Regulation of MDCK cell-substratum adhesion by RhoA and myosin light chain kinase after ATP depletion

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tum is essential for their differentiation and polarization. Despite this, the precise adhesion mechanism and its regulation are poorly under-
stood. In the kidney, an ischemic insult causes renal tubular epithelial
cells to detach from the basement membrane, even though they remain
viable. To understand this phenomenon, and to probe the regulation of
epithelial cell attachment, we used a model system consisting of
newly adherent Madin-Darby canine kidney (MDCK) cells subjected
to ATP depletion to mimic ischemic injury. We found that MDCK
cells detach from collagen I after 60 min of ATP depletion but
reattach when resupplied with glucose. Detachment is not caused by
degradation or endocytosis of β1-integrins, which mediate attachment
to collagen I. Basal actin filaments and paxillin-containing adhesion complexes are disrupted by ATP depletion and quickly reform on
removal of ATP (36, 39). However, partial preservation of basal actin
by overexpression of constitutively active RhoA does not significantly
affect cell detachment. Furthermore, Y-27632, an inhibitor of the
RhoA effector Rho-kinase, does not prevent reattachment of cells on
removal of the inhibitor but on stress fibers and adhesion complexes regulated by RhoA.

Focal complexes; focal adhesions; epithelial adhesion; stress fibers; Rho-kinase

ONE OF THE EARLY CONSEQUENCES of ischemic injury to the kidney is the detachment of tubular epithelial cells from the
basement membrane and their eventual loss by excretion (36, 38, 39, 47, 54, 59). In both acute renal failure (ARF) patients and animal models, it has been established that a large fraction of the cells voided in the urine after injury are, in fact, still viable (36–39). This observation raises the question of why living cell detach from the tubular basement membrane and suggests that reduced morbidity might result if cell detachment
can be lessened pharmacologically.

Aside from direct clinical benefits arising from a deeper understanding of how epithelial cells adhere to the extracellular matrix, there are also fundamental issues of epithelial cell biology at stake. Numerous studies suggest that epithelial cells are stimulated to polarize through signals generated in part by cell-matrix adhesion (53, 56). Despite some recent progress, however, our knowledge of events linking the assembly of mechanically stable adhesion complexes at the basal plasma membrane to later signaling events leading to full polarization is still quite limited.

Renal epithelial cells attach to the basement membrane primarily through the integrin family of extracellular matrix receptors (21, 44). Integrins are heterodimeric transmembrane glycoproteins consisting of >20 different combinations of α- and β-subunits (12, 21). In the adult kidney five or six different integrins are expressed in an overlapping but segment-specific manner (21). After ischemic injury, β1-integrins redistribute from the basal plasma membrane, possibly reflecting their inactivation (59). In detached cells accumulated in the tubular lumens, β1-integrins cannot be detected by immunocytochemistry, suggesting that they are degraded at some point during or after exfoliation (59).

Integrins mediate cell attachment through the assembly of adhesion complexes on the cytoplasmic surface (12). These are not only a locus for mechanically integrating individual adhesion
sites with the actin cytoskeleton but also serve as scaffolds for signaling molecules (5, 7, 35, 57). Altogether, more than 50 different proteins have been reported as components of cell-matrix adhesions, including cytoskeletal components, signaling
molecules, and adapters or scaffolds (57). Although a number of terms have been used to describe adhesion complexes, studies carried out primarily in migrating cells have assigned them to two main categories. Focal complexes are smaller concentrations of proteins found in lamellipodia and peripheral regions of the basal surface (7, 35). Their formation is believed to be regulated by the small GTP-binding protein Rac (7, 35, 42). Focal adhesions or contacts, on the other hand, are larger structures located mainly in the more central parts of the basal surface and regulated by RhoA (7, 35, 42). To avoid confusion, in this paper we refer to generic basal adhesion complexes unless we are specifically referring to peripheral
or central structures.

Both focal complexes and focal adhesions interact with the actin cytoskeleton, which is severely affected by renal ische-

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nia (3, 28–30). In proximal tubule cells, brush border actin disassembles, presumably contributing to the rapid disruption of the apical surface by blebbing and endocytosis (3, 19, 29, 34). In isolated proximal tubules, primary cultures of proximal tubules, or cultures of renal epithelial cell lines, ATP depletion or oxidant injury (which also results in ATP depletion) causes fragmentation of microvillar actin, dissociation of cortical actin, and disassembly of basal stress fibers (8, 22, 33, 40, 45, 48). Loss of actin stress fibers due to ATP depletion has also been correlated with cell detachment, but no causal connection is firmly established (22, 33, 52).

In this study we examined the role of integrins, the actin cytoskeleton, and RhoA in cell detachment caused by ATP depletion with newly adherent Madin-Darby canine kidney (MDCK) epithelial cells. We found that cell detachment is not caused by redistribution or degradation of integrins. Surprisingly, cell detachment as well as readhesion after replenishment of ATP also does not critically depend on the activity of either RhoA or one of its downstream effectors, Rho-kinase. Instead, adhesion of MDCK cells appears to be regulated by myosin light chain kinase (MLCK). These findings are the first to demonstrate preferential involvement of peripheral actin filaments and adhesion complexes in cell adhesion to the substratum.

MATERIALS AND METHODS

Stock MDCK cells. Stock MDCK strain II cells (low transmonolayer resistance, Heidelberg isolate) were cultured in Dulbecco’s MEM (high glucose) supplemented with 5% (vol/vol) FBS and 10 mM HEPES, pH 7.3, at 37°C in an atmosphere of 5% CO₂ as described previously (24, 27).

MDCK cells expressing the constitutively active RhoA mutant RhoAV14 under the control of the tetracycline-repressible transactivator were obtained from W. James Nelson (Stanford University Medical School, Palo Alto, CA) (15, 16). They were cultured identically to wild-type MDCK cells except that 20 ng/ml of doxycycline was added to the medium to repress mutant RhoA expression. For induction of RhoAV14, confluent cultures were trypsinized and replated in the absence of doxycycline and used the following day, usually 18 h after plating.

ATP depletion and repletion. MDCK cells were chemically depleted of ATP by a modification of the procedure of Sheridan et al. (46). Cells were incubated for various periods of time in Earle’s balanced salt solution (EBSS) containing freshly prepared 5 mM cyanide and 5 mM 2-deoxyglucose (both from Sigma). Incubations were carried out in an incubator at 37°C, 5% CO₂. To restore ATP production (repletion), the solution of metabolic inhibitors was removed and replaced without washing with an equal volume of EBSS containing 10 mM glucose. Parallel control cultures were incubated continuously in EBSS with 10 mM glucose.

ATP levels were measured in neutralized acid extracts of cells with a luciferin-luciferase assay kit (Molecular Probes). ATP concentrations were determined to be 0.74% of control values after 60 min of depletion and recovered to 32.8% of control values after 60 min of repletion with glucose.

Detachment/reattachment assay. Cell detachment after ATP depletion and reattachment after glucose repletion were measured with a modified cell adhesion assay in 96-well microtiter plates (26, 44). Wells were coated with 100 μl/well collagen I (3 μg/well; Upstate Biotechnology), vitronectin (1 μg/well; Collaborative Research), or 1% (wt/vol) BSA (negative control) and then blocked with 200 μl/well 1% (wt/vol) BSA in PBS.

To prepare the cell suspension, confluent flasks of MDCK cells were split 1:5 the day before the experiment. On the day of the assay, a single-cell suspension was prepared by detaching cells from the subconfluent cultures by washing twice with PBS without calcium or magnesium (PBS−) and once with 4 mM EGTA-1 mM EDTA-PBS− and incubating for ~40 min with EGTA-EDTA-PBS− in a 37°C incubator. Detached cells were suspended in ice-cold serum-free suspension MEM (SMEM; GIBCO-Invitrogen) at a density of 2 × 10⁶ viable cells/ml.

To initiate cell detachment, 50 μl of serum-free DMEM with 1% (wt/vol) BSA was added to each coated well, followed by 50 μl of the suspension (1 × 10⁵ cells/well). The plate was then incubated for 1 h at 37°C and 5% CO₂, and unattached cells were removed by washing twice with 200 μl/well ice-cold 0.5% (wt/vol) BSA in PBS (with calcium and magnesium; PBS+) and twice with 200 μl/well ice-cold PBS(+).

Attached cells were ATP depleted by treating for various times with 100 μl/well cyanide-deoxyglucose in EBSS at 37°C and 5% CO₂. Controls (no ATP depletion) were treated in parallel with glucose-EBSS. For samples that were repleted after depletion, depletion medium was removed from the wells and glucose-EBSS was added without washing. At the end of the incubations, wells were washed twice with 200 μl/well ice-cold 0.5% (wt/vol) BSA-PBS(+) and twice with 200 μl/well ice-cold PBS(+).

To quantitate cells remaining in the wells, plates were fixed for 10 min with 100 μl/well methanol and then stained for 5 min with filtered 1% (wt/vol) crystal violet at room temperature. After washing away of excess stain with water and drying, stained cells were solubilized in 1% (wt/vol) deoxycholate-10 mM HEPES pH 7.4 and the amount of stain was estimated in a plate reader (Molecular Devices) at 590 nm. Integrin degradation and surface expression. Integrin degradation and surface expression on detached MDCK cells were measured by metabolic labeling and immunoprecipitation of surface biotinylated proteins (44, 58). MDCK cells were labeled overnight at 37°C and 5% CO₂ with [35S]-labeled methionine-cysteine (Express Labeling Mix, New England Nuclear) in Dulbecco’s MEM containing 1/10th the normal concentration of methionine, 5% FBS, 10 mM HEPES pH 7.3, and 1% antibiotic-antimycotic mixture (Invitrogen Life Technologies), detached with EGTA-EDTA-PBS, and replated in collagen I-coated dishes for 60 min. Cultures were then treated with either depletion medium (cyanide-deoxyglucose in EBSS) or EGTA-EDTA-PBS for 1 h in the 37°C incubator. Detached cells were then collected and washed with ice-cold PBS.

To biotinylate cell surface proteins, detached cells were resuspended in 2 mg/ml freshly dissolved sulfo-NHS biotin (Pierce Chemicals) in PBS and kept on ice for 30 min with intermittent gentle agitation. At the end of the incubation period, cells were pelleted and washed twice with cold SMEM to quench the biotinylation reagent and once with ice-cold PBS.

To immunoprecipitate β₁-integrins, biotinylated cells were extracted with RIPA buffer [10 mM Tris-Cl pH 7.5, 0.5% (wt/vol) SDS, 0.5% (wt/vol) IGEPAL CA630 (Sigma), 1% deoxycholate, 0.15 M NaCl] with 1× complete protease inhibitors (Roche) by vortexing and the cell extract was incubated sequentially with anti-β₁-integrin antiserum (rabbit anti-carboxy terminal peptide) and protein A-Trisacryl beads (Pierce Chemicals). Immune complexes were released by heating the beads for 4 min at 95°C with 20 mM Tris-Cl pH 8.6, 2 mM EDTA, 1% (wt/vol) SDS, and 0.15 M NaCl. A small aliquot of this solution was saved for analysis of total β₁-integrins. The remaining solution was diluted with 10 vols of 20 mM Tris-Cl pH 8.6, 2 mM EDTA, 1% (wt/vol) Triton X-100, 0.15 M NaCl, and biotinylated β₁-integrins were precipitated by addition of streptavidin-agarose beads (Pierce). Biotinylated proteins were released from the beads by heating in complete SDS-gel sample buffer [160 mM Tris-Cl pH 8.8, 4 mM EDTA, 16% (wt/vol) sucrose, 0.2% (wt/vol) bromphenol blue, 20 mM DTT, 2% (wt/vol) SDS] and alkylated with iodoacetamide.

The biotinylated protein samples, along with reduced and alkylated total β₁-integrin samples, were analyzed by separation on 10%
Laemmli SDS polyacrylamide gels. Fixed gels were treated with Enhance (New England Nuclear, dried, and exposed to X-ray film (Kodak X-Omat) at ~80°C.

Fluorescent staining of actin, phosphorytrosine, and Paxillin. To localize actin filaments and paxillin, MDCK cells were detached from subconfluent cultures and plated on collagen-coated 22-mm-square coverslips contained in 35-mm dishes at a density of 1 × 10⁶ cells/dish. Collagen coating and cell culture and detachment were accomplished exactly as described in Integrin degradation and surface expression for integrin analysis. After cells were allowed to attach for 60 min and unattached cells were washed away, attached cells were incubated with cyanide-deoxyglucose depletion medium and/or glucose repletion medium as described for integrin analysis.

At the end of the incubation, depletion or repletion medium was removed and the coverslip cultures were fixed directly without washing with 3% formaldehyde in PBS for 20 min at room temperature. Fixation without washing permitted even loosely attached cells to be retained through the subsequent staining procedures and imaged. After washing, the fixed cells were quenched with 50 mM NH₄Cl in PBS for 10 min and permeabilized with 0.1% (wt/vol) Triton X-100 in PBS for 4 min. Permeabilized cells were blocked by incubation with several changes of 0.2% (wt/vol) fish skin gelatin (Sigma) in PBS and stained with mouse anti-paxillin (catalog no. P13520, Transduction Laboratories) at a 1:250 dilution (1 μg/ml) or mouse anti-phosphorytrosine PY20 (catalog no. P11120, Transduction Laboratories) at a dilution of 1:100 (1 μg/ml) followed by an appropriate secondary antibody conjugated to an Alexa Fluor (Molecular Probes). To detect filamentous actin, permeabilized cells were incubated with phalloidin conjugated to Alexa Fluor 488 (Molecular Probes) or Vectashield (Vector Laboratories), viewed in a Zeiss fluorescence microscope with a ×63 PlanApo objective, and photographed digitally with an Axiocam MRm camera using Axiovision 3.0 software (Zeiss). In some cases, coverslips were also imaged with a Zeiss LSM-510 confocal laser scanning microscope. Digital images were contrast adjusted with Adobe Photoshop.

Western blotting. Paxillin, RhoA, and phosphorylated MLC in cell extracts were detected by Western blotting as described previously (41). RhoA was detected by immunoblotting with mouse monoclonal anti-RhoA (Santa Cruz Biotechnology).

Western blotting. Paxillin, RhoA, and phosphorylated MLC in cell extracts were detected by Western blotting as described previously (60). Phosphorylated paxillin was detected by Western blotting with anti-phospho-paxillin (mouse monoclonal anti-phosphorytrosine PY20, Transduction Laboratories) after immunoprecipitation with rabbit polyclonal anti-paxillin (Santa Cruz Biotechnology). The immunoprecipitation was conducted exactly as outlined in Integrin degradation and surface expression for integrin analysis. Western blotting was performed exactly as described previously (60), except that the blocking and antibody diluting solution was 1% (wt/vol) BSA instead of nonfat dry milk. In some cases, membranes were stripped of antibodies after initial blotting by incubation with 62.5 mM Tris-Cl pH 6.7–2% (wt/vol) SDS-100 mM β-mercaptoethanol for 30 min at 50°C on a shaking water bath, washed and blocked with wash buffer plus milk, and reprobed with anti-paxillin.

Phosphorylated MLC was detected by Western blotting exactly as for phosphorytrosine with BSA blocking solutions. Polyclonal rabbit antibodies specific for diphosphorylated MLC and Ser19 monophosphorylated MLC were purchased from Cell Signaling Technology.

Antibody dilutions used for Western blotting were 1:5,000–1:10,000 for mouse anti-paxillin (0.025–0.05 μg/ml), 1:100 for mouse anti-RhoA (0.2 μg/ml), and 1:1,000 for anti-phosphorytrosine (1 μg/ml), anti-monophosphorylated MLC (1 μg/ml), and anti-diphosphorylated MLC (0.6 μg/ml).

Inhibitor treatments. To inhibit either Rho-kinase or MLCK, cells were treated with Y-27632 (10–50 μM; Tocris Cookson; Ref. 32) or ML-7 (25–100 μM; Calbiochem; Ref. 43), respectively. For studies during ATP repletion, drugs were usually added during the final 30 min of ATP depletion and then readded together with glucose-containing repletion medium. Y-27632 was added from a stock solution of 10 mM in PBS(−) and ML-7 from a stock solution of 20 mM in DMSO. In the case of ML-7, control cultures were given the same amount of DMSO as the highest ML-7 concentration. Both stock solutions were stored at −20°C.

RESULTS

ATP depletion leads to reversible cell detachment. When confluent cultures of MDCK cells grown in collagen-coated microtiter plates were treated with cyanide and deoxyglucose to deplete ATP and then washed, the entire cell monolayer detached as a sheet (Fig. 1A), consistent with the earlier findings of Kroshian et al. (22) with primary cultures of mouse proximal tubule cells. These results suggested that ATP depletion affected cell substratum contacts while not totally abrogating cell-cell interactions. When glucose-containing medium was added back to the depleted cultures before washing, no reattachment of the cells was observed (Fig. 1A), probably because the detached sheet floated in the dish and was unable to resettle on the substratum.

To circumvent this problem, a single-cell suspension of MDCK cells was plated in a collagen-coated dish for only 1 h and then ATP depleted. A similar approach was used by Kroshian et al. (22) in their studies. Under these conditions, nearly 100% of the added cells have sufficient time to firmly attach to the substratum but insufficient time to form extensive cell-cell contacts. This approach also had the added advantage of enabling cell-substratum interactions to be better defined. MDCK cells initially attach to collagen primarily via β₁-integrins (44). However, within ~2 h of plating, the cells begin to modify the extracellular matrix on the plastic substratum, substituting endogenously secreted proteins (B. Haus and K. S. Matlin, unpublished observations). Thus any interactions of cells with the substratum at this point would involve undefined receptors and matrix ligands.

When newly plated cells were ATP depleted for various times and washed, cell detachment was progressive, with some cells still attached after 30 min of depletion, most detached after 60 min, and nearly all detached after 90 min of depletion (Fig. 1B). Under these conditions, when cells depleted for 60 min were repleted by glucose addition for 60 min, approximately one-half of the cells reattached (Fig. 1C). This indicated first of all that the depleted cells were still viable and second that glucose readdition permitted cells to reestablish cell-substratum contacts, presumably through a mechanism dependent on ATP.

Cell detachment is not caused by changes in β₁-integrin availability. In the ischemic kidney, β₁-integrins can no longer be detected in tubular epithelial cells detached from the basement membrane (59), suggesting that the integrins are degraded either in the process of detachment or after detachment. To determine whether ATP depletion of MDCK cells also leads to β₁-integrin degradation or removal from the cell surface, cells were metabolically labeled overnight with [35S]Met-Cys, replated on collagen-coated dishes, and ATP depilated. Cells released because of ATP depletion were then collected, surface proteins were biotinylated, and β₁-integrins were immunoprecipitated. As a control, newly plated MDCK
cells were released by incubation with chelating agents and biotinylated in suspension.

As illustrated in Fig. 2, immunoprecipitates of labeled MDCK cell extracts yielded three bands on autoradiographs of SDS gels corresponding to the $\alpha_1$-subunit and the $\alpha_2$- and $\alpha_3$-subunits that form heterodimeric complexes with $\beta_1$ (44). These bands are evident both in samples of the total $\alpha_1$ immunoprecipitate (Fig. 2, lanes 3 and 4) and in samples corresponding to the biotinylated cell surface fraction of $\alpha_1$ (Fig. 2, lanes 1 and 2). Despite a slight reduction in the overall amount of $\beta_1$-integrin in samples derived from ATP-depleted cells, there is no evidence of either significant integrin degradation or removal from the cell surface. Comparable amounts of all 3 integrin subunits are also present in total immunoprecipitated samples (lanes 3 and 4), suggesting that depletion does not lead to substantive degradation of these integrins.

Fig. 2. ATP depletion does not lead to $\beta_1$-integrin endocytosis or degradation. Metabolically labeled and newly adherent MDCK cells were detached by incubation with depletion medium (Dep) or with 1 mM EGTA-4 mM EDTA in PBS (Con) for 60 min at 37°C. The suspended cells were then surface labeled with sulfo-NHS-biotin and extracted. Extracts were immunoprecipitated with anti-$\beta_1$-integrin and then with streptavidin-agarose to specifically select biotinylated integrins. The precipitated proteins were fractionated by SDS-gel electrophoresis and radioactive bands were detected by fluorography. As shown in lanes 1 and 2, bands corresponding to integrin $\alpha_2$, $\alpha_3$, and $\beta_1$-subunits are equally evident in biotinylated samples from either control or depleted cells, indicating that depletion does not remove these integrins from the cell surface. Comparable amounts of all 3 integrin subunits are also present in total immunoprecipitated samples (lanes 3 and 4), suggesting that depletion does not lead to substantive degradation of these integrins.

As mentioned above, MDCK cells attach to collagen via $\beta_1$-integrins. To determine whether ATP depletion affects adhesion mediated only by $\beta_1$-integrins or also influences adhesive contacts mediated by integrins with other $\beta$-subunits, cells were plated for 1 hour on surfaces coated with the serum

![Fig. 1. ATP depletion reversibly detaches Madin-Darby canine kidney (MDCK) cells from the substratum. Confluent (A) or newly attached (B and C) cultures of MDCK cells were ATP depleted for the indicated times and were then either washed 4 times to release loosely attached cells or had the depletion medium gently removed and replaced with glucose-containing Earle’s balanced salt solution (EBSS) to restore (replete) ATP production and were then washed. After washing, cells that remained attached to the substratum were quantitated by fixation and crystal violet staining. In A, depletion (Dep) caused all cells in the confluent culture to detach as a sheet; when repletion medium was added after depletion (Dep/Rep), no reattachment occurred. In B, a suspension of MDCK cells was plated on collagen- or BSA-coated surfaces for 60 min and then depleted. No cells attach to BSA, which serves as a negative control. Such newly adherent cells detach progressively with time, with most cells released by 60–90 min of depletion. When newly attached cells were depleted and then incubated with glucose-containing repletion medium (C; Dep/Rep), nearly half of the cells reattached in 60 min, indicating that depletion-induced detachment is reversible. Cells incubated in glucose repletion medium for 120 min after adhesion without depletion (glucose control) do not detach from the substratum.

![Fig. 2. ATP depletion does not lead to $\beta_1$-integrin endocytosis or degradation. Metabolically labeled and newly adherent MDCK cells were detached by incubation with depletion medium (Dep) or with 1 mM EGTA-4 mM EDTA in PBS (Con) for 60 min at 37°C. The suspended cells were then surface labeled with sulfo-NHS-biotin and extracted. Extracts were immunoprecipitated with anti-$\beta_1$-integrin and then with streptavidin-agarose to specifically select biotinylated integrins. The precipitated proteins were fractionated by SDS-gel electrophoresis and radioactive bands were detected by fluorography. As shown in lanes 1 and 2, bands corresponding to integrin $\alpha_2$, $\alpha_3$, and $\beta_1$-subunits are equally evident in biotinylated samples from either control or depleted cells, indicating that depletion does not remove these integrins from the cell surface. Comparable amounts of all 3 integrin subunits are also present in total immunoprecipitated samples (lanes 3 and 4), suggesting that depletion does not lead to substantive degradation of these integrins.](http://ajpcell.physiology.org/doi/abs/10.220.32.247 by 10.220.32.247 on June 25, 2017)
protein vitronectin and then ATP depleted. MDCK cells attach to vitronectin through the αvβ3-integrin (44). Although the amount of adhesion to vitronectin was reduced relative to collagen, the extent of cell detachment after 60 min of ATP depletion and washing and the approximate amount of reattachment after repletion were similar to those of cells plated on collagen (data not shown). These results indicate that ATP depletion affects either more than one class of integrin or some other aspect of the cell attachment mechanism.

**ATP depletion disassembles adhesion complexes and actin stress fibers.** Although integrins directly bind the extracellular matrix substrate, their adhesive function is also related to their interaction with adhesion complexes and actin stress fibers on the cytoplasmic side of the plasma membrane. When stress fibers were visualized by fluorescence microscopy, it was apparent that ATP depletion caused their disassembly. MDCK cells plated for 60 min on collagen-coated coverslips exhibit stress fibers extending along the base of the cell and in bundles at the cell periphery (Fig. 3b; see also Fig. 6d). After 60 min of ATP depletion, the amount of cell spreading is reduced (Fig. 3, d–f); while some filamentous actin still outlines the edges of cells (Fig. 3e; see also Fig. 6c), the majority is located in clumps in the interior of the cell. Filamentous actin is also present in long filopodia extending from the edge of the cell near the substratum up and away from the surface (Fig. 3e; see also Fig. 6c). On readdition of glucose for 60 min basal actin filaments are quickly reestablished (Fig. 3h), with a tendency for the peripheral bundles to be even more robust than before ATP depletion.

The adhesion protein paxillin also redistributes on ATP depletion, as demonstrated by immunofluorescence. In newly adherent cells, paxillin is distributed in dense basal plaques at the cell periphery (Fig. 3a), coinciding with insertion points of actin filaments (Fig. 3, b and combined image c). On ATP depletion, paxillin becomes diffusely distributed in the cytoplasm (Fig. 3d). Some coincidence of staining with residual actin filaments at the cell edge is also evident (Fig. 3f). After glucose repletion, dense plaques of paxillin staining again develop (Fig. 3, g and combined image i).

Paxillin is phosphorylated on tyrosine residues in concert with its assembly into basal adhesion complexes. To determine whether ATP depletion leads to paxillin dephosphorylation, paxillin was immunoprecipitated from detergent extracts of control MDCK cells as well as ATP-depleted and depleted/ repleted cells. SDS gels of the immunoprecipitates were then Western blotted with anti-phosphotyrosine antibodies (Fig. 4, top), and the blots were stripped and reprobed with anti- paxillin (Fig. 4, bottom). As shown in Fig. 4, paxillin migrated as a broad band at ~85 kDa on Western blots of immunoprecipitates probed with anti-paxillin (Fig. 4, bottom) (51). A second, faster-migrating band also reacted with the anti-paxillin antibody; this is probably δ-paxillin (50). When this same blot was reacted with anti-phosphotyrosine, it was evident that the most slowly migrating edge of the paxillin band corresponded to the phosphotyrosinated protein in samples from both control cells (Fig. 4, lanes 1 and 6) and cells that had been glucose repleted after ATP depletion (Fig. 4, lane 3). However, in the sample from ATP-depleted cells phosphotyrosine reactivity was absent (Fig. 4, top, lane 2) and the band in the paxillin blot lacked the slowly migrating upper edge (Fig. 4, bottom, lane 2). Thus ATP depletion resulted in reversible paxillin dephosphorylation, and this effect correlated with the paxillin redistribution seen by immunofluorescence (Fig. 3d).

In summary, these results demonstrated that ATP depletion led to disassembly of actin stress fibers as well as dissociation of paxillin from basal adhesion complexes and its dephosphorylation. Because stress fibers and adhesion complexes help to mediate cell attachment to the substratum through indirect linkage to integrins, it seemed possible if not likely that their disassembly was contributing significantly to cell detachment induced by ATP depletion. Alternatively, breakdown of basal adhesions and stress fibers might be parallel events with little importance in maintaining cell adhesion to the substratum. Rho-kinase is not required for cell detachment or reattachment. To gauge the importance of actin stress fibers and basal adhesion complexes in cell detachment, two sets of experiments were performed that took advantage of the role of RhoA in regulating stress fiber and focal adhesion formation. RhoA is a regulatory protein that activates a variety of downstream effectors on binding of GTP (4). Among these are Rho-kinase (ROCKII/ROKα), p140mDia, and the myosin binding subunit of myosin phosphatase. Through its association with these targets, RhoA stimulates the formation of stress fibers and focal adhesions (11).

To determine the effects of ATP depletion on the activity of RhoA, extracts of MDCK cells were precipitated with a rho- tekin fusion protein attached to agarose beads. Rhotekin is a RhoA effector that only associates with the active, GTP-bound form of RhoA (41). In cells that had been attached to collagen-coated dishes for 60 min, a significant fraction of RhoA was active (Fig. 5, lane 5). The amount of active RhoA declined after ATP depletion (Fig. 5, lane 6) but recovered to normal levels after glucose repletion (Fig. 5, lane 7). RhoA is normally inactivated by hydrolysis of GTP to GDP. Replacement of Gly14 with Val significantly reduces the endogenous GTPase activity, rendering the rhoAV14 mutant constitutively active (13). When MDCK cells overexpressing rhoAV14 were ATP depleted, RhoA activity was not diminished relative to control cells that had not been depleted or had been treated with glucose either continuously or after depletion (Fig. 5, lanes 1–4).

To determine whether overexpression of the constitutively active RhoAV14 mutant affected the assembly of actin stress fibers after ATP depletion, filamentous actin was visualized by staining with fluorescent phalloidin. As shown in Fig. 6, newly attached MDCK cells expressing wild-type RhoA assembled stress fibers along the basal cell surface and at the cell periphery (Fig. 6a), as described above (see Fig. 3). On ATP depletion, these actin filaments mostly disassembled and accumulated in the interior of the cell (Fig. 6c). Cells overexpressing RhoAV14 appeared less well spread than cells expressing only normal RhoA (compare Fig. 6, A and B) and exhibited dense peripheral actin filaments and interior stress fibers (Fig. 6b). On ATP depletion many actin stress fibers remained assembled, particularly along the cell periphery, along with masses of filopodia (Fig. 6d). A number of cells also exhibited a dense network of short actin filaments in the interior of the basal cytoplasm (Fig. 6d). Thus, when RhoA remained active in the face of ATP depletion, the basal actin cytoskeleton remained somewhat intact, although structurally altered (40).
Next, the adhesive characteristics of cells overexpressing RhoAV14 were examined by plating cells in collagen-coated plates followed by ATP depletion. RhoAV14 cells attached more readily to collagen than control cells but still detached after ATP depletion and washing (Fig. 7A). To compare the amount of detachment of the RhoAV14 cells to that of normal MDCK cells, the cell detachment values were normalized to the amount of initial cell adhesion before ATP depletion (Fig. 7B). With this analysis RhoAV14 cells sometimes appeared to detach less readily than normal cells, but the advantage was small and quite variable from experiment to experiment (Fig. 7, A and B, represents 1 such positive experiment). To attempt to clarify this, the standard detachment assay was modified to provide a crude estimate of the affinity of cell adhesion. In the usual assay, plates were washed four times after ATP depletion and/or repletion with glucose to remove loosely attached cells and the remaining number of attached cells was quantified. For the modified assay, plates were still washed four times but the number of cells that remained attached was quantified after each individual wash. Data were then expressed as percentage

Fig. 3. ATP depletion leads to reversible disassembly of focal adhesions and stress fibers. MDCK cells were plated for 60 min on collagen-coated coverslips and then treated first with depletion and then with glucose-containing repletion medium. Cultures were then fixed with formaldehyde, permeabilized, and stained with anti-paxillin followed by a fluorescent secondary antibody (to visualize adhesive complexes) and fluorescent phalloidin (to visualize filamentous actin). The stained specimens were examined in a confocal fluorescent microscope. All images correspond to the basal 2–3 μm of the cells; the entire cell thickness is not illustrated. As shown in a–c (Adh), prominent paxillin-containing plaques (a) and stress fibers (b) form within 60 min, with the stress fibers terminating at the adhesion plaques (c, arrows). With depletion (d–f; Dep), cells contract, paxillin (d) is dispersed in the cytoplasm, and stress fibers (b) disassemble. Some peripheral actin filaments remain (large arrow in e), and actin staining also outlines slender filopodia that arc up away from the substratum (small arrow in e). After readdition of glucose medium for 60 min (g–i; Dep/Rep), both paxillin plaques (g, arrows) and thick peripheral bundles of actin (h) terminating at the plaques (i, arrows) reform. Red, paxillin; green, F-actin. Bars: 10 μM (all images are at the same magnification).

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of control (initial) adhesion. As shown in Fig. 7C, under these circumstances it was evident that RhoAV14 cells were consistently more difficult to wash off the substratum after ATP depletion than normal cells not expressing mutant RhoA, although, as expected, the differences after four washes were often very small. Together, these studies indicated that retention of RhoA activity after ATP depletion moderately affected MDCK cell adhesion. Because the effects were not pronounced, it was possible that other factors might be more significant.

One of the downstream effectors of RhoA is Rho-kinase (also called ROCKII or ROCKα) (4). Rho-kinase is known to inactivate MLCK phosphatase, stimulating stress fiber formation or contractility, and is likely to have other targets related to stress fiber and focal adhesion assembly (1, 2, 18, 23, 25, 31, 48, 49). Rho-kinase can be effectively inhibited by the compound Y-27632 (32). When MDCK cells were pretreated and then continuously incubated with Y-27632, it was observed that the drug had no effect on either initial attachment (data not shown) or the degree of detachment after ATP depletion (Fig. 8A). The latter result was not unexpected, because ATP depletion itself might inactivate Rho-kinase. However, when depleted cells were reincubated with glucose to restore ATP levels, cells treated with Y-27632 reattached to the substratum just as effectively as untreated cells (Fig. 8A).

ATP-depleted cells treated with the Rho-kinase inhibitor during glucose repletion were also examined by immunofluorescence and fluorescent phalloidin staining to determine whether paxillin-containing adhesion complexes and stress fibers reassembled. After depletion and repletion with glucose, the reformation of paxillin-containing basal adhesion complexes and dense bundles of actin filaments was evident in controls (Fig. 8B, i–iii; see also Fig. 3). In cells treated with Y-27632 during the last 30 min of ATP depletion and then during repletion with glucose, clearly visible adhesion complexes and stress fibers or other actin bundles were largely absent (Fig. 8B, iv–vi). Some rudimentary concentrations of paxillin were evident on the peripheral edge of the basal surface, coincident with a small amount of cortical actin (Fig. 8B, iv–vi). Paxillin phosphorylation was also diminished both in cells repleted with glucose after ATP depletion in the presence of Y-27632 (Fig. 4, lanes 4 and 5) and in control cells never subjected to depletion (Fig. 4, lanes 7 and 8). From these experiments, as well as previous ones using RhoAV14-expressing cells, we concluded that readhesion of MDCK cells after ATP depletion did not depend on adhesive complexes regulated by the RhoA/Rho-kinase pathway and, in fact, did not require stress fibers or extensive basal adhesion complexes at all.

MLCK regulates MDCK cell adhesion. Recent studies in fibroblasts indicate that stress fibers in the center of cells are regulated by Rho-kinase whereas those at the periphery of cells, the so-called “cortical” or peripheral stress fibers, are regulated by MLCK (18, 49). Because MDCK cells were devoid of central stress fibers after treatment with the Rho-kinase inhibitor but still reattached to the same extent after replenishment of ATP, it seemed likely that peripheral stress fibers reassembled when ATP was restored.

Fig. 4. Paxillin tyrosine phosphorylation is blocked by ATP depletion and a Rho-kinase inhibitor. Newly adherent MDCK cells were ATP depleted for 60 min (Dep) and extracted with a detergent-containing solution or repleted for an additional 60 min with glucose medium (Dep/Rep) in the presence or absence of 10 or 50 μM Y-27632, an inhibitor of Rho-kinase (ROCK), and then extracted. Other samples that were not ATP depleted were kept in glucose medium with or without the inhibitor for 120 min (Glu) and extracted. Extracts were immunoprecipitated with anti-paxillin, and the isolated protein was separated by SDS-gel electrophoresis. After transfer of the separated proteins to Immobilon, tyrosine-phosphorylated paxillin was detected by Western blotting with anti-P-tyr (top). The developed blot was then stripped and reprobed with anti-paxillin to visualize total paxillin bands (bottom). Top: the upper band is the primary paxillin polypeptide migrating at ~85 kDa (arrow); the lower band is most likely β-paxillin, which is not tyrosine phosphorylated in MDCK cells. Paxillin is phosphorylated in control adherent cells (Adh/Con, lane 1) after 60-min adhesion. After ATP depletion no P-tyr is detectable (Dep, lane 2), but phosphorylation is restored on glucose addition (Dep/Rep, lane 3). In the presence of the ROCK inhibitor, some paxillin phosphorylation is evident, but at much lower levels than the adhesion control or repleted sample (lanes 4–5). The ROCK inhibitor also blocks paxillin phosphorylation in samples that were never ATP depleted (Glu, lanes 7–8). Note that tyrosine-phosphorylated paxillin corresponds to the most slowly migrating edge of the upper paxillin band in bottom panel (asterisk).

Fig. 5. RhoA, but not constitutively active RhoA, is inactivated by ATP depletion. Newly adherent MDCK cells inducibly expressing a constitutively active mutant of RhoA, RhoAV14, were ATP depleted and extracted (Dep) or treated with glucose repletion medium and then extracted (Dep/Rep). Control adherent cells (Adh/Con) or cells incubated for 120 min in glucose medium after cell attachment (Glu) were also extracted. Wild-type (wt) RhoA cells were not induced to express mutant RhoA and were kept in doxycycline-containing medium throughout the experiment. RhoAV14 cells were induced to express the constitutively active construct by removal of the gene suppressor doxycycline the day before the experiment. Extracts were divided into 2 parts. One was immediately incubated with rhokinin beads to capture active RhoA, and the other was used to estimate total RhoA in the extract. Both samples were separated by SDS-gel electrophoresis, and RhoA was detected by Western blotting. The intensity of the bands derived from the 2 SDS gels were separated by SDS-gel electrophoresis. After transfer of the separated proteins to Immobilon, tyrosine-phosphorylated paxillin was detected by Western blotting with anti-P-tyr (top). The developed blot was then stripped and reprobed with anti-paxillin to visualize total paxillin bands (bottom). Top: the upper band is the primary paxillin polypeptide migrating at ~85 kDa (arrow); the lower band is most likely β-paxillin, which is not tyrosine phosphorylated in MDCK cells. Paxillin is phosphorylated in control adherent cells (Adh/Con, lane 1) after 60-min adhesion. After ATP depletion no P-tyr is detectable (Dep, lane 2), but phosphorylation is restored on glucose addition (Dep/Rep, lane 3). In the presence of the ROCK inhibitor, some paxillin phosphorylation is evident, but at much lower levels than the adhesion control or repleted sample (lanes 4–5). The ROCK inhibitor also blocks paxillin phosphorylation in samples that were never ATP depleted (Glu, lanes 7–8). Note that tyrosine-phosphorylated paxillin corresponds to the most slowly migrating edge of the upper paxillin band in bottom panel (asterisk).
fibers or adhesion complexes were mediating attachment. To test this, MDCK cells were pretreated with the MLCK inhibitor ML-7 during ATP depletion and then incubated continuously with the same concentration of the drug during ATP repletion with glucose. As shown in Fig. 9A, treatment with ML-7 reduced cell reattachment initiated by repletion in a dose-dependent manner, with 100 μM ML-7 almost completely preventing reattachment. Furthermore, cells in control cultures that were not ATP-depleted detached when treated with ML-7, suggesting that MLCK was playing a fundamental role in MDCK cell adhesion independent of any perturbations elicited by ATP depletion.

ML-7 also had significant effects on the organization of adhesion complexes and the basal actin cytoskeleton (Fig. 9B). Glucose-repleted control cells exhibit intense, plaquelike staining for paxillin at the cell periphery as well as a dense peripheral bundle of actin filaments (Fig. 9B, i). Incubation with 25 μM ML-7 during repletion abolished the paxillin concentrations and reduced the amount of peripheral filamentous actin (Fig. 9B, ii). Many cells also failed to fully spread at this concentration. With 100 μM ML-7, no cells spread over the substratum; filamentous actin staining was confined to the membrane edge, and paxillin staining was only in small basal dots in the interior of the cell near the basal surface (Fig. 9B, iii). Biochemical analysis indicates that tyrosine phosphorylation of paxillin is reduced under these conditions (data not shown).

MLC is phosphorylated by MLCK and Rho-kinase at Ser19 and Thr18. To be certain that ML-7 blocked MLC phosphorylation at concentrations affecting adhesion, MDCK cells treated with ML-7 were stained with antibodies against diphosphorylated (Ser19 and Thr18) and monophosphorylated (Ser19) MLC. For comparison, cells incubated with the Rho-kinase inhibitor Y-27632 were also stained. As shown in Fig. 10A, in newly adherent MDCK cells phosphorylated MLC is predominantly localized in a dense peripheral band, with some diffuse staining in the cell interior (Fig. 10A, i and v). MLC at 50–100 μM appears to reduce this staining, particularly in the case of the diphospho-specific antibody, although quantitative conclusions are difficult because of the coincident changes in cell shape (Fig. 10A, ii, iii, vi, vii). Notably, significant monospecific staining persists at the cell periphery in cells treated with the Rho-kinase inhibitor, whereas most diphospho-specific staining is eliminated (Fig. 10A, iv, viii).

To quantitate the effects of the drugs on MLC phosphorylation, the same antibodies were used to probe Western blots of cell extracts (Fig. 10B). To control for loading, blots were simultaneously probed with an antibody against actin. Consistent with the immunofluorescence results, treatment of cells with Y-27632 significantly reduced monophosphorylation of MLC (Fig. 10B, lane 1) and nearly eliminated diphosphorylation (Fig. 10B, lane 6). In contrast, increasing concentrations of ML-7 had no effect on monophosphorylation of MLC (Fig. 10B, lanes 2–4). Diphosphorylation, however, was reduced significantly in a dose-dependent manner, although the maximal reduction at 100 μM ML-7 (52% based on scanning) was not as great as that achieved by Y-27632 (Fig. 10B, lanes 7–9).
DISCUSSION

In this study we have examined the involvement of integrins, basal adhesion complexes, and actin stress fibers in MDCK cell detachment from and reattachment to a collagen I-coated substratum. Alterations in cell adhesion were achieved by depletion and replenishment of ATP. We find that cell detachment is not caused by integrin endocytosis or degradation. Although we observe, as others have previously (8, 22, 33, 40, 45, 48, 55), that ATP depletion causes disassembly of actin stress fibers and dephosphorylation and disassembly of paxillin from basal adhesive complexes, we have been unable to clearly link these factors to either cell detachment on ATP depletion or reattachment when ATP levels are restored by glucose addition. In particular, preservation of RhoA activity in the face of ATP depletion is insufficient to completely prevent cell detachment. Furthermore, an inhibitor of the RhoA effector Rho-kinase does not block cell reattachment after restoration of ATP, even though reassembly of actin stress fibers and large adhesive complexes does not occur. Instead, we find that an inhibitor of MLCK prevents cell reattachment after ATP restoration and causes detachment of cells never subjected to ATP depletion. Because MLCK is believed to regulate the assembly of peripheral actin filaments and adhesion complexes distinct from more centrally located stress fibers and focal adhesions (18, 49), our findings are consistent with a model in which these peripheral structures mediate early, mechanical adhesion of MDCK cells to collagen I. Our results are summarized schematically in Fig. 11, which illustrates how pathways involving RhoA and MLCK might regulate both MLC phosphorylation and adhesive complex formation. This model is identical to that proposed by Totsukawa et al. (49) for the regulation of cortical actin bundle and focal adhesion assembly at the periphery of fibroblasts.

For these studies we chose to use MDCK cells newly adherent to a substratum coated with collagen I that had not yet formed stable cell-cell contacts. The reason for this was twofold. First of all, we observed that confluent MDCK cells detached as a sheet when ATP depleted and washed, as had been observed earlier for primary cultures of mouse proximal tubule cells (22). This had the effect of introducing the element of cell-cell adhesion into our assay of cell-substratum adhesion. In addition, because the detached cell sheets floated, we were unable to use confluent cultures to measure cell reattachment after readdition of glucose and restoration of ATP synthesis. As it turned out, monitoring cell reattachment provided considerable insight into the mechanisms of MDCK cell.
substratum adhesion. Second, use of newly adherent cells permitted us to define the exact nature of the cell-substratum interaction. MDCK cells plated on collagen-coated surfaces for 60 min bind via $\beta_1$-integrins; adhesion can be completely blocked by an antibody against the $\beta_1$-subunit (44). At later times, secretion of endogenous and poorly characterized matrix proteins from the attached cells modifies cell-substratum interactions (B. Haus and K. S. Matlin, unpublished observations).

After ischemic injury to the kidney, integrins of the renal tubular epithelium are known to redistribute from the basal to the lateral surfaces and may eventually appear on the apical plasma membrane of regenerating cells (59). Similar alterations in integrin distribution have also been noted in renal epithelial cells in culture subjected to either ATP depletion or oxidative injury (6, 9, 10, 33). In the rat kidney, we have shown (59) that $\beta_1$-integrins cannot be detected by immunofluorescence in exfoliated cells that accumulate in the tubular lumen.
after ischemic injury. For this reason, we suspected that MDCK cell detachment after ATP depletion might be caused by either endocytic removal of integrins from the cell surface and/or their degradation. However, examination of metabolically labeled cells by surface biotinylation soon after their ATP depletion-induced release from the substratum failed to demonstrate a significant decline in the level of β1-integrins at the cell surface, ruling out degradation or removal as possible mechanisms of detachment.

Although integrins are receptors that directly mediate interaction between cells and the substratum, they are only part of the adhesive machinery. On binding to the extracellular matrix, integrins cluster and interact with a variety of cytoskeletal and signaling molecules that link them to basal actin filaments and regulate morphogenesis, proliferation, and differentiation (7, 35). These actin-integrin adhesion complexes contain protein kinases such as Src and focal adhesion kinase (FAK), adapter proteins such as paxillin, and actin-binding proteins such as talin, vinculin, and α-actinin (7, 35, 57). The formation of these multimeric assemblies undoubtedly strengthens overall cell adhesion. For this reason, we considered the possibility that disassembly of basal adhesion complexes and the actin cytoskeleton by ATP depletion might contribute to cell detachment.

In this report we show that ATP depletion of newly adherent MDCK cells causes breakdown of basal actin filaments, confirming earlier results (22, 40, 48). We also show that, in parallel, paxillin dissociates from basal adhesion complexes and is tyrosine dephosphorylated, in agreement with others (40, 55). Raman and Atkinson (40), in particular, showed that transient expression of the RhoA constitutively active mutant RhoAV14 in LLC-PK1 cells prevented disassembly of stress fibers on ATP depletion and enhanced their reassembly during recovery of ATP levels. Conversely, expression of the dominant-negative mutant RhoAN19 or treatment of cells with the RhoA inhibitor C3 exotoxin prevented restoration of stress fibers during ATP recovery. Their results demonstrated definitively that RhoA regulates the assembly of actin stress fibers in these cells and suggested that inactivation of RhoA contributes to stress fiber disruption.

In our studies, we have correlated the inactivation of RhoA and breakdown of stress fibers with cell detachment induced by ATP depletion. We take advantage of an MDCK cell line that overexpresses constitutively active RhoA on removal of the gene suppressor doxycycline (15, 16). In agreement with Raman and Atkinson (40), we also find that expression of RhoAV14 preserves actin stress fibers in ATP-depleted cells. However, this manipulation does not completely prevent depletion-induced cell detachment, implying that there is not a strong correlation between the presence of stress fibers and the preservation of cell attachment to the substratum.

Complementary evidence consistent with this conclusion was provided by experiments using an inhibitor of Rho-kinase, Y-27632 (32). Rho-kinase is one of several direct downstream effectors of RhoA that regulate formation of focal adhesions and stress fibers (4). Rho-kinase acts, in part, by inactivating myosin phosphatase by phosphorylation and also directly phosphorylating MLC (2, 18, 49). The net effect of this is to activate myosin, enhancing stress fiber contractility. Rho-kinase may also affect actin assembly by phosphorylating LIM-kinase which, in turn, phosphorylates and inactivates collin, an actin depolymerizing factor (25). In our experiments, we found that inhibition of Rho-kinase had no effect on either initial cell adhesion or ATP depletion-induced cell detachment. When present during ATP repletion, the inhibitor blocked reformation of actin stress fibers and reassembly of paxillin into large focal adhesions, as well as reducing the level of paxillin tyrosine phosphorylation. Surprisingly, however, MDCK cells repleted under these conditions were able to reattach to the substratum just as well as cells not incubated with the Rho-kinase inhibitor. Thus the absence of actin stress fibers and an extensive network of paxillin-containing adhesive complexes did not prevent MDCK cell readhesion after ATP depletion.

In MDCK cells that reattached in the presence of the Rho-kinase inhibitor, filamentous actin and small concentrations of paxillin did remain in the extreme peripheral region of the basal surface. Recent studies in fibroblasts suggest that eukaryotic cells possess two groups of basal actin filaments or stress fibers, central and peripheral (cortical), with the former regulated by Rho-kinase and the latter by MLCK (18, 49). Through microinjection of reagents that either inhibited or activated myosin phosphatase in conjunction with specific inhibitors of either Rho-kinase or MLCK, Totsukawa and colleagues (49) found that MLC localized in the interior of the basal cell surface was phosphorylated by Rho-kinase but cortical MLC was dependent on MLCK. Furthermore, these effects on MLC led to the stimulation of stress fiber or cortical actin filament formation in their respective regions. Similarly,
Katoh et al. (18) observed that the Rho-kinase inhibitor Y-27632 blocked the incorporation of green fluorescent protein (GFP)-labeled actin into central stress fibers, whereas the MLCK inhibitor ML-7 prevented cortical actin filament assembly. Neither study related either of these two groups of stress fibers to cell-substratum adhesion. On the basis of this information and the localization of actin and paxillin in MDCK cells reattached in the presence of Y-27632, it seemed possible that...
ML-7 did inhibit reattachment of MDCK cells after replenishment of ATP. In addition, ML-7 was found to also cause detachment of cells that had never experienced ATP depletion, suggesting that the function of this adhesive system was not limited to the particular circumstances present in the ATP-depleted cell.

Our findings are consistent with the earlier results of Isemura et al. (14), who found that ML-7 inhibits attachment of mouse lung carcinoma cells to fibronectin, as well as the recent observation of Kaneko et al. (17) that ML-7 suppresses the motility and adhesion of pancreatic cancer cells. They are not, however, completely consistent with the findings of Sutton et al. (48). In their study of myosin II activation in MDCK cells subjected to ATP depletion, they reported that the Rho-kinase inhibitor Y-27632, but not the MLCK inhibitors ML-9 or ML-7, blocked MLC rephosphorylation during restoration of ATP levels after depletion. We agree that Y-27632 significantly inhibits phosphorylation of MLC. However, we find that ML-7 also reduces the amount of diphosphoryl-MLC at concentrations of 50–100 μM. At the concentration of ML-7 used in their study, 10 μM, there is no detectable effect on MLC phosphorylation.

The complexes mediating adhesion of MDCK cells were in the cortex. In fact, ML-7 did inhibit reattachment of MDCK cells after replenishment of ATP. In addition, ML-7 was found to also cause detachment of cells that had never experienced ATP depletion, suggesting that the function of this adhesive system was not limited to the particular circumstances present in the ATP-depleted cell.

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Inhibition of MLC diphosphorylation by ML-7 has the same concentration dependence as prevention of cell spreading and

Fig. 10. Effects of ML-7 and Y-27632 on MLC phosphorylation. A: immunofluorescence. MDCK cells were plated in EBSS with glucose on collagen-coated coverslips for 1 h and then incubated with DMSO (i, v), 50 μM ML-7 (ii, vi), 100 μM ML-7 (iii, vii), or 10 μM Y-27632 (iv, viii) for an additional hour. Phosphorylated MLC was then localized by staining with an antibody against Ser19 monophosphorylated MLC (i–iv) or Ser19, Thr18 diphosphorylated MLC (v–viii). Although there appears to be some reduction in MLC staining with ML-7 treatment (ii, iii, vi, vii), estimates are confounded by cell shape changes. In the case of Y-27632, however, staining of monophosphorylated MLC persists at the cell periphery (iv) but diphospho-MLC staining is almost eliminated (viii). B: Western blot of phosphorylated MLC. Western blots of cell extracts from cultures treated with ML-7 and Y-27632 exactly as described in A were reacted simultaneously with antibodies against mono- and diphosphorylated MLC and actin. The top doublet of bands is MLC, and the bottom band is actin. Y-27632 treatment significantly reduces the amount of Ser19 monophosphorylated MLC (lane 1) and nearly eliminates diphosphorylated MLC (lane 6). In contrast, ML-7 has no effect on monophosphorylation (lanes 2–4) but reduces the amount of diphosphorylation by ~50% at 100 μM (lanes 7–9).

Fig. 11. Proposed regulation of basal adhesive complex assembly in MDCK cells [after Totsukawa et al. (49)]. According to this model, adhesive complexes responsible for providing initial mechanical attachment of MDCK cells are formed in response to MLC phosphorylation. MLC phosphorylation is regulated by MLCK, but also by ROCK and RhoA acting through inhibition of myosin phosphatase. The question mark reflects the detectable but limited effect of RhoAV14 overexpression in preventing cell detachment after ATP depletion (see Fig. 7).
reattachment, suggesting that phosphorylation of MLC by MLCK regulates initial MDCK cell adhesion. A confounding observation, however, is that Y-27632, which almost eliminates diphosphorylation of MLC, has no effect on adhesion. Although we cannot absolutely rule out the possibility that ML-7 inhibits other kinases important in adhesion, a more intriguing possibility is that monophosphorylation of MLC at Thr18, which would not be detected with the phospho-specific antibodies used in this study, might play a key role in regulating adhesion. Studies to specifically examine this question are being planned.

Whereas RhoA appears to regulate the formation of central stress fibers and focal adhesions via Rho-kinase and other effectors, the identity of MLCK upstream modulators is less clear. In migrating fibroblasts, two forms of basal adhesive complexes have been distinguished, focal complexes and focal adhesions or contacts (7, 35, 42). The former are found in lamellipodia at the leading edge, where they are distinguished by concentrations of activated αβγ-integrin and the ability to apply strong traction forces to the substratum (20). Formation of focal complexes is believed to be regulated by the small GTP-binding protein Rac by a mechanism possibly involving regulation of actin dynamics (7, 35, 42). Possible effectors include type Iα phosphatidylinositol-4-phosphate 5-kinase, IRSp53, and the serine-threonine kinase PAK (4, 7). Focal complexes are thought to mature into focal contacts through a mechanism dependent on Rho (7, 35, 42). Our results examining early adhesive events of MDCK cells are consistent with this general model in that we relate mechanical adhesion to the substratum to the presence of small, less robust focal complexes at the cell cortex that are independent of the RhoA effector Rho-kinase, are not functionally preserved by constitutively active RhoA, and are inhibited by the drug ML-7 against MLCK. It remains to be seen whether this adhesive system in MDCK cells is also regulated by Rac.

The experiments described here focus on initial adhesion of renal epithelial cells to the substratum. As such, they are not directly applicable to the situation in the ischemic kidney, where cells from a fully formed epithelium detach. Instead, it is likely that more mature focal adhesions assembled from focal complexes (7, 35, 42) are the structures affected by ischemic injury. How this maturation process occurs in epithelial cells under the influence not only of changes in the extracellular matrix but also cell-cell contacts is poorly understood. Future studies in our laboratory will examine this process in detail.

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REFERENCES


