α3β1 integrin regulates epithelial cytoskeletal organization

Zemin Wang1, Jordan M. Symons1, Stuart L. Goldstein1,*, Alice McDonald1,‡, Jeffrey H. Miner2 and Jordan A. Kreidberg1,¶

1Department of Medicine, Children’s Hospital, and Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA
2Renal Division, Department of Medicine, Washington University School of Medicine, St Louis, MO 63110, USA

The first two authors contributed equally to the work presented
*Present address: Texas Children’s Hospital, Houston, TX, USA
‡Present address: Millennium Pharmaceuticals, Cambridge, MA, USA
¶Author for correspondence (e-mail: Kreidberg@a1.tch.harvard.edu)

Accepted 14 June 1999; published on WWW 12 August 1999

SUMMARY

Epithelial cell morphology and cytoskeletal organization are determined by interactions, with both adjacent cells and the extracellular matrix, which are mediated by integrins and cadherins. Little is known, however, of the relative contributions of integrins and cadherins to maintaining the sub-cortical cytoskeleton characteristic of epithelial cells. Since most studies that utilize integrin-blocking antibodies result in a loss of both cell-cell adhesion and sub-cortical cytoskeletal organization, it has been difficult to distinguish whether integrins and cadherins both mediate cytoskeletal assembly in epithelial cells. Therefore, cells derived from kidney collecting ducts of α3β1 integrin-deficient mice were used to examine the role of integrins in epithelial cell morphology and cytoskeletal organization. In primary cell culture, α3β1 integrin-deficient kidney collecting duct cells maintain cadherin-mediated cell-cell adhesions but fail to form the sub-cortical cytoskeleton that is characteristic of epithelial cells, and instead assemble actin stress fibers. Moreover, the cell-cell junctions in mutant cells were irregular, rather than being uniformly oriented perpendicular to the culture substrate. These results demonstrated that integrins have an primary and essential function in establishing and maintaining the sub-cortical cytoskeleton that is characteristic of epithelial cells. To further study the role of α3β1 integrin in establishing and maintaining cytoskeletal organization in tubular epithelial cells, we derived immortalized cell lines from wild-type and α3β1 integrin-deficient kidney collecting ducts that duplicated the cytoskeletal and cadherin organization observed in primary cells. E-cadherin and α- and β-catenin were complexed together in equal amounts in membranes of wild-type and α3β1 integrin-deficient cells. However, association of the cadherin:catenin complex with α-actinin was greatly decreased in mutant cells, indicating that integrin-mediated assembly of the sub-cortical cytoskeleton is essential for subsequent association of the cytoskeleton with the cadherin:catenin complex. These results present direct evidence for integrin:cadherin cross-regulation in which cadherin function is dependent on the presence of an integrin.

Key words: Integrin, Epithelial cell, Cytoskeleton

INTRODUCTION

The extracellular matrix (ECM), including the basement membrane, serves as an interface between epithelial cells and the surrounding mesenchyme. Therefore, the inductive interactions that occur between groups of mesenchymal and epithelial cells to regulate organ development are transmitted through the ECM. These inducing signals include both diffusible growth factors and components of the ECM itself. Integrins are heterodimeric cell surface receptors that specifically bind components of the ECM and therefore are obvious candidates to transduce signals that elicit behavioral responses determined by the composition of the surrounding ECM (Hynes, 1992). Behaviors such as cell division, migration and differentiation have either been demonstrated to be directly modulated by integrin:matrix interactions or found to be aberrant in embryos or animals carrying mutations in particular integrin genes (Hynes, 1996).

The observation that integrin:matrix interactions result in cytoskeletal rearrangements in cultured cells has fostered intense study (Clark and Brugge, 1995; Haimovich et al., 1993). Focal adhesions have been identified as points of cellular attachment to the underlying matrix, where integrin clustering results in the assembly of an array of cytoskeletal proteins (Burrige and Chrzanowska-Wodnicka, 1996). Stress fibers, composed of actin filaments, converge at focal adhesions, where they are physically linked to actin-binding proteins such as α-actinin, vinculin and talin, some of which are also physically linked to integrins (Burrige and Chrzanowska-Wodnicka, 1996). Therefore, integrins also serve...
as structural links between the ECM and the cytoskeleton. Several signal transduction proteins, such as FAK (focal adhesion kinase) and members of the Src family, have been demonstrated to associate with focal adhesions where they might regulate focal adhesion and cytoskeletal assembly or otherwise transmit signals to the cell regarding ongoing integrin:matrix interactions (Burridge et al., 1992; Calab et al., 1995; Clark and Brugge, 1995; Cobb et al., 1994; Schaller et al., 1995).

Cytoskeletal architecture in cultured cells can differ significantly between distinct cell types. Cells that grow individually without forming strong cell-cell adhesions, such as fibroblasts, tend to assemble actin stress filaments that converge at focal contacts (Burridge and Chrzanowska-Wodnicka, 1996). In contrast, cells that form well-defined cell-cell junctions, such as epithelial cells, assemble a sub-cortical actin cytoskeleton instead of focal adhesions and actin stress fibers (Carter et al., 1990; Larjava et al., 1990). Both cadherins and integrins, including α3β1, are expressed along cell-cell junctions, where they have a role in maintaining cell-cell adhesion and organization of the sub-cortical cytoskeleton (Carter et al., 1990; Kaufmann et al., 1989; Larjava et al., 1990). For example, Carter et al. (1990) showed that anti-α3β1 integrin blocking antibodies interfered with cell-cell adhesion in primary in vitro cultures of human keratinocytes. The localization pattern for α3β1 integrin in keratinocytes appeared to be dependent on the presence of Ca2+-dependent cadherin-mediated cell-cell adhesion; in the absence of Ca2+, cells assembled actin stress fibers, and α3β1 localized to focal adhesions. These results suggested an interplay between cadherins and integrins to regulate cytoskeletal assembly and cell-cell adhesion and, in particular, that cadherin-dependent cell-cell adhesion is required for the localization of integrins along cell-cell junctions. Since these early studies, it has been difficult to delineate specific roles for integrins and cadherins in cell-cell adhesion and cytoskeletal assembly (Carter et al., 1990; Larjava et al., 1990).

In order to increase our understanding of integrin-mediated cellular interactions, and how these contribute to development, we introduced a null mutation into the murine α3 integrin gene (Kreidberg et al., 1996). Our previous reports on the phenotype of mice carrying mutations in this gene demonstrated that mice homozygous for the mutation die during the perinatal period with defects in kidney, lung (Kreidberg et al., 1996) and skin development (DiPersio et al., 1997). Central to this report, fewer collecting ducts (CDs) were observed in the medullary papillae of the mutant kidneys, although those present appeared to have undergone normal differentiation.

The focus of this report is to study the role of integrin receptors in determining the pattern of cytoskeletal organization and cell-cell interaction in epithelial cells. We demonstrate that integrins have a primary role in maintaining organization of the sub-cortical cytoskeleton, even when cadherin-mediated cell-cell adhesions are present.

**MATERIALS AND METHODS**

**Materials**

ECM products, including matrigel, fibronectin, type I collagen and laminin, were obtained from Becton Dickinson-Biocoat Division and used according to the distributor’s recommendations. ECM-coated permeable membranes were also obtained from the same supplier. 8-well glass slides were obtained from LabTek.

**Antibodies**

Anti-integrin antibodies for staining and immunoprecipitation: polyclonal anti-α6α cytoplasmic domain, were obtained from V. Quarranta (Scripps Research Institute); rabbit polyclonal anti-α3, β1, α5 from R. Hynes (Massachusetts Institute of Technology); anti-human α3β1: clone P1B5 from Life Technologies, rabbit polyclonal anti-α2 from M. Hemler (Dana Farber Cancer Institute, Boston, MA); polyclonal anti-αv and β4 from Chemicon; anti-β1 for staining: clone Ha2/5 (Pharmingen). Monoclonal anti-Na+/K+-ATPase clone α5 was from the Developmental Studies Hybrida bank, University of Iowa. Rabbit polyclonal anti-aquaporin 2 was obtained from H. W. Harris (Children’s Hospital, Boston, MA). Anti-α-actinin: clone BM-75.2 was from Sigma. Anti-E-cadherin, clone 36; anti-α-catenin, clone 5; anti-β-catenin, clone 14; anti-γ-catenin, clone 15, all from Transduction Laboratories. For staining E-cadherin, clone ECD2-2 from Zymed was used. Texas Red-phalloidin was from Molecular Probes T-7471, lot 4151-2. Secondary antibodies and linking reagents were from Pharmingen, Santa Cruz Biotechnology, Jackson ImmunoResearch and Sigma.

**Mice**

Mice carrying the α3 integrin-targeted mutation (Kreidberg et al., 1996) or the temperature-sensitive T antigen transgene (Jat et al., 1991) (Immormousel, Charles River Laboratories) were housed under barrier conditions. All protocols were approved by the Institutional Animal Care and Use Committee. For the derivation of immortalized cell lines, these two strains of mice were intercrossed and the F2 generation double heterozygotes were again intercrossed to obtain embryos that were carrying homozygous wild-type or mutant alleles of the α3 integrin gene and at least were heterozygous for the T antigen transgene.

**Primary cells**

Kidneys were removed from E18 embryos obtained by Caesarian section and placed overnight in DMEM-10% fetal calf serum (FCS) (HyClone) at 4°C while the embryos were genotyped. Genotyping was done by the polymerase chain reaction, using DNA prepared according to published procedures (Laird et al., 1991). After genotyping, wild-type and homozygous mutant kidneys from embryos of multiple litters were pooled in Dulbecco’s PBS containing Mg2+ and Ca2+, and the medullary papillae were isolated by dissection using 27-gauge hypodermic needles. The papillae were minced into small pieces and incubated in DMEM containing 10% FCS with 0.2% Type I collagenase (Life Technologies 17100-017) for 45 minutes, then pelleted by low-speed centrifugation (800 rpm in a table top centrifuge) and placed in organ culture medium, containing the usual levels of Ca2+ and Mg2+ (Woolf et al., 1995) and 1% FCS. Cells were generally stained 16-20 hours after culturing, at which time epithelial cells had migrated from tubular structures and formed small monolayer patches. Cultures were done on 8-well glass slides (LabTek) coated with the appropriate extracellular matrix. Culture medium for primary and immortalized cell lines was as previously published for kidney-derived cell lines (Woolf et al., 1995) except that 1% FCS was added. ECM coating was done according to the distributor’s recommended protocols. Laminin was used at 5 μg/cm2, and matrigel was used at 25 μg/ml, which is a 1:50 dilution of the supplied solution.

**Cell lines**

Cells were obtained exactly as above and cultured in single wells of 48-well plates coated with matrigel. Early passages were especially difficult to trypsinize. The following procedure maximized cell survival. As cells approached confluence, cultures were washed twice.
with phosphate-buffered saline (PBS)/1 mM EDTA, and then a PBS-based non-enzymatic cell dissociation reagent (Sigma C5914) was placed on the cells for approximately 5 minutes, until most cells had rounded up. Then trypsin was added to a final concentration of 0.125% for an additional 3-5 minutes and cells were dislodged by gentle repetitive aspiration and pelleted by centrifugation. Cell populations were gradually expanded, using this protocol for trypsinization. Cell populations retaining the most epithelial-like morphology, as judged by a cobblestone appearance, were selected for further study. B7 and B12 are representative wild-type and mutant cell lines, respectively, derived according to this protocol. Culture medium for immortalized cells was identical as for primary cells, except that γ-interferon (Genzyme) was added at 50 units/ml to boost expression of the T-antigen, until cells had been expanded to 6-well plates, and then was gradually decreased. For the experiments in this paper, γ-interferon was not present. All experiments with cell lines were performed at 33°C, the permissive temperature for T-antigen function. Cells were transfected using calcium phosphate. The human α3 cDNA was cloned into the pcDNA 3.1 Zeo (+) vector (Invitrogen) carrying the zeocin resistance gene, and transfected cells were selected with zeocin (Invitrogen).

Immunofluorescence staining

Cells were stained 16 hours after plating on 8-chambered glass slides (LabTek) coated with various ECM products as described above. Cells were washed with PBS and fixed for 10 minutes in 3% paraformaldehyde in PBS. Cells were then washed again with PBS, permeabilized with 0.5% Nonidet P-40/PBS for 10 minutes, again washed with PBS and blocked for 30 minutes with 10% sheep serum in PBS, incubated for 1 hour at room temperature with the primary antibody diluted in PBS, washed three times with PBS, and incubated for 1 hour with the secondary antibody. FITC-coupled secondary antibodies were used. Slides were finally washed with PBS and coverslips attached with Gelmount (Biomedica, Foster City, CA).

Phalloidin staining

Cells were prepared as for immunofluorescence staining. Phalloidin-Texas Red was diluted 1:200 in PBS prior to use.

Membrane preparations

Cells were harvested by scraping off dishes in 50 mM Tris, pH 7.4, 20 mM phenylmethylsulfonyl fluoride, 25 mM Hepes, pH 7.4, 5 mM NaCl, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 10 μg/ml leupeptin. Cell suspensions were homogenized, and nuclei were removed by centrifuging at 25,000 g for 15 minutes. The supernatant was then centrifuged at 100,000 g for 1 hour, and the pellet was dissolved in 5% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, and used for immunoprecipitation after determination of protein concentrations.

Immunoprecipitation

Integrin immunoprecipitations were performed as described by Berditchevski et al. (1995) with minor modifications. Cells were surface-labelled with sulfo-NHS-Biotin, (Pierce, Rockford, IL) as described. After three washes with PBS, cells were lysed with 1% Brij 96 (Fluka), 25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 10 μg/ml leupeptin, at 4°C for 1 hour. Extracts were centrifuged at 17,000 g for 30 minutes, and protein concentrations were determined. Equal amounts of protein in supernatants were mixed with primary antibodies at appropriate dilutions, Cadherin and catenin immunoprecipitations used material from membrane extracts. Samples were incubated with gentle periodic agitation for 1 hour at 4°C and immune complexes were collected using Protein A-Sepharose beads (Pharmacia), with the exception of immunoprecipitation of β-catenin, which used Protein G-Sepharose (Boehringer-Mannheim). E-cadherin and β-catenin immunoprecipitations were performed as previously published (Calautti et al., 1998). Immune complex-bound Protein A- or G-Sepharose beads were washed four times in the above buffer, followed by elution with 0.1 M glycine, pH 2.7. Eluted proteins were electrophoresed on 5% SDS-polyacrylamide gels (3% stacking gel) under non-reducing conditions to achieve optimal separation of α and β peptides, transferred to nitrocellulose membranes, and developed using avidin-coupled horseradish peroxidase (Sigma) and Rennaissance chemiluminescent reagents (NEN, Cambridge, MA).

Western blots

Western blotting experiments were as described (Gallagher et al., 1993). HRP-conjugated secondary antibodies were used, and blots were developed using the ECL reagent, NEN (Cambridge, MA) according to the manufacturer’s protocol. For detection of aquaporin and Na⁺/K⁺-ATPase, membrane protein was prepared by homogenizing cells in 15 mM Tris, pH 7.5, 250 mM sucrose, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 10 μg/ml leupeptin. Nuclei were removed by centrifuging at 2500 g and the supernatant was then centrifuged at 100,000 g for 30 minutes. The pellets were resuspended in the above buffer and equal amounts of protein were added to electrophoresis sample buffer (Gallagher et al., 1993).

RESULTS

α5-containing laminins are expressed on collecting ducts and ureteric buds

Recent studies have shown that α5-containing laminins such as laminin-10 (α5β1γ1) and -11 (α5β2γ1) are preferred ligands for α3β1 integrin (Kikkawa et al., 1998), while laminin-1 (α1β1γ1) is a preferred ligand of α6β1 integrin (Delwel et al., 1994). Kidneys from newborn mice deficient in α3β1 integrin have marked abnormalities, including a diminution in the number of collecting ducts (Kreidberg et al., 1996). In contrast, mice deficient in α6β1 integrin have normal-appearing kidneys (Georges et al., 1996). The expression of laminin α chains was examined in newborn kidneys to determine the relative abundances of laminins-α1 and -α5, as this might provide further insight into the relative phenotypes of kidneys from mice deficient in α3β1 or α6 integrins. It has previously been demonstrated that α1-containing laminin appeared to be expressed on newly developed epithelial cells, including ureteric bud derivative nascent CDs, whereas α5-laminin appeared to be expressed on more mature epithelial cells (Sorokin et al., 1997a,b). Our studies indicated that α5-laminin was the predominant α chain expressed at equivalent levels along the full length of the CD (Fig. 1A,B). (CDs were identified in the papillary region by staining with the lectin dolichos biflorus; data not shown.) In contrast, α1 was expressed at low levels in the papillary CDs and at slightly higher levels in the ureteric bud (Fig. 1A,B). Comparison of staining intensities of proximal tubules with CDs/ureteric buds indicates that the α1-laminin antibody stains the former more intensely than the latter, whereas the inverse result is obtained with the anti-α5-laminin reagent. Laminin staining was also examined in α3 integrin mutant kidneys, and the pattern with regard to different types of tubules was unchanged from that observed in wild-type kidneys (data not shown).

Although it is difficult to make quantitative comparisons of staining intensities obtained with two different antisera, these results indicate that α5-containing laminins may predominate over α1-containing laminins on CDs/ureteric buds. The
expression of α5-containing laminins along the full length of the CDs is consistent with the hypothesis that α3β1 integrin is required by these cells to achieve their full developmental potential.

Previous studies in human fetal kidneys demonstrated basolateral expression of α3β1 integrin by collecting duct epithelial cells (Korhonen et al., 1991). In adult kidney, expression is primarily basal, with less expression along lateral membranes (Korhonen et al., 1991). We had previously demonstrated that α3β1 integrin is expressed in the developing cortex of embryonic kidneys, and that staining was negative in homozygous mutants (Kreidberg et al., 1996). In this study, we confirmed that basolateral expression of α3β1 integrin is observed in nascent collecting ducts of embryonic mouse kidneys, with basal expression observed in more mature tubules (Fig. 1C).

**Basement membrane abnormalities in α3β1 integrin-deficient CD epithelial cells**

Light microscopy did not reveal any morphological abnormalities in individual CD epithelial cells. To determine whether the absence of α3β1 integrin led to abnormal assembly of the extracellular matrix, the basement membranes of wild-type and mutant CD cells were examined by electron microscopy. Our previous report showed that there was at least a 50% decrease in the number of collecting ducts in the medullary papilla of α3β1 integrin-deficient newborn kidneys. Although examination of multiple sections by electron microscopy always reveals some degree of variation in the thickness of the basement membrane, CD epithelial cells from α3 integrin mutant kidneys characteristically had thinner basement membranes than observed in wild-type cells (Fig. 2). CD basement membranes were not as disorganized as was observed in mutant glomeruli (Kreidberg et al., 1996), presumably because CD epithelial cells express additional integrins, in contrast to glomerular podocytes that only express α3β1 (Korhonen et al., 1991). Electron microscopic examination did not reveal any other abnormalities in epithelial cell morphology or cell-cell junctions in CD epithelial cells from mutant mice.

**Actin cytoskeleton and cadherin and catenin localization are disorganized in the absence of α3β1 integrin**

Previous studies on the role of integrins in cytoskeletal organization used blocking antibodies that also interfered with cell-cell adhesion (Carter et al., 1990; Larjava et al., 1990). In these situations, cells take on a more mesenchymal appearance, with the expected assembly of actin stress fibers. These observations raise the question of whether the assembly of an epithelial sub-cortical cytoskeleton is primarily controlled by integrins or cadherins, as interactions of both receptor types with their ligands are disrupted by integrin blocking antibodies. In contrast, primary cultures of α3β1 integrin-deficient collecting duct cells exhibited the cobblestoned appearance of closely packed epithelial cells (not shown), indistinguishable from wild-type cells, indicating that cell-cell adhesions were present. This afforded the opportunity to examine the role of integrins in cytoskeletal organization in a situation where cadherin-mediated cell-cell adhesion was not disrupted. Despite the presence of cell-cell adhesions, marked differences in cytoskeletal organization were evident in cells cultured on matrigel (Fig. 3). In wild-type cells on either matrix, actin was mainly restricted to the sub-cortex along cell-cell junctions and cell edges. In contrast, prominent stress fibers that converged on focal adhesions were more evident in mutant cells, and less actin was present along the cell cortex. These results
Integrins regulate cytoskeletal assembly

demonstrate a primary role for integrins in maintaining organization of the subcortical cytoskeleton.

Cadherin-mediated cell-cell interactions are also involved in organizing F-actin into the sub-cortical cytoskeleton in epithelial cells (Braga et al., 1995, 1997; Gumbiner, 1996). When cells are cultured under low Ca²⁺ conditions that interfere with cadherin-mediated adhesion, the sub-cortical cytoskeleton is lost, and F-actin is assembled into stress fibers (Braga et al., 1995; Larjava et al., 1990). These results suggest a model whereby cadherin:catenin complexes are involved in maintaining organization of the subcortical cytoskeleton, possibly by direct association of the lateral membrane adhesion complexes with actin-binding proteins such as α-actinin and vinculin (Knudsen et al., 1995; Nieset et al., 1997). The appearance of stress fibers in α3β1 integrin-deficient cells suggested that the absence of α3β1 integrin might affect the ability of cadherin:catenin complexes to maintain organization of the sub-cortical cytoskeleton. To assess this possibility, we initially examined the localization of E-cadherin and catenins

![Fig. 2. Electron micrographs of basal surface of collecting duct epithelial cells in medullary papilla. (A) Wild-type kidney, (B) α3 integrin mutant kidney. The arrows point to the basement membrane. Arrowheads point to cell-cell junction. The basement membrane appears thinner in the mutant cell. Magnification ×2000.](image)

![Fig. 3. Staining of cytoskeletal and cell-cell adhesion-associated proteins in wild-type and α3β1 integrin-deficient cells. The protein stained is shown to the left of each row. The genotype of the cells is shown at the top of each column. Magnification ×200.](image)
were irregularly shaped instead of being uniformly oriented perpendicular to the culture substrate. These results demonstrate that while \( \alpha_3 \beta_1 \) integrin is not a priori required for localization of cadherin-catenin complexes at cell-cell junctions, it has a major role in maintaining the structural integrity of epithelial cell-cell junctions, particularly in their perpendicular orientation to the basement membrane or its equivalent in vitro.

Derivation of immortalized cell lines deficient in \( \alpha_3 \beta_1 \) integrin

In order to study the role of \( \alpha_3 \beta_1 \) integrin in a homogeneous population of epithelial cells and to perform experiments requiring larger amounts of cells than can be obtained from primary cultures, mice heterozygous for the targeted mutation in the \( \alpha_3 \) integrin gene were crossed with transgenic mice carrying a temperature-sensitive SV40 T antigen gene expressed under the control of the H-2K promoter (Jat et al., 1991). Collecting ducts were dissected from kidneys removed from wild-type and \( \alpha_3 \) integrin homozygous mutant E18 embryos, which were also transgenic for the T antigen gene, and placed in culture under conditions permissive for the temperature-sensitive T antigen (see Materials and Methods for further details of culture conditions). To maximize the survival of epithelial cells, while inhibiting the growth of mesenchyme-derived fibroblasts, cells were cultured on matrigel in a defined medium designed for the serum-free cultivation of metanephric kidney organ cultures (Woolf et al., 1995), with the addition of 1% fetal calf serum, as it improved epithelial cell survival. Matrigel was used because it had been reported to inhibit the apoptosis that normally follows culture of primary mammary epithelial cells on tissue culture plastic (Pullan et al., 1996). Indeed, we also observed that collecting duct epithelial cell survival, during an initial period, was dependent on culture on matrigel.

Several sub-populations of an initial culture of collecting duct cells from wild-type and mutant kidneys were maintained independently to develop immortalized cell lines. Identical results were obtained with several wild-type and mutant cell lines. Results are presented from two of these lines, B7 (wild type) and B12 (\( \alpha_3^-/- \)). These cell lines were morphologically similar and both had the characteristic epithelial 'cobblestone' appearance in monolayer culture, indicating that they had maintained epithelial characteristics during the immortalization process. Immunoprecipitation demonstrated abundant expression of \( \alpha_3 \beta_1 \) in the wild-type cell line and its absence in the mutant cell line (Fig. 4A). The collecting duct origin of the B7 wild-type and B12 mutant cells was confirmed by testing for the expression of aquaporin 2, a water channel specifically expressed on the apical membranes of collecting duct epithelia. Expression of Na\(^+\)/K\(^+\)-ATPase, a more general marker of epithelial cells, was also examined. As demonstrated by the western blots shown in Fig. 5A,B, both the B7 and B12 cell lines expressed aquaporin 2 and Na\(^+\)/K\(^+\)-ATPase at similar levels.

Both B7 and B12 appeared to adhere and grow with similar characteristics on matrigel, suggesting that interactions mediated by \( \alpha_3 \beta_1 \) integrin were not an absolute requirement for adhesion and growth on this matrix, although it remained possible that an alternative integrin was providing a required and perhaps identical compensatory function in B12 cells.

Integrin expression by immortalized cell lines

The expression of other integrins expressed by B7 and B12 cell lines cultured on matrigel was examined by surface labeling and immunoprecipitation. \( \alpha_2 \beta_1 \), \( \alpha_5 \beta_1 \) and \( \alpha_\nu \beta_1 \) integrins

![Fig. 4. Integrin immunoprecipitations.](image-url)
Integrins regulate cytoskeletal assembly

appeared to be expressed at equivalently low levels by both cell lines (Fig. 4A). Overall expression of β1 integrins was decreased in the B12 cell line and this appeared to be due mainly to the absence of α3β1, without an increase in other β1 integrins. These results indicate that B12 may be considered to be a β1 integrin-deficient cell line compared with wild-type cell lines. Remarkably, most α6 integrin appeared to coimmunoprecipitate with β4 rather than β1 integrin, indicating a shift in β subunit association for the α6 subunit, which primarily associates with the β1 subunit in kidney collecting ducts (Korhonen et al., 1991) (Fig. 4A). The anti-α6 antibody used only detects α6A. However, since α6 preferentially associates with β4 over β1 when both are present, the equal immunoprecipitation of an α subunit by anti-α6A or anti-β4 indicates that α6A is probably the predominant form present in these cell lines.

Although antibodies are added in excess amounts in each immunoprecipitation, it is nevertheless difficult to make direct quantitative comparisons between the expression of different α integrin subunits because each integrin is precipitated with a different antibody. Nonetheless, expression of integrins other than α3β1 and α6β4 appeared weak in both cell lines, as each of these various antibodies gives stronger signals when used to analyze other cell lines known to express the particular α integrin subunit (data not shown). These results are consistent with the general observation that many established cell lines express α3β1 as a predominant integrin (Hemler et al., 1987), although its expression is apparently not an absolute requirement for immortalization.

The localization of integrins was also assessed by immunofluorescence staining using an anti-β1 antibody. As is typical for epithelial cells in culture, β1 integrins were present along cell-cell junctions in B7, B12 and R10 (Fig. 6), although expression appeared more diffuse in B12, again suggesting that cell-cell junctions were not oriented uniformly perpendicular to the culture substrate. Staining for α3β1 integrin also revealed the same localization along cell-cell junctions in B7, and no staining of B12 (Fig. 6D,E).

**Rescue of α3β1 integrin-deficient cell lines with a human α3 integrin cDNA**

To determine that any phenotypes observed in α3β1 integrin

---

**Fig. 5.** Western blots of aquaporin 2 (A) and Na⁺/K⁺-ATPase (B). The order of B7 and B12 is reversed in A from the other Figs. An asterisk marks the expected position of the respective proteins. In A the larger band is at the expected size for aquaporin 2, and the two smaller bands are probably degradation products. The lanes marked K contain an extract of rat kidney tissue prepared identically to the cell extracts.

**Fig. 6.** Staining for β1 and human α3 integrin. (A) B7, (B) B12 and (C) R10 stained for β1 integrin. (D) B7, (E) B12 and (F) R10 stained with a rabbit polyclonal anti-α3 integrin. (G) B7, (H) B12 and (I) R10 stained with monoclonal P1B5 anti-α3β1 integrin, which detects only human α3 integrin. Magnification ×200.
deficient cell lines were specifically due to the absence of α3β1, rather than secondary changes that occurred during selection for cells able to grow in its absence, a human α3 cDNA was transfected into the B12 cell line. FACS was used to obtain the cells most highly expressing α3β1 integrin, using an antibody specific for human α3β1 (A3-X8). Several isolates, including R10, were obtained that had similar expression levels of α3β1 integrin to the B7 wild-type cell line (Fig. 4B). Immunofluorescence staining of R10 with an antibody (P1B5) that only recognizes human α3β1 demonstrated localization along cell-cell junctions of R10, but not B7 or B12 (Fig. 6G-I). R10 also stained similarly to B7 with a monoclonal anti-β1 integrin antibody (Fig. 6C) and an anti-α3 integrin polyclonal antibody (Fig. 6F).

α3β1 integrin is required to maintain epithelial cytoskeletal organization

We next examined whether immortalized cell lines maintained similar cytoskeletal organization and patterns of cadherin and catenin staining to the primary cells, and whether this was rescued by transfection of the human α3 integrin cDNA. As shown in Fig. 7, B7 and R10 assembled a sub-cortical cytoskeleton, whereas B12 displayed prominent actin stress fibers. α-actinin staining of the cell lines also resembled the primary cells (Fig. 7). These results indicated that the immortalized cell lines provide a valid model for further examination of integrin function in epithelial cells. Cadherin and catenin localization in the cell lines also duplicated the finding of decreased intensity of E-cadherin and
cortical cytoskeleton through their physical interaction with E-cadherin in the cell membrane is usually found assembled into a complex with β-catenin (Barth et al., 1997). These cadherin:catenin complexes are thought to have a major role in mediating interactions between leukocytes and target tissues (Hynes, 1992). Furthermore, in epithelial cells, both systems are thought to participate in organizing the sub-cortical cytoskeleton. In this report, we demonstrate that cadherin:catenin complexes present along intercellular junctions cannot maintain the subcortical-cytoskeleton in the absence of certain β integrins, which are represented in our experiments by α3β1.

**DISCUSSION**

Epithelial cells utilize two major adhesion systems, cadherins and integrins, both of which significantly affect cytoskeletal organization and cell morphology (Gumbiner, 1996; Hynes, 1992). Integrins are classically ascribed a function in cell-ECM adhesion, while cadherins mediate intercellular adhesion. However, in other cell types, such as those of the immune system, integrin-mediated cell-cell adhesion plays an important role in mediating interactions between leukocytes and target tissues (Hynes, 1992). Furthermore, in epithelial cells, both systems are thought to participate in organizing the sub-cortical cytoskeleton. In this report, we demonstrate that cadherin:catenin complexes present along intercellular junctions cannot maintain the subcortical-cytoskeleton in the absence of certain β integrins, which are represented in our experiments by α3β1.

**Integrins in epithelial cytoskeletal organization**

Integrin-mediated cytoskeletal organization has mainly been studied with regard to the organization of actin stress fibers at focal adhesions, where actin binding proteins are assembled into focal adhesions upon clustering of integrins at sites of interaction with the ECM (Burrage and Chrzanowska-Wodnicka, 1996). Recently, Hodivalla-Dilke et al. (1998) demonstrated that α3β1 integrin-deficient keratinocytes assembled more intensely staining actin stress fibers and focal adhesions than did wild-type cells, adding a layer of complexity to this process with the suggestion that α3β1 might transdominantly inhibit focal adhesion assembly by other integrins (Hodivala-Dilke et al., 1998). These results, along with those presented here, raise the question of whether different patterns of integrin-mediated cytoskeletal assembly might actually involve a competition between subsets of integrins that stimulate or inhibit the assembly of F-actin into different types of organized structures.

Our experiments differ from those of Hodivalla-Dilke et al. (1998) in that they cultured keratinocytes in low Ca2+ medium, so that cells would not form cell-cell junctions, whereas our experiments were performed at a Ca2+ concentration that allowed cadherin-mediated cell-cell adhesion. In other studies, addition of anti-β1 or α3 blocking antibodies to cultures of primary keratinocytes also interfered with cell-cell adhesion.
furthermore, cells so treated are unable to assemble a sub-cortical cytoskeleton and instead form actin stress fibers (Carter et al., 1990; Larjava et al., 1990). Comparisons of these different experimental conditions, along with other reports that involve altering Ca\(^{2+}\) concentrations in culture medium (Braga et al., 1995; Larjava et al., 1990), suggested that Ca\(^{2+}\)-dependent cell-cell adhesion is required for assembly of the sub-cortical cytoskeleton. More recent studies have provided a mechanistic understanding of this phenomenon by demonstrating that cadherin:catenin complexes interact with the actin cytoskeleton through associations with α-actinin and vinculin, proteins that also are found in focal contacts in close association with integrins (Hazan and Norton, 1998; Knudsen et al., 1995; Nieset et al., 1997). This has led to a model which suggests that assembly of the sub-cortical cytoskeleton is maintained by its attachment to these cadherin:catenin complexes along lateral membranes, as well as by its attachment to sites where integrins interact with the ECM. Through the use of α3β1 integrin-deficient B12 cells, we were able to examine the role of integrins in mediating organization of the sub-cortical cytoskeleton without also blocking cell-cell adhesion. Our results extend previous studies by demonstrating that a subset of integrins including α3β1, while not absolutely required for cell-cell adhesion, is required to maintain organization of the sub-cortical cytoskeleton. In the absence of α3β1 integrin, attachment of cadherin:catenin complexes to the cytoskeleton is greatly impaired. Since B12 appears to be generally deficient in β1 integrins in comparison with B7 or R10, we cannot conclude that maintenance of epithelial cytoskeletal organization is a specific property of α3β1 integrin. Indeed, the absence of α3β1 on many epithelial cell types (Korhonen et al., 1990) indicates that this is probably a general property of integrins on epithelial cells. Two possible models can be suggested to explain how integrins mediate organization of the sub-cortical cytoskeleton: (1) integrin interaction with the ECM may direct F-actin into the sub-cortical cytoskeleton, with the subsequent passive attachment of cadherin:catenin complexes to α-actinin and vinculin or (2) integrin-mediated signal transduction directly regulates attachment of cadherin:catenin complexes to the cytoskeleton. These hypotheses are currently under evaluation by further analysis of our cell lines.

Integrins are required for proper organization of cell-cell junctions

It has previously been difficult to establish whether there are any functional interactions between cadherins and integrins, or whether these represent two mutually exclusive systems that maintain cellular morphology. Additionally, it is not known whether integrins affect cell-cell adhesion entirely as a result of interactions that occur at the basement membrane, or whether they are interacting with novel ligands within intercellular junctions (Carter et al., 1990). It has been suggested that α3β1 integrin interacted homophilically (Sriramarao et al., 1993), or with α2β1 integrin (Symington et al., 1993), but these reports have not been corroborated in other systems. In this report, it is demonstrated that α3β1 is not absolutely required for cell-cell adhesion by immortalized tubular epithelial cells, but that in its absence, cell-cell junctions are not oriented uniformly perpendicular to the culture substrate. The disorganized shape of the cell-cell junction in α3β1-deficient cells is most easily attributable to the failure to assemble a rigid sub-cortical cytoskeleton.

The studies presented in this report demonstrate conclusively that integrins are required to form the sub-cortical cytoskeleton characteristic of epithelial cells. Although physical association of cadherin:catenin complexes appears to also contribute to cytoskeletal organization, it is now apparent that this interaction requires the presence of integrins, even when cadherin-mediated cell-cell adhesions are maintained. These results provide a basis for examining the role of integrin mediated signal transduction in epithelial cytoskeletal organization, and for determining whether aberrations in these signals are a component of neoplastic events that involve a loss of epithelial cell morphology.

The authors thank H. W. Harris, K. Matlin, C. M. DiPersio, R. Hynes and M. Hemler for the generous gift of antibodies, and M. Ericsson for histological preparations. M. Donovan, C. M. DiPersio, K. Hodivala-Dilke, R. Hynes and M. Hemler are thanked for valuable discussions during the course of our studies, and C. M. DiPersio, K. Hodivala-Dilke are also thanked for providing critical comments on this paper. The authors thank S. Newby for careful editing of the manuscript. This work was supported by grants from the Charles Hood Foundation, and a Young Investigator Award from the National Kidney Foundation. J.A.K. acknowledges support from a Basil O’Connor Starter Scholar Award from the March of Dimes, a Physician-Scientist Award from the Lucille Markey Foundation, and a Young Investigator of the National Kidney Foundation during the period of this work. This work was supported by a grant from the Charles Hood Foundation. J.M.S. and S.L.G. were supported by a Pediatric Nephrology Training Grant from the NIDDK.

REFERENCES


Integrins regulate cytoskeletal assembly 2935