ADF/cofilin mediates actin cytoskeletal alterations in LLC-PK cells during ATP depletion

Sharon L. Ashworth,1 Erica L. Southgate,1 Ruben M. Sandoval,1 Peter J. Meberg,2 James R. Bamburg,3 and Bruce A. Molitoris1
1Division of Nephrology, Department of Medicine, Indiana University, and Roudebush Veterans Affairs Medical Center, Indianapolis, Indiana 46202-5116; 2Department of Biology, University of North Dakota, Grand Forks, North Dakota 58201; and 3Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523-1870

Submitted 5 June 2002; accepted in final form 22 November 2002

ADF/cofilin mediates actin cytoskeletal alterations in LLC-PK cells during ATP depletion. Am J Physiol Renal Physiol 284: F852–F862, 2003. First published December 3, 2002; 10.1152/ajprenal.00210.2002.—Ischemic injury induces actin cytoskeleton disruption and aggregation, but mechanisms affecting these changes remain unclear. To determine the role of actin-depolymerizing factor (ADF)/cofilin participation in ischemic-induced actin cytoskeletal breakdown, we utilized porcine kidney cultured cells, LLC-PK1 cells, and adenovirus containing wild-type (wt), constitutively active, and inactive Xenopus ADF/cofilin linked to green fluorescence protein [XAC(wt)-GFP] in an ATP depletion model. High adenoviral infectivity (70%) in LLC-PK1 cells resulted in linearly increasing XAC(wt)-GFP and phosphorylated (p)XAC(wt)-GFP (inactive) expression. ATP depletion rapidly induced dephosphorylation, and, therefore, activation, of endogenous po2ofilin as well as aXAC(wt)-GFP in conjunction with the formation of fluorescent XAC(wt)-GFP/actin aggregates and rods. No significant actin cytoskeletal alterations occurred with short-term ATP depletion of LLC-PK1 cells expressing GFP or the constitutively inactive mutant XAC(S3E)-GFP, but cells expressing the constitutively active mutant demonstrated nearly instantaneous actin disruption with aggregate and rod formation. Confocal image three-dimensional volume reconstructions of normal and ATP-depleted LLC-PK1 cells demonstrated that 25 min of ATP depletion induced a rapid increase in XAC(wt)-GFP apical and basal signal in addition to XAC(wt)-GFP/actin aggregate formation. These data demonstrate XAC(wt)-GFP participates in ischemia-induced actin cytoskeletal alterations and determines the rate and extent of these ATP depletion-induced cellular alterations.

Ischemia; microvilli; actin-depolymerizing factor; XAC-GFP

Additional supporting information is available online only.

ADF/cofilin linked to green fluorescence protein [XAC(wt)-GFP] in an ATP depletion model. High adenoviral infectivity (70%) in LLC-PK1 cells resulted in linearly increasing XAC(wt)-GFP and phosphorylated (p)XAC(wt)-GFP (inactive) expression. ATP depletion rapidly induced dephosphorylation, and, therefore, activation, of endogenous po2ofilin as well as aXAC(wt)-GFP in conjunction with the formation of fluorescent XAC(wt)-GFP/actin aggregates and rods. No significant actin cytoskeletal alterations occurred with short-term ATP depletion of LLC-PK1 cells expressing GFP or the constitutively inactive mutant XAC(S3E)-GFP, but cells expressing the constitutively active mutant demonstrated nearly instantaneous actin disruption with aggregate and rod formation. Confocal image three-dimensional volume reconstructions of normal and ATP-depleted LLC-PK1 cells demonstrated that 25 min of ATP depletion induced a rapid increase in XAC(wt)-GFP apical and basal signal in addition to XAC(wt)-GFP/actin aggregate formation. These data demonstrate XAC(wt)-GFP participates in ischemia-induced actin cytoskeletal alterations and determines the rate and extent of these ATP depletion-induced cellular alterations.

Ischemia; microvilli; actin-depolymerizing factor; XAC-GFP

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajprenal.org

November 6, 2017
microvilli in response to ischemic injury of proximal tubule cells, direct proof for this role is lacking.

Therefore, the present studies were undertaken to directly evaluate the role ADF plays in F-actin destruction and reorganization during ischemic cell injury. To accomplish this goal, we utilized the proximal tubule cell line LLC-PK because several studies have demonstrated F-actin reorganization observed in rat proximal tubule cells in response to ischemic insult can be mimicked in LLC-PK cells by inducing ATP depletion through treatment with antimycin A in substrate-depleted medium (4, 10, 15, 24). Recently, adenoviral constructs containing CDAs of the wild-type (wt) ADF/cofilin isoform, Xenopus ADF/cofilin, XAC(wt)-green fluorescent protein (GFP), the constitutively active mutant, XAC(S3A)-GFP, and the inactive mutant, XAC(S3E)-GFP, have become available (1, 18) and allowed for expression of these proteins in LLC-PK cells. These unique tools have been successfully used for expression of the wild-type ADF/cofilin isoform to directly demonstrate XAC(wt)-GFP-mediated alterations in actin dynamics in cells (6, 18). With the use of these probes, we manipulated expression of wild-type and mutant XAC-GFP isoforms and studied the effects of their expression on the actin cytoskeleton in proximal tubule cultured cells under physiological and ATP-depleted conditions. Our data indicate a direct role for ADF/cofilin proteins in mediating the severe actin cytoskeletal alterations observed in response to cellular ATP depletion in addition to dramatically impacting the rate and extent of these cellular alterations.

**METHODS**

**Cell culture.** Cell culture experiments were performed on three proximal tubule cell lines, two of which were porcine cell lines clonally derived from LLC-PK(wt) (LLC-PK10 and LLC-PKAA8), and the S1 mouse cell line (a kind gift from Dr. G. T. Nagami, Univ. of California at Los Angeles School of Medicine, Los Angeles, CA). The LLC-PKAA8 cell line was maintained in a low-glucose (1 mg/ml glucose) DMEM (Sigma D-5523) containing 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin, pH 7.4, at 37°C in 5% CO2 incubators. The LLC-PK10 cell line was maintained and expanded on plastic tissue culture dishes in DMEM (JRH Biosciences, no. H9262) containing 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin, pH 7.4, or in substrate-free DMEM (no glucose, pyruvate, serum, or amino acids), pH 7.4, or in depletion buffer, 1× PBS containing 0.5 mM CaCl2 and 1.0 mM MgCl2, pH 7.4, for designated time intervals.

**Adenoviral construction.** XAC-GFP (wt, S3A mutant and S3E mutant) clones were constructed in the Clontech phGFP-S65T vector by H. Abe, Chiba University, and generously shared with us. The 1,300-bp XAC-phGFP (wt, S3A and S3E) inserts were removed from the phGFP plasmid with SacI and XbaI (SacI site was blunt ended by degrading the 3’

**Fig. 1.** ATP depletion activates cofilin in LLC-PKAA8 cells. Cellular homogenate proteins (equal amounts) from S1, LLC-PKAA8, and LLC-PK10 cells were probed using isoform-specific antibodies for actin-depolymerizing factor (ADF), cofilin, or the phosphoepitope of phosphorylated (p)ADF and pcofilin, which also recognize pXAC(wt)-green fluorescent protein (GFP). Under physiological conditions, the cofilin isoform was predominately expressed and phosphorylated in LLC-PKAA8 cells, whereas ADF was very low or nonexistent (A). In LLC-PK10 cells, the expression pattern was reversed, with ADF primarily expressed and phosphorylated, and neither pcofilin nor cofilin was detected. Both isoforms, ADF and cofilin, were expressed and detected in mouse proximal tubule S1 cells. Western blots of homogenates of LLC-PKAA8 cells ATP depleted for 0, 5, 15, and 30 min in depletion medium containing 0.1 µM antimycin A were probed with an antibody specific for the phosphoepitope of ADF/ cofilin (B). Equal amounts of total protein (5 µg) were loaded in each lane with 3 replicates for each time point. The concentration of the inactive pcofilin isoform decreased with increasing time of ATP depletion.
overhang with mung bean nuclease). The XAC-phGFP inserts (wt, S3A or S3E) were cloned into the XbaI and blunt ended KpnI site of the shuttle vector plasmid for adenovirus production by homologous recombination in HEK-293 cells as previously described (18). The fusion proteins were expressed under control of the immediate early promoter of the cytomegalovirus.

Adenoviral infection. The cells were infected at 40–60% confluency with a viral multiplicity of infection of 25 for 18 h with adenovirus expressing GFP, XAC(wt)-GFP, the constitutively active mutant XAC(S3A)-GFP, or the inactive mutant form XAC(S3E)-GFP. Cell cultures were harvested at 18, 28, and 51 h postinfection with cell extracts prepared and examined by SDS-PAGE, followed by Western blot analysis. By 24 h postinfection, 70–80% of the treated cells were expressing XAC-GFP isoforms as observed by epifluorescence microscopy. All studies were done at 24 h postinfection unless otherwise stated.

SDS-PAGE and Western analysis. LLC-PK or S1 cellular proteins were extracted in a 2% SDS buffer (2% SDS, 10 mM Tris, pH 7.6, 10 mM NaF, 5 mM DTT, 2 mM EGTA) and boiled. Protein concentration was determined by a filter paper dye-binding assay (19). Equal protein concentrations (5 μg of total extract protein) were loaded in each lane and separated by SDS-PAGE on 15% isocratic gels. For Western blot analysis, separated proteins were transferred to a polyvinylidene fluoride membrane, and the membrane was blocked with 5% nonfat dry milk or 10% newborn calf serum in 1× Tris-buffered saline with Tween. For immunodetection, the rabbit primary antibodies to XAC (1:10,000), to the phosphopeptide epitope of phosphorylated ADF/cofilin [pADF/pcofilin (also recognizes pXAC)] (1:1,000), and to ADF (1:10,000) or mouse primary monoclonal antibody to cofilin (also recognizes pXAC)

Microscopy. LLC-PK(A)s cells were fixed in 4% paraformaldehyde or 3.7% formaldehyde for 1 h and permeabilized with 0.1% Triton X-100. F-actin was stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR; 1:60 dilution) or Texas red-phalloidin (1:200; 1:10). Confocal images were acquired with an MRC-1024 laser-scanning confocal microscope (Bio-Rad, Hercules, CA) using a Nikon Diaphot 200 inverted microscope with a ×100, 1.4-numerical aperture (NA) oil-immersion objective or a ×60, 1.2-NA water-immersion objective. Live cell images were captured with a Nikon Diaphot inverted microscope with a ×40, 0.85-NA objective lens.
and a PXL cooled charge-coupled device camera with a Kodak 1400 chip (Photometrics, Tucson, AZ). Metamorph software (Universal Imaging, West Chester, PA) was used to process the images and to reconstruct basal-to-apical three-dimensional reconstructions.

RESULTS

**ATP depletion of LLC-PK<sub>14.8</sub> cells reduces pcofilin signal.** Initial studies were undertaken to determine the effect of ATP depletion on the phosphorylation status of cofilin in LLC-PK<sub>14.8</sub> cells. ADF and cofilin are two highly conserved and related proteins, but differentially expressed proteins with similar, but distinct, actin-binding properties belonging to the same family of actin-associated proteins (5, 32). With the use of isoform-specific antibodies and an anti-phospho-epitope antibody that recognizes the phosphorylated form of each isoform, the cellular expression of these proteins can be determined. With the use of these probes, we found that the endogenous expression of ADF and cofilin isoforms in porcine proximal tubule cell lines was not equivalent. The LLC-PK<sub>14.8</sub> cells had ample expression of cofilin and little or no expression of ADF (Fig. 1A). The LLC-PK<sub>10</sub> cell line expressed ADF with no expression of cofilin, whereas the S<sub>1</sub> mouse cell line expressed both isoforms.

As shown in Fig. 1B, antimycin A-induced ATP depletion of LLC-PK<sub>14.8</sub> cells diminished the pcofilin signal in a time-dependent manner consistent with previously published in vivo rat kidney data that demonstrated ischemia induced a time-dependent dephosphorylation of phosphorylated ADF (29). Induction of ATP depletion for 5 min had no effect on cofilin phosphorylation, but within 15 min, a 60% decrease was observed in pcofilin concentration. By 30 min of ATP depletion, pcofilin had been reduced to 10%. As there was no change in total cellular cofilin (data not shown), these data imply that ATP depletion induced a rapid duration-dependent dephosphorylation of cofilin.

**Expression of XAC-GFP through adenoviral infection of LLC-PK<sub>14.8</sub> cells.** To obtain direct evidence regarding the role of cofilin-mediated cellular actin destruction and reorganization and microvillar F-actin core...
degeneration during ATP depletion, we utilized adenoviral vectors containing GFP, XAC(wt)-GFP, XAC(S3A)-GFP, or XAC(S3E)-GFP cDNA to express GFP or ADF/cofilin protein isoforms in LLC-PK A4.8 cells. In characterization studies, LLC-PKA4.8 cells infected with adenovirus containing the cDNA for XAC(wt)-GFP demonstrated expression of the XAC(wt)-GFP fusion protein as early as 18 h postinfection, as detected by GFP fluorescence (Fig. 2A) and Western blotting techniques (Fig. 2B). The fraction of GFP-expressing cells increased from -70 to 90% over the 51-h period postinfection (Fig. 2A). The level of XAC(wt)-GFP expression increased linearly over the 51-h period postinfection (Fig. 2B). The level of phosphorylated XAC(wt)-GFP, as detected by Western blot analysis, also increased linearly over the 51-h period postinfection (Fig. 2B). Therefore, as the wild-type XAC-GFP protein was expressed, it was regulated through phosphorylation by a cellular kinase. The level of endogenous cofilin, as detected by Western blot analysis, remained constant during the first 18 h postinfection of XAC(wt)-GFP, but decreased endogenous cofilin levels were observed at 28 and 51 h postinfection.

ATP depletion resulted in rapid dephosphorylation of pXAC(wt)-GFP (Fig. 2C), as was seen for endogenous cofilin (Fig. 1B). Compared with control levels, there was a 60% decrease in pXAC(wt)-GFP in response to 5 min of ATP depletion, which was further reduced to 10% pXAC(wt)-GFP at 30 min of ATP depletion, showing dephosphorylation by an endogenous phosphatase. Together, these data indicate that ample expression, physiological phosphorylation, and dephosphorylation, in response to ATP depletion of XAC(wt)-GFP, occurred at the cellular level. Endogenous cofilin was also downregulated in response to XAC(wt)-GFP expression.

XAC-GFP expression did not alter F-actin under physiological conditions. To determine the effect of XAC(wt)-GFP expression on the F-actin cytoskeleton of LLC-PKA4.8 cells, cells were stained with rhodamine or Texas red-phalloidin 24 h postinfection with adenovirus containing XAC(wt)-GFP. In Fig. 3, A–F, reconstructed basal-to-apical images and single-plane basal images of uninfected control cells (A and B) and XAC(wt)-GFP-expressing cells (C–F) are presented. In Fig. 3, C–F, comparison of actin cytoskeletal stress fibers, microvillar microfilaments, and cortical actin network can be drawn between high (a), medium (b), and low (c) XAC(wt)-GFP-expressing cells and nonexpressing cells (d). These data demonstrate that adenoviral infection and XAC(wt)-GFP expression did not affect the distribution or composition of the dense F-actin bundles that compose basal stress fibers, apical microvillar microfilament cores, or the cortical actin orientation of LLC-PKA4.8 cells, suggesting XAC(wt)-GFP expression does not alter cellular actin architecture under physiological conditions, implying physiological regulation and function of the XAC(wt)-GFP proteins.

XAC(wt)-GFP translocates to the surface membrane domain in response to ATP depletion. Basal-to-apical reconstructions (x-z axes images) demonstrate that, under physiological conditions (Fig. 4A), F-actin primarily located to basal and lateral aspects of the cell and in the microvilli at the apical surface. The expression of XAC(wt)-GFP under physiological conditions (Fig. 4B) was primarily detected in the cytoplasm of the LLC-PKA4.8 cells with little or no fluorescence in the apical aspects of the cell (B). XAC(wt)-GFP-expressing cells that were ATP depleted for 25 min demonstrated a significant increase in the cytoplasmic XAC(wt)-GFP fluorescence colocalizing (yellow) with F-actin in the surface membrane regions and in aggregates in the cytosol (C).
XAC mediates F-actin aggregation and rod formation during ATP depletion. We next sought to determine the effect of XAC expression and ATP depletion on the F-actin cytoskeleton. Cell monolayers infected with the XAC(wt)-GFP adenovirus were ATP depleted with antimycin A in depletion media for 25 min and stained for F-actin using Texas red-phalloidin (1:10). In Fig. 5, A–C, both XAC(wt)-GFP-expressing and uninfected cells (arrows) were present in the same monolayer. Uninfected cells, ATP depleted for 25 min (Fig. 5, A–C, arrows), were characterized by minimal disturbance in the fine-mesh cortical and stress fiber F-actin staining (Fig. 5A, arrow). These data are similar to what we previously described under physiological or short-term, ATP-depleted conditions (24). However, in XAC(wt)-GFP-expressing neighboring cells undergoing ATP depletion, intracellular F-actin disruption and aggregation were readily seen, with higher XAC(wt)-GFP-expressing cells being disrupted to a greater extent than cells with lower expression levels. Colocalization of XAC(wt)-GFP and F-actin, as demonstrated by intense yellow fluorescence (Fig. 5C, open square), was apparent in XAC(wt)-GFP-expressing cells. The F-actin- and XAC-GFP-stained aggregates had a much brighter GFP signal than the Texas red-phalloidin F-actin signal. We believe this difference in staining properties results from the known competition between XAC-GFP and phalloidin for F-actin binding (5, 17).

To evaluate whether rod and aggregate formation, in response to ATP depletion in XAC(wt)-GFP-expressing cells, was a direct result of XAC(wt)-GFP-mediated actin alterations, we expressed either the constitutively active mutant XAC(S3A)-GFP, the inactive mutant XAC(S3E)-GFP, or GFP in LLC-PK1 cells. No rods or aggregates formed in the GFP (Fig. 6, A–C) or S3E mutant-infected cells (Fig. 6, D–F), even when ATP depleted for 30 min. These data support the hypothesis that the active ADF/cofilin isozyme directly mediates breakdown of the actin cytoskeleton, leading to formation of ADF/cofilin aggregates and rods, whereas the inactive isozyme cannot induce these events. These data further support a role for the dephosphorylated and activated ADF/cofilin proteins mediating the cellular actin changes observed with ATP depletion during renal ischemia. Expression of the constitutively active isozyme led to spontaneous disruption of the actin cytoskeleton, with formation of rods and aggregates often resulting in detachment or cell death by 24 h postinfection (Fig. 6, G–I). In addition, we observed a reduction in cellular stress fibers in cells expressing the constitutively active mutant. ATP depletion induces rapid formation of aggregates and rods in cells expressing XAC(wt)-GFP. Next, we sought to determine the time course of F-actin alterations in control and XAC(wt)-GFP-expressing cells in response to ATP depletion. Rapid and extensive appearance of XAC-GFP/F-actin aggregates and rods would directly indicate an important and early role for ADF/cofilin proteins in mediating F-actin disruption. To test this hypothesis, we undertook ATP depletion studies of cells infected with either XAC(wt)-GFP, GFP, or XAC(S3E)-GFP. In GFP- and XAC(S3E)-GFP-expressing cells, as well as in uninfected cells, we did not observe alterations to the actin cytoskeleton comparable with the severe alterations observed in XAC(wt)-GFP-expressing cells in response to the same time of ATP depletion (Figs. 5 and 6). XAC(wt)-GFP-, GFP-, and XAC(S3E)-GFP-expressing cells all demonstrated a high percentage of GFP signal, indicating a
similar level of infection and GFP protein expression. In addition, the wild-type XAC(wt)-GFP-expressing cells appeared similar in morphology to uninfected cells or cells infected with GFP or the inactive S3E mutant. During ATP depletion, the GFP intensity and distribution at 2 min were comparable in the XAC(wt)-GFP-, GFP-, XAC(S3E)-GFP-expressing cells (Fig. 7, A, D, and G). A homogenous cytosolic distribution of GFP was observed, and nuclear localization was also noted. By 10 min of ATP depletion, localization of the GFP signal began to change in the XAC(wt)-GFP-expressing cells but not in the GFP- or XAC(S3E)-GFP-expressing cells (data not shown). By 20 min, cells expressing XAC(wt)-GFP had a reduction in the homogenous cytosolic XAC(wt)-GFP signal and an accumulation of cytoplasmic XAC(wt)-GFP-stained aggregates (Fig. 7B,
arrows). By 40 min, this effect was further enhanced in the XAC(wt)-GFP-infected cells (Fig. 7C), but the GFP- and XAC(S3E)-GFP-expressing cells still demonstrated no change in the diffuse GFP fluorescence (Fig. 7, F and I).

DISCUSSION

This is the first study to directly demonstrate that the ADF/cofilin family of proteins mediates dramatic alterations to actin filament cytoarchitecture in response to ATP depletion. The ADF/cofilin family of proteins orchestrates actin dynamics primarily through accelerating the rate of pointed-end F-actin depolymerization and by severing long F-actin filaments (5). To mediate cellular changes in actin dynamics, these stimulus/responsive proteins preferentially bind ADP-charged F-actin in a pH-dependent manner (7, 8, 11, 16). The ADF/cofilin proteins substantially
increase the polymerization rate of actin, with ADP-actin polymerization affected to a greater extent than ATP-actin polymerization (11). The actin-binding properties of this family of proteins are primarily regulated by phosphorylation and dephosphorylation. Also, ADF/cofilin proteins compete for F-actin binding with other actin-binding proteins and phalloidin. Two kinase families have been identified to specifically phosphorylate ADF/cofilin on serine-3, each with different upstream regulators. The Lim kinase family, the first identified ADF/cofilin-specific kinase, is phosphorylated, and its kinase activity is significantly increased through downstream effects of the Rho family of small GTPases, Rac, Rho, and Cdc42. In turn, the activated Lim kinase phosphorylates and inactivates the ADF/cofilin protein family (2, 34). The second family of ADF/cofilin-specific kinases, the testicular protein kinase family (TESK1 or TESK2), includes serine/threonine kinases stimulated through the integrin-mediated signaling pathway (31). Phosphorylated ADF/cofilin proteins can no longer bind F- or G-actin to regulate actin dynamics (9, 25). Recently, the ADF/cofilin-specific phosphatase slingshot has been shown to dephosphorylate and activate ADF/cofilin at serine-3 (26).

Our previous studies suggested the ADF/cofilin family of proteins played a significant role in ischemia-induced renal cell injury of proximal tubule cells (3, 29). Acute renal failure mediates functional changes in the biochemical, physiological, and morphological aspects of proximal tubule cells (30). The extent of these cellular alterations depends on the time and severity of the cellular injury, with apical membrane microvilli being extremely sensitive because they contain the majority of F-actin in these cells (14, 15, 24). Clinical consequences resulting from ischemic injury include tubular obstruction from apical membrane blebbing, back-leak between cells that have lost their junctional complex integrity, reduced Na\(^{+}/H\(^{+}\) reabsorption from redistribution of ion pumps in the membrane, and abnormal tubuloglomerular feedback (30).

Changes in the actin cytoarchitecture occur early and precede the other observed biochemical, functional, and structural alterations, suggesting actin changes are, in part, responsible for the subsequent destructive cellular changes. Within 5 min of renal artery clamping, we observed dephosphorylation/activation of ADF, along with localization of this small protein into the apical microvillar region of the proximal tubule cell, where F-actin staining patterns show initial alterations (3). By 15 min of ischemia-induced injury, the apical membrane begins to coalesce and form luminal or cytoplasmic blebs or vesicles containing high concentrations of ADF and G-actin. In addition, microvillar microfilament destruction is concurrent with increased G-actin concentration in the apical membrane region. These events occur in a time frame
to suggest that ADF locates to this region to participate through F-actin severing and depolymerization in the breakdown of the microvillar microfilament core. In addition to microvillar microfilament changes, aggregates of F-actin have been observed in the cytoplasm of injured proximal tubule cells (12, 15, 24).

Although our previous studies suggested dephosphorylation/activation and relocalization of ADF were coincident with microvillar microfilament core disintegration in response to ischemic injury, we could not directly test the involvement of ADF in this process. Therefore, to directly evaluate the role of the ADF/cofilin family of proteins in proximal tubule cell actin alterations, we expressed the ADF/cofilin isoform XAC(wt)-GFP by adenoviral infection in the proximal tubule cultured cell line LLC-PK1A4.8. In these cells, endogenous cofilin expression is <0.1% of the total protein concentration (data not shown). With expression of XAC(wt)-GFP, we observed a decrease in endogenous cofilin levels, suggesting that endogenous cofilin played a minimal role in actin alterations in response to ATP depletion in XAC(wt)-GFP LLC-PK1A4.8-expressing cells. Although expression of GFP, XAC(S3E)-GFP, or XAC(wt)-GFP in these cells did not alter the integrity of their actin cytoskeleton, inducing ATP depletion in the XAC(wt)-GFP-expressing cells resulted in extremely rapid and extensive changes in the actin cytoarchitecture (Figs. 3, 5, and 6) comparable to the phenotype observed in uninfected cells that underwent a much longer ischemic insult (12). XAC(wt)-GFP-containing aggregates and rods appeared within 10 min of ATP depletion and increased in number and size with depletion time. Actin aggregates were not observed in uninfected cells until after >30 min of ATP depletion. These aggregates were primarily located in the cytoplasm, although rods were also observed in the nucleus. As the number of XAC(wt)-GFP/actin aggregates increased, stress fibers and the fine meshwork of the cortical F-actin disappeared, suggesting XAC(wt)-GFP bound F-actin to depolymerize, sever, and redistribute the characteristic F-actin meshwork into dense aggregates of F-actin bound by XAC(wt)-GFP. Because XAC(wt)-GFP competes with phalloidin for F-actin binding, increased concentrations of Texas red-phalloidin were utilized to insure phalloidin binding and, therefore, visualization of F-actin. Also, with ATP depletion, the XAC(wt)-GFP relocalized into basal and apical regions of the cells. Therefore, with ATP depletion, XAC(wt)-GFP signal significantly increased and rapidly moved from a diffuse cytoplasmic distribution into aggregates along with F-actin. To achieve this remodeling, XAC(wt)-GFP must be activated from its predepletion state and relocalized to bind F-actin with subsequent F-actin depolymerization and severing activity, followed by localization of XAC(wt)-GFP along with F-actin to new abnormal actin aggregate and rod structures (Fig. 8). These data extend our kidney in vivo studies by providing direct evidence that XAC(wt)-GFP relocalizes and participates in F-actin destruction and remodeling. Finally, in cells infected with the constitutively active form of XAC(S3A)-GFP, spontaneously occurring aggregates and rods were seen postinfection, and 24 h later, the entire actin cytoskeleton was disrupted. This resulted in cell detachment and death (Fig. 6). These data, and the lack of F-actin disruption in response to ATP depletion in GFP- and XAC(S3E)-GFP-expressing cells (Fig. 6), further demonstrate that activation of ADF/cofilin is required to bring about these cytoskeletal alterations.

The mechanism for formation of ADF/cofilin rods and aggregates is unknown, although recent studies by Pfannstiel and coworkers (27) suggest cofilin oligomers may induce actin bundling activity, leading to aggregate formation. At present, there are no data to support this in LLC-PK1A4.8 cells that have been ATP depleted. Although it is possible that XAC(wt)-GFP proteins may form oligomers in response to long-term ATP depletion in oxidizing conditions, short-term ATP depletion results in a drop in intracellular pH that is not consistent with reported conditions for cofilin oligomer formation (27).

In summary, these studies strongly suggest ATP depletion induced ADF dephosphorylation/activation and relocalization to mediate F-actin alterations. By expressing the ADF/cofilin protein, and through its GFP fluorescent tag, we were able to follow its activity in response to ATP depletion. With the use of this powerful tool, we demonstrated that ATP depletion rapidly stimulated movement of the XAC(wt)-GFP signal from a diffuse cytoplasmic distribution to localize at sites of F-actin and to newly formed actin aggregates and rod structures. These data strongly suggest XAC(wt)-GFP bound, depolymerized, and severed F-actin to remodel actin into XAC(wt)-GFP-containing aggregates and rods. These data further substantiate a mechanistic role for ADF/cofilin proteins in mediating the rapid actin cytoskeletal remodeling that leads to the functional changes observed in the biochemical, physiological, and morphological aspects of the proximal tubule cells in response to ischemia-induced injury.

We thank Laurie Minamide and Melanie Hosford for technical expertise and helpful discussions.

This research was supported by National Institutes of Health (NIH) Grants 1P01-DK-53465, 1R01-DK-41126, and Veterans Affairs Merit Review grants (to B. A. Molitoris), American Paralysis Association Grant BB2-9601 (to P. J. Meberg), and NIH Grants GM-35126 and NS-40371 (to J. R. Bamburg).

REFERENCES


