The Actin Released from Profilin—Actin Complexes Is Insufficient to Account for the Increase in F-Actin in Chemoattractant-stimulated Polymorphonuclear Leukocytes

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Abstract. Chemoattractant stimulation of polymorphonuclear leukocytes is associated with a nearly twofold rise in actin filament content. We examined the role of the actin monomer sequestering protein, profilin, in the regulation of PMN actin filament assembly during chemoattractant stimulation using a Triton extraction method. Poly-L-proline-conjugated Sepharose beads were used to assess the relative concentration of actin bound to profilin with high enough affinity to withstand dilution (profilin—actin complex) and DNase I-conjugated beads to measure the relative concentration of actin in the Triton-soluble fraction not bound to profilin. Actin associated with the Triton-insoluble fraction (F-actin) was also measured. In unstimulated PMN, the relative concentration of actin bound to profilin was maximum. After FMLP stimulation, profilin released actin monomers within 10 s, with the profilin—actin complex concentration reaching a nadir by 40 s and remaining low as long as the cells were exposed to chemoattractant (up to 30 min). If FMLP was dissociated from PMN membrane receptors using t-BOC, actin reassociated with profilin within 20 s. Quantitative analysis of these reactions, however, revealed that profilin release of and rebinding to actin could account for only a small percentage of the total change in F-actin content. Determination of the total profilin and actin concentrations in PMN revealed that the molar ratio of profilin to actin was 1 to 5.2. When purified actin was polymerized in PMN Triton extract containing EGTA, removal of profilin from the extract minimally affected (12% reduction) the high apparent critical concentration at which actin began to assemble. Although profilin released actin at the appropriate time to stimulate actin assembly during exposure to chemoattractants, the concentration of profilin in PMN was insufficient to explain the high unpolymerized actin content in unstimulated PMN and the quantity of actin released from profilin too small to account for the large shifts from unpolymerized to polymerized actin associated with maximal chemoattractant stimulation.

Polymorphonuclear leukocytes undergo rapid changes in morphology in response to various stimuli. Chemoattractants induce polarization and formation of lamellipodia; phagocytic particles induce formation of pseudopods and attachment to surfaces induce spreading and lamellipodia formation. All of these stimuli also induce a nearly twofold rise in actin filament content (11, 16, 36, 40, 45, 46). In unstimulated PMN, a high percentage of the total actin (60-70% or ~200 μM, assuming a uniform distribution) is in an unpolymerized state. After chemoattractant stimulation there is rapid filament assembly, F-actin content reaching a maximum (60-70% of total actin, 200 μM) and G-actin content reaching a nadir (30-40% of total actin, 100 μM) within 30-60 s. These observations indicate that the high concentrations of unpolymerized actin in resting PMNs are capable of being incorporated into filaments following stimulation.

Profilin is an attractive candidate for maintaining the high concentrations of monomeric actin in resting PMN and for regulating the release of actin monomers after stimulation. This protein is a 15,500-kD polypeptide that binds actin in a 1:1 complex and prevents monomers from being incorporated into filaments. Profilin was initially purified from spleen (7). Subsequently a protein of similar molecular weight and function has been purified from brain, thymus (4), platelets (14, 28), and macrophages (10). The human profilin gene has recently been cloned and Northern blot analysis has revealed that this protein is expressed in a wide variety of tissues including liver, uterus, kidney, and heart (18). A protein with a lower molecular weight, 12,800 kD, but similar function, has also been purified from Acanthamoeba (35, 43) and Physarum (34).

Two major questions concerning the role of profilin in regulating the state of actin in nonmuscle cells have not been
clearly answered by these investigators. First, in stimulated cells is the release of actin monomers from profilin temporally associated with stimulus induced actin filament assembly? The possible role of profilin in regulating the shift from G to F-actin has been recently addressed by Lasing and Lindberg (23) who have found the profilin-actin complexes can be dissociated by phosphoinositol bisphosphate, a phosphatidyl inositol by-product whose turnover increases after chemotactic stimulation. Using platelets two groups of investigators studied the effects of thrombin stimulation, a stimulus that induces rapid actin filament assembly in platelets. Their results were contradictory. Markey et al. (29) found maximal concentrations of actin bound to profilin in resting platelets and observed a rapid dissociation of actin from profilin in response to thrombin stimulation, whereas Lind et al. (25) found low concentrations of actin bound to profilin in resting platelets and during initial thrombin stimulation observed an increase in the concentration of actin bound to profilin. These latter findings suggested that profilin might not play a role in regulating the initial rise in actin filament content induced by thrombin, since profilin was shown to be sequestering monomers at the same time actin filaments were assembling.

A second question to be clarified is whether or not the concentrations of profilin found in the cytoplasm can totally account for the high concentrations of unpolymerized actin found in unstimulated nonmuscle cells. Estimates in nonmuscle cells of the contribution of profilin-actin complexes to the total unpolymerized actin pool have ranged from 14-100% (4, 7, 14, 29, 35, 44).

We have shown that human PMN also contain a protein with physical–chemical and functional characteristics similar to previously purified mammalian profilins. Using poly-l-proline-conjugated Sepharose beads we have examined the effects of chemotactic stimulation on high-affinity profilin-actin complex concentration in human PMN, as well as attempted to determine the contribution of profilin in maintaining the high concentration of monomers found in PMN. In unstimulated PMN, like Markey et al., we have found profilin-actin complex concentration to be maximal. Following stimulation at all time points studied (10 s to 30 min) profilin released actin monomers. The concentrations of profilin found in PMN, however, could account for less than one third of unpolymerized actin in resting PMN. In addition during chemoattractant stimulation the relative concentrations of monomeric actin released by profilin were too small to account for the concomitant large increases in F-actin content.

**Materials and Methods**

**Purification of Profilin from PMN**

200 ml of freshly expired pharesis granulocytes (3.5-5 × 10⁷ cells/ml WBC) were obtained from the American Red Cross. The remaining red cells were removed by dextran sedimentation (one part 4 g/dl dextran T500 to four parts PMN solution) and hypotonic lysis as previously described (39). Wright-stained specimens were shown to contain >90% neutrophils. PMNs were pretreated with disopropyfluorophosphate as previously described (1) and washed with PBS containing 5 mM N-ethylmaleimide (NEM) before lysis. We discovered that this condition dissociated profilin-actin complexes (see Results). The cell pellets were next suspended in a 1:1 vol of homogenization buffer containing 0.34 M sucrose, 5 mM DTT, 5 mM ATP, 5 mM EGTA, 1 mM PMSF, 0.075 mM benzamidine, 0.01 mg/ml leupeptin, 0.04 mg/ml aprotonin and 20 mM imidazole-HCl, pH 7.5 and broken by a Dounce homogenizer as previously described (40). The mixture was centrifuged at 12,000 g for 30 min. The resulting supernatant was incubated overnight at 4°C after the addition of final concentrations of 0.1 M KCl and 2 mM MgCl₂. The solution was then subjected to ultracentrifugation at 100,000 g for 1 h. This supernatant was applied directly to a 0.5 × 7.0 cm poly-l-proline Sepharose column equilibrated in homogenization buffer, washed with five column volumes of buffer A (10 mM imidazole chloride, pH 7.4, 1 mM DTT, 0.1 M KCl), then eluted with the same buffer containing 5 mM poly-l-proline and washed finally with additional buffer A. The 3.5-ml eluate was concentrated with a microconcentrator (CentriPREC-10; Amicon Corp., Danvers, MA) to 0.5 ml and applied to a 1.0 × 20 cm ACA-54 ultragel column (IBF Biotechniques, Villeneuve-la-Garenne, France) in buffer A. Protein concentrations were determined by UV absorption using the previously reported extinction coefficient for mammalian profilin (22) or by the Bradford method (5) using an immunoglobulin standard. Simultaneous determination of profilin concentration using both assays resulted in nearly identical values.

Peak fractions, identified by absorbance at 280 nm, were analyzed by SDS-PAGE on 12.5% gels and for their ability to prolong the lag period for the polymerization of gel filtered G-actin containing 6% pyrene-labeled actin. The concentration of pyrenyl-actin was kept below the critical concentration for actin filament assembly to reduce the artifact resulting from the low affinity of profilin for pyrenyl actin (19, 27).

**Isoelectric Focusing**

Isoelectric focusing was performed as described by O’Farrell (33) using amphotoline polyacylamide gels, pH 3.5-9.5 (PAGplates; LKB, Bromma, Sweden) using a Multiphor II system. 4.5 μg of purified profilin was applied to paper ploquets placed at two locations on the gel, and focused for 3/2 h at 10 W.

**Purification of Human PMN for Triton Extract Studies**

40 ml of whole blood from healthy adult volunteers was drawn into syringes containing 3.2 ml of a 10 g/dl EDTA (Sigma Chemical Co., St. Louis, MO) solution. In the majority of experiments neutrophils were purified by centrifugation through Ficoll/Hypaque solution (36). In some experiments PMN were purified by hypotonic lysis and differential centrifugation as previously described (36). Purified cells were suspended in a modified Hanks’ solution containing 3.2 ml of a 10 g/dl EDTA (Sigma Chemical Co., St. Louis, MO) solution. For PMN treated with 1 mM EGTA, 200 ml of freshly expired pharesis granulocytes (3-5 × 10⁷ cells/ml WBC) were obtained from the American Red Cross. The mixture was centrifuged at 12,000 g for 2 min at 12,000 g in a tabletop centrifuge. The supernatant was removed by dextran sedimentation (one part 4 g/dl dextran T500 to four parts PMN solution) and hypotonic lysis as previously described (39). Wright-stained specimens were shown to contain >90% neutrophils. PMNs were pretreated with disopropylfluorophosphate as previously described (1) and washed with PBS containing 5 mM N-ethylmaleimide (NEM) before lysis. We discovered that this condition dissociated profilin-actin complexes (see Results). The cell pellets were next suspended in a 1:1 vol of homogenization buffer containing 0.34 M sucrose, 5 mM DTT, 5 mM ATP, 5 mM EGTA, 1 mM PMSF, 0.075 mM benzamidine, 0.01 mg/ml leupeptin, 0.04 mg/ml aprotonin and 20 mM imidazole-HCl, pH 7.5 and broken by a Dounce homogenizer as previously described (40). The mixture was centrifuged at 12,000 g for 30 min. The resulting supernatant was incubated overnight at 4°C after the addition of final concentrations of 0.1 M KCl and 2 mM MgCl₂. The solution was then subjected to ultracentrifugation at 100,000 g for 1 h. This supernatant was applied directly to a 0.5 × 7.0 cm poly-l-proline Sepharose column equilibrated in homogenization buffer, washed with five column volumes of buffer A (10 mM imidazole chloride, pH 7.4, 1 mM DTT, 0.1 M KCl), then eluted with the same buffer containing 5 mM poly-l-proline and washed finally with additional buffer A. The 3.5-ml eluate was concentrated with a microconcentrator (CentriPREC-10; Amicon Corp., Danvers, MA) to 0.5 ml and applied to a 1.0 × 20 cm ACA-54 ultragel column (IBF Biotechniques, Villeneuve-la-Garenne, France) in buffer A. Protein concentrations were determined by UV absorption using the previously reported extinction coefficient for mammalian profilin (22) or by the Bradford method (5) using an immunoglobulin standard. Simultaneous determination of profilin concentration using both assays resulted in nearly identical values.

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**Actin Association with the Triton-insoluble Cytoskeleton**

A modification of the method described by White et al. (46) was used. Purified human PMN, final concentration of 1.5 × 10⁷ cells per ml, were prewashed at 37°C for 10 min before stimulation and then stimulated with FMLP (Sigma Chemical Co., St. Louis, MO) (final concentration 5 × 10⁻⁹ M). In some experiments FMLP binding was reversed using the antagonist t-butoxycarbonyl-leu-leu-ple-leu-phe (t-BOC). For these experiments, PMN were stimulated with a final concentration of 1 × 10⁻⁷ M for 40 s followed by the addition of a final concentration of 1 × 10⁻⁷ M t-BOC for 20 s. The reaction was stopped by the addition of 1/10 vol of a stock solution containing 10% Triton X-100, 10 mM dithiobisulfite, 0.075 mg/ml benzamidine, 0.04 mg/ml aprotonin, 0.01 mg/ml leupeptin, and 1 mM PMSF in modified Hanks’ buffer solution. After mixing by two gentle inversions, the solution was left standing for 2 min at 25°C, and then centrifuged for 2 min at 12,000 g in a tabletop centrifuge. The supernatant was decanted and placed on ice (see below). The pellet, representing the Triton-
Determination of Actin and Profilin Content in Triton-treated PMN Supernatants

The Triton-soluble supernatant, described above, was added to 60-90 μl of a 1:1 mixture of poly-L-proline (PLP) beads and modified Hank’s buffer, rotated at 4°C for 3 h, and then centrifuged at 12,000 g for 2 min. Previous experiments using purified profilin demonstrated that this volume of beads bound 22-33 μg of profilin, a capacity considerably higher than the total profilin content of our PMN extracts (see Results). In some experiments the supernatant fluid was added to a second set of PLP beads. This step failed to find significant concentrations of either profilin or actin. The supernatant was next incubated for 3 h with 60 μl of a 1:1 mixture of DNase I-Sepharose beads and modified Hank’s buffer, followed by centrifugation at 12,000 g.

After incubation in the supernatants, the beads were mixed with 30 vol of 0.3 M MgCl₂; in modified Hank’s buffer, mixed and centrifuged. Beads were then washed a second time with 30 vol of modified Hank’s solution. MgCl₂ was used in our initial wash because this ionic condition has been shown to depolymerize actin filaments associated with Sepharose beads (9, 25). MgCl₂ has also been shown to reduce the affinity of sperm profilin for actin (22), therefore PLP beads were exposed to our MgCl₂ solution for <3 min. To determine if MgCl₂ treatment dissociated PMN profilin-actin complexes, on several occasions duplicate sets of PLP beads incubated in PMN Triton extract were washed with 0.1 M KCl or 0.3 M MgCl₂. In the presence of KCl, the percentage of profilin molecules bound to actin was the same as for the wash in MgCl₂; however, KCl-treated beads also contained higher concentrations of other less tightly bound polypeptides. Further controls were also performed using profilin-actin complexes purified as described in Fig. 1. Purified PA (final concentration 12.2 μM) was incubated in 0.1 M KCl or 0.3 M MgCl₂; or in low ionic strength buffer for 2 h at 25°C. Purified actin alone (final concentration 12.2 μM) was treated identically. Samples were then centrifuged in an Airfuge (Beckman Instruments Co., Fullerton, CA) at 160,000 g for 30 min to sediment any polymerized actin. No actin polymerized in any of the PA solutions while a high percentage of the purified actin incubated in the first two solutions formed sedimentable actin filaments. Purified PA complexes (1.4 and 0.7 μM final concentrations) were also incubated with or without 0.3 M MgCl₂ or for 2 h at 4°C followed by absorption of poly-L-proline. The ratio of actin to profilin was identical in the presence or absence of MgCl₂. These findings all indicate that MgCl₂ does not destabilize high-affinity PA complex under the conditions of our experiments.

We also examined the effects of serum on profilin-actin complexes. Incubation of PMN supernatants containing 25% serum did not interfere with binding of profilin to PLP; however the relative amount of actin bound to profilin was reduced by 60%. This finding may be explained by the presence of vitamin D binding protein in serum which can remove actin from profilin-actin complexes (30). All subsequent experiments were performed in serum-free media.

Washed PLP- and DNase I-beads and lyophilized supernatant were combined with gel sample buffer and boiled for 5 min. Triton-insoluble PMN pellets, beads, and supernatant were next electrophoresed into 5-15% polyacrylamide slab gels followed by staining with Coomassie blue. Gels were scanned with a laser densitometer (Zeinith; Biomed Instruments, Inc., Fullerton, CA) and the relative peak areas were determined by integration using an Apple II computer. The relative peak areas of the ~42,000-D polypeptide in the various fractions: Triton-insoluble pellet, PLP beads, DNase I-beads, and final supernatant, were determined for each experiment. The sum of these areas was defined as the total actin content. The percent of total PMN actin in each fraction was calculated for each experiment. The sum of the areas of the individual fractions was equal to the area of the ~42,000-D polypeptide in whole cell extract. The relative increase in F-actin content after chemoattractant stimulation was also calculated as previously described (46).

Using known concentrations of purified actin, we found a linear relationship between the area of the actin peak on Coomassie blue-stained polyacrylamide slab gels and actual protein content in the range of 2.5-30 μg of actin. Using mixtures of purified profilin and skeletal muscle actin, we also determined that under the conditions of our experiments, profilin bound Coomassie blue 90%, as well as actin on a weight basis. This difference was taken into account in calculating actin/profilin molar ratios.

Determination of the Total Content of Profilin and Actin in Unstimulated Human PMN

Total protein concentration of PMN (1 x 10⁶) was determined using the Biuret method (21) and the concentrations of actin and profilin calculated based on scanning densitometry. The denominator for determining the percentage of the total protein represented by actin and profilin was the total integration units found in whole cell extract or the sum of the areas of the polypeptides found in the four fractions of Triton-extracted PMN. Similar values were derived using either denominator. Total actin content was defined as either the area under the 42 Kd polypeptide in whole cell extract or sum of the 42-Kd polypeptide areas from the Triton-soluble and insoluble fractions. Similar values for actin content were determined using either method.

Total profilin content was defined as the amount of profilin bound to PLP beads incubated in the Triton-soluble fraction. To assure that no profilin was trapped in the Triton-insoluble fraction, in several experiments the samples were vigorously sonicated on ice immediately after addition of the Triton solution. No further profilin was released by sonication. The affinity of PLP beads for profilin-actin complexes has been shown to be somewhat lower than for free profilin (42); therefore it was possible that during our wash steps profilin-actin complexes were inadvertently removed. To assure that profilin-actin complexes were not underestimated in our experiments, on several occasions the order of incubation of beads in the Triton-soluble fraction was reversed, samples first being incubated with DNase I-conjugated Sepharose beads to bind all monomeric actin including actin bound to profilin. Samples were subsequently incubated with PLP beads. The total amount of profilin bound to conjugated beads was comparable to that found

Figure 1. Coomassie blue-stained SDS-PAGE of gel filtration fractions from untreated and NEM-treated human PMN. Lane 1, representitive gel filtration fraction from extract derived from PMN not treated with NEM. The extract was first passed over a PLP-conjugated Sepharose column that was eluted with PLP. The eluted proteins were concentrated by pressure dialysis and loaded on an ACA-54 Sephacryl column. Many fractions contained a 1:1 molar ratio of actin to profilin. Others contained lower molar ratios of actin to profilin (0.5). In the later fractions, very dilute free profilin were also found. The 34-Kd polypeptide probably represents a degradation product of actin. The protein load was ~10 μg with profilin representing 2.5 μg. Lane 2, representative gel filtration fraction of PMN treated with NEM as described in Materials and Methods and then treated identically to extract shown in lane 1. The total protein load was similar to lane 2; however, since actin was bound to this preparation of profilin, the 15-KD band appears denser than in lane 1, since this polypeptide accounts for the total protein added. The yields of profilin in the non-NEM and NEM preparations depicted were nearly identical, 0.57 and 0.6 mg, respectively. Numbers and arrows represent the molecular weights in kilodaltons of known standards: Bovine plasma albumin (66 Kd), ovalbumin (45 Kd), pepsin (34.7 Kd), trypsinogen (24 Kd), β-lactoglobulin (18.4 Kd), and lysozyme (14.3 Kd). Slab gels contained 12.5% acrylamide.
Pyrenyl–Actin Studies of PMN Triton Extracts

Triton-soluble supernatants from human PMN were prepared as previously described (8). The extract was diluted with suspension buffer containing 0.1% Triton X-100, 138 mM KCl, 2 mM MgCl2, 1 mM EGTA, 1 mM CaCl2, 0.2 mM DTT, 10 mM imidizole, pH 7.4) to achieve a final protein concentration of 0.1 M KCl and 2 mM MgCl2 in the presence (solid squares) or absence (open squares) of a final concentration of 4 μM purified human PMN profilin. Actin polymerized in the absence of profilin had a lag phase of ~3 min after which the fluorescence intensity began to increase reaching a steady-state relative fluorescence intensity of 79 (read at 24 h). The same concentration of actin polymerized in the presence of profilin demonstrated a marked prolongation of the lag phase, >70 min. At 24 h, the relative fluorescence intensity was 13. The critical concentration of the actin used in these experiments was 0.36 μM. The Kd of the profilin-actin complex was calculated to be 1.1 μM. In a duplicate experiment the Kd of the PA complex was 0.9 μM.

using our standard method, with the amount of profilin–actin bound to the DNase I beads being offset by a reduction in the amount of profilin–actin complex bound to PLP beads. This finding indicated that the difference in affinity of PLP beads for profilin and profilin–actin complexes did not significantly affect our ability to quantitate the percentage of actin bound to profilin.

In some experiments the DNase I fractions were associated with a small polypeptide or peptides whose molecular weight was <15 kDa (see Fig. 3 B; lanes 5 and 6). When DNase I beads preceded PLP beads, two bands were seen, one distinct band at 15 kD and a second more diffuse band clearly migrating below 15 kDa. If the Triton-soluble supernatant was first absorbed with PLP beads followed by DNase I beads, the 15-kD band was no longer seen in the DNase fraction, indicating all PA complex was re-absorbed by PLP beads.

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Triton-soluble supernatants from human PMN were prepared as previously described (8). The extract was diluted with suspension buffer containing 0.1% Triton X-100, 138 mM KCl, 2 mM MgCl2, 1 mM EGTA, 1 mM ATP, 10 mM imidizole, pH 7.4 to achieve a final protein concentration of 1–2 mg/ml containing between 5 and 11 μM PMN actin. Extract was combined with varying concentrations of purified skeletal muscle actin containing a subcritical concentration of pyrene-labeled actin (66%) and kept at 37°C. The fluorescence intensity of these mixtures were read at 0 and 16 h. Parallel experiments were performed by incubating similar concentrations of actin in the Triton-containing buffer. Skeletal muscle actin and pyrene-conjugated actin were prepared by previously described methods (32, 41). Human PMN actin was purified using DNase I-Sepharose chromatography and formamide as previously reported (47).

Statistical Analysis

The statistical significance of differences for the means of samples were analyzed using the paired and nonpaired t test.

Results

Purification of Human PMN Profilin

PMN profilin was purified by PLP affinity chromatography and gel filtration. In the absence of NEM nearly all the profilin copurified as a 1:1 complex with actin (see Fig. 1), however, after PMN were treated with NEM, the major species purified by the same procedure was free profilin. As shown in Fig. 1, PMN profilin could be purified to apparent homogeneity after NEM treatment. 100 mg of PMN extract yielded between 0.6 mg and 1 mg of profilin. The molecular weight of PMN profilin was found to be 15,100 D by SDS polyacrylamide gel electrophoresis on 5–17% gradient gels when compared to known protein standards (see Fig. 1). The protein had a pi of 8.8. PMN profilin isolated by this method prolonged the lag period for actin polymerization (Fig. 2) and reduced the extent of actin filament assembly as observed with other mammalian profilins. The calculated dissociation constant of the PMN profilin–actin complex was 1 μM.

Partitioning of Actin in Unstimulated PMN

Using SDS-PAGE and scanning densitometry (see Figs. 3, A and B), the relative concentrations of actin associated with the Triton-insoluble cytoskeleton, actin bound with high affinity to profilin, and unpolymerized actin not complexed with profilin were estimated (see Materials and Methods). In unstimulated PMN 42.6% ± 2.0% (mean, SEM n = 14) of the total actin was associated with the Triton-insoluble cytoskeleton (F-actin), 6.8 ± 0.05% (n = 14) of the actin was bound to profilin (PA-complex) and 48.9 ± 2.1% (n = 14) was found in the Triton-soluble fraction not complexed to profilin. Assuming profilin binds with 1:1 stoichiometry, 44 ± 5% (n = 12) of the profilin molecules collected by PLP bead absorption were complexed with actin. This value is lower than would have been predicted based on the high yield of 1:1 profilin–actin complex during purification of PMN profilin (see Fig. 1, lane f). Unlike the experiments described above which used PMN within 3 h of phlebotomy, PMN used for profilin purification had been stored by the Red Cross for 24–48 h before being released for experimental use. The difference in the percentage of profilin complexed to actin in these two experimental conditions suggests that prolonged storage of PMN may increase the formation of high-affinity PA complexes.

To exclude the possibility that PMN had been inadvertently stimulated during Ficoll/Hypaque purification and to more accurately determine the true relative percentage of actin bound to profilin in “resting cells,” PMN were rapidly purified by differential centrifugation and hypotonic lysis (see Materials and Methods). Cells were suspended in modified Hanks’ solution for 30 min before Triton extraction. All procedures were performed at 4°C. The relative percentage of actin associated with the Triton-insoluble fraction was somewhat lower than Ficoll/Hypaque purified PMN 34.5 ± 1.3% (n = 4) vs. 42.6 ± 2.0% (n = 14, P = 0.06), however the percentage of actin bound to profilin did not differ significantly under the two conditions 6.4 ± 0.8% (n = 4) (Differential centrifugation) vs. 6.8 ± 0.05% (n = 14) (Ficoll/Hypaque). The lower F-actin content of these more rapidly purified PMN was associated with a significantly higher unpolymerized fraction 59.1 ± 1.6% vs. 48.9 ± 2.1%
Human PMN (7.5 × 10^6 in 500 ul) were exposed to FMLPprofilin-actin complex dissociation after exposure to chemoattractant from unstimulated PMN (lane 5). After removal of the DNase I beads the supernatant was electrophoresed on the same gel (lane 7). The G-actin fraction in supernatants from stimulated PMN is also shown: the fraction bound to DNase I beads (lane 6) and the final supernatant (lane 8). Numbers represent the molecular weights of PMN actin and profilin in kilodaltons. The low molecular weight polypeptide seen in the DNase I bead lanes migrated below profilin.

In other experiments in which samples were electrophoresed a greater distance this difference in molecular weights was even more apparent.
Figure 4. The effects of prolonged chemoattractant stimulation on the percentage of actin assembled into filaments and complexed to profilin. The same methods were used as described in Fig. 3. PMN were exposed to FMLP for the durations shown on the graph before the addition of Triton stop solution. Although F-actin content decreased with prolonged chemoattractant stimulation, the percent of actin bound to profilin remained low, whereas the percent of total actin in the unbound fraction increased.

The effects of t-BOC on profilin actin-complex formation. PMN were treated as described in Fig. 3 A except PMN were stimulated with a final concentration of $1 \times 10^{-4}$ M for 40 s followed by the addition of final concentration of $1 \times 10^{-4}$ M t-BOC for 20 s. The percentage of actin bound to profilin at each time point is depicted on the graph. Addition of t-BOC resulted in an increase in the percent of actin bound to profilin, which was comparable to unstimulated cells. Bars represent the SEM of four to seven experiments. The final point represents the mean of two experiments, values differing by only 0.3% of the total actin content.

Determination of Whole Cell Content of PMN Profilin and Actin

The number of PMN used in our experiments, $7.5 \times 10^6$, contained $1.12 \pm 0.05$ mg (SEM of $n = 6$ separate preparations of PMN) of total protein of which $222.9 \pm 4.5$ mg ($n = 52$) or $5.3$ nmol was actin and $15.8 \pm 0.5$ mg ($n = 52$) or 1.0 nmol was profilin. Only 44% of profilin molecules were bound to actin in unstimulated PMN. Because the volume of our cell preparations was $500 \mu l$, the concentration of actin in the Triton-soluble fraction was $6.1 \mu M$ in resting PMN and $3.2 \mu M$ in stimulated PMN; and the concentration of profilin $2 \mu M$. If it was assumed that 100% of the profilin molecules bound actin monomers in resting PMN, profilin could bind to and prevent polymerization of 19–20% of the total actin or 33–35% of the unpolymerized actin in unstimulated PMN. Vigorous sonication of the Triton-insoluble fraction before centrifugation failed to increase the concentration of profilin in the Triton-soluble fraction. Repeated absorption with PLP beads also failed to increase the profilin yield.

Polymerization of Purified Skeletal Muscle Actin in Triton-solubilized PMN Extracts

High concentrations of actin remained in the supernatants of Triton-solubilized extracts of unstimulated PMN. To determine the apparent critical concentration (true critical concentration plus unpolymerized actin) of monomeric actin required to initiate assembly of the Triton-soluble PMN actin, increasing amounts of purified actin containing a low concentration of pyrene-labeled actin (6.6%) and physiologic concentrations of salt (0.138 M K+1, 2 mM MgCl2) were added to the extracts. We found that the Triton buffer alone increased the critical concentration of purified actin from 0.2 $\mu M$ to $0.37 \pm 0.05$ $\mu M$ (SEM, $n = 7$), however when purified actin was combined with PMN extract, a 1 mg/ml concentration increased the concentration at which actin monomers began to polymerize to 5.8 $\mu M$. Higher concentrations of extract caused a proportionally greater increase in apparent critical concentration (see Fig. 6, inset). As shown in a representative experiment in Fig. 6, at extract concentrations of 1 mg/ml or higher in many of the experiments the slope of the line relating fluorescence (F-actin concentration) to the total starting actin concentration was flatter in extract, than in buffer alone. More prolonged incubation of the samples did not result in a change in the steady-state fluorescence of extract samples.

To determine to what extent profilin in PMN extracts contributed to the marked inhibition of actin assembly, extract was absorbed with an excess concentration of PLP beads to remove profilin. PLP treatment of extract was associated with a small reduction ($\sim 12\%$ decrease) in the concentration at which actin began to assemble, reflecting the concentration of PMN actin removed as profilin-actin complex. (Example: 1 mg/ml untreated PMN extract, unpolymerized actin $= 5.75 \mu M$; after absorption with PLP beads the same concentration of extract, unpolymerized actin $= 5.05 \mu M$; actin polymerized in buffer, unpolymerized actin $= 0.2 \mu M$.)
The final pool of unpolymerized actin not bound to profilin was measured by absorbing the Triton-soluble fraction with PLP beads plus any 42,000-D peptide (generally <3\% of the total actin, see Fig. 3 B, lanes 7 and 8) remaining in the final supernatant.

In unstimulated granulocytes the highest percentage of total actin was found in the third pool, unpolymerized actin not bound to profilin, whereas 30–40\% was associated with the Triton-insoluble cytoskeleton and only 6.8\% was represented by high affinity PA complex. Unstimulated PMN contained the highest observed concentrations of PA complex, 44\% of the profilin molecules being complexed with actin. After stimulation with chemoattractant the relative concentration of actin bound to profilin decreased to 3.4 and only 27\% of the profilin molecules were bound to actin. The relative PA-complex concentration remained low with continued stimulation. However if FMLP was displaced by t-BOC, PA complex rapidly increased to unstimulated values, indicating that chemoattractant receptor occupancy was required to maintain the dissociation of actin from profilin.

Our findings were similar to platelet studies of Markey et al. (29), but differed from those of Lind et al. (25) who found very low concentrations of PA complex (only 3–10\% of profilin was complexed to actin) in unstimulated platelets. Upon thrombin stimulation Lind et al. observed a rapid increase in high-affinity complex (70\% of profilin bound to actin) which with continued thrombin stimulation was then followed by a second decline in PA complex concentration. There are several possible reasons for the differences between Lind et al. and our findings: (a) Variations in buffer conditions could have altered PA complex concentration; (b) PMN may not have been in a true resting state; and (c) Platelet and PMN profilin function differently.

Our buffers and Triton solution were nearly identical to those used by Lind et al. with the exception that platelets were incubated in plasma in some experiments. Since serum and plasma contain vitamin D binding protein which can dissociate actin monomers from profilin (30), this condition could falsely lower the concentration of actin bound to profilin (see Materials and Methods) and might explain the low resting concentrations of profilin–actin complex in some of our experiments. We performed all of our experiments in serum free buffers. To address the second possibility that PMN were not in a true resting state, PMN were purified using minimal perturbation and prolonged incubation on ice to minimize inadvertent stimulation. Also PMN were purified in lipopolysaccharide-free media to eliminate inadvertent priming. Under these conditions high-affinity PA complex concentrations were nearly identical to Ficoll/Hypaque-purified PMN. In addition, extracts derived from human PMN stored in the cold and subjected to PLP affinity chromatography and gel filtration, yielded primarily profilin complexed to actin. Only by using NEM treatment, a chemical that binds to cysteine 374 in actin and weakens profilin's affinity for actin (20, 27), were we able to purify higher concentrations of free profilin. Finally as discussed above, when FMLP was displaced from its PMN membrane receptors, an increase in actin associated with profilin was observed. These findings all suggest that PA complex concentration is maximal in unstimulated PMN.

Although PMN profilin–actin complexes dissociated at the appropriate time during stimulation, a condition that would be expected to enhance actin filament assembly, the concentration of profilin present in human PMN relative to the total

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**Figure 6.** Determination of the concentration of unpolymerized actin \((C_u)\) in the Triton-soluble fractions from PMN. Increasing concentrations of purified rabbit skeletal muscle actin containing 6.6\% pyrenyl actin were added to 1.8 mg/ml of Triton-soluble PMN extract \((closed~squares)\) prepared as described in the methods or to the Triton buffer solution alone \((open~squares)\). Samples were incubated at 25°C and fluorescence intensities read after 12 h. Previous experiments revealed that fluorescence intensity values reach a steady state by 6 h. The buffer contained final concentrations of 0.138 M KCl and 2 mM MgCl\(_2\). For clarity, the G-actin fluorescence intensities have not been included. The critical concentration of actin polymerized in the Triton buffer was 0.4 \(\mu M\), whereas the concentration of unpolymerized actin in the extract was nearly 10 \(\mu M\). The insert shows the effect of increasing concentrations of extract on the concentration of unpolymerized actin \((C_u)\). The slope of the line is 5.8 \(\mu M/mg/ml\) extract.

To exclude the possibility that the Triton-soluble PMN actin had been denatured, the Triton-soluble fraction from PMN was subjected to DNase I chromatography followed by elution with formamide and dialysis (see Materials and Methods). As compared with the native actin in a 1 mg/ml PMN extract, apparent critical concentration = 5.8, purified PMN actin had a critical concentration of 0.6 \(\mu M\) (two separate purifications).

**Discussion**

Profilin is thought to play a major role in maintaining the high concentrations of monomeric actin in unstimulated cells and in regulating the assembly of these monomers into filaments. For profilin to fulfill these roles in the PMN, the profilin–actin complex must dissociate at the same time as F-actin content is increasing and secondly, profilin must be present in sufficient quantities to bind the high concentrations of monomers found in the resting PMN. Our investigations indicate that PMN profilin fulfills the first but not the second of these requirements.

Methods similar to those used by Lind et al. (25) were used to examine profilin function in human PMN. Data were analyzed by defining three pools of actin. The first pool consisted of actin associated with the Triton-insoluble cytoskeleton. This actin was assumed to be primarily F-actin. The other two pools of actin were derived from the Triton-soluble fraction. The profilin–actin complex (PA complex) pool was measured by absorbing the Triton-soluble fraction with PLP beads followed by extensive washing. Profilin–actin complex as measured by our methods represented a high-affinity complex since the complex remained intact after incubation for 7 min in very dilute solutions (see Materials and Methods). The final pool of unpolymerized actin not bound to profilin was defined as the percentage of the total actin absorbed by DNase I-beads incubated in the PMN Triton-soluble solu-
actin concentration (molar ratio profilin to actin 1:5.3) was too low to account for a major portion of the actin found in the Triton-soluble fraction, both before and after stimulation. A similarly low molar ratio of profilin to actin (1:6) was also recently reported in platelets (25). In PMN there was sufficient PA complex to account for only 12–14% of the actin found in the Triton-soluble fraction of unstimulated as well as PMN stimulated with chemoattractant (assuming PLP beads bound all the profilin molecules, see below). If the contribution of low-affinity profilin–actin complex were included in our calculations, assuming a $K_d$ of 1 μM (see Fig. 2), these complexes could account for only an additional 2.5–5% of the unpolymerized actin. If it were assumed that each molecule of profilin bound an actin molecule in unstimulated PMN (i.e., the percent of profilin bound to actin with high affinity was 100%, rather than 44%), actin bound to profilin could still only account for a minority (approximately one-third) of the Triton soluble pool. However, in this last circumstance, the profilin–actin pool could theoretically contribute nearly half of the actin monomers for chemoattractant stimulated actin filament assembly.

Addition of purified actin to the Triton-soluble PMN extract also suggested that profilin alone could not account for the high concentrations of unpolymerized PMN actin. The apparent critical concentration under the conditions of our experiments, physiologic salt and low Ca$^{++}$ was 5.8 μM for each mg/ml concentration of extract. Preabsorption of extract with PLP beads had only a small affect on this value, decreasing the concentration of unpolymerized actin by only a small fraction (12%). Purification of polymerization competent actin from Triton-soluble extract excluded the possibility that PMN actin had been denatured during extract preparation.

It is possible that our methods underestimated the true profilin content; however, attempts to increase the yield of profilin by repeated absorption with PLP beads, by initially mixing supernatant with DNase I beads to enhance recovery of profilin–actin complexes, and by vigorously sonicating the samples in Triton before addition of PLP beads all failed to increase the profilin yield. It remains possible that there is a large population of profilin in PMN that fails to bind to PLP beads. If, however, it is assumed this affinity method binds a large population of profilin in PMN that fails to bind to PLP, PLP beads bound all the profilin molecules, see below). If, however, it is assumed this affinity method binds only a minority (approxi-

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