Isolation and Characterization of Cytoplasmic Cofilin-Actin Rods

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Cofilin-actin bundles (rods), which form in axons and dendrites of stressed neurons, lead to synaptic dysfunction and may mediate cognitive deficits in dementias. Rods form abundantly in the cytoplasm of non-neuronal cells in response to many treatments that induce rods in neurons. Rods in cell lysates are not stable in detergents or with added calcium. Rods induced by ATP-depletion and released from cells by mechanical lysis were first isolated from two cell lines expressing chimeric actin-depolymerizing factor (ADF)/cofilin fluorescent proteins by differential and equilibrium sedimentation on OptiPrep gradients and then from neuronal and non-neuronal cells expressing only endogenous proteins. Rods contain ADF/cofilin and actin in a 1:1 ratio. Isolated rods are stable in dithiothreitol, EGTA, Ca2+, and ATP. Cofilin-GFP-containing rods are stable in 500 mM NaCl, whereas rods formed from endogenous proteins are significantly less stable in high salt. Proteomic analysis of rods formed from endogenous proteins identified other potential components whose presence in rods was examined by immunofluorescence staining of cells. Only actin and ADF/cofilin are in rods during all phases of their formation; furthermore, the rapid assembly of rods in vitro from these purified proteins at physiological concentration shows that they are the only proteins necessary for rod formation. Cytoplasmic rod formation is inhibited by cytochalasin D and jasplakinolide. Time lapse imaging of rod formation shows abundant small needle-shaped rods that coalesce over time. Rod filament lengths measured by ultrastructural tomography ranged from 22 to 1480 nm. These results suggest rods form by assembly of cofilin-actin subunits, followed by self-association of ADF/cofilin-saturated F-actin.

Microischemia (mimicked by transient ATP depletion), oxidative stress (mimicked by peroxide or NO), excessive glutamate or AMPA4 stimulation, and small soluble forms of amyloid β peptide (Aβ-(1–42)) cause within neurites of cultured hippocampal or cortical neurons the formation of rodlike inclusions (rods) composed of actin and the actin assembly-regulatory proteins, actin-depolymerizing factor (ADF) and/or cofilin (1–3). These neuronal treatments induce the dephosphorylation (activation) of ADF/cofilin in neurons in which rods form. Neuronal rods failed to stain with fluorescent derivatives of phalloidin, a mushroom toxin widely used to identify filamentous actin (F-actin) structures (4). However, ADF/cofilins bind to a slightly twisted form of F-actin in which the phalloidin binding site is eliminated (5, 6), suggesting that rods might be composed of ADF/cofilin-saturated F-actin.

ADF/cofilin-containing rods and aggregates were also observed in hippocampal neurons treated with Pak (p21-activated kinase) inhibitor, PAK18 (7). Down-regulation of Pak activity occurs in human Alzheimer disease (AD) brain and correlates with cognitive deficits in AD mice (7). Rod-shaped cofilin-immunostained structures were found in human AD brains but not in human control brain (1) and can be induced in organotypic hippocampal cultures (8). Significantly, brains from perfusion-fixed transgenic mice expressing human amyloid precursor protein with familial AD mutations also contain rods (2, 7), demonstrating that rods are not post-mortem artifacts. Mature rods can completely occlude the neurite, blocking transport and causing distal atrophy and synaptic dysfunction (1, 2, 9).

Actin-containing rodlike structures are not restricted to neurons. They were first identified in the nucleus of cultured Dictyostelium and HeLa cells treated with high concentrations of DMSO (10); DMSO-induced rods were later shown to contain cofilin (11). Actin-containing rods also have been identified in the nucleus of muscle cells of patients with nemaline myopathy (12). Expression in cultured cells of human skeletal muscle actin containing a nemaline myopathy mutation results in formation of nuclear rods, only some of which stain for cofilin (13). Therefore, there are multiple ways to form rod-shaped actin inclusions with different compositions. Thus, knowing the components of rods is an important step in understanding the mechanism of their formation and in finding ways to inhibit or reverse their formation in neurons before permanent damage ensues.

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4 The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; ADF, actin-depolymerizing factor; AD, Alzheimer disease; GFP, green fluorescent protein; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; MOPS, 4-morpholinopropanesulfonic acid; MS, mass spectrometry; MS/MS, tandem MS; DTT, dithiothreitol; XAC, Xenopus ADF/cofilin 1.
Because actin rod formation in response to a decline in cellular ATP is a general phenomenon that affects a high percentage of many different types of cells (14), including neurons (1), we utilized ATP depletion to generate large numbers of rods in non-neuronal cells expressing fluorescent ADF/cofilins. Live cell time lapse imaging showed that rods formed rapidly as short needle-like aggregates, which could be blocked from forming with cytochalasin D or jasplakinolide. Electron microscopic tomographic reconstruction of a mature rod demonstrated that it was composed of variable length filaments. We developed an isolation protocol for rods formed from fluorescent proteins and applied it to lysates of cells, including cortical neurons, in which rods were induced without exogenous protein expression. We developed a quantitative sedimentation method to assess stability of rods in response to different agents and applied this to rods made from cofilin-GFP or endogenous proteins. Rod-associated proteins were identified through mass spectrometric analysis of their peptides and quantified by SDS-PAGE and Western blot analysis. Immunofluorescence methods were used to determine if these proteins were components of rods formed in neurons. Whereas some of these proteins were found associated with mature rods, none other than ADF/cofilin and actin appeared to be necessary to initiate rod formation, a finding confirmed by the in vitro assembly of rods from these purified proteins at physiological concentrations.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Porcine proximal tubule LLCPK A4.8 cells (a kind gift of Dr. Bruce Molitoris) were maintained in low glucose Dulbecco’s modified Eagle’s medium (Invitrogen), 10% fetal bovine serum supplemented with 12.5 mM Hepes. Human epidermoid carcinoma A431 cells (ATCC, Manassas, VA) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. HeLa cells transfected with human cofilin-GFP (15) were maintained in high glucose Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum.

**Rod Isolation**—Four 10-cm dishes of A4.8 cells at 10–15% confluence were each infected 3–4 days prior to harvest with adenovirus expressing *Xenopus* ADF/cofilin (XAC1) (wild type)-GFP (approximate multiplicity of infection of 200) (16, 17). The cells were washed with 3 × 10 ml of calcium- and magnesium-free phosphate-buffered saline (PBS) and then ATP-depleted in 10 mM sodium azide, 6 mM 2-deoxyglucose in PBS for 1 h at 37 °C. Preparations of rods from neurons, A431 cells, and HeLa cells were performed in the presence of nocodazole (supplemental Fig. S1) as described under “Results.” The cells from each dish were scraped into 200 μl of 20 mM Pipes, pH 6.8, 140 mM NaCl, 1 mM EGTA (Pipes buffer) at 4 °C and transferred to separate microcentrifuge tubes. Cell lysis and all subsequent steps as described under “Results” were performed at 4 °C. Rods were separated by two gradient centrifugation steps on OptiPrep (Axis-Shield PoC AS, Oslo, Norway) in polyallomer tubes (11 × 34 mm; Beckman, Palo Alto, CA) in a swinging bucket rotor (TLS55) in a TL-100 ultracentrifuge.

**Electron Microscopy**—Samples (10 μl) of rods from the OptiPrep gradients were fixed on ice by the addition of an equal volume of 2% glutaraldehyde in MOPS buffer for 10 min (18). The fixed rods (5 μl) were applied to a Formvar-coated grid (kept on Parafilm-covered glass on ice) for 10 min, and the excess liquid was wicked off, followed by the addition of a drop of aqueous 1% phosphotungstic acid for 5 min. The phosphotungstic acid was wicked off, and after a quick rinse with a drop of water, the grid was wicked dry and stored at room temperature. Negatively stained samples were viewed on a JEOL 2000 transmission electron microscope operating at 80 kV.

**Tomography**—A4.8 cells infected with adenovirus for expressing XAC-GFP were grown at 37 °C on 0.7% Formvar-coated 75-mesh gold grids in 95% air, 5% CO2. The cells were ATP-depleted for 30 min in PBS containing 10 mM sodium azide and 6 mM 2-deoxyglucose, fixed in 2% glutaraldehyde for 45 min, rinsed briefly in PBS, placed in 8% sucrose in PBS, and plunge-frozen and freeze-substituted as previously described (19). Briefly, cells on grids were fixed with glutaraldehyde, washed free of most liquid and plunge-frozen in liquid propane (−174 °C). Cells on grids were quickly transferred to liquid nitrogen and freeze-substituted into 1% glutaraldehyde in acetone and maintained at −90 °C for 2 days, after which the solution was replaced with a −90 °C solution of 1% osmium tetroxide and 0.1% uranyl acetate. After warming to 4 °C over 24 h, grids with cells were infiltrated with increasing concentrations of Epon-Araldite resin, which was polymerized at 60 °C for 2 days. Areas of well preserved cells identified by phase microscopy were excised and remounted on resin stubs with epoxy adhesive MS-907 (Miller-Stephenson, Morton Grove, IL), and 200-nm sections were collected on 0.7% Formvar-coated copper slot grids, poststained with 1% aqueous uranyl acetate and Reynolds’ lead citrate and imaged in a Tecnai F30 electron microscope operating at 300 kV. Dual axis tomography (20) was used to generate a three-dimensional image from a section of a rod, and computer visualization of the three-dimensional image data was accomplished using the software program IMOD (21).

**Proteomic Analysis of Rod Components**—Rod proteins from A431 cells were dissolved in 8 M urea, 100 mM Tris-Cl, pH 8.5, reduced by adding Tris(2-carboxyethyl)phosphine to 5 mM, incubated at 20 °C for 30 min, and then carboxamidomethylated by adding iodoacetamide to 10 mM and incubating at 20 °C for 30 min in the dark. Samples were diluted to 2 M urea with 100 mM Tris-Cl, pH 8.5, brought to 1 mM CaCl2, and further digested with trypsin by using an enzyme/substrate ratio of 1:20 overnight at 37 °C. The digestion reaction was quenched by the addition of formic acid to 5%. Rod proteins from neurons were treated similarly, except reduction was with dithiothreitol.
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(DTT), and trypsin digestion was in ammonium bicarbonate buffer.

Liquid Chromatography-MS/MS—Peptides from the A431 rods were analyzed as follows. A 100-μm inner diameter capillary with a 5-μm pulled tip was packed with 10 cm of 5-μm Aqua C18 material (Phenomenex, Ventura, CA). The desalting column was then equilibrated for 30 min with Buffer A (5% acetonitrile, 0.1% formic acid), and the protein digest was pressure-loaded onto it. The column was placed inline with an Agilent 1100 quaternary high pressure liquid chromatograph (Palo Alto, CA) and analyzed by using a one-step separation, which consisted of a 120-min gradient from 0 to 100% Buffer B (80% acetonitrile, 0.1% formic acid). As peptides eluted from the microcapillary column, they were electrospayed directly onto an LTQ mass spectrometer (ThermoFinnigan, Palo Alto, CA) with the application of a distal 2.4-kV spray voltage. A cycle of one full-scan mass spectrum (400–1,400 m/z) was followed by three data-dependent MS/MS spectra at a 35% normalized collision energy. Applications of MS scan functions and high pressure liquid chromatography solvent gradients were controlled by the XCalibur data system.

Peptides from the neuronal rods were analyzed similarly by the Proteomics and Metabolomics Facility at Colorado State University. They were purified and concentrated using an online enrichment column (Agilent Zorbax C18, 5 μm × 5 μm × 0.3 mm). Subsequent chromatographic separation was performed on a reverse phase nanospray column (Agilent 1100 nanoHPLC, Zorbax C18, 5 μm × 75 μm inner diameter × 150-mm column) using a 42-min linear gradient from 25 to 55% Buffer C (90% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min. Peptides were eluted directly into the Thermo Scientific LTQ linear ion trap mass spectrometer, and spectra were collected over an m/z range of 200–2000 Da using a dynamic exclusion limit of two MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Bioworks version 3.0 software (Thermo Scientific) with an intensity threshold of 5,000 and 1 scan/group.

Tandem MS spectra of A431 peptides were analyzed by using the following software analysis protocol. Poor quality spectra were removed from the data set by using an automated spectral quality assessment algorithm. Tandem MS spectra remaining after filtering were searched with the SEQUEST algorithm against a protein sequence data base (NCBI) with an intensity threshold of 0.25 m Tris, pH 6.8, 1% SDS, 10% 2-mercaptoethanol, 10% glyc erol, and bromophenol blue were loaded onto SDS-containing polyacrylamide slab gels (7.5, 10, 12.5, or 15% isocratic) (23). Western blots (nitrocellulose membrane) were probed with antibodies specific for total ADF and cofilin (rabbit 1439) (24), cofilin (ACFL02, Cytoskeleton Inc. (Denver, CO)), actin (clone C4, MP Biomedicals (Irvine, CA)), 14-3-3ζ (which also recognizes β and σ isoforms), tropomyosin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Hsp60 (Cell Signaling, Danvers, MA), and annexin A2 (ProteinTech Group, Chicago, IL). Bound fluorescent secondary antibodies were detected with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Quantitative measurements from Western blots were performed with Total Lab one-dimensional gel software (Nonlinear Dynamics, Durham, NC). To be within the linear range of the standard curves (supplemental Fig. S2C), different amounts of the same samples were used to quantify actin and the ADF/cofilin or its chimera with GFP. Actin was quantified using the actin monoclonal C4 antibody with purified muscle actin as a standard, whereas different antibodies and standards (see below) were used for the different ADF/cofilin proteins.

We tested three different antibodies against human cofilin-GFP: a mouse monoclonal antibody (MAb22) (25), the rabbit 1439 pan-ADF/cofilin antibody, and a commercial anti-cofilin antibody (ACFL01, Cytoskeleton Inc.), but none of them gave adequate sensitivity to both the cofilin-GFP and the human cofilin standard. Thus, we quantified the human cofilin-GFP/actin ratio in HeLa cell rods from silver-stained gels by comparison with actin and XAC-GST standards (see supplemental Fig. S2C).

Fixation and Immunostaining of Cultured Cells—Cells were fixed for 45 min at room temperature with prewarmed (37 °C) 4% paraformaldehyde with or without 0.1% glutaraldehyde in PBS, permeabilized with methanol (prechilled at −20 °C) for 3 min at room temperature, and blocked in 2% goat serum, 1% bovine serum albumin in Tris-buffered saline before immunostaining. Primary antibodies were the same as used for immunoblotting plus peroxiredoxin 1 antibody (Abcam, Cambridge, MA). ProLong Gold Antifade (Invitrogen) was used for the mounting medium.

Fixation and Immunostaining of Isolated Rods—Initially, isolated and fixed rods were allowed to settle on glass coverslips coated with nitrocellulose (10 mg/ml in amyl acetate) and immunostained (see below) but were later sedimented onto ACLAR™ (Ted Pella, Redding, CA) embedding film. ACLAR™ circles (4-mm diameter) were coated with 5 μl of...
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Rod Isolation—To develop an isolation protocol for AC-actin rods, we used porcine LLCPK A4.8 cells (A4.8 cells). This kidney-proximal tubule cell line expresses low levels of ADF/cofilin (<0.1% of protein; 99% as cofilin and 1% or less as ADF from two-dimensional immunoblots; data not shown), whereas actin comprises 2.7% of total protein, corresponding to a molar ratio of total cofilin (endogenous cofilin + XAC-GFP) to actin is 0.07. Small aggregates containing actin and cofilin are expressed proteins in epidermoid carcinoma A431 cells (Fig. 6a), and ADF and cofilin were concentrated on Milipore Ultrafree centrifugal filters to a final concentration of about 1.5 mg/ml (80 μM). For some experiments, an equal volume of ADF and actin was mixed, and 5 μl was mixed with the ADP-Pipes buffer containing 20% polyethylene glycol 400, and the drop was suspended over a 24-well crystallization dish containing the ADP-Pipes buffer with 20% polyethylene glycol and 1% sodium azide. The drops were examined every 2 days by dark field microscopy. When fibrous material was observed (about 48 h), the protein from the hanging drop was transferred to a Formvar-coated electron microscopy grid, and material was allowed to adhere for 30 s before wicking off the solution, negatively staining the rods with filtered 1% uranyl acetate, air drying, and observing by transmission electron microscopy on a JEOL 2000 as described above.

For other experiments, the actin was supplemented with 1% Alexa 488 actin or 1% Alexa 594 actin (Invitrogen), and an equal volume of actin and either ADF or cofilin were mixed with an assembly buffer containing 10 mM Pipes, pH 6.8, 2 mM MgCl₂, 1 mM EGTA, and KCl amounts between 0 and 100 mM. For long term studies, the mixture was suspended as a drop over a well containing ADP-Pipes buffer, and the dish was maintained at room temperature for hours or days before the drop was transferred to a glass slide, covered with a coverslip, and observed by epifluorescence microscopy. For short term studies, the mixtures were incubated at room temperature for 15–30 min and centrifuged through 5% OptiPrep onto nitrocellulose-coated ACLA™. While still on the polycrylamide plug, rods adhered to nitrocellulose-coated ACLA™ discs were fixed with 25 μl of 2% formaldehyde, 0.1% glutaraldehyde in 20 mM Pipes, pH 6.8, 140 mM NaCl, 1 mM EGTA. After 5 min at room temperature, the samples were centrifuged for 1 min, the supernatants were discarded, and the in vitro assembled rods were immunostained for cofilin as above.

RESULTS

Rod Isolation—To develop an isolation protocol for AC-actin rods, we used porcine LLCPK A4.8 cells (A4.8 cells). This kidney-proximal tubule cell line expresses low levels of ADF/cofilin (<0.1% of protein; 99% as cofilin and 1% or less as ADF from two-dimensional immunoblots; data not shown), whereas actin comprises 2.7% of total protein, corresponding to a molar ratio of total cofilin (endogenous cofilin + XAC-GFP) to actin is 0.07. Small aggregates containing actin and cofilin, but not rods, form in A4.8 cells that are ATP-depleted for 1 h (29). A4.8 cells infect readily with recombinant adenovirus expressing the wild type XAC as a GFP chimera. Rods similar in morphology to those found in cultured neurons and AD brain form in ATP-depleted A4.8 cells expressing XAC-GFP (Fig. 1a) when the molar ratio of total cofilin (endogenous cofilin + XAC-GFP) to actin is >0.5 (between 36 and 48 h after infection). Rod size and rod number reach a maximum between 72 and 96 h post-infection following 60 min of ATP depletion. Rods were also induced by 60 min of ATP depletion in HeLa cells stably transfected with a plasmid expressing human cofilin-GFP (Fig. 1b). Rods form readily from endogenously expressed proteins in epidermoid carcinoma A431 cells (Fig.
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FIGURE 1. ATP depletion induces rod formation in cultured cells. a, XAC-GFP rods in A4.8 cells; b, cofilin-GFP rods in HeLa cells; c, cofilin-immunostained rods in A431 cells; d, cofilin-immunostained rods in primary cultures of rat cortical neurons. All panels show inverted fluorescence images. Bars, 10 μm.

1c) and rat cortical neurons (Fig. 1d), where they can be visualized by immunostaining for cofilin.

To determine optimal conditions for isolating rods, we exposed mechanical cell lysates of ATP-depleted A4.8 cells expressing XAC-GFP to different detergents, pH, ionic strengths, reducing conditions, ATP, calcium, and temperatures and examined rod stability qualitatively by fluorescence microscopy. Rods were most stable in physiological salt at pH 6.5–6.8 but were not stable in any ionic or non-ionic detergent tested (Triton X-100, Nonidet P-40, saponin, and CHAPS). The addition of 1 mM EGTA or 10 mM DTT did not affect rod stability, but added CaCl2 caused loss of rods in the cell lysate. Rods were stable at 4 °C and room temperature. Protease inhibitors were used initially, but they were found to be unnecessary. Based upon these findings, the rod isolation buffer contained 20 mM Pipes, pH 6.8, 140 mM NaCl, and 1 mM EGTA and was used at 4 °C. Because rods were unstable in detergents, different mechanical cell lysis treatments were utilized, including sonication, homogenization, and repetitive passage through a 30-gauge needle. Sonication was easiest but resulted in significantly smaller rods. Repetitive passage (40 times) through a 30-gauge syringe needle resulted in lysates with the largest average rod size. However, proteomic analysis (see below) of rod samples first isolated by this method identified many isoforms of tubulin as major proteins in the rod fraction, although rods in vivo do not immunostain for tubulins (1). Thus, we surmised that cold-stable microtubules were being sheared by the needle cell lysis procedure and that microtubule pieces co-fractionated with rods. In subsequent preparations, cells were treated with 10 μM nocodazole for 10 min prior to and during the ATP depletion. Nocodazole treatment eliminated microtubules in tubulin-immunostained cells and substantially reduced tubulin contamination of rods without any obvious effects on rod numbers, size, or isolation from any cell type used (supplemental Fig. 1).

After cell lysis by repetitive passage through a 30-gauge needle, nuclei and large cellular debris were removed by centrifugation at 325 × g for 3 min. Rods were then isolated from the supernatant by two centrifugation steps on OptiPrep gradients. Centrifugation of the supernatant for 10 min at 6,650 × g at 4 °C on a two-step (10 and 15%) OptiPrep gradient separated the rods from the soluble proteins, which did not enter the 10% OptiPrep layer. Maximal loading on the two-step gradient was lysate-derived from one 10-cm dish of nearly confluent non-neuronal cells or from two 10-cm dishes of cortical neurons plated at a density of 250,000 cells/cm². The rods collect above the interface between the 10 and 15% OptiPrep layers and were harvested in ~200 μl by side puncture of the tube with an 18-gauge needle on a 1-ml syringe. Collected samples from two gradient tubes (~400 μl) were combined and mixed with 1.7 ml of 13% OptiPrep in the Pipes lysis buffer and centrifuged at 166,000 × g for 2 h at 4 °C. The self-forming continuous OptiPrep gradient separated free rods from rods adhered to cellular components. Fractions (0.2 ml) were collected from the top of the gradients. Typically, the rods were contained in fractions 9–10. When fraction 10 (0.15 ml) was harvested, care was taken to avoid disturbing or collecting the pelleted material, which contained rods associated with other cellular debris. The rods were concentrated by diluting the rod-containing OptiPrep fractions 1:1 with lysis buffer and centrifuging. When collected at the bottom of a tube, the rods formed an amorphous mass, but when sedimented onto 15% OptiPrep in the Pipes lysis buffer at 4 °C, the rods could be isolated in suspension in about 35 μl of 15% OptiPrep. The rods could also be centrifuged onto either nitrocellulose-coated ACLAR™ discs or Formvar-coated copper grids supported on 10% polyacrylamide cushions in microcentrifuge tubes. For these centrifugations, the tubes were centrifuged horizontally at ~8,000 × g. Rods induced by ATP depletion were isolated from A431 cells, rat cortical neurons, and HeLa cells stably expressing human cofilin-GFP using identical methods.

The isolated XAC-GFP rods from A4.8 cells and cofilin-GFP rods from HeLa cells are shown in Fig. 2. a and b, respectively. Isolated rods were typically 2–5 μm in length, about the same length as rods found in the cells and also those in neurons and organotypic brain slices (1, 8). A low magnification electron micrograph of negatively stained rods from A431 cells is shown in Fig. 2c. The rod fraction from A431 cells contains a pool of morphologically similar rod-shaped structures that are surprisingly homogeneous in size. However, the lengths of these negatively stained rods are only about 0.1 times the average length of rods immunostained in intact A431 cells. It is not clear if this difference is due to the inability of the larger rods to adhere to the grids, the processing for the negative staining, or fragmentation and a selection of shorter rods during the isolation procedure. In Fig. 2d, a magnified image of a negatively stained XAC-GFP rod from A4.8 cells is shown, which is about 0.7 μm long, 3–4-fold longer than most of the rods in Fig. 2c.

Lysates of neuronal cultures before or after ATP depletion were put through the rod isolation protocol. The final fractions were fixed in suspension and allowed to settle onto nitrocellulose-coated glass coverslips and immunostained for cofilin. Immunofluorescent rods (Fig. 2e) were isolated from the ATP-depleted cell extracts but were only occasionally observed in the
non-ATP-depleted sample. It is not surprising to find some rods in the untreated cortical cultures because these cells have undergone considerable stress in their preparation and large scale culturing, and often 2–4% of these neurons have rods (2). Rod lengths observed by immunostaining on nitrocellulose were up to 5 μm, similar to the average lengths of the rods observed in neurites of fixed cells. The fluorescent rod in Fig. 2e is also visible by phase contrast (Fig. 2f).

**ADF/Cofilin and Actin as Core Rod Components—**SDS-PAGE of isolated rods shows that ADF/cofilin and actin are the main structural components. A silver-stained gel of the XAC-GFP rod-containing fractions from A4.8 cells after the first and second OptiPrep gradient spins shows that actin and XAC-GFP (doublet shown magnified) are the major components (Fig. 3a). Rods isolated from HeLa cells contain human cofilin-GFP and actin as the major components (Fig. 3b). Because cofilin-GFP and actin migrate as a single band on 12.5% gels, a 7.5% gel was used to separate cofilin-GFP from actin (Fig. 3c), in which the upper band was identified as cofilin-GFP from immunoblots. Actin is the predominant band in A431 rod preparations as well (Fig. 3b), but many contaminants, especially keratins in the 50 kDa range, were observed in rods prepared from these cells. A431 cells are derived from an epidermoid carcinoma, and their high level of keratin expression may be responsible for the greater difficulty in obtaining a cleaner rod preparation from these cells. Rods isolated from cortical neurons (Fig. 3d) also contained actin, cofilin, and ADF (silver-stained bands were identified from corresponding immunoblots; data not shown) as primary rod components.

To determine the ADF/cofilin to actin ratios in rods isolated from the different cell types, quantitative Western blot analyses were performed. None of the three cofilin antibodies we tested gave adequate sensitivity to both the cofilin-GFP and the human cofilin-GFP/actin ratio in HeLa cell rods from silver-stained gels by comparison with actin and XAC-GST standards (supplemental Fig. S2C). The ADF/cofilin to actin ratios obtained for XAC-GFP rods from A4.8 cells and human cofilin-GFP rods from HeLa cells were 1.2 ± 0.3 (S.D.) and 1.0 ± 0.17 (variation of duplicates), respectively, values close to the 1:1 ratio expected from cofilin-saturated actin filaments (5, 30, 31). Rods isolated from A4.8 cells expressing different amounts of XAC-GFP maintained a consistent ADF/cofilin to actin ratio of about 1:1, suggesting that cofilin levels are limiting for rod formation until they reach saturation. However, the cofilin/actin ratio for rods isolated from A431 cells is only 0.2 ± 0.05 (S.D.). This low value can be explained by the co-isolation of other actin bundles from these epidermoid carcinoma cells (see “Discussion”).

The 1:1 ratio of ADF/cofilin to actin in neuronal rods (1.2 ± 0.2 (S.D.):1) was readily quantified from silver-stained gels in which ADF, cofilin, and actin are well separated (Fig. 3d) and by comparisons with silver-stained standard curves (supplemental Fig. S2, B and C). In multiple rod preparations from cortical neurons, we found ADF present at levels from 29 to 68% those of cofilin.

**Stability of Isolated Rods—**To quantify the stability of rods after different treatments, we developed a sedimentation assay in which isolated rods were treated for 10 min with the different test agents, fixed in solution, and then centrifuged onto nitrocellulose-coated ACLAR™ discs, as described previously. We first showed by fluorescence microscopy of the supernatant that centrifugation of cofilin-GFP rods for 2.5 min at −8,000 × g was sufficient to pellet completely all fluorescent rods in the sample. By digital imaging of the rod fluorescence on the disc, an intensity threshold was obtained that excluded low level...
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FIGURE 4. Stability of isolated rods to 10-min exposure to different reagents. Shown are plots of relative rod index (rod fluorescence area/total field area for a particular treatment divided by rod fluorescence area/total field area for control) × 100 for ADP/cofilin-GFP-containing rods (A) and cofilin-immunostained endogenous rods (B). Rods did not survive exposure to Triton X-100. The cofilin-GFP rods in A are from HeLa cells, and the XAC-GFP rods are from A4.8 cells. All rods were isolated at pH 6.8. The XAC and XAC-GFP rod stability results are the mean values of triplicate samples from a single experiment. The decreased stability of rods composed of endogenous proteins to high salt suggests that the fluorescent proteins play a role in stabilizing the cofilin-GFP-actin rods. Error bars, S.D. values from 3–7 different rod preparations from cortical neurons, HeLa, and A431 cells that were normalized to the control rod index. Only the Triton X-100 and 0.5 M NaCl treatments for the rods made from endogenous proteins (cortical neurons and A431 cells) showed a statistically significant difference from controls (*, p < 0.01 in a one-sample t test).

TABLE 1

Mass spectral identification of rod components from A431 cells

| Protein | Mass spectrometry | For each of three preparations | Average | Rank |
|---------|------------------|-------------------------------|---------|------|
| β-Actin | 2                | 2                             | 9       | 4    |
| γ-Actin | 3                | 3                             | 10      | 5    |
| Cofilin | 4                | 9                             | 8       | 7    |
| Peroxiredoxin 1 | 17           | 13                            | 10      | 4    |
| Annexin A2 | 28            | 4                             | 11      | 5    |
| Hsp60   | 8                | 10                            | 18      | 12   |
| 14-3-3 (any isoform) | 25 | 16                           | 15      | 19   |

a After elimination of tubulin and keratin isoforms.  
b Final rank based upon lowest average ranking.

background fluorescence and allowed us to quantify the area of a field occupied by rods per total field area (supplemental Fig. S3). This value was relatively consistent for different fields from a disc and across discs made from the same rod preparation but varied between different rod preparations.

Cofilin-GFP-containing rods were compared for stability under different buffer conditions with rods formed from endogenous proteins isolated from both A431 cells and cortical neurons. Multiple preparations of rods were used from each source, so all values were normalized to the lysis buffer control in each set to create the relative rod index, which allowed us to combine data between sets. The stability of the rods exposed to 0.05% Triton X-100, 5 mM DTT, 5 mM ATP, 5 mM CaCl2, and 500 mM NaCl is shown in Fig. 4. Rods were not stable to Triton X-100. Rods from the different cell types were stable to added DTT, ATP, and CaCl2. Rods composed of cofilin-GFP-actin were significantly more stable to high salt (0.5 M NaCl) than were rods formed from endogenous proteins. To test if the salt stability differences between cofilin-GFP- and endogenous cofilin-containing rods could be due to different rod-associated proteins from the different cell types used, we expressed XAC and XAC-GFP in the same cell type (A4.8 cells) and isolated rods that were compared side-by-side. As shown in Fig. 4, rods isolated from A4.8 cells containing XAC showed greater sensitivity to 500 mM NaCl than those containing XAC-GFP. Thus, rods made from endogenous proteins are more labile in high salt than are rods made from cofilin-GFP.

Proteomic Analysis of Rods—Because the presence of GFP on the cofilin might alter its association with other possible rod components, we limited the mass spectrometric proteomic analysis to rods formed from endogenous proteins isolated from ATP-depleted A431 cells and cortical neurons. Three separate preparations of A431 rods, one performed without nocodazole treatment and two performed following nocodazole treatment of the cells, showed cytoplasmic actins and cofilin as the top ranked proteins identified by the mass spectrometry analyses, after eliminating contaminating tubulins and keratins (Table 1). The rankings are based on the frequency with which each of the peptides generated by the tryptic digests was detected and the percentage of each protein’s sequence that was identified.

In addition to the cytoplasmic β- and γ-actins and cofilin-1, only four other proteins appeared among the top 30 in all three preparations: peroxiredoxin 1, an antioxidant enzyme; annexin A2, an actin- and phospholipid-binding protein; heat shock protein 60 (Hsp60), a folding and stress response protein; and several isoforms of 14-3-3, a scaffolding protein. Antibodies against Hsp60, peroxiredoxin 1, and annexin A2 did not immunostain rods (data not shown) in either A431 cells or neurons under staining conditions that are best for rods (4% paraformaldehyde, 0.1% glutaraldehyde fix and cold methanol membrane permeabilization) or when fixed in the absence of glutaraldehyde followed by Triton X-100 permeabilization, a non-optimal procedure for rod preservation but one that is often better for immunostaining by some antibodies (32).

Although the scaffolding protein 14-3-3 generally interacts with phosphorylated proteins, it can bind both phosphorylated and dephosphorylated cofilin (33) and thus was considered a strong possibility as a structural component of rods. To test this, rods were induced in A431 cells and in hippocampal neurons by ATP depletion. The rods were then fixed and immunostained for 14-3-3 (Fig. 5, a and b, arrowheads), but most do not. In hippocampal neurons, some transient rods, formed immediately in response to ATP depletion, contain 14-3-3ζ (Fig. 5, c and d, arrowheads), but some do not (Fig. 5, c and d, arrows). Some persistent rods, those present 24 h after a 30-min ATP depletion and 24-h wash out, immunostained with the 14-3-3ζ antibody (Fig. 5, e and f). However, 14-3-3ζ is not always restricted to the cofilin-stained rod region (Fig. 5, g–i). Thus, 14-3-3 is not a core structural component of rods but appears to accumulate at rods over time.
ATP depletion in A431 cells also immunostain for 14-3-3 (d,e); depletion and 24-h recovery immunostain for both cofilin (f,g) and 14-3-3 (h,i). The 14-3-3 immunostaining within processes where rods form is not restricted to the cofilin-rod region (arrowheads). Some rods present in neurites of 5 days in vitro hippocampal neurons following 30-min transient ATP depletion and 24-h recovery immunostain for both cofilin (e) and 14-3-3 (f). Again, 14-3-3 immunostaining is not always restricted to the cofilin-rod region (g and h). Bars, 10 μm.

**In Vitro Assembly of Rods**—ADF and cofilin bind to Mg-ADF-actin with $K_D$ values of about 0.5–1.0 μM (28). In an attempt to crystallize a complex between chick ADF and actin, we mixed chick ADF and muscle actin at a 1:1 ratio in a hanging drop containing 20% polyethylene glycol in a crystallization dish as described under “Experimental Procedures.” The final ADF concentration of 40 μM is within its physiological range in some cell types (34, 35). Even at the low ionic strength used here, light scattering by fibrous material was visible by 48 h in the drop at the light microscopic level using dark field observation. Negative stain electron microscopy of the fixed material in this drop showed the presence of several rod-shaped structures, which at high magnification appeared to contain longitudinal filaments (Fig. 6f).

To determine if rods would form in vitro from the cofilin-actin complex, we mixed chicken muscle Mg-ADF-actin, added to 1% Alexa 488-actin, with chick cofilin at a 33 μM final concentration of each protein in 10 mM Pipes, pH 6.8, 0–100 mM KCl, 2 mM MgCl₂, and 1 mM EGTA. The drops were suspended over wells containing the Pipes buffer on a 12-well crystallization dish as described under “Experimental Procedures.” The hanging drops were kept at room temperature. Aliquots of each drop were transferred to a glass slide, and a coverslip was added to observe the sample by epifluorescence and phase microscopy. Within the background of fluorescent actin filaments, distinct rodlike structures were observed in both phase and fluorescence, similar in dimensions to rods observed in cells (Fig. 6, a and b). In the absence of cofilin, the actin alone showed swirls of filaments but no condensed rodlike structures at any ionic strength. At the lowest ionic strength (no added KCl), we observed wavy actin filament bundles coalescing into a central rodlike structure in samples containing actin and cofilin (Fig. 6e). To confirm that the in vitro rods contained both actin and cofilin, chick muscle actin spiked with Alexa 594 actin (1%) and chick cofilin were incubated together for 15–30 min, centrifuged through 5% OptiPrep onto nitrocellulose-coated ACLAR™ discs, and immunostained for cofilin. Rods containing both actin and cofilin were observed (Fig. 6, c and d). Together, these experiments demonstrate that ADF/cofilin-actin rods form rapidly in vitro and do not need additional proteins for their formation.

**Mechanism of in Vivo Rod Formation**—Several different mechanisms for in vivo rod formation come to mind. Activated ADF/cofilin binds cooperatively (31, 36) to long actin filaments, which could cause their self-association. During activation of ADF/cofilin, filaments may be severed, and then these shorter filaments could become saturated with ADF/cofilin and self-associate into rods. Because ADF/cofilin-ADF-actin subunits also add on to filaments and may even nucleate new filament growth (28, 37, 38), the depolymerized pool of actin may asso-
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ciate with ADF/cofilin and assemble into ADF/cofilin-saturated filaments that bundle into rods. To distinguish between some of these mechanisms, we followed rod formation in HeLa cells expressing coflin-GFP by live cell imaging in the presence and absence of cytochalasin D, a drug that inhibits barbed end growth; latrunculin B, a drug that binds monomeric actin and inhibits its assembly; and jasplakinolide, an F-actin-stabilizing drug that inhibits coflin-dependent turnover.

In cultured neurons, latrunculin was previously shown to induce rods and jasplakinolide to inhibit rod formation in response to ATP depletion with antimycin (32). These results are consistent with the effects of these drugs on rod formation in HeLa cells expressing coflin-GFP (supplemental Fig. S4), where latrunculin B induced rods in otherwise untreated cells and jasplakinolide blocked rod formation induced by ATP depletion. Cytochalasin D also inhibited rod formation induced by ATP depletion. The cytochalasin D effects were also studied in ATP-depleted A431 cells by fixing and staining at different times during early stages of rod formation (data not shown).

Prior to ATP depletion, most coflin is diffusely distributed within the cell (Fig. 7). Within 5 min, small needle-shaped rods begin to form (in HeLa cells, this started within 1 min). The cytochalasin D inhibition of their formation suggests that filament growth into rods depends on subunit addition onto barbed ends of filaments. These small rods coalesce into fewer but larger rods with time, suggesting that rods may form from association of shorter filaments that are saturated with ADF/cofilin. Thus, the intracellular mechanism of rod formation appears to mimic what we observed in vitro (Fig. 6e), where coflin-actin assembles into filaments, which coalesce into bundles that condense into rods.

To assess the stability of rods to cytochalasin D and jasplakinolide, rod formation in HeLa cells expressing coflin-GFP was initiated by 30-min ATP depletion and observed continuously following the drug addition. Surprisingly, both cytochalasin D and jasplakinolide treatment eliminated coflin-GFP rods during continued ATP depletion (supplemental Fig. S5).

DISCUSSION

Previously, we demonstrated the inability of fluorescent phalloidins to bind to rods (1, 8), suggesting that they may contain F-actin saturated with ADF/cofilin, a prediction confirmed by the findings here. Cofilin has its optimal F-actin severing activity at relatively low stoichiometry to actin (1:800) and has been shown to be an F-actin-binding and -stabilizing protein when present in nearly equal stoichiometry to actin subunits (38). Rods isolated from ATP-depleted neurons and coflin-GFP-expressing cells contain ADF/cofilin and actin in a ratio of 1:1; supporting this finding, rodlike structures form in vitro when the two proteins are incubated together in equal molar ratios. However, rods isolated from A431 cells had lower ADF/cofilin to actin ratios, suggesting contamination with other actin structures that contain little or no coflin. A431 cells are an epidermoid carcinoma, and the high cytokeratin contamination of the rod preparation might increase cosedimentation of other non-cofilin-containing actin structures. Some carcinomas of this type have up-reg...
lated tropomyosin 3 (39). Many tropomyosins compete with cofilin for binding to actin filaments (40), so we hypothesized that tropomyosin-bound actin filaments may be co-purifying with the rods from A431 cells and, thus, lowering the cofilin/actin ratio. To test this hypothesis, we applied the rod isolation protocol to lysates from non-ATP-depleted A431 cells and obtained a fraction from the final gradient, which gave faint keratin and actin bands when fractionated by SDS-PAGE and silver-stained. Proteomic analysis of this fraction identified tropomyosin 3 isoforms near the top of the list (data not shown). Thus, the lower cofilin/actin ratio in rods prepared from A431 cells probably reflects contamination with other actin structures that do not contain cofilin.

Of interest in the rod stability studies is the fact that rods in cell lysates appear to be sensitive to added calcium, whereas isolated rods are stable to calcium. This suggests that some calcium-dependent factor may be involved in rod dissolution in vivo. Although we have not identified this factor, having isolated rods and a quantitative assay for their stability should now allow us to add back fractions to test for the presence of this calcium-dependent factor and isolate it.

The ability of latrunculins to induce cytoplasmic rods in neurons was previously reported (32). Latrunculin works at low micromolar concentrations to sequester actin monomers and depolymerize actin in cells, but latrunculin has a much higher ED$_{50}$ (~50 µM) for inhibiting cofilin-actin binding (41) and thus actually provides a cofilin-reactive monomer pool for rod assembly.

It was surprising to find that both jasplakinolide and cytochalasin D caused the loss of cofilin-GFP from rods formed in HeLa cells, even in the presence of continuous ATP depletion. Because jasplakinolide stabilizes F-actin in an untwisted form, as does phalloidin, and phalloidin binding competes with cofilin (31), it is not difficult to explain how cofilin-GFP could be displaced from the actin within rods by jasplakinolide. However, cytochalasin D is thought to bind only barbed ends of F-actin and prevent further subunit addition. Because it is very unlikely that filament dynamics would play a role in turnover of rod components in these ATP-depleted cells, and indeed fluorescence recovery after photobleaching studies previously showed virtually no cofilin recovery over 6 min (41), it is more likely that the barbed end binding of cytochalasin D has longer range effects on filament structure than previously appreciated, causing release of cofilin-GFP. Because the majority of filaments within a rod are quite short (average of less than 100 subunits/filament; see Fig. 8) and cofilin binding is cooperative (42), disruption near the filament barbed ends could help strip the filaments of the cofilin-GFP. Alternatively, the structure of actin in rods may not be normal F-actin saturated with cofilin but instead some alternative 1:1 complex.

Proteomic analysis of rods showed the presence of many other proteins. However, none of the more abundant contaminating proteins localized to rods during their initial formation, although some are recruited after rod maturation. Surprisingly, we did not detect the microtubule binding protein Tau by proteomic analysis of the neuronal rods, although specific phosphorylated epitopes of Tau (those recognized by the 12E8 antibody) associate with cofilin-actin rods in neurons (32). However, the strength of this interaction has not been measured, and it is conceivable that associated Tau and perhaps other weakly associated proteins are removed during the rod isolation process.

Cofilin dimerization or oligomerization through sulphydryl oxidation was previously shown to promote F-actin bundling in vitro, resulting in rods that rapidly disappeared when treated with DTT (43). However, rods isolated from ATP-depleted cells are not destroyed by DTT, suggesting that they do not require oxidized forms of cofilin for their stabilization or cross-linking. This does not exclude the possibility of cross-linked cofilin in rods induced by other means; nor does it mean that disulfide-cross-linked forms of cofilin or cofilin-actin do not occur in rods. In fact, different oxidized forms of cofilin have been identified in rods from ATP-depleted cells.5

Although our interest is primarily in neuronal rod formation, which is important in Alzheimer disease and probably in many other neurodegenerative disorders, isolating rods from neurons is a more difficult undertaking than obtaining them from non-neuronal cells. Neuronal rods form principally in neurites, where ADF/cofilin concentrations are highest (1). Rods in neuronal cultures are present in far fewer numbers per unit area of culture than in confluent cultures of non-neuronal cells (see Fig. 1). This has limited us so far to the isolation of neuronal rods induced by ATP depletion, during which >80% of the neurons form rods. In future studies, we would like to compare rod composition and structure when induced by other stimuli (e.g. amyloid β peptide, excitotoxic glutamate, and peroxide).

Although we have followed rod formation in live neurons expressing cofilin-GFP, the growth properties of rods are more difficult to analyze because by the time they are visible as rods, they are all aligned parallel to the neurite axis, often in tandem arrays. Thus, we have not been able to differentiate if rods grow from small needle-shaped rods through coalescence or by the addition of cofilin-actin subunits onto nucleation sites. The inability of HeLa cells to form cofilin-GFP rods in the presence of cytochalasin D also suggests that subunit addition or filament annealing through barbed end growth is required for rod formation. In the non-neuronal cells, it is quite clear from live cell imaging that large rods form from many smaller needle-shaped rods by coalescence, but the small rods do not form in the presence of cytochalasin D.

There are numerous signaling pathways that regulate cofilin activity in cells through kinases and phosphatases (44–46). Indeed, the variety of ways in which rod formation can be induced might explain the complexity of sporadic AD through age-related changes in pathways dealing with oxidative stress, glutamate metabolism, ischemic injury, and production and clearance of β-amyloid. Although it might be expected that ATP depletion is a more global effector of multiple pathways, recent studies indicate that the drop in ATP initially activates the cofilin phosphatase, chronophin, by releasing it from binding to Hsp90 (15). Treating cultured neurons with the Hsp90 inhibitor 17AAG caused the rapid formation of rods in a chronophin-dependent manner. In preliminary studies, we

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5 B. W. Bernstein, A. E. Shaw, L. S. Minamide, and J. R. Bamberg, manuscript in preparation.
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have utilized 17AAG to induce rods in cofilin-GFP-expressing HeLa cells and have applied the rod isolation scheme developed here to isolate a rod fraction that appears to be of a composition similar to that of rods formed by ATP depletion. Thus, this method for rod isolation might ultimately prove useful in isolating rods induced by many different stimuli for comparing protein composition, modifications, and oxidation states.

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