Actin cytoskeleton regulates extracellular matrix-dependent survival signals in glomerular epithelial cells

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Bijian, Krikor, Tomoko Takano, Joan Papillon, Ludmilla Le Berre, Jean-Louis Michaud, Chris R. J. Kennedy, and Andrey V. Cybulsky. Actin cytoskeleton regulates extracellular matrix-dependent survival signals in glomerular epithelial cells. Am J Physiol Renal Physiol 289: F1313–F1323, 2005. First published July 12, 2005; doi:10.1152/ajprenal.00106.2005.—Adhesion of rat glomerular epithelial cells (GEC) to collagen activates focal adhesion kinase (FAK) and the Ras-extracellular signal-regulated kinase (ERK) pathway and supports survival (prevents apoptosis). The present study addresses the relationship between actin organization and the survival phenotype. Parental GEC (adherent to collagen) and GEC stably transfected with constitutively active mutants of mitogen-activated protein kinase (R4F-MEK) or FAK (CD2-FAK) (on plastic) showed ERK activation, low levels of apoptosis, and a cortical distribution of F-actin. Parental GEC adherent to plastic showed increased apoptosis, disorganization of cortical F-actin, and formation of prominent stress fibers. Assembly of cortical F-actin was, at least in part, mediated via ERK. However, disruption of the actin cytoskeleton with cytochalasin D or latrunculin B in parental GEC (on collagen) and in GEC that express R4F-MEK or CD2-FAK (on plastic) decreased ERK activation and increased apoptosis. Expression of a constitutively active RhoA (L63RhoA) induced assembly of cortical F-actin, promoted ERK activation, and supplanted the requirement for collagen for survival. Adhesion of GEC to collagen increased phosphatidylinositol-4,5-bisphosphate (PIP2). Downregulation or sequestration of PIP2 by transfection with an inositol 5'-phosphatase or the plextrin-homology domain of phospholipase C-β1 decreased F-actin content and survival. Moreover, overexpression of wild-type or K256E mutant α-actinin-4 with increased affinity for F-actin increased apoptosis. These results demonstrate a reciprocal relationship between collagen-induced cortical F-actin assembly and collagen-dependent survival signaling, including ERK activation. Appropriate remodeling of the actin cytoskeleton may be necessary for facilitating survival, as both disassembly and excessive crosslinking affect survival adversely.

α-actinin-4; apoptosis; collagen; extracellular signal-regulated kinase; RhoA

ADHESION OF CELLS to extracellular matrix (ECM) via integrins modulates cell growth, differentiation, survival, and morphology (20, 21). Integrins are heterodimeric transmembrane molecules consisting of α- and β-subunits. In addition to providing a structural link between ECM proteins and the actin cytoskeleton, integrins are often involved in signal transduction pathways in cooperation with growth factor receptors. Among the many intracellular signaling pathways that may be activated independently or synergistically by integrins and growth factor receptors, the Ras-extracellular-signal-regulated kinase (ERK) pathway is one of the central pathways regulating cell proliferation and survival. The level(s) at which cross talk occurs may be dependent on the cell type, i.e., cross talk has been demonstrated at the level of growth factor receptors, upstream of Ras, from Ras to Raf-1, from Raf-1 to mitogen-activated protein kinase kinase (MEK), and at the nuclear translocation of activated ERK (29).

Glomerular epithelial cells (GEC) or podocytes are highly differentiated cells that play a key role in maintaining glomerular permeability (19, 27, 32, 35). The major integrin expressed in GEC is α3β1, which preferentially binds to laminin, as well as collagen, fibronectin, and entactin/nidogen (1, 2, 17, 18, 24, 28). GEC are anchorage-dependent cells, as they require attachment to ECM via integrins for survival, and soluble growth factors for proliferation. Under normal conditions, where GEC are in contact with ECM, there is little GEC turnover. Podocyte injury occurs in many forms of human and experimental glomerular diseases (5, 6, 33, 42). Injury, leading to foot process effacement and proteinuria, may be caused, at least in part, by disruption of the actin cytoskeleton. In addition, mutations in a gene encoding for α-actinin-4, which increase the affinity of α-actinin-4 for filamentous (F)-actin, have been associated with a familial form of autosomal dominant focal segmental glomerulosclerosis (25, 30). Altered expression of another actin binding protein, synaptopodin, has been observed in various forms of glomerular disease (5, 26, 37). Other factors that have been shown to affect the organization of the actin cytoskeleton include Rho GTPases (8) and phosphatidylinositol-4,5-bisphosphate (PIP2) (43). Mice deficient in Rho GDP dissociation inhibitor-α (which maintains Rho family members in their GDP-bound inactive state) develop massive proteinuria and glomerulosclerosis, demonstrating the importance of signaling pathways regulated by Rho GTPases in maintaining glomerular permeability (39). PIP2 has been shown to modulate the activity and targeting of actin regulatory proteins, promoting actin polymerization (43). In addition to proteinuria, podocyte injury may also result in apoptosis or proliferation and in glomerulosclerosis (19, 27, 32, 35).

We have previously demonstrated that adhesion of GEC to collagen results in the activation of focal adhesion kinase (FAK), a key mediator of integrin signaling. In GEC, FAK activates the ERK pathway, which supports survival (prevents apoptosis) (7). In contrast, apoptosis is markedly enhanced in GEC adherent to plastic substratum. Stable expression of
constitutively active mutants of FAK (CD2-FAK) or MEK (R4F-MEK) in GEC activated the ERK pathway and supplanted the requirement of collagen for survival, whereas inhibition of the ERK pathway decreased survival of GEC on collagen. Analogous signals for GEC survival were activated in experimental focal segmental glomerulosclerosis, a model of GEC injury in vivo (7). Earlier, we showed that adhesion to ECM increased PIP2 levels in GEC, possibly through the activation of phosphatidylinositol 4-phosphate 5-kinase, although the involvement of PIP2 in the regulation of GEC actin cytoskeleton has yet to be demonstrated (14).

The aim of the present study was to address the relationship between GEC actin organization and the survival phenotype. We demonstrate that in GEC adherent to plastic (i.e., the apoptosis phenotype), F-actin is organized predominantly into stress fibers. On collagen, and in GEC that express CD2-FAK, R4F-MEK, or a constitutively active mutant of RhoA (i.e., the survival phenotype), there is a switch to cortical actin organization. Disruption of the actin cytoskeleton with actin-depolymerizing drugs decreased ERK activation and promoted GEC death. Moreover, F-actin content and survival were decreased by downregulating/sequestering PIP2 levels through the expression of an inositol 5′-phosphatase (Pase) or the plextrin homology domain of phospholipase C-ε1 [PH(PLC)]. Stable expression of wild-type α-actinin-4 or a mutant α-actinin-4 with increased affinity for F-actin decreased GEC proliferation while increasing GEC apoptosis.

MATERIALS AND METHODS

Materials. Tissue culture reagents, Transfectin CaPO4, transfection system, and G418 (geneticin) were obtained from Invitrogen (Burlington, Ontario). NuSerum was obtained from BD Biosciences (Mississauga, Ontario). Eugene 6 transfection reagent was obtained from Roche Applied Science (Laval, Quebec). Pepsin-solubilized bovine dermal collagen I (Vitrogen) was from Cohesion (Palo Alto, CA). Epidermal growth factor (EGF), LY-294002, cytochalasin D, latrunculin B, caspase-3 substrate, and mouse anti-vinculin antibody were obtained from Sigma-Aldrich Canada (Mississauga, Ontario). Bisbenzimide H33342 fluorochrome, propidium iodide, caspase-8 substrate (granzyme B substrate I), caspase-9 substrate II, PD-98059, and Y27632 were obtained from Calbiochem (La Jolla, CA). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Mississauga, Ontario). Rhodamine-conjugated phalloidin and Alexa-Fluor 488-conjugated deoxyribonucleic acid (DNAse I) were obtained from Molecular Probes (Eugene, OR). Rabbit anti-phospho-ERK1/2 (threonine202/tyrosine204) and rabbit anti-ERK1/2 antibodies were purchased from New England Biolabs (Mississauga, Ontario). Mouse anti-RhoA and mouse anti-green fluorescent protein (GFP) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). R4F-MEK, CD2-FAK, and L63-RhoA cDNAs have been described previously (7, 16). Briefly, GEC on collagen were cotransfected with the expression vector of interest (2 μg of DNA per 100-mm plate) and a neomycin resistance gene pRc/RSV (molar ratio 12.5:1), using the CaPO4 technique, as described previously (15). GEC were then cultured on collagen in K1 medium containing 0.5 mg/ml G418. GEC clones resistant to G418 were isolated and replated onto plastic substratum. Clones that proliferated on plastic in K1 medium were selected, passaged, and assessed for the expression of the specific protein by immunoblotting. A soft agar assay was used to test for anchorage-independent proliferation (15). GEC on collagen were also transfected with wild-type or mutant mouse GFP-α-actinin-4 cDNAs (10 μg of plasmid DNA containing a neomycin resistance gene per 100-mm plate) or cotransfected with GFP-PH(PLC) or GFP-Pase (10 μg of plasmid DNA per 100-mm plate) and a neomycin resistance gene pRc/RSV (2.6 μg) using Eugene 6 transfection reagent, according to the manufacturer’s instructions. GEC were then cultured on collagen in K1 medium containing 0.5 mg/ml G418. GEC clones resistant to G418 were isolated, sorted by flow cytometry, and GFP-positive GEC were expanded on collagen.

Measurement of GEC proliferation. Cell number was determined by visual counting. Cells adherent to collagen gels (35-mm plates) were placed into single-cell suspension by incubation with collagenase and trypsin-EDTA. Cells on plastic substratum were placed into suspension by incubation with trypsin-EDTA. Suspended cells were then counted in a hemacytometer (12, 15).

Immunoblotting. GEC lysates were subjected to SDS-PAGE under reducing conditions, as detailed previously (7). Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 3% BSA, and incubated with primary antibody, and then with horseradish peroxidase-conjugated secondary antibody. The blots were developed using the enhanced chemiluminescense technique (ECL, Amersham Pharmacia Biotech AB). Protein content was quantified by scanning densitometry, using National Institutes of Health Image software (7). Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

Hoechst H33342 staining. Staining with Hoechst H33342 dye was used to quantify apoptosis, as described previously (7). Briefly, adherent cells were stained with H33342 (1 μg/ml) for 10 min at 37°C without fixation. After being washed with PBS, cells were stained with propidium iodide (5 μg/ml) to identify necrotic or late-apoptotic cells. Cells were examined using a Nikon Diaphot fluorescence microscope, and the number of H33342-positive/propidium iodide-
negative cells was quantified by visual counting. Previously, we demonstrated that apoptosis, as assessed by H33342 staining, correlated with the DNA laddering assay (7).

**Caspase activity assay.** Intracellular caspase-3, -8, and -9 activities were measured using a colorimetric assay with p-nitroaniline (pNA)-labeled substrates, as described previously (7). Adherent cells were lysed in buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 100 µM EDTA, pH 7.4 (4°C). The mixture was centrifuged at 14,000 g for 10 min. Supernatants were collected and adjusted for protein concentration. The reaction mixture contained 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 µM EDTA, 10% glycerol, pH 7.4 (37°C) with 2 mM substrate, i.e., Ac-Asp-Glu-Val-Asp-pNA (caspase-3), Ac-Leu-Glu-His-Asp-pNA (caspase-9), or Ac-Ile-Glu-Thr-Asp-pNA (caspase-8). The absorbance of the reaction was read at 405 nm every 5 min for 2 h. After subtraction of background activity, the slope of absorbance vs. time is proportional to caspase activity.

**Fluorescence microscopy.** Cells adherent to uncoated or collagen-coated glass coverslips were fixed with 3% paraformaldehyde in PBS and were permeabilized with 0.5% Triton X-100. After being washed, cells were incubated with rhodamine-phalloidin (0.043 µg/ml) to visualize F-actin and Alexa Fluor 488-conjugated-DNase I (9 mg/ml) to visualize G-actin for 20 min. Coverslips were mounted onto glass slides and were examined using both rhodamine and fluorescein filters in a Nikon Diaphot fluorescence microscope. Slides were photographed using a Nikon Coolpix 995 digital camera locked at a constant exposure. Quantification of fluorescence intensity and merging of fluorescence images were carried out with Adobe Photoshop software. Immunofluorescence staining with antibody to vinculin was carried out according to a protocol described previously (16).

**Statistics.** Data are presented as means ± SE. The t-statistic was used to determine significant differences between two groups. One-way ANOVA was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t-statistic and adjusting the critical value according to the Bonferroni method.

**RESULTS**

**Role of F-actin in GEC survival.** In keeping with previous results (7), ERK phosphorylation (which correlates with activation) was greater in GEC adherent to collagen, compared with plastic (Fig. 1A). GEC on plastic underwent apoptosis at a rate more than 10-fold greater than GEC on collagen (7) (also see below Fig. 4B), and the anti-apoptotic effect of collagen was dependent on ERK activation. Analogous signals for GEC survival were activated in glomeruli of rats with experimental focal segmental glomerulosclerosis, an in vivo model of GEC injury (7). Stable expression of constitutively active mutants of FAK (CD2-FAK) or MEK (R4F-MEK) in GEC both activated the ERK pathway (Fig. 1, B and C) and supplanted the requirement of collagen for survival (7). To study the relationship between actin organization and the survival phenotype, we analyzed the actin cytoskeleton in various GEC lines. Parental GEC adherent to plastic had significantly greater levels of F-actin, compared with parental GEC on collagen (Fig. 2A). In

![Fig. 1. Survival signals emanating from collagen are dependent on an intact actin cytoskeleton. A-C. ERK1/2 activation. Adhesion of parental (Par) GEC to collagen (COL) stimulates ERK phosphorylation, compared with GEC on plastic (PL) (A, lanes 1 and 2). Expression of R4F-MEK (B, lanes 1 and 2) or CD2-FAK (C, lanes 1 and 2) increases ERK phosphorylation, compared with parental GEC on PL. GEC lysates were immunoblotted with antibodies to ERK1/2, phosphorylated at threonine 187 and tyrosine 189 (pERK; top) or ERK1/2 (bottom). Parental GEC on collagen (A), R4F-MEK GEC on PL (C) or CD2-FAK GEC on PL (B) were untreated (Untr) or were treated with cytochalasin D (CD; 20 µM) or latrunculin B (LtB; 1 µM) for 24 h in serum–poor medium. Cell lysates were immunoblotted with antibodies to pERK (top) or ERK (bottom). Representative immunoblots are shown on the left (lanes 3–8) and densitometric quantification on the right. ERK activation was decreased significantly in CD- or LtB-treated GEC, compared with untreated. * P < 0.002 CD vs. untreated. ** P < 0.001 LtB vs. untreated (3–5 experiments). B: * P < 0.001 CD or LtB vs. untreated (6 experiments). C: * P < 0.001 CD or LtB vs. untreated (6 experiments). There were no significant differences in ERK protein expression. D: apoptosis was monitored as H33342 positively stained nuclei in the 3 GEC lines treated with or without CD or LtB. There were significant increases in apoptosis in CD- or LtB-treated GEC, compared with untreated. * P < 0.001 CD or LtB vs. untreated (4 experiments). E: caspase-3 activity was monitored in parental GEC on collagen treated with or without CD or LtB (top). Caspase-3 activity (expressed in arbitrary units) was significantly increased in CD- or LtB-treated GEC, compared with untreated control (* P < 0.006 CD vs. untreated. ** P < 0.03 LtB vs. untreated, 4 experiments). Caspase-3 activity was increased significantly in parental GEC plated on plastic substratum, compared with GEC plated on collagen (bottom; * P < 0.003 PL vs. COL, 3 experiments) F: caspase-8 and -9 activities were monitored in GEC expressing R4F-FAK or CD2-FAK, treated with or without CD or LtB. There were significant increases in caspase-8 and -9 activities in GEC expressing R4F-FAK or CD2-FAK, treated with or without CD or LtB, compared with untreated. * P < 0.02 CD vs. untreated (R4F-FAK, caspase-8). ** P < 0.03 LtB vs. untreated (R4F-FAK, caspase-8). *** P < 0.001 CD or LtB vs. untreated (CD2-FAK; caspase-8 and -9). CD vs. untreated (R4F-FAK; caspase-9). *** P < 0.004 LtB vs. untreated (R4F-FAK; caspase-9). 4–6 experiments).
parental GEC on collagen, F-actin was distributed in a cortical pattern (Fig. 2B), whereas parental GEC adherent to plastic demonstrated a complete loss of the cortical F-actin structure and formation of extensive stress fibers (Fig. 2B). GEC expressing CD2-FAK or R4F-MEK on plastic had levels of F-actin comparable with parental GEC on collagen (Fig. 2A). In addition, the GEC expressing CD2-FAK or R4F-MEK on plastic showed a clearer cortical actin distribution, comparable to parental GEC on collagen, and in keeping with their survival phenotype (Fig. 2B). These results demonstrate the association of a cortical actin cytoskeletal structure with GEC survival.

In a previous study, we demonstrated that the MEK inhibitor PD-98059 induced apoptosis in parental GEC adherent to collagen, and in CD2-FAK- and R4F-MEK-expressing GEC (on plastic) (7). Thus survival of GEC was dependent on the activation of ERK. To determine whether ERK pathway activation was associated with reorganization of the cytoskeleton, we examined F-actin staining in collagen-adherent GEC that were treated with PD-98059. Inhibition of the ERK pathway in parental GEC on collagen resulted in a partial loss of the cortical F-actin staining pattern, and formation of stress fibers (Fig. 2B), resembling F-actin staining in parental GEC adherent to plastic (Fig. 2B). This result suggests that the ERK pathway contributes toward cortical F-actin organization, which is associated with the survival phenotype.

In the next series of experiments, we addressed the importance of the cytoskeleton in facilitating ECM-induced survival signaling. GEC were treated with two distinct inhibitors of actin polymerization, cytochalasin D or latrunculin B. Cytochalasin D binds to the growing end of actin filaments, whereas latrunculin B sequesters G-actin monomers. In GEC, both compounds were shown to disassemble F-actin extensively (16). Incubation of GEC with cytochalasin D or latrunculin B for 24 h significantly reduced ERK activation in parental GEC, as well as in R4F-MEK- and CD2-FAK-expressing clones, compared with untreated cells (Fig. 1A-C). Thus there appears to be a reciprocal relationship between ERK activation and cortical F-actin assembly, i.e., ERK activation is associated with cortical F-actin organization (Fig. 2B), while disassembly of F-actin impairs ERK activation.

F-actin disruption also led to increased levels of apoptosis in parental GEC, as well as in R4F-MEK- and CD2-FAK-expressing clones (Fig. 1D). Moreover, analysis of intracellular caspase activities revealed increased caspase-3 activity in parental GEC on collagen treated with cytochalasin D or latrunculin B (Fig. 1E, top), similar to the increased caspase-3 (Fig. 1E, bottom) observed when parental GEC are plated on plastic substrate. The change in caspase-3 activity is in keeping with changes in caspase-8 and -9 activities, demonstrated previously (7). In addition, caspase-8 and -9 activities were increased significantly in R4F-MEK- and CD2-FAK-expressing GEC treated with cytochalasin D or latrunculin B, compared with untreated cells (Fig. 1F).
Expression of constitutively active RhoA (L63RhoA). Rho GTPases are known to regulate the structure of the actin cytoskeleton (8). To address the effects of RhoA on GEC survival and cytoskeletal structure, GEC were stably transfected with constitutively active RhoA (L63RhoA). Two representative clones are demonstrated in Fig. 3A. In keeping with previous results, parental GEC proliferated only on collagen, but not plastic substratum (Fig. 3B). In contrast, the two L63RhoA-expressing clones (as well as several other L63RhoA clones that are not shown) were also able to proliferate on plastic substratum (Fig. 3B). Proliferation of L63RhoA-expressing GEC on plastic was dependent on the presence of growth factors in the medium (Fig. 3C, 2 left bars) and on the activation of the ERK pathway (Fig. 3C, PD-98059). Moreover, proliferation of L63RhoA-expressing GEC on plastic was adhesion dependent, as demonstrated by inability to form colonies in soft agar (not shown). Thus expression of L63RhoA suppled the requirement of GEC for collagen in supporting survival and proliferation.

GEC expressing L63RhoA (on plastic) showed levels of F-actin comparable to parental GEC on collagen (Fig. 2A). Moreover, the GEC expressing L63RhoA (on plastic) showed a cortical actin distribution, comparable to parental GEC on collagen (Fig. 2B) and in keeping with the survival phenotype. In addition, the L63RhoA clones had some stress fiber formation, although these stress fibers were substantially smaller than those observed in parental GEC adherent to plastic. Incubation of L63RhoA GEC with PD-98059 (75 μM for 24 h) resulted in a decrease in cortical actin and an increase in stress fibers (not shown). The changes were similar to those observed in parental GEC on collagen treated with PD-98059 (Fig. 2B). The result suggests that L63RhoA facilitates formation of cortical actin via the ERK pathway.

In the absence of mitogens (serum-poor medium), basal ERK phosphorylation in the L63RhoA-expressing clones on plastic was approximately fourfold greater compared with parental GEC on plastic, reaching ~50% of the level seen in parental cells on collagen (Fig. 3D). Moreover, basal ERK phosphorylation in L63RhoA GEC on plastic could be further

Fig. 3. Stable transfection of GEC with constitutively active RhoA (L63RhoA). A: 2 representative GEC clones (clones 13 and 22) that stably express L63RhoA, adherent to plastic (PL), and parental (Par) GEC on collagen (COL). Equal amounts of total protein were loaded into each lane. Endogenous RhoA was not detectable by immunoblotting at this exposure. B: parental or L63RhoA-transfected GEC (clones 13 and 22) were plated at 2 x 10^4 cells/well and cultured on collagen or plastic in growth factor-replete (K1) medium for 72 h. Parental GEC proliferated only on collagen (*P < 0.005 parental COL vs. parental PL, 11 experiments). L63RhoA clones proliferated on collagen and plastic (P < 0.005, + P < 0.041 L63RhoA PL vs. parental PL, 11 experiments). C: L63RhoA transfected GEC (50,000 cells) on plastic were cultured in serum-poor (SP) medium or K1 medium for 5 days. Cell number increased in the presence of K1 (*P < 0.001 K1 vs. SP, 8 experiments). Addition of PD-98059 (75 μM) abolished proliferation. D: parental GEC on collagen or plastic in K1 or L63RhoA-transfected GEC on plastic (clone 13) were cultured in serum-poor medium for 24 h. Lysates were immunoblotted with antibodies to ERK1/2 phospho-threonine202/tyrosine204 (pERK; top) or to ERK1/2 (bottom; representative immunoblots and densitometric quantification). ERK activation was low in parental GEC on plastic, while ERK was activated in GEC on collagen. ERK phosphorylation was increased in L63RhoA GEC, compared with parental GEC on plastic (*P < 0.004 L63RhoA PL vs. parental PL, 4 experiments). E: L63RhoA-transfected GEC on plastic were cultured in serum-poor medium for 24 h. Cells were then treated with or without EGF (100 ng/ml) for 30 min. Equal amounts of total protein were loaded into each lane before immunoblotting with anti-phosphoERK antibody. Basal L63RhoA ERK phosphorylation was further stimulated by EGF (*P < 0.002 EGF vs. untreated, 6 experiments). F: apoptosis (H33342 positively stained nuclei) in L63RhoA GEC on plastic was monitored after treatment with or without PD-98059 (PD: 75 μM), the phosphatidylinositol 3-kinase inhibitor, LY-294002 (LY; 10 μM), or the Rho kinase (ROCK) inhibitor, Y27632 (Y27; 10 μM) in serum-poor medium for 24 h. There was a significant increase in apoptosis only in PD-98059-treated cells (*P < 0.001 vs. untreated, 8 experiments). G: caspase-3 activity was monitored in L63RhoA-transfected GEC on plastic cultured in serum-poor medium with or without PD-98059 (75 μM), LY-294002 (10 μM), Y27632 (10 μM), cytochalasin D (CD; 20 μM), or latrunculin B (LtB; 1 μM) for 24 h. Caspase-3 activity was increased in the L63RhoA GEC treated with PD-98059, cytochalasin D, and latrunculin B, compared with untreated (*P < 0.001 vs. untreated, 4 experiments). H: apoptosis of parental GEC on collagen and L63RhoA GEC on plastic was monitored after treatment with or without cytochalasin D (4 or 20 μM) significantly increased apoptosis in both cell lines, whereas treatment with 1.0 μM LtB significantly increased apoptosis only in parental GEC (*P < 0.001 vs. untreated parental or untreated L63RhoA; 8 experiments).
stimulated by EGF (Fig. 3E), similar to parental GEC adherent to collagen, but unlike parental GEC on plastic, where lack of signals emanating from the ECM prohibited efficient activation of the EGF receptor and ERK pathway by EGF (7, 12). The L63RhoA GEC did not demonstrate phosphorylation of FAK (result not shown), implying that FAK was not involved in ERK activation. Survival of L63RhoA GEC was dependent on the ERK pathway, as inhibition of MEK with PD-98059 increased apoptosis and caspase-3 activation (Fig. 3, F and G). L63RhoA-expressing GEC on plastic were relatively resistant to cytochalasin D- or latrunculin B-induced apoptosis, demonstrated by reduced levels of apoptosis at similar drug concentrations, when compared with parental GEC on collagen (Fig. 3H). Nevertheless, cytochalasin D and latrunculin B treatments led to increased caspase-3 activity in L63RhoA-expressing GEC (Fig. 3G). For comparison, L63RhoA-expressing GEC were also treated with the Rho kinase (ROCK) inhibitor, Y27632 (41), or with the phosphatidylinositol 3-kinase inhibitor, LY-294002 (7). Y27632 and LY-294002 had no significant effects on apoptosis or caspase-3 activation (Fig. 3, F and G), and did not affect ERK phosphorylation (not shown). Thus stable expression of L63RhoA in GEC induced an actin organization that permitted efficient activation of ERK in the absence of ECM and thus supplanted the requirement of collagen for survival. It should also be noted that in L63RhoA-expressing GEC adherent to collagen, levels of phosphoERK (not shown) were comparable to the levels observed on plastic (Fig. 3D). The amount of apoptotic cells in L63RhoA-expressing GEC on collagen (<0.5% of total) was also similar to the level on plastic (Fig. 3F, untreated). Finally, the L63RhoA GEC on collagen displayed predominantly a cortical F-actin staining pattern (not shown), which was indistinguishable from the pattern on plastic (Fig. 2B), although the small/scant stress fibers were present in these cells only on plastic.

**Downregulation of PIP2 in GEC.** Adhesion of GEC to collagen was shown to increase the mass of PIP2 more than twofold, compared with GEC on plastic, and this effect of collagen was correlated with increased activity of membrane phosphatidylinositol 4-phosphate 5-kinase activity (14). To address the role of PIP2 in regulating cell survival, PIP2 was downregulated or sequestered by stably transfecting GEC on collagen with GFP fused with an inositol 5' specific phosphatase (GFP-Pase), or the plextin-homology domain of phospholipase C-δ1 [GFP-PH(PLCδ)], which binds to PIP2 with high affinity and specificity (36, 38). The expression of GFP-PH(PLCδ) and GFP-Pase is demonstrated in Fig. 4A. We observed a significant increase in apoptosis in GFP-PH(PLCδ) and GFP-Pase GEC compared with parental cells, although the level of apoptosis observed in GFP-PH(PLCδ) or GFP-Pase GEC was less than in parental GEC adherent to plastic substratum (Fig. 4B). There were no significant differences observed between the proliferation rates (i.e., cell counts) of GFP-PH(PLCδ) or GFP-Pase-expressing GEC and parental cells (data not shown); however, this assay may not have been sufficiently sensitive to detect small differences. Intra-cellular caspase-3 activity was significantly increased in GFP-PH(PLCδ) and GFP-Pase-expressing GEC, compared with parental GEC (Fig. 4C), in keeping with apoptotic cell counts (Fig. 4B). In addition, basal ERK activity was not significantly different among the three cell lines (not shown). The importance of the ERK pathway in promoting cell survival was further demonstrated by treating GEC with the MEK inhibitor, PD-98059, and quantifying caspase-3 activity. In addition to the increased levels of caspase-3 observed in GFP-PH(PLCδ) and GFP-Pase-expressing GEC, there was a further increase in caspase-3 activity in the PD-98059-treated GEC, compared with untreated (Fig. 4D). This result suggests that sequestration of PIP2 and inhibition of the ERK cascade
may lead to apoptosis via separate pathways, as the inhibitory effects on the two pathways were additive.

GEC expressing GFP-PH(PLCδ) adherent to collagen had reduced levels of F-actin, compared with parental GEC on collagen (Fig. 2A). A trend toward lower F-actin content was observed in GEC expressing GFP-Pase, although the result was not statistically significant (Fig. 2A). GEC expressing GFP-PH(PLCδ) or GFP-Pase demonstrated cortical actin staining, however, at a somewhat reduced intensity, compared with parental GEC on collagen (Fig. 2B).

Expression of wild-type and mutant α-actinin-4. Focal segmental glomerulosclerosis may be associated with GEC apoptosis, and with mutations in the F-actin-binding protein, α-actinin-4. To determine whether α-actinin-4 affects survival, GEC on collagen were stably transfected with GFP-tagged wild-type and K256E mutant α-actinin-4 cDNAs. The K256E mutant has been shown to bind to F-actin in vitro with greater affinity, compared with wild-type (31). Expression of wild-type and mutant α-actinin-4 proteins in GEC on collagen is demonstrated in Fig. 5A. We observed a significant decrease in proliferation of both wild-type and mutant GFP-α-actinin-4-expressing GEC, compared with parental cells (Fig. 5B). Moreover, wild-type and mutant GFP-α-actinin-4 GEC demonstrated increased levels of apoptosis (Fig. 5C), as well as increased caspase-3 activity (Fig. 5D), compared with parental cells. It should be noted that despite significantly lower expression levels of the mutant GFP-α-actinin-4, compared with the wild-type (Fig. 5A), GEC expressing the mutant demonstrated significantly decreased proliferation, as well as greater apoptosis and caspase-3 activity, compared with the wild-type GFP-α-actinin-4-expressing GEC (Fig. 5, B-D), highlighting the detrimental consequences of this mutation. Basal ERK phosphorylation was not significantly different among the three cell lines adherent to collagen (not shown).

The subcellular distribution of wild-type and mutant GFP-α-actinin-4 proteins and F-actin was analyzed by fluorescence microscopy. The latter was visualized by staining with rhodamine-phalloidin. Some focal colocalization of F-actin with wild-type and mutant GFP-α-actinin-4 was observed in GEC on collagen (Fig. 6, arrows), but F-actin and wild-type or mutant GFP-α-actinin-4 were largely dissociated from one another. Both wild-type and mutant GFP-α-actinin-4 were primarily found in the cytoplasm, and there were variable levels of expression among cells. In addition, a subpopulation of GEC expressed wild-type GFP-α-actinin-4 exclusively in the nucleus (Fig. 6, arrowhead). GEC expressing wild-type and to a lesser extent mutant GFP-α-actinin-4 also displayed an increase in stress fibers (Fig. 6, middle), compared with parental GEC (Fig. 2B), reminiscent of an apoptotic phenotype.

Effect of collagen and L63RhoA expression on focal adhesion complexes. Under some conditions, cells in culture adhere to substrata via structurally defined adhesion sites known as focal adhesion complexes (44). We examined GEC for the presence of focal adhesion complexes by immunostaining for vinculin (44). The results demonstrate the presence of focal adhesion complexes in parental GEC adherent to plastic (Fig. 7, A-C). Focal adhesion complexes were, however, not detected in parental GEC adherent to collagen (Fig. 7D) nor in GEC that express L63RhoA (Fig. 7E). Thus the presence of focal adhesion complexes correlates with the presence of actin

![Fig. 5](http://ajprenal.physiology.org/)

Fig. 5. Stable transfection of GEC with GFP-tagged wild-type (WT-ACTN-4) or K256E mutant α-actinin-4 (MUT-ACTN-4). A: lysates of parental GEC and of GEC that stably express GFP-WT-ACTN-4 or GFP-MUT-ACTN-4 were immunoblotted with anti-GFP antibody. Equal amounts of total protein were loaded into each lane. B: parental (Par) GEC, GEC expressing GFP-WT-ACTN-4 or GFP-MUT-ACTN-4 (50,000 cells) were cultured in K1 medium. Cell number was quantified at 72 h. GEC expressing GFP-WT-ACTN-4 or GFP-MUT-ACTN-4 had reduced levels of proliferation, compared with parental cells (*P < 0.03 WT-ACTN-4 vs. parental, **P < 0.001 MUT-ACTN-4 vs. parental, 4 experiments). GEC expressing the GFP-MUT-ACTN-4 had significantly reduced proliferation, compared with GFP-WT-ACTN-4 (‘P < 0.01 MUT-ACTN-4 vs WT-ACTN-4). C: apoptosis of parental GEC and of GEC expressing GFP-WT-ACTN-4 or GFP-MUT-ACTN-4 cultured in serum-poor medium for 24 h was quantified by monitoring H33342 staining of nuclei. GEC expressing GFP-WT-ACTN-4 or GFP-MUT-ACTN-4 had increased levels of apoptosis, compared with parental cells (*P < 0.03 WT-ACTN-4 vs. parental, **P < 0.001 GFP-MUT-ACTN-4 vs. parental, 3 experiments). GEC expressing GFP-MUT-ACTN-4 had significantly greater levels of apoptosis compared with GFP-WT-ACTN-4 (‘P < 0.01 MUT-ACTN-4 vs WT-ACTN-4). D: caspase-3 activity was monitored in parental, and GFP-WT-ACTN-4- and GFP-MUT-ACTN-4-expressing GEC. Caspase-3 activity was significantly increased in GEC expressing GFP-WT-ACTN-4 or GFP-MUT-ACTN-4, compared with parental cells (*P < 0.04 WT-ACTN-4 vs. parental, **P < 0.001 MUT-ACTN-4 vs. parental, 4 experiments). GEC expressing GFP-MUT-ACTN-4 had significantly increased caspase-3 activity, compared with GFP-WT-ACTN-4 (‘P < 0.04 MUT-ACTN-4 vs WT-ACTN-4).
stress fibers, reduced cortical actin, and proapoptotic phenotype.

**DISCUSSION**

This study demonstrates that organization of the actin cytoskeleton is critical for transmission of survival signals from ECM. Adhesion of GEC to collagen induced FAK and ERK activation, which supports survival (7), and ERK activation was associated with assembly of a cortical actin cytoskeleton (Figs. 2 and 8). By analogy, ERK was activated and facilitated survival, as well as cortical actin assembly in GEC that express constitutively active FAK or MEK (Figs. 2 and 8) (7). In contrast, adhesion to plastic, which enhanced apoptosis (Fig. 4B) (7), was associated with disorganization of cortical actin and formation of prominent stress fibers (Fig. 2). There was a reciprocal relationship between ERK activation and cortical F-actin assembly (Fig. 8). Thus disruption of the actin cytoskeleton by cytochalasin D or latrunculin B in parental GEC adherent to collagen as well as in GEC that express R4F-MEK and CD2-FAK led to decreased activation of ERK and reduced cell survival (increased apoptosis) (Fig. 1). In GEC, collagen-induced FAK activation was dependent on Src and resulted in binding of Grb2 to FAK, and activation of the Ras-ERK cascade (Fig. 8) (7). Cytochalasin D and latrunculin B had no effect on FAK activation in parental GEC (data not shown), suggesting functional integrin-FAK signaling despite cytoskeletal disruption. Thus the cytoskeleton-depolymerizing drugs most likely acted downstream of FAK, in particular, at the level of ERK activation by MEK (Fig. 1B). Together, the results show that efficient Ras-ERK pathway activation and transmission of survival signals from collagen depend on the proper organization of the actin cytoskeleton.

A role for the cytoskeleton in ERK activation has been addressed in other cells. In fibroblasts, EGF-dependent ERK activation was dependent on adhesion to ECM and an intact cytoskeleton (4). In cells in suspension or in cells treated with cytochalasin D, the nuclear translocation of activated ERK was absent because of the disruption of the actin cytoskeleton (4). Insulin-induced Ras and ERK activation were inhibited by cytochalasin D in L6 myotubes (40). However, in human intestinal epithelial cells (HT-29 or Caco-2), disruption of the actin cytoskeleton with cytochalasin D induced the activation of NF-κB and p38 kinase.

![Fig. 6. Localization of GFP-tagged wild-type (WT-ACTN-4) and K256E mutant α-actinin-4 (MUT-ACTN-4) and F-actin. GEC expressing GFP-WT-ACTN-4 (top) and GFP-MUT-ACTN-4 (bottom) were fixed, stained with rhodamine-phalloidin (to visualize F-actin), and examined by fluorescence microscopy. GFP-WT- and MUT-ACTN-4 showed cortical (arrows) as well as cytoplasmic staining (left). In addition, WT-ACTN-4 was present in the nuclei of some cells (arrowheads). F-actin showed a cortical distribution together with the presence of stress fibers (middle). Right: merged images of GFP-WT- or MUT-ACTN-4 (green fluorescence) and F-actin (red fluorescence). There was some colocalization of F-actin with GFP-WT- and MUT-ACTN-4 in a cortical distribution (yellow fluorescence, arrows).]
increased in L63RhoA-GEC, compared with parental GEC on requirement for ECM (Fig. 3). Basal ERK phosphorylation was cells to proliferate on plastic substratum, thus supplanting the phosphorylation of ezrin-radixin-moesin proteins (8). L63RhoA could constitutively active RhoA was reported to induce phosphorylation of filaments at the cell periphery near the plasma membrane, as resulted from actin disassembly (34).

Fig. 7. Focal adhesion complexes. GEC were immunostained with antibody to vinculin, a component of focal adhesion complexes. A–C: 3 examples of parental GEC adherent to plastic. These cells revealed elongated or dotlike structures (arrows), mainly at the edges of monolayers, in keeping with focal adhesion complexes. Similar structures were not detected in parental GEC adherent to collagen (D), and in L63RhoA GEC on plastic (E), as vinculin staining was mainly cytoplasmic.

demonstrating the concomitant activation of certain pathways resulting from actin disassembly (34).

Signaling, survival, and organization of the actin cytoskeleton in GEC were modulated by stable expression of L63RhoA. Survival of L63RhoA-expressing cells adherent to plastic was comparable to that of parental GEC on collagen (Fig. 3). In the presence of growth factors, the L63RhoA expression allowed cells to proliferate on plastic substratum, thus supplanting the requirement for ECM (Fig. 3). Basal ERK phosphorylation was increased in L63RhoA-GEC, compared with parental GEC on plastic (Fig. 3), indicating that L63RhoA stimulated a pathway that resulted in ERK activation (Fig. 8). Also, stable expression of L63RhoA in GEC induced cortical distribution of F-actin, as well as some stress fiber formation (Figs. 2 and 8), in keeping with the results of a recent study (16). L63RhoA may have acted through a mechanism involving stabilization of actin filaments at the cell periphery near the plasma membrane, as constitutively active RhoA was reported to induce phosphorylation of ezrin-radixin-moesin proteins (8). L63RhoA could potentially activate various effectors, which include the myosin-binding subunit of myosin phosphatase, protein kinase N/PRK1, p140 mDia, or phosphatidylinositol 4-phosphate 5-kinase; failure to block the action of L63RhoA with an inhibitor of Rho kinase/ROK/ROCK suggests that this pathway was not involved (23).

Disassembly of the actin cytoskeleton with cytochalasin D or latrunculin B led to increased apoptosis in parental GEC on collagen (Fig. 3), while L63RhoA GEC proved to be more resistant to these drugs, displaying relatively lower levels of apoptosis at every concentration of cytochalasin D or latrunculin B (Fig. 3). In our earlier study, treatment of L63RhoA GEC with latrunculin B resulted in relatively less disassembly of the cortical actin structure, although the stress fibers disappeared (16). This result reinforces the importance of the cortical actin structure in maintaining cell survival. In L63RhoA GEC, apoptosis and the activity of caspase-3 (an instigator of apoptosis) were increased after inhibition of the ERK pathway, but not after inhibition of phosphatidylinositol 3-kinase-Akt and Rho kinase pathways (Fig. 3). This result is in keeping with previous observations in parental GEC and further supports the view that the ERK cascade is the major pathway mediating survival signaling emanating from collagen (7). It should be noted that we were unable to detect activation of endogenous RhoA in parental GEC on collagen or plastic (results not shown). Lack of detection may be related to a low expression level of endogenous RhoA (Fig. 3), or other technical reasons, but it is also possible that assembly of cortical F-actin and survival signaling in parental GEC may not involve RhoA. Thus the expression and function of L63RhoA may be unlike those of CD2-FAK, and R4F-MEK, both of which appear to be supplanting the endogenous GEC kinases activated by ECM.

It is becoming increasingly apparent that phosphoinositides, particularly PIP₂, may regulate the actin cytoskeleton by modulating the activity and targeting of actin regulatory proteins. PIP₂ promotes actin polymerization by interacting with proteins, including WASP, gelsolin, and cofillin. Moreover, PIP₂ interacts with many actin cross-linking proteins (e.g., α-actinin, filamin, ezrin/radixin/moesin, talin, vinculin), further facilitating actin organization (43). We previously determined that adhesion of GEC to collagen increases PIP₂ levels, prob-

Fig. 8. Schematic representation of the reciprocal relationship between ERK activation and the cortical F-actin structure involved in the intracellular survival signals emanating from ECM. In GEC adherent to collagen, there is activation of FAK (involving Src and Grb2), and ERK, as well as an increase in PIP₂. Collagen induces a cortical distribution of F-actin, in part, mediated via ERK and PIP₂. α-Actinin-4 K256E disrupts cortical F-actin. There is a reciprocal relationship between ERK activation and the cortical F-actin structure, and both are associated with a survival phenotype. Overexpression of CD2-FAK, R4F-MEK, or L63RhoA supplants the requirement for collagen and leads to ERK activation, cortical actin assembly, and the survival phenotype. In parental GEC on plastic, FAK and ERK are not activated, and there is loss of cortical actin.
ably through activation of phosphatidylinositol 4-phosphate 5-kinase (Fig. 8) (14). In the present study, we reduced/sequestered PIP2 by stable transfection with GFP-Pase or GFP-PH(PLCδ). GEC expressing either of these two proteins demonstrated increased levels of apoptosis (Fig. 4), although the levels of apoptosis were substantially lower, compared with parental GEC on plastic. In addition, the GFP-PH(PLCδ) and GFP-Pase cell lines had reduced levels of F-actin, compared with parental GEC, although the cortical F-actin pattern was maintained (Fig. 2). These results indicate that PIP2 facilitated survival and was involved in F-actin organization (Fig. 8). The results are in agreement with a recent study in 3T3-L1 adipocytes, where the induction of insulin resistance in these cells was paralleled by a marked loss of cortical F-actin and a decrease in PIP2 levels. Cortical F-actin and insulin responsiveness was restored after replenishing the plasma membrane with PIP2 (9).

Mutations in the gene encoding α-actinin-4, which increase the affinity of α-actinin-4 for F-actin and exaggerate its actin crosslinking potential, have recently been associated with an autosomal dominant familial form of focal segmental glomerulosclerosis (25). Mice with podocyte-specific expression of K256E mutant α-actinin-4 develop focal sclerosis-like features similar to the human lesion (31). The pathogenesis of sclerotic lesions may, at least in part, involve loss of GEC due to apoptosis (‘podocytopenia’) (32, 35). Thus we hypothesized that expression of mutated α-actinin-4 may enhance apoptosis. Interestingly, mutant and wild-type GFP-α-actinin-4 colocalized focally with F-actin, predominantly in a cortical distribution (Fig. 6), and in keeping with a F-actin crosslinking function. The majority of mutant and wild-type GFP-α-actinin-4 was, however, dissociated from F-actin and was found in the cytoplasm, while the wild-type was also found exclusively in the nucleus of some cells (Fig. 6). Our results in GEC are in keeping with the study that originally characterized α-actinin-4 (22), and additional studies will be required to determine the significance of α-actinin-4 subcellular localization, particularly in the cytoplasmic and nuclear compartments. In keeping with our hypothesis, overexpression of mutant or wild-type α-actinin-4 reduced GEC survival, although overexpression of the wild-type was reasonably well tolerated by GEC considering the much higher expression level, compared with the mutant (Fig. 5). Furthermore, GEC expressing wild-type GFP-α-actinin-4, and to a lesser extent the mutant displayed an increase in stress fibers (Fig. 6), compared with parental GEC (Fig. 2B), in keeping with an apoptotic phenotype.

The results show that actin depolymerization or inhibition of polymerization (cytochalasin D, latrunculin B, PIP2) as well as excessive crosslinking (K256E α-actinin-4) affect survival adversely. Appropriate remodeling of the actin cytoskeleton may be necessary for facilitating survival, perhaps via proper compartmentalization of intracellular signals. For example, RhoA inhibition increased the amount of activated ERK found in the nucleus of smooth muscle cells, suggesting that regulation of the actin cytoskeleton by RhoA may determine the subcellular localization of ERK (45). The importance of ERK localization was recently demonstrated in a study that employed constitutively active forms of ERK2, whose expression was restricted to the nucleus or to the cytoplasm. Cytoplasmic ERK2 protected against apoptosis caused by prolonged serum starvation, whereas ERK2 restricted to the nucleus protected against apoptosis induced by the Bcr-Abl inhibitor STI571. Furthermore, neither cytoplasmic nor nuclear ERK2 was able to inhibit apoptosis induced by UV light (3). Moreover, cytoskeletal remodeling may influence the temporal activation of ERK by regulating the subcellular localization of ERK scaffold proteins. It should be noted that in GEC, survival signals dependent on the cytoskeleton may also involve non-ERK effectors, as mutant α-actinin-4 and reduction of PIP2 enhanced apoptosis independently of ERK (Fig. 8).

The structure of podocyte foot processes is dependent on the actin cytoskeleton. GEC injury affecting the cytoskeleton or genetic alterations in the expression of certain cytoskeleton-modulating proteins are observed in different forms of focal segmental glomerulosclerosis, in association with proteinuria and foot process effacement (32, 35). Expanding our knowledge of factors that control GEC survival will improve our understanding of the pathophysiology of GEC injury in vivo and will eventually provide novel therapeutic approaches to preserving glomerular perme selectivity and function.

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