. 1, compare lanes 1 and 6). Occasionally, a polypeptide of slightly less than 200 kDa was also observed (Fig. 1, lanes 4 and 6, arrowhead). When cells were extracted under denaturing conditions (see Materials and Methods) and precipitated, two polypeptides were seen (Fig. 1, lane 2). The faster migrating of these was apparently the biosynthetic precursor of β_1, since digestion of immunoprecipitates with endoglycosidase H caused a shift in the mobility of this band but not the more slowly migrating band (Fig. 1, lane 3). Under nonreducing conditions, the polypeptide identified as mature β_1 shifted to a slightly faster mobility that coincided with the mobility of the Endo H precursor, consistent with the behaviour reported for β_1 from other cell types (data not shown).

To identify the other polypeptides, labeled cell extracts were

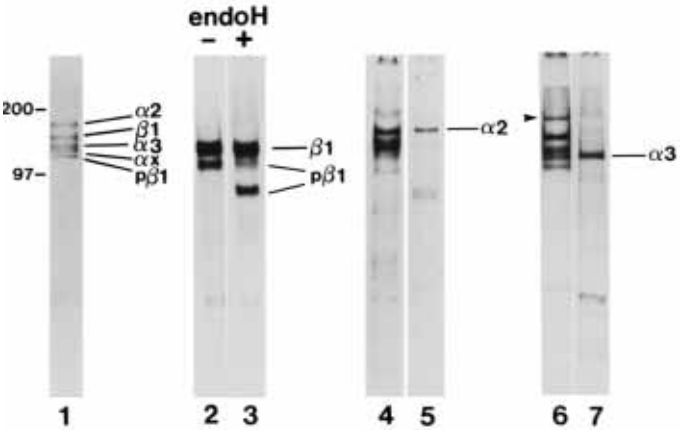


Fig. 1. Immunoprecipitation of β_1 integrins from MDCK cells. MDCK cells cultured on permeable filter supports were labeled overnight with [^{35}S]methionine and extracted under either nondenaturing (lanes 1,4 and 6) or denaturing conditions (lanes 2,3,5 and 7). The extracts were immunoprecipitated with polyclonal antibodies against the β_1, α_2 and α_3 integrin subunits. Nondenaturing conditions preserve interactions between the integrin α and β subunits; under denaturing conditions, only the polypeptide that directly interacts with the antibody is precipitated. The immunoprecipitates were analyzed by SDS-gel electrophoresis and fluorography. The data in lanes 1, 2 and 3, 4 and 5, and 6 and 7 are all derived from separate experiments. Relative molecular masses ($\times 10^{-3}$) determined by electrophoresis of marker proteins are indicated on the left side of lane 1; these are only approximately valid for the other lanes. Under nondenaturing conditions, a polyclonal anti-β_1 antibody precipitates five polypeptides (lane 1). Two of these are identified as β_1 and its Endo-H-sensitive precursor ($p\beta_1$) by immunoprecipitation under denaturing conditions and digestion with Endo H (lanes 2 and 3). Two others are identified as α_2 and α_3 by immunoprecipitation with monospecific anti-peptide antibodies (lanes 5 and 7, respectively); immunoprecipitates with anti-β_1 under nondenaturing conditions are shown in lanes 4 and 6 for comparison. The fifth polypeptide, which often appears to comigrate with α_3, has not been identified (α_x, lane 1). In some cases, a polypeptide of approximately 200×10^{-3}, which may be α_1 is also precipitated by anti-β_1 (lane 6, arrowhead).

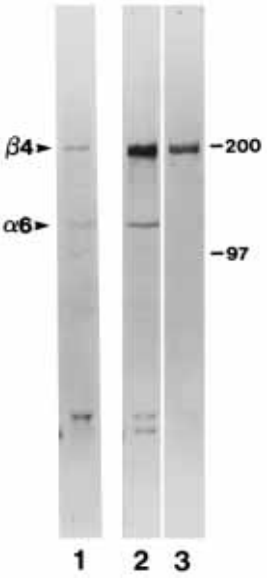


Fig. 2. Immunoprecipitation of $\alpha_6\beta_4$ from MDCK cells. Labeled MDCK cells were extracted under nondenaturing and denaturing conditions and immunoprecipitated with monoclonal antibody GoH3 to the α_6 subunit (lane 1) and polyclonal antibody to the β_4 subunit (lanes 2 and 3). Relative molecular masses ($\times 10^{-3}$) are indicated on the right. Under nondenaturing conditions, both anti-α_6 and anti-β_4 precipitate the same two polypeptides whose mobilities correspond to those of α_6 and β_4 (lanes 1 and 2). Under denaturing conditions, anti-β_4 precipitates a closely spaced doublet corresponding to β_4 (lane 3).

immunoprecipitated with polyclonal anti-peptide and monoclonal antibodies against a number of α subunits known to associate with β_1 (see Table 1). Of these, polyclonal antibodies directed against α_2 and α_3 precipitated single polypeptides under denaturing conditions, whose mobilities corresponded to those of two of the five polypeptides precipitated by anti-β_1 under nondenaturing conditions (Fig. 1, lanes 4,5 and 6,7). Antibodies against α_4 and α_5 (Table I) failed to precipitate any bands in the expected molecular mass range, suggesting that these integrins were not expressed by MDCK cells. The possibility that the high molecular mass polypeptide sometimes detected (Fig. 1, lane 6, arrowhead) was the α_1 subunit was not tested because an appropriate antibody was not available.

To determine whether the fifth polypeptide (α_x) coprecipitated with β_1 was α_6 (Fig. 1, lane 1), labeled extracts were precipitated under nondenaturing conditions with either GoH3 or J1B5, monoclonal antibodies against α_6 (Table 1). Under these conditions two polypeptides were detected. The apparent molecular mass of one of these corresponded to that expected for α_6 (Fig. 2, lane 1). Because the epitopes of GoH3 and J1B5 are sensitive to SDS (unpublished observations), it was not possible to perform an imunoprecipitation under denaturing conditions. The mobility of the second polypeptide precipitated by anti-α_6 corresponded to a molecular mass of approximately 200 kDa, suggesting that it was the integrin subunit β_4, which is known to associate with α_6 (Fig. 2, lane 1). This was confirmed using a polyclonal antibody against β_4. Under nondenaturing conditions this antibody precipitated the same two polypeptides while under denaturing conditions it precipitated only the 200 kDa polypeptide (Fig. 2, lanes 2 and 3). To provide additional evidence that no $\alpha_6\beta_1$ was expressed by MDCK cells, labeled extracts were immunoprecipitated under nondenaturing conditions with anti-α_6, and the immunoprecipitates resolubilized and reprecipitated with polyclonal anti-β_1. Under these conditions, no β_1 was detected (data not shown).

Extracts of MDCK cells were also precipitated with a polyclonal antibody against the vitronectin receptor, $\alpha_v\beta_3$. Under nondenaturing conditions this antiserum precipitated two polypeptides whose apparent molecular masses (Fig. 3, lane 1) and shifts in mobility in the absence of reduction (data not shown) were characteristic of the α_v and β_3 subunits. Under denaturing conditions, only presumptive β_3 was precipitated (Fig. 3, lane 2).

Based on these findings, we concluded that MDCK cells express the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ heterodimers, but not β_1 integrins with the α_4, α_5 or α_6 subunits. The identity of α_x, which is precipitated by anti-β_1 under nondenaturing conditions, remains unclear. It is also possible that $\alpha1\beta_1$ is expressed under some conditions, but this has not yet been tested. The α_6 subunit is

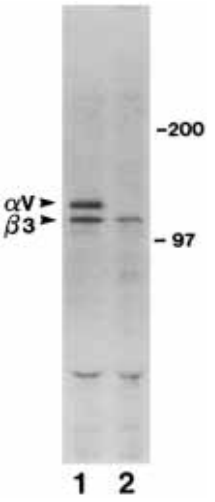


Fig. 3. Immunoprecipitation of presumptive $\alpha_v\beta_3$ from MDCK cells. Labeled MDCK cells were extracted under nondenaturing and denaturing conditions and immunoprecipitated with a polyclonal antibody to a vitronectin receptor. Relative molecular masses ($\times 10^{-3}$) are indicated on the right. Under nondenaturing conditions (lane 1) two polypeptides are precipitated, while only one is precipitated under denaturing conditions (lane 2). Based on mobilities, these polypeptides are tentatively identified as β_3 and α_v.

expressed, but exclusively in a complex with β_4. In addition, MDCK cells express another integrin, tentatively identified as $\alpha_v\beta_3$.

Integrins in MDCK cells are found on both basal and lateral surfaces

All of the integrins expressed by MDCK cells are known to be receptors for the extracellular matrix (Hemler, 1991; Hynes, 1992; Ruoslahti, 1991). As such, they should be localized to the basal plasma membrane. However, recent evidence suggests that integrins are also on the lateral surface in some epithelial cells where they may participate in cell-cell interactions (Carter et al., 1990; Larjava et al., 1990; Symington et al., 1993). To determine where integrins are found in MDCK cells, β_1, β_3 and β_4 integrins were localized by immunofluorescence microscopy. Localization of both β_1 integrins and $\alpha_v\beta_3$ was accomplished with polyclonal antisera directed against the β subunits; $\alpha_6\beta_4$, on the other hand, was localized using the GoH3 monoclonal antibody against α_6.

As shown in Fig. 4, staining of frozen sections revealed that all three groups of integrins were localized to not only the basal but also the lateral aspects of the plasma membrane (Fig. 4A, E and I). These findings were confirmed and extended by confocal microscopy (Fig. 4B-D, F-H and J-L). Using this technique, staining of the integrins on the basal surface was in no case localized to focal adhesions but instead appeared to be unevenly distributed with no apparent pattern. The basal staining was most intense with GoH3 and less with anti-β_1 (Fig. 4D and H); anti-β_3 staining, on the other hand, was more finely granular and concentrated near the cell borders (Fig. 4L). On the lateral surfaces, staining with all three antibodies appeared intense (Fig. 4C, G and K). The lateral staining with anti-α_6, and to a lesser extent anti-β_1, appeared somewhat punctate, suggesting focal concentration of the integrins (Fig. 4C and G). With all the antibodies, staining on the apical surfaces appeared faint. However, it was difficult to decide if this represented a low concentration of apical integrins, or simply background from the primary antibodies.

The polar distribution of the integrins was also examined biochemically by surface biotinylation. MDCK cells on permeable supports were labeled overnight with [^{35}S]methionine and then treated from either the apical or basolateral surfaces, or both, with sulfo-NHS biotin. After immunoprecipitation of integrins, the biotinylated proteins were recovered with streptavidin-agarose and analyzed by SDS-gel electrophoresis and fluorography. As illustrated in Fig. 5, in all cases biotinylated integrins were detected only basolaterally (b) and not apically (a), consistent with the immunolocalization data. The intensity of the bands biotinylated from both sides of the monolayer (ab) was identical to that biotinylated basolaterally.

MDCK cell integrins are receptors for laminin, collagen and vitronectin

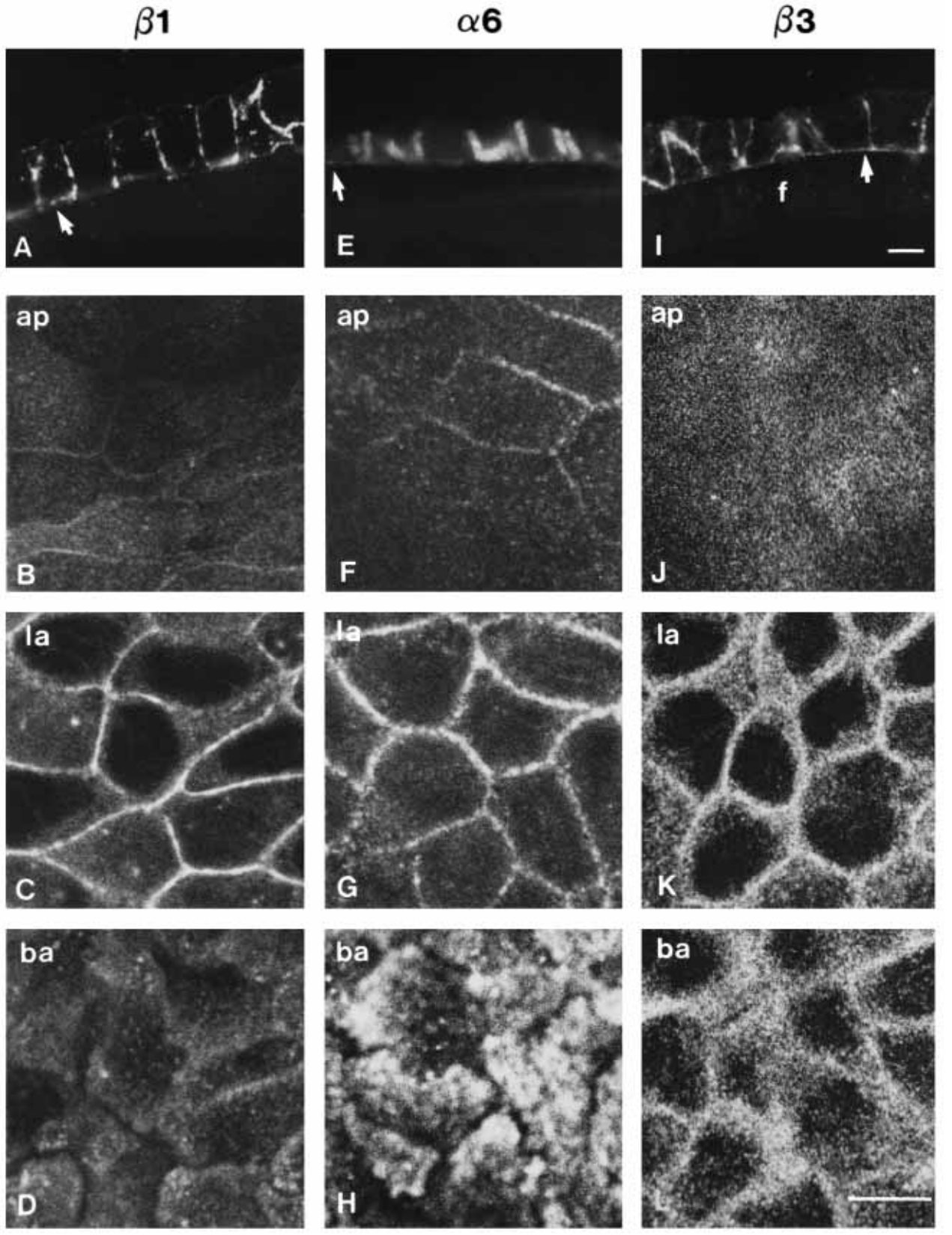
To determine the ligand specificities of the MDCK cell integrins, adhesion assays were conducted on extracellular matrix-coated dishes with and without the addition of adhesion-blocking antibodies. Polylysine-coated dishes served as positive controls for complete cell attachment. MDCK cells attached well to Matrigel, an extract of the EHS sarcoma that is enriched in laminin-nidogen, collagen IV and proteoglycans (data not shown). They also attached readily to laminin (Fig. 6A), collagen IV (Fig. 6B) purified from the EHS sarcoma matrix, and collagen I (Fig. 6C). MDCK cells failed to adhere to dishes coated with heat-denatured bovine serum albumin. Adhesion to laminin, collagen IV and collagen I could be completely inhibited by the monoclonal antibody AIIB2 against the β_1 subunit (Fig. 6A-C). The blocking anti-α_6 antibody GoH3 had no effect on adhesion of MDCK cells to laminin, even at concentrations that significantly inhibited the adhesion of other cells (data not shown) (Lee et al., 1992).

MDCK cells also adhered well to the serum adhesion protein vitronectin (Fig. 6D). This could not be blocked by anti-β_1, consistent with vitronectin adhesion being mediated by $\alpha_v\beta_3$ (Fig. 6D). In addition, MDCK cells attached to fibronectin-coated plates, but less efficiently than to other components (Fig. 7). This is clearly evident when adhesion to different con-

Fig. 4. Immunolocalization of integrins in MDCK cells. Frozen sections (A,E,I) or Triton-permeabilized whole mounts (B-D, F-H, J-L) were stained with antibodies to the β_1 (A-D), α_6 (E-H), or β_3 (I-L) subunits followed by fluorescent secondary antibodies. Frozen sections were examined in the fluorescence microscope; whole mounts were imaged with a confocal laser scanning microscope. Bars, 10 μm. In the frozen sections, it is apparent that staining of all three integrin subunits is primarily basolateral. In (A) and (E) the lateral staining is more prominent because the basal fluorescence is slightly out-of-focus (arrows). In addition, staining with the anti-α_6 antibody (E) is more intense because the section is thicker (1.0 μm vs 0.5 μm in (A) and (I)). In (I) anti-β_3 stains the basal (arrow) and lateral surfaces; f indicates the filter in this image. In confocal optical sections, only slight staining is apparent at the apical surfaces (ap) with all three antibodies (B,F,J). In (B) and (F) lateral staining is evident where the optical section has penetrated just below the apical surface. Note the focal concentration of α_6 integrin on the lateral (la) surface (G) and the granular (L) to matt-like (H) staining of the basal (ba) surface with the β_3 and α_6 antibodies, respectively.

centrations of fibronectin and laminin are compared. Adherence to laminin is maximal at 1.5 μg, while adherence to fibronectin is barely saturated at 6.0 μg (Fig. 7A). Adhesion to fibronectin was not inhibited by anti-β_1, suggesting that it was also mediated by $\alpha_v\beta_3$ (Fig. 7B).

In summary, adhesion assays and antibody inhibition exper-



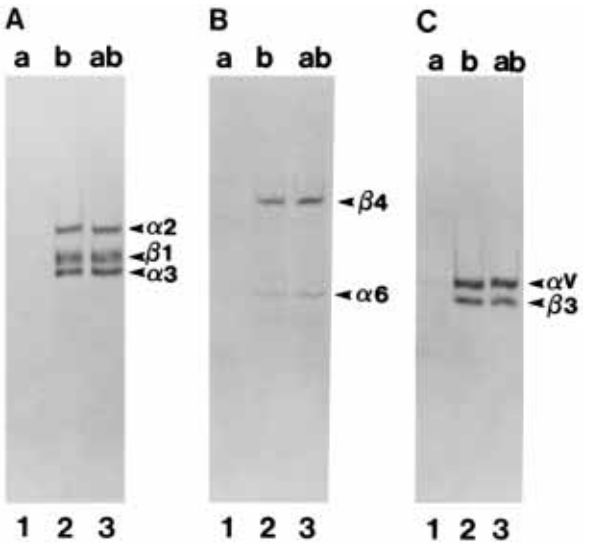


Fig. 5. Surface labeling of MDCK cell integrins. MDCK cells cultured on permeable supports were labeled overnight with [^{35}S]methionine. Replicate filter chambers were then reacted from the apical (a), basolateral (b), or apical and basal (ab) sides of the cell monolayer with sulfo-NHS biotin. The cells were extracted under nondenaturing conditions and immunoprecipitated with polyclonal anti-β_1 (A), monoclonal GoH3 anti-α_6 (B), or polyclonal anti-vitronectin receptor (C). Immunoprecipitates were resolubilized and reprecipitated with streptavidin/agarose to capture the surface biotinylated proteins. As shown in (A), (B) and (C), β_1, β_4 and β_3 integrins can be detected from only the basolateral surface (compare lanes 1 with lanes 2). Because no integrins can be detected on the apical surface, the intensities of the bands resulting from biotinylation from both sides of the filter chamber (ab) are identical to those due to basolateral biotinylation (b). Note also that the β_1 precursor (pβ_1, see Fig. 1) is not detected by biotinylation (A, lanes 2 and 3), confirming that it is an intracellular form of the protein.

iments indicated that MDCK cells adhere to components of the basal lamina and to collagen I via β_1 integrins. They also adhere readily to vitronectin, most likely through the $\alpha_v\beta_3$ vitronectin receptor, but less well to fibronectin. These findings, along with the observations that the anti-β_1 antibody AIIB2 has no effect on fibronectin adhesion and that the anti-α_6 antibody GoH3 does not block laminin adhesion, are consistent with our inability to identify either $\alpha_5\beta_1$ (a fibronectin receptor) or $\alpha_6\beta_1$ (a laminin receptor) in MDCK cell immunoprecipitates. They also suggest that MDCK cells do not express the fibronectin/vitronectin receptor $\alpha_v\beta_1$.

Transformed MDCK cells exhibit altered integrin expression and localization

Our laboratory has recently described MDCK cell lines transformed by the Kirsten *ras* oncogene (Schoenenberger et al., 1991). These cells exhibit a lack of apical polarity and tendency to form multilayers which is correlated with the amount of K-*ras* expression. Nevertheless, the transformants develop functional tight junctions and retain basolateral proteins in regions of cell-cell contact (Schoenenberger et al., 1991). The formation of multilayers suggests that the balance between cell-substratum and cell-cell adhesion, which normally results in a monolayer, has been disturbed. To examine whether these changes in cell adhesion and polarity could be correlated with alterations in integrin expression, we compared the integrin composition of the transformed cell lines with that of normal MDCK cells.

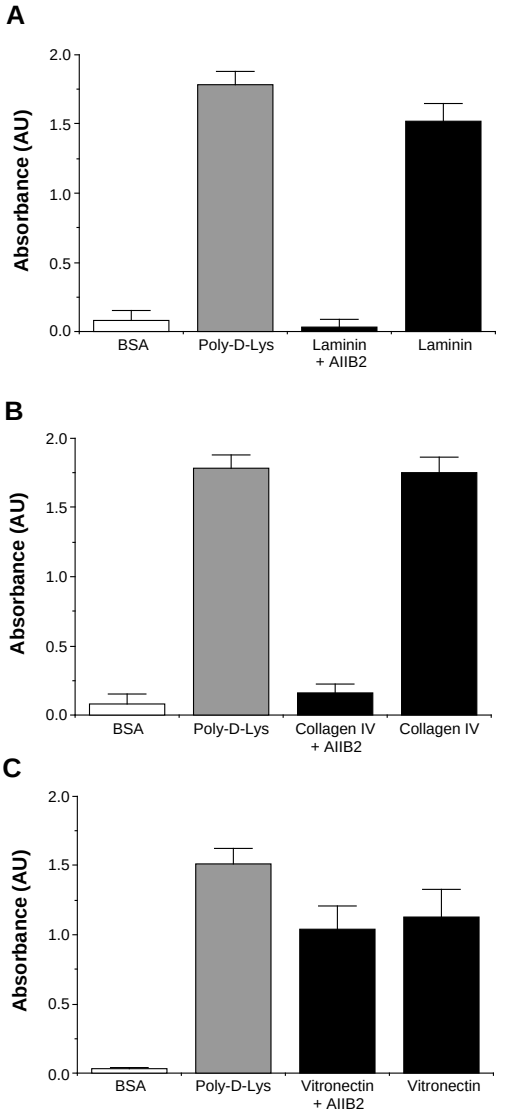


Fig. 6. MDCK cell adhesion to laminin, collagen IV and collagen I, but not vitronectin is blocked by AIIB2, an anti-β_1 antibody. MDCK cells were challenged to adhere to heat-treated BSA, poly-D-lysine and 3 μg/well laminin (A), 1.5 μg/well collagen IV (B), 3 μg/well collagen I (C), or 1 μg/well vitronectin in the presence or absence of the function-blocking monoclonal antibody AIIB2 diluted 1:10. The cells were allowed to adhere for 90 minutes before washing away non-adherent cells. Adherent cells were fixed, stained with crystal violet, and quantitated with a microplate reader. Each graph summarizes three separate trials. Bars represent standard error of the means. AU, arbitrary units.

Three lines of transformed MDCK cells as well as the control cell line C6 were labeled overnight with [^{35}S]methionine, extracted under non-denaturing conditions, and immunoprecipitated with polyclonal anti-β_1. Under these conditions, transformants showed a significant reduction in some of the β_1 integrins correlated with the amount of *ras* expression (Fig. 8A). In higher-resolution gels, R5 cells were seen to almost completely lack two polypeptides with the highest mobility

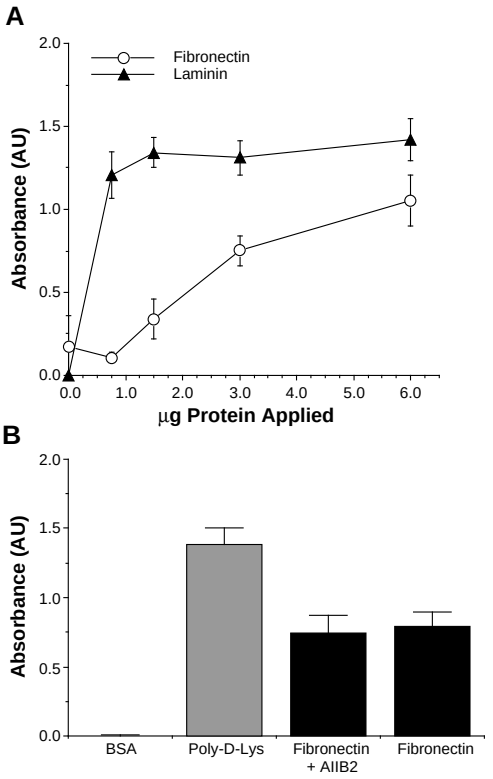


Fig. 7. MDCK cell adhesion to fibronectin is inefficient and is not inhibited by the anti-β_1 antibody AIIB2. (A) To determine the amount of fibronectin necessary for maximal attachment, MDCK cells were plated onto various quantities of fibronectin (○) in 96-well plates. As a comparison, they were also plated onto plates coated with different amounts of laminin (▲). After a 90 minute incubation non-adherent cells were washed away and adherent cells were fixed with crystal violet before being stained and quantitated with a microplate reader. Note that maximal MDCK cell adhesion occurs at very low concentrations of laminin while adhesion to fibronectin is relatively inefficient. (B) MDCK cells were challenged to adhere in 96-well plates to heat-treated BSA, poly-D-lysine or 3 μg fibronectin/well in the presence or absence of a 1:10 dilution of AIIB2. The cells were allowed to adhere for 90 minutes before washing, fixing, staining with crystal violet and quantitating with a microplate reader. Each graph summarizes three separate trials. Bars represent standard error of the means. Note that adhesion to fibronectin is not perturbed by the AIIB2 antibody.

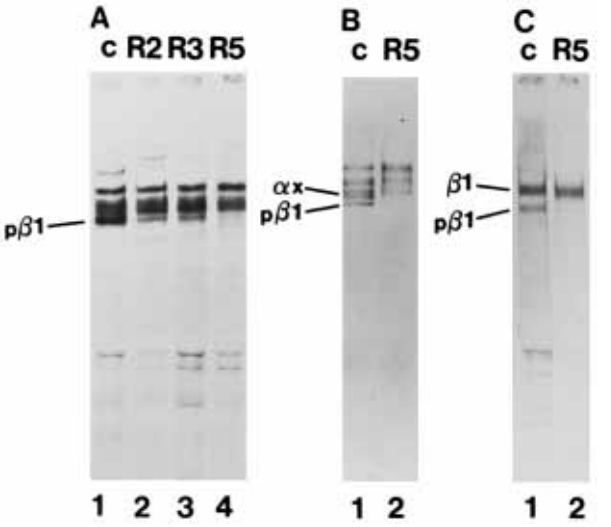


Fig. 8. Immunoprecipitation of β_1 integrins from transformed MDCK cells. Normal MDCK cells (C6, c) and K-*ras* transformed lines (R2, R3, R5) cultured on permeable supports were labeled overnight with [^{35}S]methionine, extracted under nondenaturing (A,B) or denaturing (C) conditions, and immunoprecipitated with a polyclonal anti-β_1 antibody. In A, different transformed lines that express increasing amounts of *ras* p21 (R2<R3<R5) show a gradual loss of polypeptides comigrating with the β_1 precursor (pβ_1, compare control lane 1 with lanes 2-4). In more favourable gels (B), the transformant R5 can be seen to lack α_x and express diminished amounts of the β_1 precursor (pβ_1). The latter is even more evident when immunoprecipitations are conducted under denaturing conditions (*C*). Only one major polypeptide corresponding to mature β_1 is precipitated by anti-β_1 from R5 cells, while both mature β_1 and the β_1 precursor are precipitated from the C6 controls.

(Fig. 8B). On the basis of previous experiments (see Fig. 1), the fastest migrating of these was identified as the β_1 biosynthetic precursor (pβ_1). The other band corresponded to α_x, the putative integrin subunit precipitated by anti-β_1 (Fig. 1). The absence of the β_1 precursor was more clearly evident in immunoprecipitates carried out under denaturing conditions (Fig. 8C). In this case, anti-β_1 precipitated two polypeptides from normal cells but only one from the transformant (Fig. 8C).

With integrins, as with many other oligomeric proteins, transport out of the endoplasmic reticulum to the plasma membrane is dependent on formation of the correct oligomeric structure: namely, the $\alpha\beta$ heterodimer (Bodary and McLean, 1990; Cheresh and Spiro, 1987; Hemler, 1991; O'Toole et al., 1989). Normal MDCK cells appear to synthesize more β_1 subunits than compatible α subunits. This excess, which is evident as the Endo-H-sensitive β_1 detected under denaturing conditions (Fig. 1, lanes 2,3), apparently permits all of the α subunits that can associate with β_1 to form heterodimers and be transported to the plasma membrane. In transformed MDCK cells this does not seem to be the case, since little or no Endo-H-sensitive β_1 precursor can be detected (Fig. 8C).

If excess β_1 is not present in transformed cells, then it is possible that not all of the synthesized α subunits are able to exit from the endoplasmic reticulum. To test this, labeled extracts from transformed and normal cells were immunoprecipitated under denaturing conditions with antibodies against the α_2 and α_3 subunits. In the case of α_2, only one Endo-H-resistant polypeptide was detected in both normal and transformed cells (data not shown). In the case of α_3, on the other hand, two polypeptides were seen in the transformed cells as compared to one detected in normal cells (Fig. 9, lanes 1,2). Of the two, the more slowly migrating was sensitive to Endo H (Fig. 9, lanes 3,4).

Immunoprecipitation of the transformants with antibodies directed against the $\alpha_v\beta_3$ vitronectin receptor and both the α_6 and β_4 subunits uncovered no significant differences between the normal and transformed cells (data not shown).

Because changes in β_1 integrins were detected in the transformed cells, it was possible that their surface expression was also altered. This was examined by immunofluorescence microscopy and surface labeling. As illustrated in Fig. 10, β_1 integrins were distributed mainly to the parts of the plasma

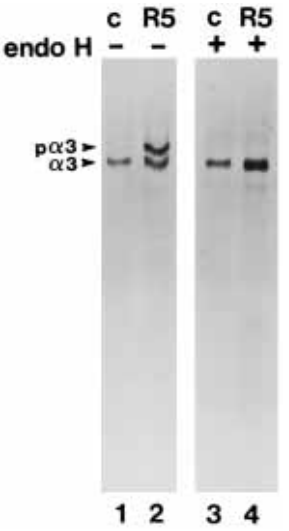


Fig. 9. Immunoprecipitation of α_3 from transformed MDCK cells. Normal MDCK cells (C6, c) and the K-*ras*-transformed line R5 cultured on permeable supports were labeled overnight with [^{35}S]methionine, extracted under denaturing conditions, and immunoprecipitated with polyclonal anti-α_3 antibodies. Half of each sample was then digested with Endo H (lanes 3 and 4) or incubated without the enzyme (lanes 1 and 2). In normal MDCK cells, anti-α_3 precipitates a single polypeptide (lane 1) whose mobility is unaltered by Endo H digestion (lane 3). In the R5 transformed line, however, two polypeptides are precipitated (lane 2); the more slowly migrating of these is sensitive to Endo H and comigrates with the Endo-H-resistant band after digestion (lane 4).

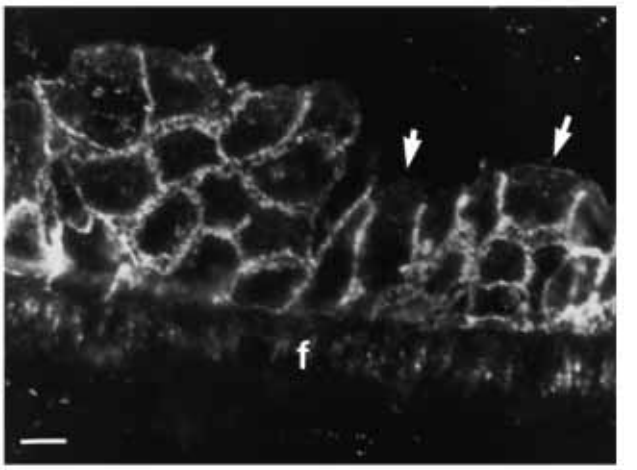


Fig. 10. Immunolocalization of the β_1 integrin subunit in transformed MDCK cells. Frozen sections (0.5 μm) of fixed R5 transformed cells cultured on permeable supports were stained with anti-β_1 antibodies and observed in the fluorescent microscope. Multilayers of cells can be seen attached to the filter substratum (f). The β_1 subunit is localized predominantly to areas of cell-cell contact throughout the multilayer. Staining of the basal surface is also evident as an out-of-focus glare adjacent to the filter. In some cells, a small amount of staining can also be detected on the free or apical surface of the topmost layer of cells (arrows). Fluorescence within the filter is presumably due to blebbing of the basal plasma membrane of R5 cells. Bar, 10 μm.

membrane involved in cell-cell contacts in multilayers of the R5 line. A small amount of staining could be seen on the apical surface in some cells (Fig. 10). For surface biotinylation, the R5 transformant cultured on permeable supports was reacted with sulfo-NHS biotin as described previously. To make sure that the labeling reagent had access to all cells, cultures were grown for only 4 days to make sure that multilayering had not yet commenced. Examination of parallel cultures by light microscopy of thick plastic sections indicated that the cells grown under these conditions formed a confluent monolayer (data not shown). Measurement of the transepithelial resistance of other cultures indicated that they were tight and thus not likely to permit the labeling reagent to penetrate from one side of the culture to the other (data not shown). When surface biotinylation was conducted under these conditions, both the α_3 and the α_2 subunits were detected on the basolateral cell surface together with β_1 (Fig. 11, lane 5) as was also seen in the C6 control line (Fig. 11, lane 2). Surprisingly, however, a fraction of these integrins was also detected on the apical surface (Fig. 11, lane 4); as before, no integrins were detected on the apical plasma membrane of the control (Fig. 11, lane 1). When R5 cultures were biotinylated simultaneously from both the apical and basolateral sides (Fig. 11, lane 6), the intensities of the bands corresponded to approximately the sum of the intensities derived from separate apical and basal biotinylation. This indicated that the apical signal was actually due to apical integrins and not the result of leakage of biotinylation reagent from the apical to the basal compartment.

In summary, analysis of the integrin composition of transformed MDCK cells indicated that they expressed less of the β_1 subunit relative to the amount of α subunits. This apparently resulted in a change in the subunit balance such that Endo-H-sensitive α_3 but no Endo-H-sensitive β_1 could be detected in the transformed cells. In addition, in gels with favourable resolution, no α_x could be detected in the R5 transformant. Finally, in cultures of R5 transformants that had not yet formed multilayers, a significant amount of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ could be detected not only on the basolateral domain but also on the apical domain by surface biotinylation.

DISCUSSION

Integrin expression in MDCK cells and the kidney

The results presented here establish that normal MDCK cells adhere to the substratum via a variety of integrins arrayed on the basolateral surface. These include the laminin and collagen receptors $\alpha_2\beta_1$ and $\alpha_3\beta_1$, and the vitronectin receptor $\alpha_v\beta_3$. In addition, MDCK cells express $\alpha_6\beta_4$ and possibly other integrin α subunits that associate with β_1. Given that MDCK cells resemble cells of the kidney distal tubule (Meier and Insel, 1985), our findings are generally consistent with those of Korhonen et al. (1990a), who reported that human distal tubular cells express the α_2, α_3 and α_6 integrin subunits. Our observations disagree, however, with their conclusion that β_4 is not expressed by kidney tubular cells and that, as a result, α_6 must form a heterodimer with β_1. In contrast, our immunoprecipitations under nondenaturing conditions with antibodies directed against either α_6 or β_4 suggest that MDCK cells express α_6 exclusively in a complex with β_4. The conclusion that kidney epithelial cells express not only β_4 but also α_6 is

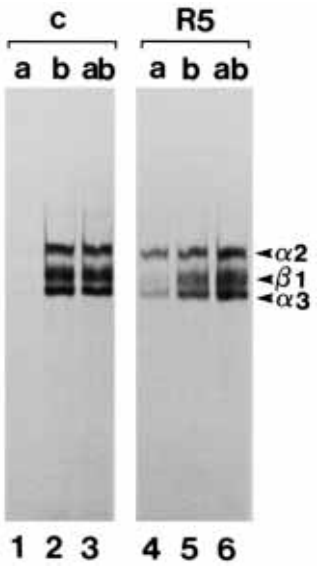


Fig. 11. Surface labeling of β_1 integrins in transformed MDCK cells. Normal (C6,c) and R5 transformed MDCK cells cultured on permeable supports were labeled overnight with [^{35}S]methionine. Replicate filter chambers were then reacted from the apical (a), basolateral (b), or apical and basolateral (ab) sides of the cell monolayer with sulfo-NHS biotin. The cells were extracted under nondenaturing conditions and immunoprecipitated with a polyclonal anti-β_1 antibody. Immunoprecipitates were resolubilized and reprecipitated with streptavidin agarose to capture the surface biotinylated proteins. In normal MDCK cells, β_1 integrins can only be detected from the basolateral surface (compare lanes 1 and 2; see also Fig. 5A). In contrast, in R5 cells, both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ can be detected not only from the basolateral side but also from the apical side (lanes 4 and 5). Note that the intensities of the bands representing biotinylation of both apical and basolateral compartments of R5 cultures (lane 6) appear to be the sum of the intensities of the apical bands and the basolateral bands (i.e. lanes 4+5=6). This indicates that biotinylation from the apical side is not due to leakage of the biotinylation reagent from the apical to basolateral compartment.

supported by the recent studies of Natali et al. (1992), who found that α_6 and β_4 staining was evident in kidney tubules.

No polypeptides were immunoprecipitated from MDCK cells with antibodies against α_4 and α_5 subunits. While these data are not definitive, because we cannot be certain that the anti-peptide antibodies employed cross-react with the equivalent canine integrins, they are consistent with the observed adhesion preferences of MDCK cells. Both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are fibronectin receptors (Hemler, 1991; Hynes, 1992; Ruoslahti, 1991). However, not only do MDCK cells adhere relatively poorly to fibronectin, but this small adherence is not inhibited by a specific function-blocking antibody to β_1.

The identity of the fifth polypeptide (α_x) sometimes resolved in SDS-gels of anti-β_1 immunoprecipitates is unknown but is of great interest due to its apparent absence from transformed MDCK cells. We have not yet tested antibodies against α_v, α_1, $\alpha 7$ and $\alpha 8$, which are known to associate with β_1. The unknown polypeptide is, however, not likely to be either α_1 or $\alpha 7$ because these differ considerably in molecular mass from α_x (Hemler, 1991). It is also probably not α_v, a fibronectin receptor, because anti-β_1-blocking antibodies do not inhibit MDCK cell adhesion to fibronectin. In addition, when immunoprecipitates containing α_v are electrophoresed on the same gel as β_1 integrins, α_x and α_v do not co-migrate (unpublished results). At present we cannot exclude the possibility that this polypeptide is a splice variant or modified form of one of the other integrin α subunits, although the data from denaturing immunoprecipitations would argue against this. Ultimately, identification of this polypeptide will depend on more detailed analysis by two-dimensional electrophoresis and examination of mRNAs.

The β_1 integrins expressed in MDCK cells appear to be the sole receptors for adhesion to both laminin and collagens I and IV, since adhesion to these ligands is completely blocked by an antibody against the β_1 subunit. Which α subunits are involved is not clear, since α_2 and α_3 can each act as both laminin and collagen receptors (Chan and Hemler, 1993; Elices and Hemler, 1989; Elices et al., 1990; Gehlsen et al., 1988; Hemler, 1991; Hynes, 1992; Kirchofer et al., 1990; Languino et al., 1989; Ruoslahti, 1991; Staatz et al., 1989; Wayner and Carter, 1987). Other α subunits, such as α_x and the high molecular mass polypeptide possibly corresponding to α_1 might also be collagen or laminin receptors (Hall et al., 1990). Salas recently reported that MDCK cells express a 37/67 kDa laminin-binding protein (Salas et al., 1992). Whether this functions independently or in conjunction with laminin-binding integrins remains to be determined.

In addition to the β_1 integrins, MDCK cells also express a receptor for vitronectin, which we have tentatively identified as $\alpha_v\beta_3$. This identification is based upon immunoprecipitation data using a commercial polyclonal antiserum directed against the vitronectin receptor $\alpha_v\beta_3$. Because subsequent characterization of lots of this antibody have indicated that it may also react with $\alpha_v\beta 5$ (Telios Pharmaceuticals, Technical Literature), we cannot rule out the possibility that it is this integrin and not $\alpha_v\beta_3$, which is expressed by MDCK cells. In any case, it is likely that adherence to both vitronectin and fibronectin are mediated by the same integrin, since a blocking antibody against β_1 has no effect on adhesion to either substratum.

The evidence for expression of a β_3 integrin in kidney tubular cells is inconclusive. Korhonen et al. (1990b) were unable to detect the β_3 subunit in human kidney using a monoclonal anti-human antibody. They did, however, find staining in the glomerulus and proximal tubule with a polyclonal anti-vitronectin receptor antiserum. It is possible that this polyclonal antiserum, which was purchased from the same source as the anti-vitronectin receptor antiserum used in our experiments (Korhonen et al., 1990b), actually reacted with $\beta 5$ and not β_3. A recent report indicating that $\beta 5$ is expressed in the glomerulus, proximal tubule and collecting duct makes this even more likely (Pasqualini et al., 1993).

On the role of $\alpha_6\beta_4$ in MDCK cells

Although $\alpha_6\beta_4$ has been reported to be a laminin receptor in human colon carcinoma cells (Lee et al., 1992), in MDCK cells it does not seem to be responsible for adhesion to laminin. Even at high concentrations, GoH3, a blocking anti-α_6 antibody, fails to inhibit attachment to laminin-coated substrata. It is possible that in MDCK cells, $\alpha_6\beta_4$ is either inactive or mediates attachment to some other unidentified ligand. One possibility is kalinin, a member of the laminin family, which codistributes with $\alpha_6\beta_4$ in keratinocytes (Marchisio et al., 1993). In the future, it will be important to

determine if MDCK cells synthesize or attach to kalinin or other alternative forms of laminin (Paulsson, 1993), which may serve as kidney-specific ligands for $\alpha_6\beta_4$.

Our observations concerning the α_6 subunit are significant because it has been implicated in the formation of the first polarized epithelium during kidney development. Ekblom and colleagues reported that GoH3 prevents differentiation of the condensed mesenchyme to an epithelium by inhibiting its interaction with the laminin A chain (Klein et al., 1988; Sorokin et al., 1990). Because development of the epithelium is also blocked by antibodies to the E8 fragment of laminin (Sorokin et al., 1990), whose receptor is $\alpha_6\beta_1$ and not $\alpha_6\beta_4$ (Sonnenberg et al., 1990b), it is believed that the integrin involved in the differentiation event is $\alpha_6\beta_1$. To our knowledge, however, this has not been directly proven. In fact it is likely that the situation in the developing kidney is even more complex, with more than one kind of laminin and more than a single α_6-bearing integrin involved in epithelial polarization.

Localization of integrins in MDCK cells

Localization of integrins in MDCK cells by immunofluorescence microscopy demonstrated that β_1, β_3 or β_4 (as revealed by an antibody to α_6) integrins are all found not only on the basal surface, but also on the lateral surface. It is possible that this distribution occurs because there are insufficient ligands deposited on the basal substratum to interact with the cell surface integrins; under these conditions the uncomplexed integrins might remain diffusely distributed over both the basal and lateral surfaces. Our results also do not rule out the possibility that there are extracellular matrix proteins between the cells to which the integrins could attach, although we have not detected any ultrastructurally.

Alternatively, the lateral integrins could be involved in cell-cell interactions. Although the integrins of three β-subunit families found in MDCK cells are primarily known as receptors for extracellular matrix molecules, there is considerable evidence that some of them also participate in cell-cell interactions (Carter et al., 1990; Larjava et al., 1990; Symington et al., 1993). Studies of keratinocytes, in particular, have implicated $\alpha_2\beta_1$ and $\alpha_3\beta_1$ in cell-cell adhesion (Carter et al., 1990; Larjava et al., 1990; Symington et al., 1993). Both have been localized to regions of cell-cell contacts, and antibodies against β_1 cause cells to detach from each other in suspension culture and when adherent to a vitronectin substratum (Carter et al., 1990; Larjava et al., 1990). More recently, biochemical experiments have suggested that keratinocyte $\alpha_2\beta_1$ and $\alpha_3\beta_1$ might interact, implying that they participate in heterotypic cell-cell contacts (Symington et al., 1993). Because MDCK cells express both of these integrins, it will be of great interest to determine whether function-blocking anti-β_1 antibodies disrupt cell-cell as well as cell-substratum contacts.

The localization of $\alpha_6\beta_4$ in MDCK cells is of particular interest. In skin and cornea, $\alpha_6\beta_4$ is found exclusively in hemidesmosomes where it may interact both with the extracellular anchoring filaments and the intermediate filament cytoskeleton (Quaranta and Jones, 1991; Sonnenberg et al., 1991; Stepp et al., 1990). In MDCK cells, which do not form hemidesmosomes, $\alpha_6\beta_4$ is found not only on the basal surface, where it is distributed unevenly over the entire surface, but also on the lateral surface, where its distribution is interrupted and possibly focal. Given the propensity of $\alpha_6\beta_4$ to interact with hemidesmosomes on the basal surface of external epithelia, it is tempting to speculate that in MDCK cells, the $\alpha_6\beta_4$ found on both the basal and lateral surface also participates in junctional complexes involving intermediate filaments. Resolution of this issue will require ultrastructural immunolocalization.

Integrins and transformation

Our results demonstrate that transformed MDCK cells, while generally displaying the same integrin profile as normal MDCK cells, have some deficiencies in the expression and localization of β_1 integrins. In addition to lacking α_x, transformed MDCK cells do not express significant amounts of the Endo-H-sensitive β_1 precursor. In normal MDCK cells an excess of β_1 is synthesized relative to the stoichiometric amounts of α subunits. All of the synthesized α subunits are then able to associate with β_1 and exit the endoplasmic reticulum on their way to the plasma membrane (Hemler, 1991). Although we have not localized β_1 to the endoplasmic reticulum in MDCK cells, this scenario is consistent not only with present knowledge of glycoprotein transport and processing (Hurtley and Helenius, 1989), but also with our surface labeling data showing only mature β_1 at the cell surface. This interpretation is also consistent with the lack of Endo-H-sensitive precursors of α_2 and α_3 in normal cells. Because the amount of β_1 is relatively less in transformed MDCK cells, α subunits compatible with β_1 presumably compete for available β_1 on the basis of affinities. Under these circumstances, α_2 seems to win out over α_3 in the race for a partner, resulting in some α_3 being left behind as an Endo-H-sensitive precursor. Interestingly, the α_6 subunit does not seem to even enter the race for β_1, although it can associate with it in other cells. Instead, it appears to have such a great affinity for β_4 that in the normal cells, where excess β_1 remains in the endoplasmic reticulum, no $\alpha_6\beta_1$ is detectable.

The effect of the disturbance in processing and transport of β_1 integrins that accompanies transformation on MDCK cell differentiation is difficult to assess. If the morphogenesis of polarized epithelial cells is dependent only qualitatively on the complement of integrins expressed on the cell surface, then this apparently minor alteration would not be expected to be important. If, on the other hand, correct morphogenesis depends on the exact balance of various integrins at the plasma membrane, then a change in relative amounts could be quite significant. Current experiments in our laboratory are attempting to address this issue.

In multilayers of the R5 transformant, there was only a small amount of apical staining by antibodies to β_1 that did not seem to exceed the amount of apical β_1 staining seen in the normal cells. This was consistent with the previous observation that proteins that are usually apical are randomly distributed in the transformed cells while normally basolateral proteins such as the Na^+,K^+-ATPase are found on the basal and lateral domains (Schoenenberger et al., 1991). In contrast, significant quantities of both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ were detected on the apical surface by biotinylation in R5 cultures that had not yet formed multilayers while nothing was found apically with the same technique in normal cells.

The discrepancy between the immunofluorescence and biochemical data is difficult to explain. One possibility is that the polarity of the transformed cells is not uniform and the signal

from some completely depolarized cells skewed the biotinylation data. Alternatively, surface biotinylation could simply be more sensitive than immunofluorescence. A third explanation is that significantly more integrins are found on the apical surface in monolayers of the R5 line than on multilayers.

If there is mislocalization of β_1 integrins in the transformed cells, then it may have come about through a variety of mechanisms. Basolateral proteins are known to polarize slowly in MDCK cells after the formation of cell-substratum and cell-cell contacts (Rodriguez-Boulan and Nelson, 1989). In the transformant, it is possible that the β_1 integrins had not yet shifted from a random to a basolateral distribution at the time when the surface labeling was conducted, even though the monolayer was electrically tight. Another possibility is that the localization of different integrins depends on the localization of their ligands. Thus, it is possible that transformation causes laminin or collagen to be secreted randomly from both sides of the cells, with the integrins simply following their lead. This option can be tested by examining integrin localization in transformed cells cultured on exogenous extracellular matrix proteins. Indeed, preliminary experiments have suggested that Matrigel may partially revert some aspects of the transformed phenotype (A. Zuk and K. S. Matlin, unpublished results). Conversely, if transformation leads to mistargeting of integrins to the apical surface, then these cells may not adhere properly to the basal extracellular matrix. Such weak attachment to the substratum might then make cell-cell contacts paramount, leading to multilayering, and signalling a loss of apical polarity.

Numerous changes in both integrin expression and localization have been previously noted in both tumor cells and oncogenically transformed cells in culture. In tumors, reduced expression of some integrins normally associated with the well-differentiated phenotype, such as $\alpha_2\beta_1$, and increased expression of others, such as $\alpha_v\beta_3$, are correlated with cellular dedifferentiation, loss of polarity and increased invasiveness (Koretz et al., 1991; Koukoulis et al., 1991; Pignatelli et al., 1990, 1991; Weinel et al., 1992). Although no causal relationships have been established, it is suggested that changes in integrins might allow tumor cells to more readily cross the basal lamina and invade the interstitial matrix (Pignatelli and Bodmer, 1990; Ruoslahti, 1991). In cultured fibroblastic cells transformed by v-*src* or v-*ras*, the reduction in expression of $\alpha_5\beta_1$ and two other unidentified integrins correlates well with observed decreases in binding to fibronectin-coated substrata (Plantefaber and Hynes, 1989). Conversely, overexpression of $\alpha_5\beta_1$ in transformed Chinese hamster ovary cells results in the loss of some features of the transformed phenotype (Giancotti and Ruoslahti, 1990). Thus, it seems likely that changes in integrins actually drive generation of the transformed phenotype and are not just side-effects of oncogenesis.

Integrins and epithelial cell polarization

In MDCK cells contact with the substratum is sufficient to initate formation of an apical pole, which then reaches maturity after cell-cell contacts are established (Rodriguez-Boulan and Nelson, 1989). In suspension culture in normal medium, MDCK cells form aggregates that eventually develop into cysts (Wang et al., 1990a). Under these conditions, polarization of both apical and basolateral proteins can occur in the aggregates in the apparent absence of an extracellular matrix substratum, but correct placement of the tight-junctional protein ZO-1 is dependent on deposition of collagen IV in the lumen following cyst formation (Wang et al., 1990a). In addition, in MDCK cells and other epithelia, the layering of a collagen gel over the apical surface results in dramatic reorganization of the cells into tubular structures and cysts, and inversion of the axis of polarity in response to the new situation (Hall et al., 1982; Nitsch and Wollman, 1980; Wang et al., 1990b; Chambard et al., 1981 p. 540). Finally, as discussed previously, events mediated by a laminin-integrin interaction are important in polarization of the murine kidney epithelium during development (Klein et al., 1988; Sorokin et al., 1990).

From all of these phenomena, two themes emerge. One is that extracellular matrix in general, and laminin and collagen in particular, are important for the correct morphogenesis and polarization of an epithelium. Second, cell-substratum and cell-cell contacts appear to be interdependent, sometimes working together and at other times, in the absence of the other, working alone while performing the other's function (Rodriguez-Boulan and Nelson, 1989). Thus, apical polarity can form in isolated attached cells in the absence of cell-cell contacts, but fully develops only with the cooperation of cell-cell adhesion. Conversely, when contact with the substratum is missing, as in suspended aggregates of cells, then polarization can occur, but requires the synthesis of and interaction with extracellular matrix proteins to reach maturity (Wang et al., 1990a).

Our results demonstrate that MDCK cells depend on integrins for interaction with both laminin and collagen, as well as with vitronectin. Integrins are, therefore, clearly important in polarization. In addition, our observations raise the possibility that integrins also participate in cell-cell interactions. Thus, the interdependence of cell-substratum and cell-cell contacts in epithelial polarization could be due at least partially to the fact that the integrins participate in both. Future work in our laboratory will examine the validity of this hypothesis.

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