Mechanisms Responsible for F-actin Stabilization after Lysis of Polymorphonuclear Leukocytes

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Abstract. While actin polymerization and depolymerization are both essential for cell movement, few studies have focused on actin depolymerization. In vivo, depolymerization can occur exceedingly rapidly and in a spatially defined manner: the F-actin in the lamellipodia depolymerizes in 30 s after chemoattractant removal (Cassimeris, L., H. McNeill, and S. H. Zigmond. 1990. *J. Cell Biol.* 110:1067–1075). To begin to understand the regulation of F-actin depolymerization, we have examined F-actin depolymerization in lysates of polymorphonuclear leukocytes (PMNs). Surprisingly, much of the cell F-actin, measured with a TRITC-phalloidin–binding assay, was stable after lysis in a physiological salt buffer (0.15 M KCl): ~50% of the F-actin did not depolymerize even after 18 h. This stable F-actin included lamellar F-actin which could still be visualized one hour after lysis by staining with TRITC-phalloidin and by EM. We investigated the basis for this stability. In lysates with cell concentrations > 10^7 cells/ml, sufficient globular actin (G-actin) was present to result in a net increase in F-actin.

However, the F-actin stability was not solely because of the presence of free G-actin since addition of DNase I to the lysate did not increase the F-actin loss. Nor did it appear to be because of barbed end capping factors since cell lysates provided sites for barbed end polymerization of exogenous added actin. The stable F-actin existed in a macromolecular complex that pelleted at low gravitational forces. Increasing the salt concentration of the lysis buffer decreased the amount of F-actin that pelleted at low gravitational forces and increased the amount of F-actin that depolymerized. Various actin-binding and cross-linking proteins such as tropomyosin, α-actinin, and actin-binding protein pelleted with the stable F-actin. In addition, we found that α-actinin, a filament cross-linking protein, inhibited the rate of pyrenyl F-actin depolymerization. These results suggested that actin cross-linking proteins may contribute to the stability of cellular actin after lysis. The activity of crosslinkers may be regulated in vivo to allow rapid turnover of lamellipodial F-actin.

CELLS moving on surfaces depend on the extension of lamellipodia for motility. Lamellipodia are dynamic, protruding in the direction of movement and withdrawing from other directions. The lamellipodia are sites of rapid actin polymerization: within 30 s after addition of chemoattractant to a polymorphonuclear leukocyte (PMN)[1] at 23°C, the F-actin content of the cell doubles with most of the newly polymerized F-actin located in lamellipodia. The presence of cytochalasin or ADP-ribosylated actin, both filament barbed end caps, inhibit chemoattractant-induced polymerization suggesting that elongation occurs at the barbed ends (Wegner and Aktories, 1988; White et al., 1983). Various studies have localized a major site of actin polymerization to the tip of lamellipodia; the F-actin then moves rearward (Glacy, 1983; Okabe and Hirokawa, 1989; Wang, 1985;

Forscher and Smith, 1988; Heath, 1983; Symons and Mitchison, 1991; Svitkina et al., 1986; Small et al., 1978).

During cell locomotion, the rate of depolymerization of F-actin must equal the rate of polymerization since the total F-actin present remains constant. 30 s after removal of chemoattractant from PMNs (at 23°C), the lamellipodial F-actin declines to basal levels, while the cortical F-actin remains constant (Cassimeris et al., 1990). The lamellipodial F-actin probably depolymerizes from its pointed ends since depolymerization occurs in the presence of cytochalasin, which blocks depolymerization from the barbed end (Sklar et al., 1985; Cassimeris et al., 1990).

Were an actin filament to extend across the 3–5 μm from the front of the lamellipodium to the junction of the lamellipodium with the endoplasm, and "treadmill" at the rate of locomotion, its rate of depolymerization would need to equal the rate of movement. If the rate of filament growth at the cell front occurs at a rate equal to the rate of cell movement, PMNs moving at rates up to 30 μm/min (0.5 μm/s) would

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need to incorporate 180 actin monomers/s/filament. This would require a dissociation rate constant at the pointed end ~1800 times higher than those measured for purified muscle F-actin in vitro (using a dissociation rate constant of 0.1 s^{-1} and 360 monomers per μm of actin filament [Weber et al., 1987; Korn et al., 1987; Mitchison and Kirschner, 1988; Cassimeris et al., 1990]).

Morphological and kinetic data suggest that a given actin filament does not extend across the length of the lamellipodium. Electron micrographs show that in the cortical meshwork of *Dictyostelium*, macrophages, and PMNs, intersections between actin filaments occur at intervals between 0.05 and 0.6 μm (Hartwig and Shevlin, 1986; Rubino and Small, 1987; Ryder et al., 1984). Kinetic evidence from studies on the depolymerization of F-actin of *Dictyostelium* and PMNs indicates that most filaments are <0.2-μm long (Podolski and Steck, 1990; Cano et al., 1991). If lamellipodia are composed of small 0.1-μm filaments, and if all the filaments depolymerize simultaneously, as suggested by Symons and Mitchison (1991), the required dissociation rate at the pointed end would still be 36 times faster than the dissociation rate measured in vitro (see above). Proteins that can increase the rate of depolymerization, such as actin depolymerizing factor (destrin), actophorin, and depactin, have been identified in various systems and may contribute to this rapid rate of depolymerization (Bamburg et al., 1991; Cooper et al., 1986; Moriyama et al., 1990; Mabuchi, 1983).

To identify factors that regulate actin disassembly in vivo, we have begun to characterize factors that affect F-actin depolymerization in PMN lysates. Rather than finding that these filaments depolymerized more rapidly than pure actin, we found that they depolymerized more slowly. While it has been known for many years that actin filaments in the cytoskeleton are unusually stable, the reasons for this stability have not been identified. We found that the F-actin in lysates existed in two pools: one depolymerized if the lysate cell concentration was sufficiently low; the other was stable for long periods of time. These pools did not correspond to the labile and stable pools observed in vivo (Cassimeris et al., 1990). Increasing the KCl concentration released actin filaments from a cross-linked meshwork and increased the amount of F-actin that depolymerized. This suggested that filament binding and cross-linking proteins might inhibit F-actin depolymerization. We therefore examined the effects of a filament cross-linking protein, α-actinin, and found that it inhibited F-actin depolymerization. Thus, the rapid turnover of F-actin observed in vivo may be more complicated than previously anticipated and require not only an increase in the monomer dissociation rate but also a decrease in the binding of factors that inhibit filament depolymerization.

Materials and Methods

Reagents

N-formylnorleucylleucylphenylalanine (FNLLP), t-Boc-norleu-leu-phe (t-BocNLLP), deoxyribonuclease I (DNase I) (type II from bovine pancreas chromatographically purified), TRITC-phalloidin, and phalloidin were all obtained from Sigma Chemical Co. (St. Louis, MO).

Cells

Rabbit peritoneal cells were obtained as described previously (Zigmond and Sullivan, 1979). The cells were washed two times with saline (0.9%) and suspended in cell buffer (HBSS without phenol red, bicarbonate, or calcium and magnesium (Gibco Laboratories, Grand Island, NY), but with the addition of 10 mM Hepes, pH 7.2).

Proteins

Pyrenyl Actin. Rabbit skeletal muscle actin was isolated (Spudich and Watt, 1971; Murray et al., 1981) and labeled with pyrene (50–80% labeled in different preparations) as previously described (Northrop et al., 1986; Carson et al., 1986). Pyrenyl actin was stored as G-actin at 4°C until use. Pyrenyl F-actin was prepared by incubating pyrenyl G-actin (2.0 μM) overnight in 0.14 M KCl, 2 mM MgCl$_2$, 1 mM ATP, 25 mM Tris-HCl (pH 7.4), 1 mM EGTA, and 0.2% NP-40. Depolymerization of pyrenyl F-actin was followed by decreases in pyrene fluorescence (excitation 370 nm/emission 410 nm) in a fluorescence spectrophotometer (model LS-5; Perkin-Elmer Cetus Instruments, Norwalk, CT).

Rhodamine Actin. Rabbit skeletal muscle actin was labeled with lissamine rhodamine (Molecular Probes, Inc., Eugene, OR) essentially as described by Sanger et al. (1984) with the following further purification steps. After labeling and separation of actin from free dye on a Sephadex G-25 column, the actin was purified by gel filtration chromatography on a Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) column (MacLean-Fletcher and Pollard, 1980). Actin containing fractions were pooled, the actin polymerized by addition of KCl to 0.1 M and MgCl$_2$ to 2 mM, and the F-actin pelleted (80,000 g for 2 h at 15°C). The pellets were homogenized in calcium depolymerizing solution (CDS) (10 mM Tris-HCl, pH 8.0, 0.2 mM CaCl$_2$, 0.5 mM ATP, 50 μM CaEDTA, 1 mM DTT, 1 mM Azide), dialyzed overnight against CDS, and the G-actin solution was clarified by centrifugation (80,000 g for 3 h at 4°C). The G-actin solution was frozen in liquid nitrogen in 100-μl aliquots and stored at -80°C until use. The preparation used in this study was ~30% labeled (based on an extinction coefficient of 77 mM^{-1} cm^{-1} at 570 nm for rhodamine).

α-Actinin. Chicken gizzard α-actinin was purified using a combination of published methods. Glycerination of gizzards, extraction, ammonium sulfate fractionation, and chromatography on DEAE-cellulose and Sepharose 4B were performed as described by Craig et al. (1982). The α-actinin was further purified by chromatography on hydroxylapatite, rechromatographed on DEAE-Sephacel as described by Feramisco and Burridge (1980), and finally chromatographed on butyl-Sepharose (Pharmacia Fine Chemicals) using conditions previously described for butyl-toyopearl (Mimura and Asano, 1986). The α-actinin was then dialyzed in 20 mM Tris, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 0.02% sodium azide, pH 7.4, sterilized by passage through a 0.2-μm pore filter, and stored at 4°C.

Cell Lysis

Detergent Lysis. Cells were suspended in cell buffer with or without 2 × 10^{-8} M FNLLP for 90 s and then the cells were lysed by addition of lysis buffer (final concentrations unless stated otherwise): 2 mM MgCl$_2$, 5 mM EGTA, 1% NP-40, 0.15 M KCl, 2 mM KPO$_4$, 10 mM β-glycerophosphate, 5 mM ATP, 10 mM Hepes, protease inhibitors (1 μg/ml leupeptin, 1 μg/ml benzamidine, 10 μg/ml aprotinin, 10 μg/ml TAME-HCl, added from a 100× stock), and 1 mM PMSF (added just before use) at pH 7.2. When noted, the lysis buffer was modified by increasing the KCl concentration and/or by addition of 10 μM DNase I (Sigma Chemical Co.). In some experiments cells were lysed in PHEM buffer of Schliwa and van Berklom (1981) (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl$_2$, pH 6.9).

Detergent lysis was used in most studies since it is nearly instantaneous and fairly synchronous; it also seemed less likely to break actin filaments than mechanical methods (homogenization, sonication, or filter lysis) or to cause depolymerization of existing filaments at the low temperature or high pressure (required for lysis by freezing and thawing or nitrogen cavitation, respectively). Detergent also offered the advantage of reducing nonspecific binding of TRITC-phalloidin to the centrifuge tubes. Nevertheless, to investigate the role of detergent, we also lysed cells mechanically.

Mechanical Lysis. Cells were lysed by rapid passage of 20 ml of cell suspension (1 × 10^6 cell/ml) through a 2.5-cm diameter, 3-μm pore size, Millipore filter (Millipore Corporation, Bedford, MA) attached to a 20-ml syringe. This procedure resulted in breakage of >99% of the cells. By comparing detergent lysates with filter lysates to which detergent was added before or after filtration, we estimated that ~13% of the F-actin remained on the Millipore filter.

Morphological Examination of Cellular F-Actin

Fluorescence Microscopy and TRITC-Phalloidin Staining. Cells (2 × 10⁵) attached to coverslips were incubated in 3 × 10⁻⁹ M FNLLP at 37°C to obtain a population of cells with a polarized morphology. They were then lysed by placing the coverslip in a Coplin jar containing 10 ml of lysis buffer or PHEM buffer (final G-actin concentration <0.002 μM). Coverslips were transferred to a Coplin jar containing TRITC-phalloidin (0.6 μM) at various times after lysis. The cells were then examined and photographed using an epifluorescent microscope (Carl Zeiss, Inc., Thornwood, NY).

Rhodamine Actin Polymerization from PMN Cytoskeletons. Cells attached to coverslips were incubated with peptide as described above. Cells were lysed by draining off the buffer and placing coverslips into 10 ml of either (*a*) lysis buffer for 1 h or (*b*) lysis buffer plus 1 μM unlabeled phalloidin for 30 s. After lysis by either protocol, the fluid around the cells was carefully removed and 50 μl of lysis buffer containing 1 μM rhodamine actin (diluted into lysis buffer immediately before use from a 16 μM stock) and 1 μM unlabeled phalloidin was applied to the coverslips. In some cases 2 μM cytochalasin B or 35 nM gelsolin/actin complex (a generous gift from Dr. Annemarie Weber, University of Pennsylvania, Philadelphia, PA) was included to block barbed end polymerization. After incubation for 2 min, the coverslips were drained, rinsed in lysis buffer containing 1 μM unlabeled phalloidin, and then mounted on slides and viewed immediately with a camera (Quantex Corp., Sunnyvale, CA) on an epifluorescent microscope (Carl Zeiss, Inc.). Images were collected with an Image One image analysis system (Universal Imaging Corporation, West Chester, PA). Micrographs were made by photographing the image from a video monitor using T-MAX 100 ASA film (Eastman Kodak Co., Rochester, NY).

Electron microscopy. Cells attached to coverslips were incubated in peptide as described above. The coverslips were then either (*a*) lysed in PHEM buffer containing 1 μM unlabeled phalloidin for 30 s before fixation with 1% glutaraldehyde or (*b*) lysed in PHEM for 30 min before fixation in lysis buffer containing 1 μM unlabeled phalloidin and 1% glutaraldehyde. In some experiments, the lysates were incubated for 10 min in 2 mg/ml of the S₁ fragment of myosin (a generous gift from Dr. Lew Tilney, University of Pennsylvania, Philadelphia, PA). After infiltration in 70% ethanol, the coverslips were carefully drained and frozen in liquid nitrogen–cooled propane. The cells were freeze dried at −100°C, rotary shadowed with platinum at 25°C, and replicated with carbon in a Balzer 400 freeze fracture apparatus (Balzers S. p. A., Milan). The replicas were detached from the glass slide, sometimes with the help of 5% sodium hypochlorite, mounted on naked grids, and examined in an electron microscope (model 410; Philips Electronic Instruments Co., Mahwah, NJ).

F-actin Quantitation by TRITC-Phalloidin Staining

The F-actin in the cell lysates was measured by addition of 0.6 μM TRITC-phalloidin (Howard and Oresajo, 1985; Cassimeris et al., 1990; Cano et al., 1991). Control experiments documented the ability of TRITC-phalloidin to rapidly stop depolymerization and to bind quantitatively to F-actin (Cano et al., 1991). In preliminary experiments, glutaraldehyde and/or formaldehyde were used to stop depolymerization; however, parallel experiments following pyrene actin depolymerization and TRITC-phalloidin binding indicated that addition of glutaraldehyde caused anomalously low values in the TRITC-phalloidin assay.

When F-actin was measured in lysates incubated at high cell concentrations (>2 × 10⁶ cells/ml), the lysate was diluted to a cell concentration of 2 × 10⁶ cells/ml at the time of addition of TRITC-phalloidin to prevent phalloidin-induced polymerization during staining. Control experiments indicated that no polymerization occurred under these staining conditions. Each sample contained 5 × 10⁶ cells. After staining for 60 min, the samples were spun at 4°C for 15 min at 80,000 rpm in a 100.3 rotor in a tabletop ultracentrifuge (model TL-100; Beckman Instruments, Inc., Fullerton, CA) (346,000 *g*). The supernatant was removed and the pellet was extracted for 24 to 48 h in 1 ml of 100% methanol. The TRITC fluorescence was read in a fluorescence spectrophotometer (ex 540 nm, em 575 nm). Under these conditions, the fluorescence was proportional to the amount of pure F-actin added. Nonsaturable staining (staining in the presence of 6 μM unlabeled phalloidin) was subtracted from all data.

Assays of F-actin Depolymerization

F-actin depolymerization was followed by losses in TRITC-phalloidin staining of F-actin in lysates (Cano et al., 1991). Depolymerization was stopped at various times after lysis by adding 0.6 μM TRITC-phalloidin as described

above. Several factors were considered while developing the depolymerization conditions. To prevent myosin rigor bonds from inhibiting depolymerization, ATP was present in all media. To insure that the G-actin present in lysates did not affect the depolymerization kinetics, it was necessary either to dilute the cells to <3 × 10⁵ cells/ml (total actin concentration <0.05 μM) or to include DNase I to complex any G-actin present. The TRITC fluorescence signal obtained after high cell dilution (lysing cells in either 8- or 30-ml tubes) was noisy, primarily because the background from nonspecific binding of TRITC-phalloidin to the large tubes was high. Furthermore, the cost of adding 0.6 μM TRITC-phalloidin to large volumes of lysate became a consideration. Thus, to insure that G-actin concentrations were low, we included 10 μM DNase I in the medium and used lysates at 2 × 10⁶ cells/ml (total actin concentration ~0.2 μM). This allowed us to perform the assays in 3-ml tubes while keeping total cell number constant at 5 × 10⁶ cells.

Since DNase I binds G-actin with a dissociation constant of 10⁻⁹ M, it effectively sequesters the G-actin (Podolski and Steck, 1990). DNase I binds to the pointed end of actin filaments and prevents polymerization at this end (Podolski and Steck, 1990). However, it does not inhibit depolymerization from the pointed end (Cano et al., 1991). Under our conditions (lysis buffer containing either 0.15 or 1.2 M KCl), the presence of 10 μM DNase I increased the net depolymerization rate by about 1.5-fold. The increases in rate appeared independent of KCl concentrations (Cano et al., 1991). The increase was independent of filament length indicating that the increase in rate was because of an increase in the dissociation rate constant and not to filament cutting (Cano et al., 1991).

Cytoskeletal-Associated F-actin

Lysates were spun at low *g* forces (1 min in a Beckman 100.3 rotor in a Beckman TL-100 tabletop ultracentrifuge [Beckman Instruments, Inc.] during which time the centrifuge achieved 40,000 rpm; average *g* ~86,000) to determine what fraction of F-actin was present in a large complex. Total F-actin was determined after a high speed spin (15 min at 80,000 rpm, *g* = 346,000) using the TRITC-phalloidin binding assay described above. Only 3% of pure 2 μM F-actin (polymerized overnight in 0.14 M KCl, 2 mM MgCl₂, 1 mM ATP, 25 mM Tris-HCl (pH 7.4), 1 mM EGTA, and 0.2% NP-40) that pelleted in the high *g* spin was pelleted in the low *g* spin.

SDS Gel Electrophoresis and Immunoblotting

Cells were lysed at 5 × 10⁶ cells/ml and cytoskeletons were pelleted at various times after lysis by centrifugation at 13,000 rpm for 2 min in a Hill microfuge (Hill Scientific, Inc., Derby, CT). After removal of the supernatant, the cytoskeletons were resuspended in sample buffer (containing 1% β-mercaptoethanol and 1% SDS) at 0.05× the initial lysate volume. 10 and 3–12.5% gradient SDS-PAGE gels were run according to Laemmli (1970). For quantitation of F-actin, the gels were stained with Coomassie blue. Bands on the gels were quantified with an Image One image analysis system. For immunoblotting, gels were transferred to Immobilon-P (Millipore Corp.). After blocking in PBS containing 5% nonfat milk (Carnation) and 0.05% Tween 20 for 1 h, the immobilon was incubated in primary antibody overnight, then washed (PBS plus 0.05% Tween 20) and incubated in peroxidase secondary antibody (Sigma Chemical Co.) for 1–2 h. After washing, blots were developed using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) on XRP film (Eastman Kodak Co., Rochester, NY). Tropomyosin antibody (rabbit polyclonal) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA); Anti-actin binding protein (mAb) was a generous gift of Dr. John Hartwig (Massachusetts General Hospital, Boston, MA); and α-actinin antibody (rabbit polyclonal, affinity purified) was a gift from Dr. Kyoko Imamaka-Yoshida (University of Pennsylvania, Philadelphia, PA).

Results

Morphological Examination of the F-actin Remaining after Lysis

In vivo, the F-actin in the lamellipodium is labile (Cassimeris et al., 1990). However, we find that after lysis, much of the lamellipodial F-actin is stable. The TRITC-phalloidin staining pattern of F-actin was similar in cells lysed directly
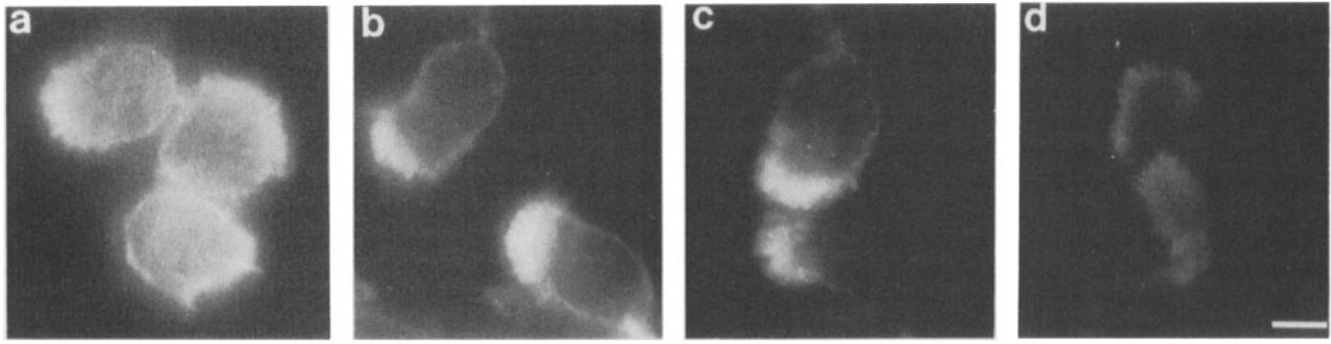
Figure 1. Distribution of the TRITC-phalloidin staining in polarized cells. Cells were allowed to attach to coverslips and develop a polarized morphology by incubating in the presence of 3×10^{-9} M FNLLP for 10 min at 37°C. The coverslips were then placed in lysis buffer (0.15 M KCl) and 0.6 μM TRITC-phalloidin (*a*) or in lysis buffer with 0.15, 0.6, or 1.2 M KCl but without TRITC-phalloidin for 60 min before transfer to lysis buffer containing 0.6 μM TRITC-phalloidin (*b*, *c*, and *d*). After staining for 30 min, the cells were then rinsed in lysis buffer without TRITC-phalloidin, examined, and photographed immediately. Bar, 5 μm.

into TRITC-phalloidin (with or without fixation with 1% glutaraldehyde) (Fig. 1 *a*) and in cells lysed for various periods of time before addition of TRITC-phalloidin. While there was a decrease in the diffuse staining in the body of the cell, staining of the lamellipodia and cell cortex remained prominent in cells lysed for 60 min before adding TRITC-phalloidin (Fig. 1 *b*). Neither removal of chemoattractant 5 s before lysis, nor addition of cytochalasin B at the time of lysis, increased the lability of lamellipodial F-actin after lysis. The staining pattern and the persistent ability to bind TRITC-phalloidin was similar in cells lysed in suspension and in cells attached to coverslips. Thus, substrate attachment was not responsible for the stability of the lamellipodial filaments after lysis. Varying the lysis buffer by omitting Mg^{2+} or ATP, adding back calcium after lysis in EGTA, varying the pH between 6 and 8, lysing at 4°C, including millimolar glucose or polyethylene glycol, or by lysing in a PHEM buffer (Schliwa and van Berklom, 1981) yielded cells with similar amounts of TRITC-phalloidin staining.

Increasing the KCl concentration in the lysate decreased the TRITC-phalloidin staining. After lysis for 60 min in a KCl concentration of 0.6 M, the TRITC staining of the cytoskeleton was fainter, the actin pattern was fragmented, and some cell fragments containing F-actin were released from the substrate and formed clumps (Fig. 1 *c*). In 1.2 M KCl, the TRITC-phalloidin staining was further reduced. Occasionally, clumps of F-actin were observed (Fig. 1 *d*) that were similar in size and shape to the F-actin staining of lamellipodia in physiological KCl.

A meshwork of lamellipodial F-actin could be seen in electron micrographs of platinum shadowed carbon replicas of rapidly frozen cells that had been lysed for 30 s in the presence of phalloidin before fixation (Fig. 2 *a*), or lysed for 30 min before addition of phalloidin and fixation in glutaraldehyde (Fig. 2 *b*). Similar filamentous structures could be decorated with myosin S_1 (not shown) indicating that the structures in Fig. 2, *a* and *b* were actin filaments. Thus, even the fine structure of the F-actin meshwork was stable after lysis.

F-actin Depolymerization Occurs in Two Phases

To study quantitatively the depolymerization of F-actin after cell lysis, we followed the time course of F-actin depolymer-

ization of cells lysed in suspension. TRITC-phalloidin added at various times after lysis prevented further F-actin depolymerization and stained the F-actin present (Cano et al., 1991). The TRITC-phalloidin extracted from a high speed pellet served as a quantitative measure of the F-actin present as described in Materials and Methods. As shown in Fig. 3, in lysates of 3×10^5 cells/ml, the loss of pelletable TRITC phalloidin occurred in two phases. One pool of F-actin, the labile pool, depolymerized rapidly; the half time for the rapid loss of TRITC-phalloidin staining was ∿1 min. This rapid depolymerization was nevertheless slower than the rapid depolymerization in vivo at 23°C (half time <15 s). The stable F-actin pool consisted of F-actin remaining 5 min after lysis; more than half of the original amount of pelletable TRITC-phalloidin was in this pool. There was no further loss of this stable pool of F-actin over the next 18 h. We found that ∿50% of the initial F-actin level remained after an 18-h depolymerization.

The time courses of depolymerization of F-actin in lysates of control PMNs and of PMNs stimulated for 90 s with chemoattractant (at room temperature) before lysis were similar (Fig. 3). Unstimulated cells contained cortical F-actin, while stimulated cells contained both lamellipodial and cortical F-actin. In both cases the F-actin was distributed about equally into labile and stable pools. Since the peptide-stimulated cells had about twice as much F-actin at time zero as control cells, the amount of F-actin remaining in the stable pool of peptide stimulated cells exceeded that in the stable pool of unstimulated cells. This stability in vitro contrasts with the situation in vivo, where all of the peptide-induced F-actin is in the labile pool, primarily in the lamellipodium (Cassimeris et al., 1990). This confirms the morphological observations that in vitro stable F-actin was located in both the lamellipodia and cortex. Addition of 6.7 $\times 10^{-5}$ M t-BocNLLP (a competitive antagonist of FNLLP which in vivo induces a rapid depolymerization of F-actin from cells incubated in FNLLP) (Sklar et al., 1985) 10 s before lysis did not increase the rate of depolymerization of the F-actin remaining at the time of lysis (data not shown). Thus, even when depolymerization is occurring in the cell at the time of lysis, after lysis the filaments are stable.

To determine if the presence of detergent inhibited the rate or extent of F-actin depolymerization, cells were lysed
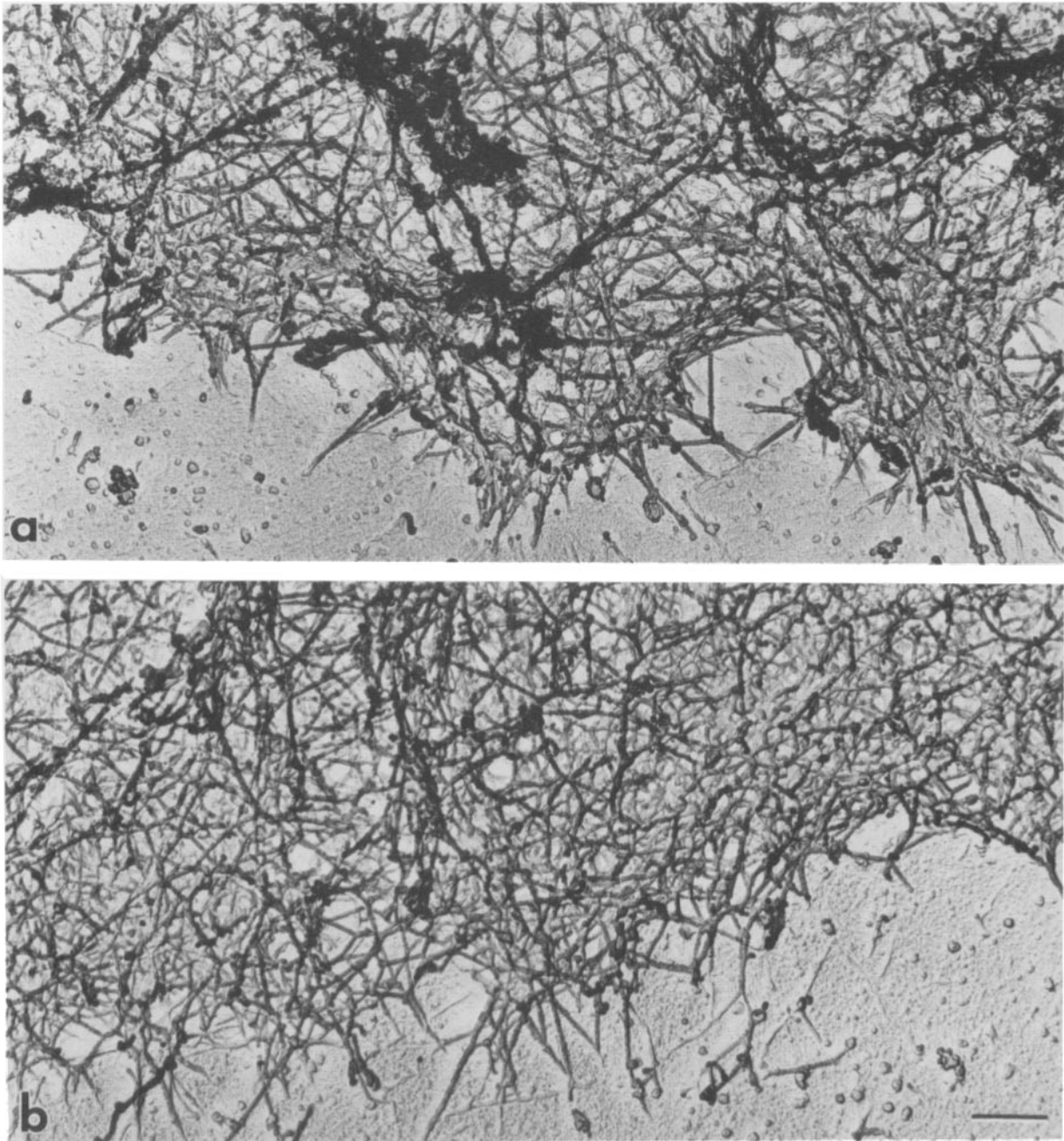
Figure 2. Electron micrographs of lamellipodial F-actin lysed directly into TRITC-phalloidin (*a*) or lysed for 30 min in PHEM buffer before addition of TRITC-phalloidin (*b*). Samples were fixed with 1% glutaraldehyde and processed for EM as described in Materials and Methods. Bar, 0.2 μm.

mechanically by rapid filtration through a millipore filter. These lysates behaved similarly to detergent-lysed cells (i.e., they exhibited a rapid but partial loss of TRITC-phalloidin staining). The depolymerization of the F-actin in control and peptide-stimulated PMNs lysed by filtration at 1×10^6 cells/ml is shown in the inset to Fig. 3.

Depolymerization of F-actin Depends On Cell Concentration in the Lysate

Changes in the levels of F-actin in cell lysates depended on the cell concentration. At concentrations above 10^7 cells/ml, the total F-actin level actually increased after lysis while at concentrations below 10^7 cells/ml the F-actin level decreased (Fig. 4 *a*). The maximal loss of F-actin occurred in cell concentrations $\leqslant 3 \times 10^5$ cells/ml and was ~50% in 5 min. Increasing the KCl concentration allowed net F-actin depolymerization in lysates at higher cell concentrations. In 1.2 M KCl, depolymerization occurred up to cell concentrations of 10^8 cells/ml (Fig. 4 *b*).

The increases in F-actin observed at the higher cell concentrations in Fig. 4, *a* and *b* were surprising. The total G-actin in a lysate of 10^7 cells/ml is ~0.5 μM; were this actin free, it would polymerize. However, the G-actin in the cell is likely buffered by monomer binding proteins, thus, reduc-
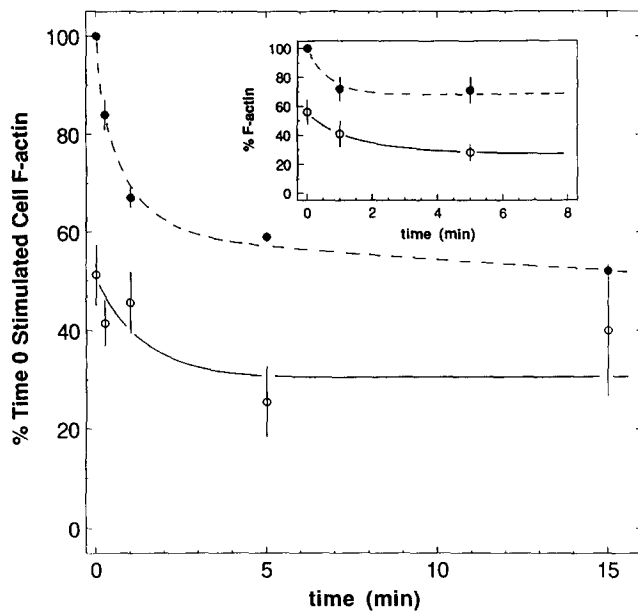
Figure 3. Time course of F-actin depolymerization in control and peptide stimulated cells. Cells were incubated for 90 s at room temperature in modified Hank's buffer with (●) or without (○) 3 × 10^{-8} M FNLLP and then lysed at 3 × 10^5 cells/ml in detergent lysis buffer containing 0.15 M KCl. At various times, TRITC-phalloidin (0.6 μM final concentration) was added and the samples were processed as described in Materials and Methods. The data presented are the mean ± SEM for duplicate samples from a representative experiment. Data are plotted as percent time 0 stimulated cell F-actin based on the time 0 TRITC fluorescence of stimulated cells (see Materials and Methods). The nonsaturable fluorescence has been subtracted from all data. (*Inset*) Cells (1 × 10^6 cells/ml) were lysed by rapid filtration (without detergent) and depolymerization stopped by addition of 0.6 μM TRITC-phalloidin at various times after lysis. The data presented are pooled from duplicate samples from three experiments with control cells (○) and four experiments with cells incubated with 2 × 10^{-8} M FNLLP for 90 s before lysis (●). The data are normalized with 100% representing the staining of peptide-stimulated cells at the time of lysis. Data plotted are the means ± SEM where N = 6 for unstimulated and N = 7 for stimulated cells. The lines are drawn to approach 30-min time points (not shown).
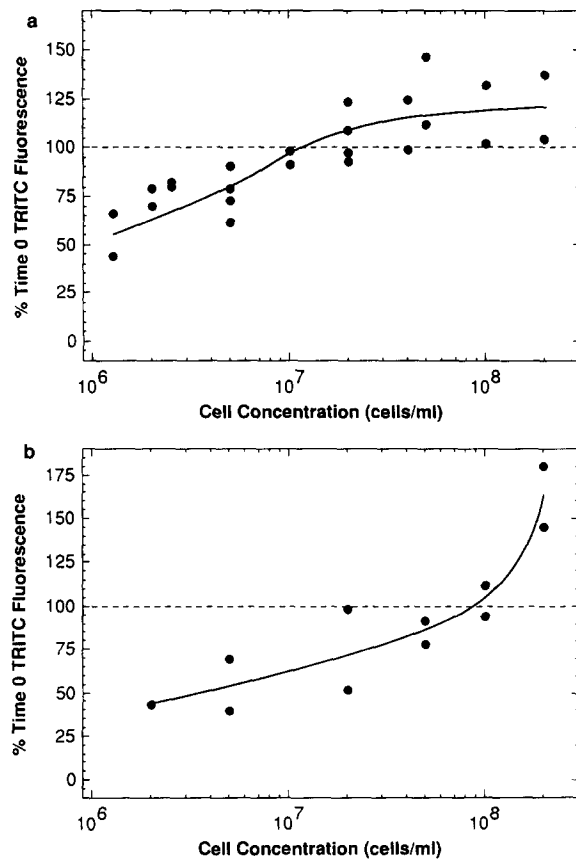

Figure 4. (*a*) Effect of lysate cell concentration on the amount of F-actin present 5 min after lysis in 0.15 M KCl. Cells were lysed into lysis buffer (0.15 M KCl). TRITC-phalloidin (0.6 μM) was present either at the time of lysis (T_0, here set to 100%), or added at 5 min. At the time of adding the TRITC-phalloidin, the cells were all diluted to 2 × 10^6 cells/ml so the staining was performed under identical conditions and at a G-actin concentration of about 0.05 μM, low enough that no polymerization was induced by the TRITC-phalloidin. After staining for 1 h, the F-actin was pelleted and the TRITC-phalloidin extracted with methanol as described in Materials and Methods. The percentage of T_0 staining was calculated after samples were corrected for nonsaturable staining. Duplicate samples from each of two experiments are shown. (*b*) Effect of lysate cell concentration on the amount of F-actin depolymerized 5 min after lysis in 1.2 M KCl. Cells were lysed into lysis buffer containing 1.2 M KCl. The experiment was performed as described in *a*. Duplicate samples from a typical experiment are shown.

ing the concentration of free G-actin. Merely diluting these buffers, whatever their affinity for G-actin, would result in a decrease in the concentration of free G-actin. Therefore, an increase in F-actin would not be expected. It appears that lysis disrupts some regulatory mechanism which normally limits actin polymerization in the cell. Furthermore, at intermediate cell concentrations (2 × 10^6 cells/ml) there is little actin depolymerization. Several factors could be affecting the F-actin levels in these studies: modification of the actin itself, or, the presence of G-actin, capping proteins that block filament ends, and factors that bind to the sides of actin filaments (i.e., tropomyosin and filament cross-linking factors).

G-actin Can Contribute to the Inhibition of Depolymerization in Lysates at High Cell Concentrations but not at Low Cell Concentrations

The G-actin in the lysate of cells at concentrations >2 ×

10^6 cells/ml is >0.1 μM (measured by DNase inhibition [Cano et al., 1991] and thus greater than the critical concentration of actin in vitro. At these lysate concentrations, G-actin could inhibit F-actin depolymerization. The presence of 10 μM DNase in the lysate of 1 × 10^6 cells/ml did not decrease significantly the TRITC-phalloidin staining 5 min after lysis (Table I) indicating that G-actin was not inhibiting F-actin depolymerization at this concentration. In 0.15 M KCl, even in lysates at high cell concentrations, the presence of DNase did not result in net depolymerization. However, DNase inhibited the net polymerization observed in these lysates. In 1.2 M KCl, the presence of DNase did allow depolymerization in lysates at 1 × 10^8 cells/ml but not at 3 × 10^8 cells/ml. Table I summarizes the changes in F-actin 5 min after lysis in 0.15 or 1.2 M KCl and at various cell concentrations in the presence and absence of DNase I. When the cells

Table I. Characterization of F-actin in Cell Lysates

Cell concentration[¶]	Percent change in F-actin in 5 min[‡]	
	(−DNase)	(+DNase)[§]
	Lysis buffer containing 0.15 M KCl[*]	
3×10^5	−50	−50
1×10^6	−45	−45
1×10^7	0	0
1×10^8	+20	0
	Lysis buffer containing 1.2 M KCl[*]	
1×10^6	−55	−55
1×10^7	−50	−50
1×10^8	0	−55
3×10^8	+60	0

[*] Actin critical concentration in 0.15 M KCl = 0.1 μM; in 1.2 M KCl $\sim$ 1 μM.
[‡] Cells were lysed in lysis buffer containing 0.15 or 1.2 M KCl with or without DNase I. TRITC-phalloidin (0.6 μM) was added either at the time of lysis (T$_0$) or after 5 minutes (T$_5$). The percent change in F-actin after 5 min was the ratio of the TRITC-phalloidin staining at T$_5$ relative to the T$_0$.
[§] DNase concentrations adequate to bind all G-actin were added (10 μM up to 10^7 cells/ml, 40 μM for 10^8 cells/ml, and 120 μM for 3 × 10^8 cells/ml).
[¶] Cell concentration is in cells per ml in lysis buffer.

were lysed for 24 h before the addition of TRITC-phalloidin, the F-actin level decreased by $\sim$50% in 0.15 M KCl and by 100% in 1.2 M KCl for all cell concentrations examined (3 × 10^5 to 3 × 10^8 cells/ml in the presence of DNase).

Some Filament Barbed Ends Appear to Be Free and Can Nucleate Actin Elongation

The ability of lysates to nucleate the polymerization of exogenous actin suggested that capping of barbed ends could not account for the stability of the F-actin remaining in lysates of 2 × 10^6 cells/ml. As shown in Fig. 5, rhodamine actin polymerized from the cytoskeleton of cells attached to coverslips (Fig. 5 a). The addition of cytochalasin (Fig. 5 b) or 35 nM gelsolin/actin complex (not shown) inhibited this polymerization suggesting that the growth was from barbed ends. The amount of polymerization was decreased on cells lysed for 1 h before addition of rhodamine actin (Fig. 5 c), but sites that initiate growth in a cytochalasin-sensitive manner were still present (Fig. 5 d). Quantitative studies of cells lysed in suspension have also shown that after 1 h of depolymerization, the lysates can still nucleate elongation of exoge-

nous pyrenyl actin (Cano et al., 1991). In these studies, the time course of the loss of nucleation sites paralleled the loss of filaments as determined from depolymerization kinetics (Cano et al., 1991).

Furthermore, simply blocking barbed ends would be insufficient to account for the stability of the stable pool of F-actin. For example, if the pointed ends were free to depolymerize, a 1.5-μm filament would depolymerize in 3 h. Filaments are much shorter than 1.5 μm ($\sim$80% are <0.2 μm [Cano et al., 1991]) and yet, in lysates at 0.15 M KCl, the F-actin persisted overnight (see below). Even though no evidence can be found in the literature for a pointed end capping factor, our data does not rule out the possibility that some of the pointed ends in the lysates may be capped. A pointed end capping activity could contribute to the F-actin stability. For cappers to account for the F-actin stability, filaments would have to be capped at both their barbed and pointed ends. Alternatively, lateral binding factors could be inhibiting F-actin depolymerization from both ends while maintaining available sites for polymerization as indicated in Fig. 5.

In summary, at a cell concentration of 3 × 10^5 cells/ml in 0.15 M KCl and at cell concentrations up to 10^7 cells/ml in 1.2 M KCl, neither G-actin nor barbed-end–capping factors appear able to account for the stability of the F-actin.

Increasing KCl Concentration Increases the Rate and Extent of Depolymerization in Lysates at Low Cell Concentrations

Increasing the KCl concentration in the lysates of control and peptide-stimulated cells increased both the rate and extent of F-actin depolymerization (Fig. 6, a and b). At each time point, the fraction of F-actin that had depolymerized was greater at the higher salt concentrations.

The rate of F-actin depolymerization at infinite dilution is proportional to the number of filaments depolymerizing:

$$\frac{dF}{dt} = -k_{off,b}N_b - k_{off,p}N_p \qquad (1)$$

where F is the F-actin; $k_{off,b}$ and $k_{off,p}$, the dissociation rate constants for the barbed and pointed ends, respectively; N_b, the number of barbed ends; and N_p, the number of pointed ends. When all of the actin filaments are free to depolymerize, $N_b = N_p = N$, Eq. 1 can be written as:
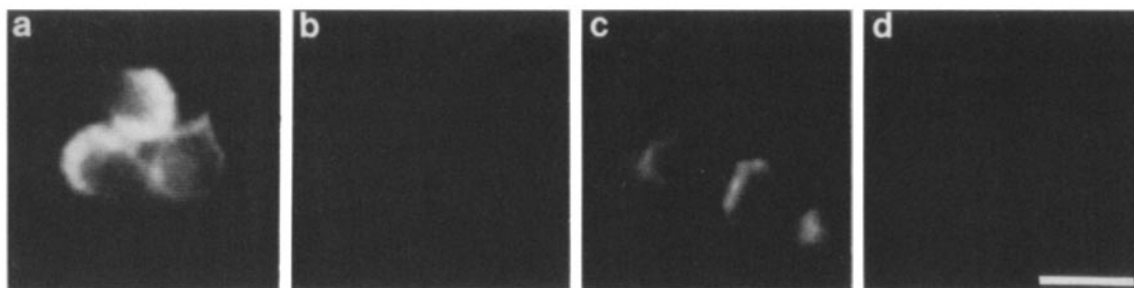

Figure 5. Polymerization of rhodamine actin from cytoskeletons. Cells attached to coverslips as in Fig. 1 were lysed for 30 s in lysis buffer containing 1 μM unlabeled phalloidin (a and b) or 1 h in lysis buffer without phalloidin (c and d) before incubation for 2 min in 1 μM rhodamine actin with (b and d) or without (a and c) 2 μM cytochalasin B. All cells were photographed and printed under identical conditions. This resulted in prints which did not show the faint rhodamine fluorescence of cytochalasin treated samples that could be seen under the microscope. Bar, 10 μm.
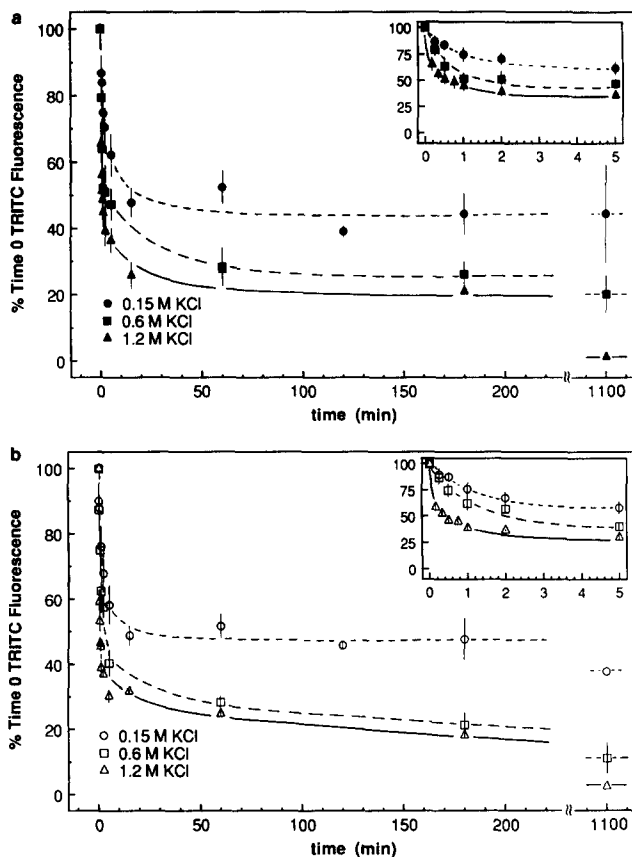
Figure 6. Effect of KCl concentration on the depolymerization time course of F-actin in cell lysates. (*a*) Cells were incubated at room temperature for 90 s in cell buffer and then lysed at 2×10^6 cells/ml in buffer containing 10 μM DNase I and either 0.15 (●), 0.6 (■), or 1.2 M KCl (▲). At various times after lysis, 0.6 μM TRITC-phalloidin was added to stop depolymerization. Samples were then processed as described in Materials and Methods for quantitation of F-actin. The data (mean ± SEM of N experiments with duplicate samples, N = 3, 3, and 5 for 0.15, 0.6, and 1.2 M KCl, respectively) are plotted as percent fluorescence relative to the amount of fluorescence at time zero. The inset shows the first five minutes of the time courses. (*b*) Cells were incubated at room temperature for 90 s in cell buffer containing 0.02 μM FNLLP and then lysed as described in (*a*). Symbols for different salt concentrations are given in (*a*). The data (mean ± SEM of N experiments with duplicate samples, N = 3, 3, and 5 for 0.15, 0.6, and 1.2 M KCl, respectively) are plotted as percent fluorescence relative to the amount of fluorescence at time zero. The inset shows the first five minutes of the time courses.

$$\frac{dF}{dt} = -(k_{off,b} + k_{off,p})N \tag{2}$$

where N is the number of filaments depolymerizing and $(k_{off,b} + k_{off,p})$ is the overall dissociation rate constant. Following the depolymerization of pyrene-labeled muscle actin, we determined that the overall dissociation rate constants increased 1.5- and 2.5-fold as the KCl concentration in the lysis buffer increased from 0.15 to 0.6 and 1.2 M KCl, respectively. The increase in rate of depolymerization of cellular F-actin in lysates in high KCl was greater than could be accounted for by these increases in dissociation rate constants. Increasing the KCl in the lysis buffer from 0.15 to 0.6 and 1.2 M increased the cell F-actin depolymerization rates by
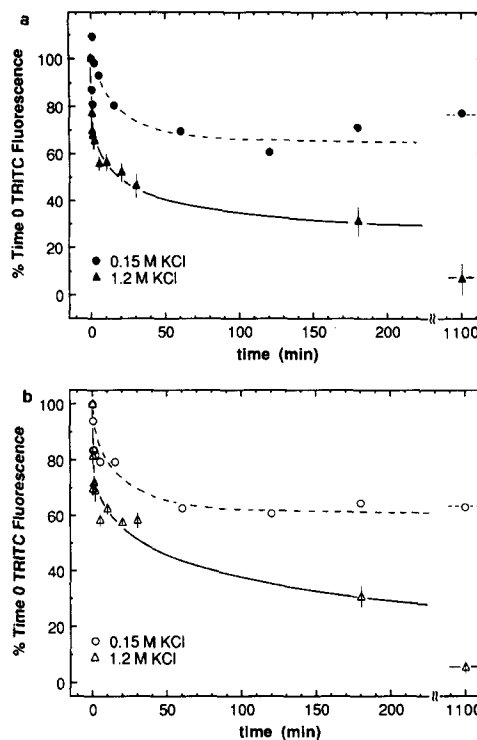

Figure 7. Effect of cytochalasin and KCl on time course of depolymerization. (*a*) Cells were incubated at room temperature for 90 s in cell buffer and then lysed at 2×10^6 cells/ml in buffer containing 0.2 μM cytochalasin D, 10 μM DNase I and either 0.15 (●) or 1.2 M KCl (▲). At various times after lysis, 0.6 μM TRITC-phalloidin was added to stop F-actin depolymerization. Samples were then processed as described in Materials and Methods for quantitation of F-actin. The data (mean ± SEM of N experiments with duplicate samples, N = 1 and 4 for 0.15 and 1.2 M KCl, respectively) are plotted as percent fluorescence relative to the amount of fluorescence at time zero. (*b*) Cells were incubated at room temperature for 90 s in cell buffer containing 0.02 μM FNLLP and then lysed and processed as described in (*a*). The data (mean ± SEM of N experiments with duplicate samples, N = 1 and 4 for 0.15 and 1.2 M KCl respectively) are plotted as percent fluorescence relative to the amount of fluorescence at time zero.

1.6- and 3.9-fold for unstimulated cells and 1.7- and 6.0-fold for stimulated cells, respectively. Thus, increasing the KCl concentration must increase the initial depolymerization rate by increasing the number of filaments (N) able to depolymerize. This increase could be because of the removal of barbed-end–capping factors and/or side-binding factors which were inhibiting depolymerization.

Increasing salt concentration also increased the rate of depolymerization in the presence of 0.2 μM cytochalasin D (CD) (Fig. 7, *a* and *b*). This further indicated that increased salt was not merely removing a barbed end cap. The initial rate of depolymerization (0 to 5 min) was CD sensitive in both 0.15 and 1.2 M KCl, suggesting that barbed ends were contributing to the depolymerization observed in Fig. 6, *a* and *b* (compare Fig. 6, *a* and *b* with Fig. 7, *a* and *b*). The rate of depolymerization at later times (60–180 min) was only slightly CD sensitive (compare Fig. 6, *a* and *b* with Fig. 7, *a* and *b*). However, the fact that even after 60 min we observed barbed end elongation sites suggested that the inhibition of depolymerization at this time cannot be completely
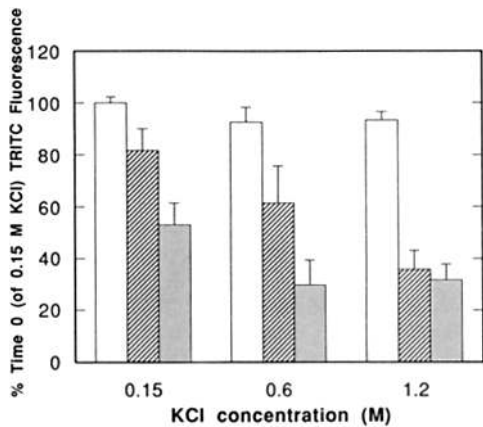
Figure 8. Increasing KCl releases F-actin from complexes that pellet in low gravitational forces. Cells at 2×10^6 cells/ml were lysed in lysis buffer containing 0.15, 0.6, or 1.2 M KCl. To some samples, TRITC-phalloidin was added at the time of lysis. After 1 h of staining, the lysates were spun at low g ($\sim$86,000 $g \times$ min) to determine what fraction of F-actin was associated with the Triton insoluble cytoskeleton (▨) or at high g (5,190,000 $g \times$ min) to determine the total F-actin present initially (□). The F-actin remaining after 60 min of depolymerization was determined from samples to which the TRITC-phalloidin was added 60 min after lysis. After staining for 1 h, these samples were also spun at high g (▧) or at low g (not shown). Data show the fluorescence of the TRITC-phalloidin extracted from the pellets (mean ± SEM) for duplicate samples normalized to the fluorescence extracted from the high speed spin of cells lysed directly into TRITC-phalloidin in 0.15 M KCl.

explained by capping of barbed ends. The presence of CD during lysis of unstimulated cells in 0.15 and 0.6 M KCl frequently resulted in a transient increase in F-actin for reasons unknown (e.g., see Fig. 7 *a*).

High KCl Decreased the Fraction of F-actin that Pelleted at Low Gravitational Forces

It is possible that filament crosslinking or lateral binding factors contribute to the stability of cellular F-actin in vitro. Increasing salt concentration appeared to increase actin depolymerization by releasing filaments from the cytoskeleton (Fig. 8). In 0.15 M KCl, 82% of the TRITC staining that pelleted at high g (5.19 $\times$ 10^6 $g \times$ min) also pelleted at low g (8.6 $\times$ 10^4 $g \times$ min) (see Materials and Methods). Increasing the KCl concentration to 0.6 and 1.2 M KCl decreased the fraction of cellular F-actin that could be pelleted at low g to 62 and 36%, respectively. In all salt conditions, more than 80% of the F-actin that remained after 60 min of depolymerization (i.e., pelleted at high g) could be pelleted at low g (not shown) indicating that this stable F-actin was present primarily in large complexes.

Examination of Proteins in the Cytoskeletal Fraction

To determine factors that may be responsible for the F-actin stabilization, the proteins present in the F-actin pellet were examined by quantitative gel scanning and by Western blots at 1, 5, and 60 min after lysis of stimulated cells in lysis buffer containing 0.6 M KCl. The Coomassie blue–stained actin band on the SDS gel showed 64, 45, and 34% of the
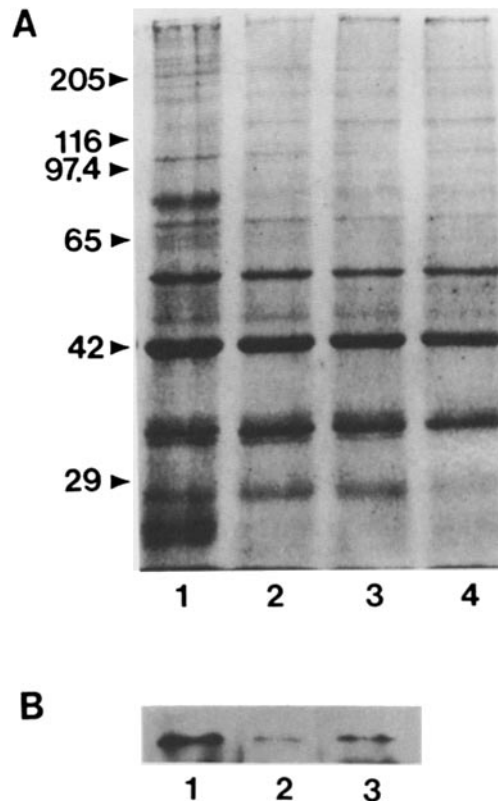

Figure 9. Protein composition of cytoskeletons after lysis. (*a*) SDS-polyacrylamide gel (10%) of cytoskeletal fractions (prepared from lysates as described in Materials and Methods) 0 (lane *1*), 5 (lane *2*), 55 (lane *3*), or 180 min (lane *4*) after cell lysis. The positions of the molecular weight standards are shown to the left. (*b*) Immunoblot showing association of α-actinin with the cytoskeleton 1 h after lysis in buffer containing 0.6 M KCl. Cytoskeletons were prepared either immediately after lysis (lane *1*), 10 μl load; lane *2*, 5 μl load) or 1 h after lysis (lane *3*, 10 μl load).

initial F-actin at 1, 5, and 60 min after lysis (Fig. 9 *A*) (comparable to the 63, 41, and 28%, respectively, determined by TRITC staining, Fig. 6 *b*). Numerous other proteins, in addition to actin, remain in the pellet and may be associated with the nucleus or intermediate filaments rather than actin. However, immunoblots indicated that tropomyosin, actin-binding protein, and α-actinin were present with the stable F-actin in the pellet even after 1 h in lysis buffer containing 0.6 M KCl (data not shown). Furthermore, immunoblots for α-actinin (Fig. 9 *B*) indicated that after 1 h in lysis buffer containing 0.6 M KCl the amount of α-actinin that pelleted at low speed decreased by only 10 to 25%. Since more than 60% of the actin was lost, the F-actin remaining after 60 min may be more highly bound by α-actinin than that which had depolymerized.

Pyrenyl F-actin Depolymerization Is Inhibited by α-Actinin

To test if filament crosslinking proteins could be responsible for the F-actin stabilization, we examined the ability of α-actinin to inhibit the rate of pyrenyl F-actin depolymerization (Fig. 10). α-Actinin has been shown to crosslink actin filaments (for review see Blanchard et al., 1989). We incubated 1 μM pyrenyl F-actin with and without 1 μM α-actinin for
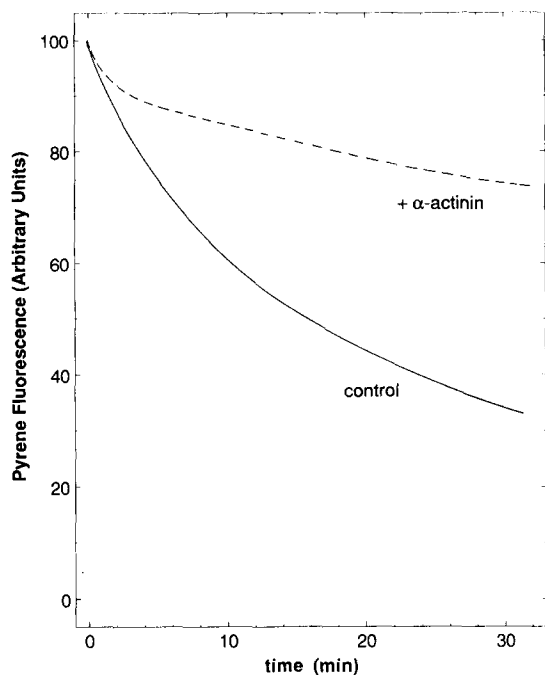
Figure 10. F-actin depolymerization is inhibited by α-actinin. Pyrenyl F-actin (1 μM) was preincubated with and without 1 μM α-actinin for 2 h at room temperature. DNase I (5 μM) was then added to induce depolymerization. The pyrenyl F-actin depolymerization was followed by losses in pyrene fluorescence. The data are plotted as the percent fluorescence relative to the amount of fluorescence at time zero.

2 h and then initiated depolymerization of the F-actin by adding 5 μM DNase I. We found that α-actinin did inhibit the rate of F-actin depolymerization. Thus, it is possible that filament cross-linking proteins such as α-actinin contribute to the F-actin stabilization observed in our lysates.

Discussion

Polymorphonuclear leukocyte F-actin in vivo and in vitro exists in stable and labile pools. However, the labile pool in vivo does not correspond to the labile pool in vitro. In vivo, the labile pool is induced by chemoattractant, is localized in the lamellipodia, and has a half-life of ~15 s at room temperature (Cassimeris et al., 1990). In lysates, the labile pool is present in control as well as peptide-stimulated cells, does not correlate with lamellipodial F-actin and has a half-life of about 1 min at room temperature. Thus various factors that regulate F-actin in the cell must be altered upon lysis.

In some cases (e.g., the regulation of the cell cycle) increasing the concentration of cells in a lysate allows continuation of cell processes in a cell-free system (for reviews see Lohka, 1989; Murray and Kirschner, 1989). However, while we have not yet achieved the degree of lysate concentration used in the cell-cycle studies, the alterations in F-actin depolymerization occurring upon lysis do not appear to be simply a result of dilution since increasing the cell concentration in the lysate decreased rather than increased the rate of depolymerization. It is likely that for F-actin, which is spatially regulated in the cell, mere concentration will not be sufficient to mimic in vivo actin dynamics.

In many studies of lysed cells, a pool of F-actin has been observed that fails to depolymerize even after prolonged incubations. For example, membranes from HeLa cells dialyzed 18 h at 4°C were shown to retain ~40% of their actin (Gruenstein et al., 1975). Because a portion of the F-actin does not readily depolymerize, investigators have resorted to various techniques such as: gelsolin to fragment the F-actin (Otto and Schroeder, 1990), trypsin to degrade actin-binding proteins (Atkinson et al., 1982), and denaturing agents such as ammonium sulfate (Fey et al., 1984), urea (Nelson and Veshnock, 1986), 1% Tween with 0.5% deoxycholate (Fey et al., 1984), and potassium iodide (Fath and Lasek, 1988) to remove the F-actin from their cell fractions.

Factors that Contribute To F-actin Stability

We have examined factors that contribute to this F-actin stability. Were the actin filaments in a cell lysate free to depolymerize from either end, in 18 h all filaments ≤9 μm would have depolymerized completely in 0.15 M KCl (or ≤24 μm in 1.2 M KCl) (pointed end off-rates = 0.05 and 0.13 s⁻¹ in the two salt concentrations, respectively) [Weber et al., 1987; our own unpublished results). Since most filaments in cells are ~0.2 μm (Cano et al., 1991) they would have depolymerized in about 0.3–0.7 min if they were completely free or 9–24 min if only the pointed end was free. Thus, the cellular F-actin must not be free to depolymerize readily from either the barbed or the pointed end.

At high cell concentrations, the concentration of G-actin is well above the critical concentration. Thus, if it is not sequestered, it should polymerize. Indeed, we find G-actin does polymerize, and this polymerization can be inhibited by the presence of DNase. However, merely removing G-actin does not lead to depolymerization (Table I).

At some cell lysate concentrations, barbed end capping factors may be contributing to F-actin stabilization. Although we could not determine whether all of the filament barbed ends were free, after lysis at low cell concentrations, some filaments in the stable pool do not appear to be blocked at their barbed ends since lysates are able to serve as elongation sites for polymerization of exogenous actin and this polymerization is severely inhibited by the presence of cytochalasin (Fig. 5) or gelsolin/actin complex (not shown) suggesting that it occurs at the filament barbed ends. That these nucleation sites are free barbed ends is suggested by studies showing the number of barbed end growing sites is similar to the number of free barbed ends as assayed by depolymerization, i.e., depolymerization inhibited by CD (Cano et al., 1991). Thus, while the number of elongation sites in the lysate decreased with time after lysis, some sites remained even when the cytoskeleton was undergoing almost no depolymerization, i.e., only the stable pool of F-actin remained (Cano et al., 1991). Thus, additional factors appear to inhibit this depolymerization.

Increasing the salt concentration from 0.15 to 1.2 M KCl increased both the number of filaments undergoing depolymerization and the extent of F-actin depolymerization. Thus, the increased fraction of the total F-actin that depolymerized in 15 min (from 50% in 0.15 M KCl to 70% in 1.2 M KCl) was not merely because of an increase in the length of each filament depolymerizing. While it was not possible to determine conclusively whether the length distribution of the filaments released by high KCl was the same as those depolymerizing in 0.15 M KCl, were salt merely removing some

block to depolymerization along the length of the filament, one would expect to see an increase in the duration of depolymerization with no increase in the number of filaments (i.e., the initial rate of depolymerization). This was not observed. This suggests that increasing salt concentration increases the number of filaments depolymerizing without significant changes in the mean length of the depolymerizing pool.

Several pieces of evidence suggest that stable F-actin is linked to itself and/or other cross-linking factors. These may be contributing to the stability of F-actin in vitro. (a) Lysed cells retain the morphology of their actin meshwork suggesting that the stable filaments are crosslinked in the meshwork (Figs. 1 and 2). (b) Increasing salt concentration decreased the fraction of cellular F-actin that could be rapidly pelleted (i.e., filaments were detached from the cytoskeleton) and also increased the fraction that rapidly depolymerized (Figs. 6, 7, and 8). (c) Many actin cross-linking proteins including α-actinin and actin-binding protein are known to be present in Triton insoluble cytoskeletons and high salt is known to increase their release into the supernatant (Fig. 9; Schliwa and van Blerkom, 1981; Rosenberg et al., 1981; Pacaud, 1986).

We have not attempted to assign the inhibition to a particular actin cross-linking protein since a number of F-actin-binding and cross-linking proteins are present and they all may contribute to the inhibition (Pacaud, 1986). Removal of any one factor may have only a small effect on the stability of the F-actin. Presumably, F-actin cross-linking factors contribute to the stability of echinoderm sperm acrosomal processes (Tilney, 1973). It is known that myosin rigor complexes can inhibit depolymerization (Detmers et al., 1981). These results led us to the idea that filament cross-linking proteins may be responsible for the F-actin stabilization observed in PMN lysates. We have evidence that α-actinin inhibits the rate of F-actin depolymerization (Fig. 10). Further experiments characterizing this inhibition are in progress. Tropomyosin also inhibits depolymerization of F-actin, particularly from the pointed end of the filament (Broschat et al., 1989; Weight et al., 1990). Thus, tropomyosin and α-actinin, two proteins found associated with the stable F-actin, probably contribute to the stabilization observed here. In addition, we now have evidence that the 30-kD actin cross-linking protein from *Dictyostelium* (Fechheimer et al., 1991) is a potent inhibitor of actin depolymerization (Zigmond, S. H., R. Furukawa, and M. Fechheimer. 1991. *J. Cell Biol.* 115:329a).

Physiological Significance of F-actin Stabilization

The dramatic inhibition of depolymerization in lysates suggests that the binding properties of the inhibitory factors must be modified in vivo to allow the dynamic properties of F-actin. An increase in the affinity of cross-linking proteins in lysates could account for the inhibition of depolymerization. The conclusion that in vitro complexes of F-actin with actin-binding proteins are different than the complexes formed in vivo was also reached by Janmey et al. (1990) when considering the very slow deformability of gels made of F-actin and actin-binding protein in vitro. Such gels could not accommodate the rapid shape changes seen in vivo (Janmey et al., 1990). In vivo, the actin meshwork must be sufficiently crosslinked to give it mechanical strength but

must also be able to change shape and readily disassemble through depolymerization.

In summary, we have examined F-actin depolymerization in PMN lysates to begin to understand the regulation of F-actin depolymerization in vivo. We have identified several factors that contribute to F-actin stability in lysates. By examining the F-actin in lysates of various cell concentrations, we have characterized the effects of G-actin, barbed end capping factors, and cross-linking factors on the F-actin stability in the lysate. The effects of these stabilizing factors should be considered in in vivo studies of cell F-actin depolymerization.

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