Mechanisms for Actin Reorganization in Chemotactic Factor-Activated Polymorphonuclear Leukocytes

By Raymond G. Watts and Thomas H. Howard

Cytoskeletal structure in polymorphonuclear leukocytes (PMNs) is thought to reflect a simple equilibrium between two actin pools (globular [G]- and filamentous [F] actin). Recent description of two distinct F-actin pools in PMNs (Triton-insoluble [stable] and Triton-soluble [labile] F-actin pools) (Watts and Howard, *Cell Motil Cytoskeleton,* 21:25, 1992) suggest a tripartite equilibrium between these F-actin pools and G-actin and multiple possible mechanisms for polymerization. To study the contribution of each actin pool to actin dynamics in PMNs, changes in actin content of the Triton-soluble and -insoluble F-actin pools and G-actin in chemotactic factor (CTF)-activated PMNs were measured by NBDphallacidin binding and by gel scans of Triton-lysed PMNs. From 0 to 30 seconds after CTF activation, PMNs rapidly increase total (Triton-soluble + Triton-insoluble) F-actin content (maximum = 1.7- ± 0.10-fold basal at 30 seconds). Concurrent measures of the actin content of individual actin pools (Triton-soluble and -insoluble F-actin and G-actin) show that at all times (0 to 30 seconds) only the Triton-insoluble F-actin pool grows (maximum = 2.81- ± 0.73-fold basal at 30 seconds), whereas both the Triton-soluble and G-actin pools simultaneously decrease (50% decrease at 30 seconds). Concurrent growth of one F-actin pool (Triton-insoluble) and loss of another F-actin pool (Triton-soluble) emphasize the functional uniqueness of the F-actin pools and can occur only if the Triton-soluble F-actin anneals or cross-links filament-to-filament with the Triton-insoluble fraction or if the Triton-insoluble F-actin pool first depolymerizes to monomer, which is then added to the Triton-insoluble pool. Because from 0 to 30 seconds after FMLP activation G-actin never increases, but, like the Triton-soluble F-actin progressively decreases, the results suggest that F-actin growth results from simultaneous new filament growth by monomer addition to the Triton-insoluble F-actin and cytoskeletal remodelling by Triton-soluble F-actin annealing or cross-linking to Triton-insoluble F-actin. These findings offer important new insights into the mechanism(s) of actin polymerization in CTF-activated human PMNs.

THE MICROFILAMENTOUS cytoskeleton of polymorphonuclear leukocytes (PMNs) and other nonmuscle cells consists of a complex framework of filamentous actin and associated proteins.[1,2] Dynamic assembly, disassembly, and reorganization of this cytoskeleton is required for cellular motile processes such as shape change,[3] diapedesis, chemotaxis, secretion, and phagocytosis.[4,5] The actin of the nonmuscle cytoskeleton exists in an equilibrium between two forms, a 43-Kd monomeric form (G-actin) and a polymeric form (F-actin). Rapid conversion of G-actin to F-actin (polymerization) and/or F-actin to G-actin (depolymerization) in response to soluble chemotactic factors (CTFs) or cellular activators permits the PMN to rapidly and reversibly reorganize its cytoskeleton and to perform required motile functions.[6,7] Thus, elucidation of the mechanisms for regulation of actin polymerization/depolymerization is fundamental to an understanding of PMN motility and function.

In vitro, the equilibrium between pure G- and F-actin is dependent on G-actin concentration, divalent cation concentration, the availability of adenosine triphosphate (ATP) or adenosine diphosphate (ADP), and other factors.[5] In vitro, when the actin monomer concentration exceeds the critical concentration (Ccr) for actin assembly, G-actin rapidly polymerizes to form filaments. Resultant actin filaments have intrinsic polarity, ie, a barbed and a pointed end, as defined by heavy meromyosin decoration of actin filaments. The barbed or plus (+) end of the actin filament is the preferred site for monomer addition and filament growth, although monomer can also associate with the opposite pointed or minus (−) end of the filament. Monomer addition to the barbed end of F-actin is stoichiometrically favored by at least a factor of 10.[2] Therefore, in vitro the vast majority of filament growth occurs by monomer addition to the barbed filament end in a concentration-dependent manner. In contrast, early studies of actin concentrations in the cytoplasm of fibroblasts showed that in cells, G-actin exists in concentrations far in excess of the in vitro Ccr for actin polymerization in a buffer that mimics the intracellular milieu.[8] This surprising finding suggested that cellular systems contain components that regulate the state of actin polymerization. These modifying components include a variety of actin binding and regulatory proteins.[2]

The relative amounts of G- or F-actin within individual cells depends on the state of cell activation and the state of activation of actin binding and regulatory proteins.[9,10] In basal PMNs, like fibroblasts, the majority of actin exists as G-actin.[11-15] After cellular activation with CTFs such as formyl-met-leu-phe (FMLP), there is a rapid, transient increase in F-actin temporally associated with a decrease in G-actin.[3,12,13] Extrapolation of in vitro observations with pure actin suggests that FMLP-induced actin polymerization in PMNs is a simple conversion of G-actin to F-actin. This conceptualization of actin dynamics in the cellular cytoskeleton limits the mechanisms for actin polymerization to processes of de novo F-actin formation from monomeric G-actin or addition of G-

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actin to pre-existing F-actin. However, the existence of two recently described distinct F-actin pools in basal PMNs (a Triton-insoluble or stable F-actin pool and a Triton-soluble or labile F-actin pool) challenge the notion of a simple G-actin to F-actin equilibrium in PMNs.

The Triton-insoluble F-actin pool accounts for a minority of cellular F-actin, is sedimented by low-speed centrifugation (15,900*g*), is cold insensitive, associated with the actin-regulatory proteins actin-binding protein (filamin), tropomyosin, and α-actinin, but not gelsolin, and thus likely represents a cross-linked supramolecular meshwork of F-actin.[7,16-18] The Triton-soluble F-actin pool accounts for the majority of cellular F-actin, requires high centrifugal force to sediment (366,000*g*), is sensitive to cold but not dilutional depolymerization, and is associated with gelsolin, but not filamin, tropomyosin, or α-actinin, and thus likely represents short actin filaments.

Because distinct Triton-insoluble and -soluble F-actin pools and G-actin all exist simultaneously in a tripartite equilibrium in the PMN, actin polymerization in cells can no longer be conceptualized as a simple equilibrium between a monomer and a single class of polymer. This tripartite equilibrium suggests additional unique mechanisms for F-actin reorganizations and actin polymerization in PMNs.[10,16,19] Studies seeking to elucidate the mechanisms of actin polymerization/depolymerization in cells must therefore take into account the existence of distinct F-actin pools and should measure not only changes in total F-actin, but also concurrent changes in Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin.

In the study reported here, simultaneous measurement of actin in the three distinct pools examines the mechanisms of actin polymerization in CTF- (FMLP) activated human PMNs. The results show that (1) the Triton-insoluble and -soluble F-actin pools respond differently to FMLP, (2) FMLP activation leads to growth of F-actin exclusively in the Triton-insoluble F-actin pool and concurrent loss of actin from both the Triton-soluble F-actin and G-actin pools, and (3) the Triton-insoluble F-actin growth includes and exceeds new F-actin polymerization. The results suggest that Triton-insoluble F-actin pool growth in response to FMLP occurs via at least two mechanisms: (1) reorganization of F-actin by filament-to-filament binding of Triton-soluble F-actin to Triton-insoluble F-actin, and (2) new F-actin growth by the addition of G-actin to the Triton-insoluble F-actin pool. Both processes are inhibited by the barbed end capping alkaloid cytochalasin D, suggesting that filament-to-filament binding occurs preferentially at the barbed end of the filament. These findings offer important new insights into the mechanisms of actin polymerization in CTF-activated human PMNs and document increased complexity of dynamic reorganizations of the microfilamentous cytoskeleton.

MATERIALS AND METHODS

Materials. Standard stock chemicals were purchased from J. T. Baker chemical company (Phillipsburg, NJ), Fisher Scientific (Fair Lawn, NJ), or Sigma (St Louis, MO). Triton X-100 was obtained from Mallinckrodt, Inc (Paris, KY). NBDphallacidin was supplied by Molecular Probes (Eugene, OR). Stock solutions were prepared as follows: FMLP, cytochalasin D (CD) and phalloidin were stored at 0°C as 10^{-2} mol/L stocks in dimethylsulfoxide (DMSO); DNase I was stored at 0°C as a 1 mg/mL stock in Hanks'/HEPES buffer with 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF).

Isolation and purification of human PMNs. Human PMNs were isolated under endotoxin-free conditions (ETF) over Percoll gradients as previously described.[9] Cells were studied in suspension in Hanks'/HEPES buffer (25 mmol/L HEPES, 50 mmol/L phosphate, 150 mmol/L NaCl, 4 mmol/L KCl, pH 7.15) to a final cell concentration of 2×10^6 cells/experiment.

Quantification of F-actin by flow cytometry. F-actin content as NBDphallacidin by flow cytometry was determined in fixed PMNs as previously described[13] with the modification that fixation (3.7% formalin), cell lysis (1% Triton), and NBD staining (NBDphallacidin, 1.7×10^{-7} mol/L) were performed in three separate steps. All cells were basal ETF human PMNs in suspension. After fixation, lysis, and staining, 5,000 cells were analyzed on a Becton Dickinson FACStar flow cytometer (Becton Dickinson, Mountain View, CA) to produce a quantitative measure of F-actin content, as previously described.[6] Samples in which fixation precedes cell lysis contain both the Triton-soluble and -insoluble F-actin pools, whereas cell lysis before fixation releases the Triton-soluble F-actin pool and is thus a measure of Triton-insoluble F-actin.[16]

Quantification of F-actin by gel scanning. F-actin was also determined as cytoskeletal-associated actin (CAA) by a modification of the technique of Phillips[20] (see also White et al[11] and Watts and Howard[16]). Figure 1 is a schematic representation of the separation of distinct F-actin pools. The Triton-insoluble F-actin pool is that F-actin retained in the low-speed pellet (15,900*g* for 2 minutes) after cell lysis with Triton X-100 for 15 minutes at 25°C (1% in imidazole 10 mmol/L KCl 40 mmol/L, and EGTA 10 mmol/L, pH 7.15, with 7 mmol/L diisopropylfluorophosphate [DFP] to prevent proteolysis). The supernatant after low-speed centrifugation is separated into Triton-soluble F-actin and G-actin by ultracentrifugation in a TL 100 ultracentrifuge (Beckman, Palo Alto, CA) at 366,000*g* for 5 minutes as previously described.[16] The supernatant after high-speed centrifugation contains G-actin and is concentrated by precipitation with 20% trichloroacetic acid.[21] Samples were solubilized in a Tris buffer (0.625 mol/L in 2% sodium dodecyl sulfate [SDS], in 10% glycerol,

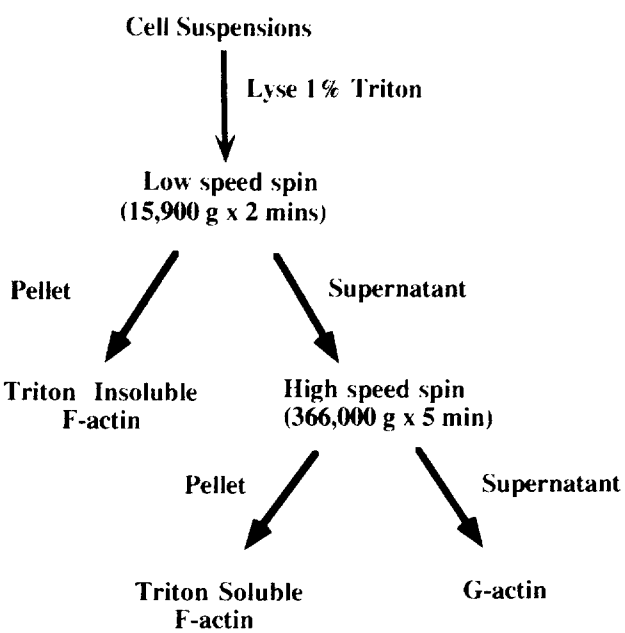

Fig 1. Schematic of separation of distinct actin pools.

and 5% 2-mercaptoethanol) and evaluated by gradient (5% to 15%) SDS-polyacrylamide gel electrophoresis (SDS-PAGE)[22] using the Mini Protean II system (BioRad, Richmond, CA). Protein bands were quantified by densitometric gel scans of Coomassie blue-stained gels via laser densitometry (LKB Ultrascan XL, Bromma, Sweden).

Purification of actin. Actin was purified from rabbit skeletal muscle by the technique of Spudich and Watt[23] and further purified in a chromatographic step over a Sephadex G-150 column to remove filaments and endogenous nucleating activity.[24,25]

RESULTS

Chemotactic peptide activation of PMNs results in new F-actin growth. The increase of F-actin in CTF-activated PMNs is presumed to occur primarily by new F-actin formation from actin monomer released by monomer sequestering proteins such as profilin.[1,5] To evaluate new actin filament growth after chemotactic peptide activation, the total F-actin content of FMLP-activated PMNs was determined by two complementary techniques, quantification of NBDphallacidin binding and of CAA.

Measurement of F-actin content in PMNs by NBDphallacidin binding in fixed PMNs is a quantitative measure of total cellular F-actin (both Triton-insoluble and Triton-soluble F-actin pools).[6,16] Quantification of F-actin as CAA by gel scanning of the actin retained in the operationally defined Triton-insoluble cytoskeleton (TICS) is equivalent to the NBDphallacidin binding assay only when both Triton-insoluble and -soluble F-actin are retained in the TICS by fixation before Triton treatment.[16] However, as originally described with Triton treatment before fixation or gel analysis,[11,20] the assay for CAA in the low-speed sedimented TICS excludes the Triton-soluble F-actin pool and discards it with the G-actin pool in the low-speed supernatant. Subsequent high-speed centrifugation of the initial low-speed supernatant pellets the Triton-soluble F-actin pool (Fig 1), and the total cellular F-actin content (Triton-insoluble + Triton-soluble F-actin) measured by gel scanning is quantitatively similar to the total cellular F-actin content measured by NBDphallacidin binding in a cell fixed with formalin before Triton lysis.[16] Therefore, the correlation of the two measures of F-actin content allows total cellular F-actin content to be measured either by NBDphallacidin binding or as CAA by gel scans of the total F-actin pools (sum of Triton-insoluble plus Triton-soluble F-actin pools) and concurrent measure of changes in actin content in distinct actin pools (Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin).

As shown in Fig 2, activation of endotoxin-free PMNs with FMLP (2×10^{-9} mol/L at 25°C) causes a net increase in F-actin content (polymerization) to maximal levels approximately 1.6× basal at 30 seconds after activation, followed by depolymerization to basal levels by 120 seconds. The kinetics and extent of F-actin growth in response to FMLP activation is similar whether F-actin is measured by NBDphallacidin binding or by gel scans of the Triton-insoluble F-actin plus Triton-soluble F-actin pools. The observed magnitude of F-actin growth in response to FMLP in these experiments is in agreement with previous reports in endotoxin-free human PMNs.[3,6,26-28] The results show that new filament growth accounts for an approximate 60% increase

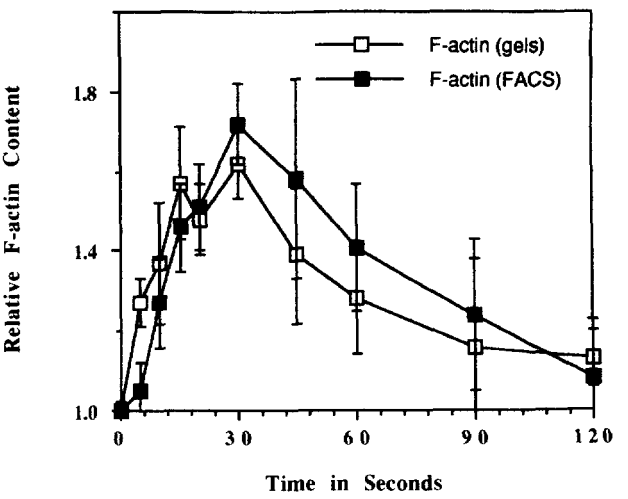

Fig 2. New filament growth after FMLP activation. Shown is the F-actin content of 2×10^6 PMNs/mL in suspension after activation with FMLP (2×10^{-9} mol/L at 25°C) measured at 5-second intervals by NBDphallacidin binding (FACS; ■) and by gel scanning of the Triton-insoluble F-actin plus Triton-soluble F-actin pools (gels; □). F-actin content is expressed relative to basal cells. The results represent the mean ± SD of five separate experiments. The results show that FMLP activation results in a 1.6- to 1.7-fold increase in total F-actin content within PMNs, which is maximal at approximately 30 seconds. The two measures of total F-actin content either as total F-actin by NBDphallacidin binding in prefixed PMNs or as the sum of Triton-insoluble F-actin plus Triton-soluble F-actin in PMNs by gel scanning are quantitatively similar.

in total F-actin content in FMLP-activated PMNs. The ability to individually measure the Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin pools by gel scans offers the opportunity to directly evaluate kinetic changes in these actin pools and examine the mechanisms of actin polymerization in cells.

CTF-induced actin polymerization occurs exclusively in the Triton-insoluble F-actin pool. To describe kinetic changes in each actin pool and elucidate the mechanisms for pool interaction during CTF-induced actin polymerization in PMNs, the quantity of actin in each actin pool (Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin) was measured simultaneously in FMLP-activated PMNs at 5-second intervals. As shown in Fig 3, FMLP activation results in an increase in the amount of actin exclusively in the Triton-insoluble F-actin pool. There is concurrent decrease in amounts of actin in the Triton-soluble F-actin and G-actin pools. At the time of maximum F-actin growth (30 seconds at 25°C) the Triton-insoluble F-actin pool attains a maximum value of 2.81 ± 0.73-fold basal, whereas at the same time the Triton-soluble F-actin and G-actin pools approach nadirs of 0.44- ± 0.09-fold and 0.49- ± 0.17-fold basal, respectively. Note that the relative increase in amount of actin in the Triton-insoluble F-actin pool (2.8-fold) exceeds the increase in new F-actin (1.6-fold measured as total F-actin as in Fig 2). By quantitative comparison, the majority of Triton-insoluble F-actin growth is not accounted for by new filament formation from monomer.

The actin content of the three distinct pools is also quantifiable as moles of actin per cell number, allowing a direct

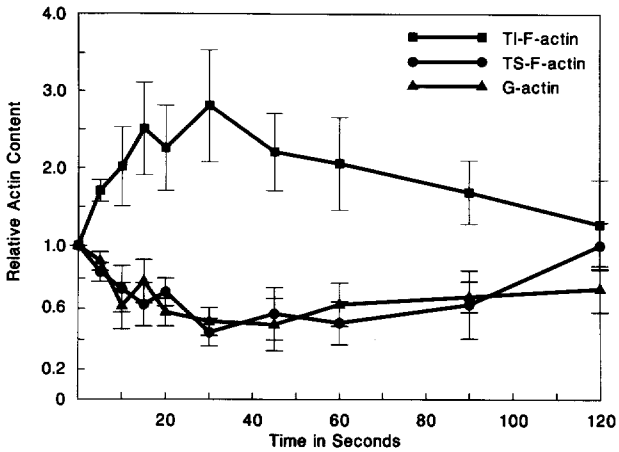

Fig 3. Effect of FMLP activation on the amount of actin in Triton-insoluble F-actin and Triton-soluble F-actin and G-actin pools. Shown is the time course of relative changes in amount of actin in the Triton-insoluble F-actin (TI-F-actin; ■), Triton-soluble F-actin (TS-F-actin; ●), and G-actin (▲) pools in FMLP (2×10^{-9} mol/L at 25°C)-activated PMNs (2×10^6 cells). The actin content in each pool was measured by densitometric gel scans of Coomassie blue-stained polyacrylamide gels. The actin content in each pool is expressed relative to baseline values. The results represent the means ± SD of 10 separate experiments. The results show that FMLP activation results in actin growth only in the Triton-insoluble F-actin pool and a concomitant decrease in actin content in both Triton-soluble F-actin and G-actin pools. At no time (0 to 120 seconds) does the actin content of the Triton-soluble or G-actin pools exceed basal levels. Also note that the relative growth in the Triton-insoluble F-actin pool exceeds the amount of new F-actin formation shown in Fig 2.

confirmation of the relative actin dynamics shown in Fig 3. The total protein concentration measured in 2×10^6 PMNs in suspension is 84 μg and the percent total protein as actin is 13.2%.[16] The calculated moles of total actin per 2×10^6 PMNs is, therefore, 2.58×10^{-10} moles of actin (84×10^{-6} g of total cellular protein × 13.2% actin = 11.1×10^{-6} g of actin/4.3×10^4 g of actin/moles of actin = 2.58×10^{-10} moles of actin). In a representative experiment, the distribution of total cellular actin between the Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin pools is 17%, 40%, and 44%, respectively. Therefore, the moles of actin in basal PMN pools is 0.44, 1.023, and 1.135×10^{-10}, respectively, in the Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin pools. After FMLP activation, the respective distribution of moles of actin is 1.41, 0.25, and 0.69×10^{-10} in the Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin pools. The increase in Triton-insoluble F-actin content can be directly accounted for mole to mole by the concurrent decrease in Triton-soluble F-actin and G-actin content.

To ensure that the observed shifts in actin between the three pools is physiologically relevant and not due to nonspecific rapid depolymerization in the extraction buffer during pool separation, the experiments described above were also performed with phalloidin present in the lysis buffer to stabilize filaments and prevent nonspecific exchange of actin between pools during separation. The mushroom-derived phallotoxin, phalloidin, binds to actin filaments and inhibits nonspecific dissociation of subunits from the filament ends.[29] As shown in Fig 4, the presence of 10^{-6} mol/L phalloidin in the extraction buffer did not prevent the observed growth in Triton-insoluble F-actin and concurrent decrease in Triton-soluble F-actin and G-actin, but, in fact, slightly accentuated the effect. The return of the actin pools to basal levels was delayed in the phalloidin-exposed cells, but returned to basal levels by 5 minutes. Likewise, the inclusion of DNase I alone or in combination with phalloidin in the extraction buffer failed to inhibit the observations (data not shown). These important control experiments further validate the pool specific shifts in actin content after CTF activation.

Temporally and quantitatively, the growth of the Triton-insoluble F-actin pool can be accounted for by the loss of actin from the Triton-soluble F-actin and G-actin pools. Importantly, at no time after FMLP activation (0 to 120 seconds) does the content of actin in the Triton-soluble F-actin or G-actin pools exceed basal levels. Conversion of one F-actin pool to another would occur without a net increase or decrease in total cellular F-actin. Conversion of G-actin to F-actin would obviously result in the generation of measurable new F-actin. The absence of an increase in G-actin above basal levels suggests that the Triton-insoluble F-actin pool grows not by Triton-soluble F-actin depolymerization to G-actin and then polymerization into the Triton-insoluble F-actin, but likely by direct addition of Triton-soluble F-actin to Triton-insoluble F-actin. Conversely, failure of Triton-soluble F-actin to increase above basal levels suggests that G-actin adds predominantly to new F-actin in the Triton-insoluble F-actin pool. The results are consistent with Triton-insoluble F-actin growth by monomer addition to create new F-actin and by direct addition of Triton-soluble F-actin to Triton-insoluble F-actin. If Triton-soluble F-actin adds directly to Triton-insoluble F-actin, this could occur by either end-to-end filament annealing or by side-to-side filament cross-linking. To distinguish between annealing and cross-linking of F-actin as mechanisms for simultaneous Triton-insoluble F-actin growth and Triton-soluble F-actin loss, the effect of CD on Triton-insoluble F-actin growth was determined. CD should inhibit end-to-end filament annealing, but not side-to-side filament cross-linking.

The addition of Triton-soluble F-actin to Triton-insoluble F-actin is CD inhibitable. CD binds to the barbed end of actin filament (the stoichiometrically preferred end for filament growth) and inhibits both the association and dissociation of subunits at that end.[24,30,31] Micromolar concentrations of CD inhibit barbed end growth by 90% in vitro.[29] The effect of CD on new F-actin polymerization from monomer in endotoxin-free PMNs is well documented.[3] CD inhibits approximately 75% of FMLP-induced new filament growth from G-actin in PMNs. However, the effect of CD on filament-to-filament annealing or cross-linking is unknown. If Triton-soluble F-actin to Triton-insoluble F-actin conversion occurs via end-to-end annealing, CD would be expected to inhibit a majority of the process. Conversely, if Triton-soluble F-actin to Triton-insoluble F-actin addition occurs via side-to-side binding or cross-linking, the barbed end capping of CD should have little inhibitory effect.

As shown in Fig 5, CD inhibits 72% ± 5.5% of FMLP-induced new filament growth by monomer addition (total F-

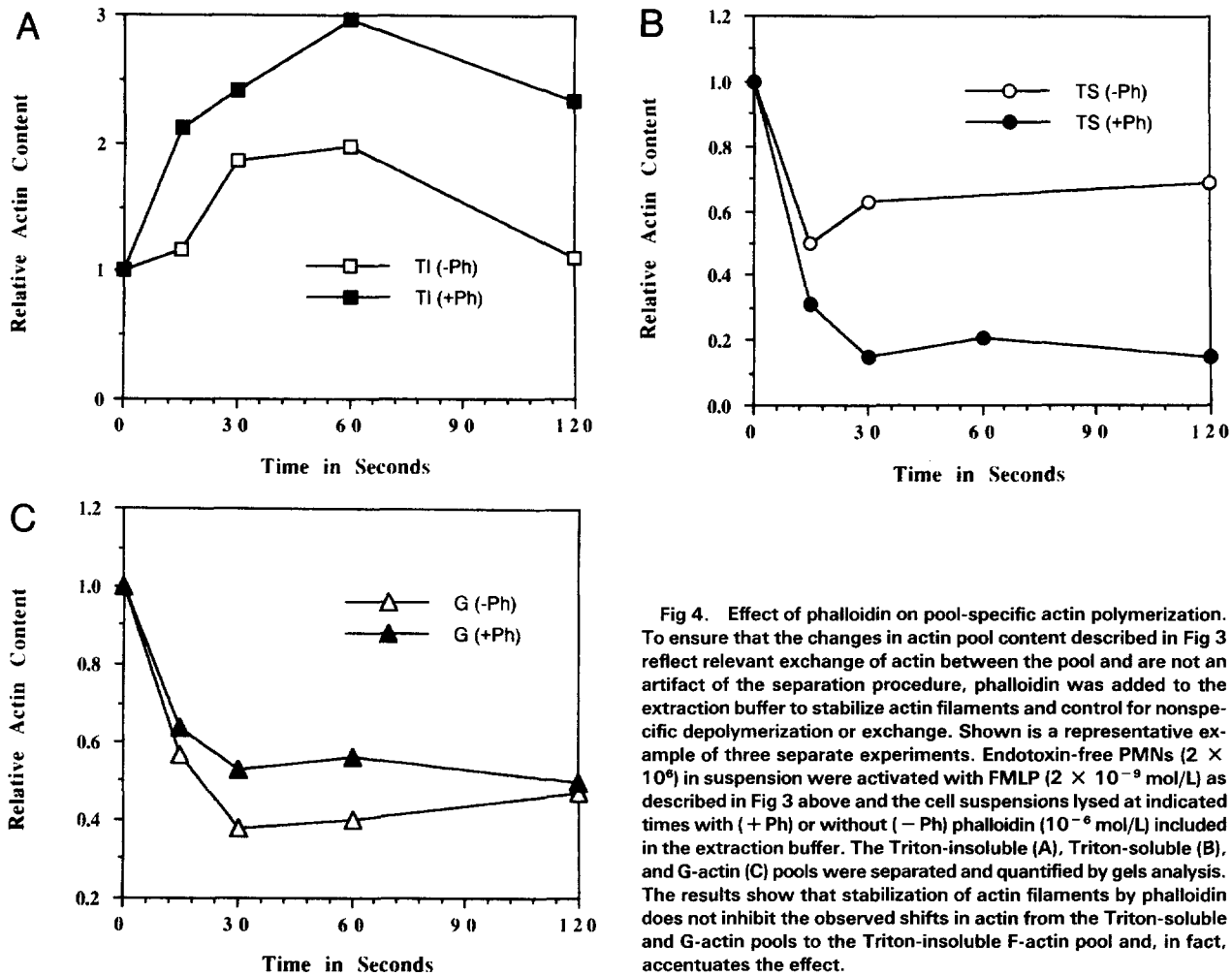

Fig 4. Effect of phalloidin on pool-specific actin polymerization. To ensure that the changes in actin pool content described in Fig 3 reflect relevant exchange of actin between the pool and are not an artifact of the separation procedure, phalloidin was added to the extraction buffer to stabilize actin filaments and control for nonspecific depolymerization or exchange. Shown is a representative example of three separate experiments. Endotoxin-free PMNs (2×10^6) in suspension were activated with FMLP (2×10^{-9} mol/L) as described in Fig 3 above and the cell suspensions lysed at indicated times with (+ Ph) or without (− Ph) phalloidin (10^{-6} mol/L) included in the extraction buffer. The Triton-insoluble (A), Triton-soluble (B), and G-actin (C) pools were separated and quantified by gels analysis. The results show that stabilization of actin filaments by phalloidin does not inhibit the observed shifts in actin from the Triton-soluble and G-actin pools to the Triton-insoluble F-actin pool and, in fact, accentuates the effect.

actin). Similarly, when Triton-insoluble F-actin growth alone is measured, CD inhibits 77% ± 6.0% of growth of the Triton-insoluble F-actin pool. Likewise, CD inhibits the loss of actin from the Triton-soluble F-actin and G-actin pools equally (76% *v* 72% inhibition). Because Triton-insoluble F-actin growth in response to FMLP is secondary to both G-actin addition and direct addition of Triton-soluble F-actin to Triton-insoluble F-actin, the results show that CD inhibits both Triton-insoluble F-actin growth from G-actin addition and Triton-insoluble F-actin growth from direct Triton-soluble F-actin addition. The CD sensitivity of Triton-soluble F-actin addition to Triton-insoluble F-actin suggests that the process of filament to filament addition of the Triton-soluble to the Triton-insoluble F-actin pool occurs predominantly via end-to-end annealing.

DISCUSSION

The studies reported here enhance our understanding of the structure and dynamics of the microfilamentous cytoskeleton in human PMNs. Recent reports document the existence of distinct F-actin pools in PMNs[7,16-19] and platelets.[32] A Triton-insoluble F-actin pool and Triton-soluble F-actin and G-actin pools coexist in basal PMNs. The studies reported here are the first to focus on the kinetic interaction of these pools in PMNs during CTF-induced cytoskeletal reorganization and elucidate the possible mechanisms of actin dynamics within the three pools.

The results show that after CTF (FMLP) activation, F-actin polymerizes. This result is expected and well documented.[12,13] The amount and extent of actin polymerization can be directly correlated with important PMN motile processes, including shape change[3] and chemotaxis.[27] Surprisingly, however, when the content of actin within the three distinct pools is measured after FMLP activation, F-actin is found to increase only in the Triton-insoluble F-actin pool. Furthermore, the relative growth of Triton-insoluble F-actin exceeds the growth in total new F-actin and Triton-insoluble F-actin growth occurs coincident with a decrease in both the Triton-soluble F-actin pool and G-actin. Taken together, the results show the functionally distinct dynamics of F-actin within the two F-actin pools and provide evidence for specific mechanisms of actin polymerization after FMLP activation.

The schema in Fig 6 illustrates the theoretically possible actin polymerization mechanisms between the distinct actin pools. Previous models of actin interaction that assumed a simple G- to F-actin equilibrium limit potential mechanisms

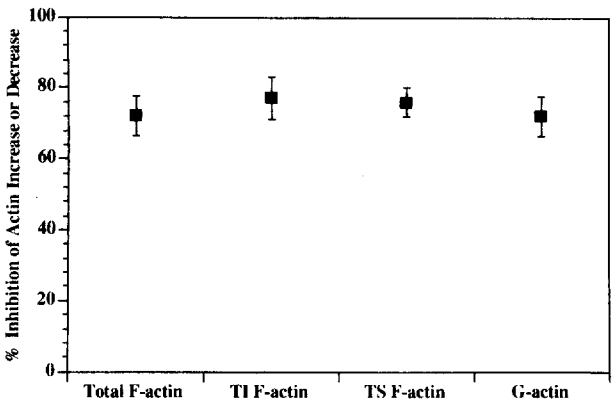

Fig 5. Effect of CD on pool-specific actin polymerization. Shown is the effect of CD on FMLP-induced actin polymerization in the Triton-insoluble F-actin pool and total F-actin pools of PMNs and actin loss from the Triton-soluble F-actin and G-actin pools. PMN suspensions (2×10^6 cells/mL) at 25°C were exposed to CD (2×10^{-5} mol/L for 5 minutes) or solvent control before FMLP activation (2×10^{-9} mol/L). At 30 seconds after FMLP activation, the cell suspensions were lysed and the actin pools separated as described. Change in actin content of the Triton-insoluble, Triton-soluble, and G-actin pools was quantified by gel scanning or NBDphallacidin binding relative to basal PMNs. The results are expressed as the percent inhibition of maximal actin increase or decrease of test versus control cells. Note that CD has the same relative inhibitory effect on Triton-insoluble F-actin growth as total F-actin growth. Likewise, CD inhibits the loss of actin from the Triton-soluble F-actin and G-actin pools equally. The results suggest that direct filament addition is CD inhibitable and thus occurs predominantly at the barbed end of the actin filament by end-to-end annealing.

of actin polymerization to direct addition of monomer to pre-existing filament or nucleation of monomer to new filament. However, the delineation of at least three actin pools in PMNs suggests several alternative polymerization mechanisms, including direct filament-to-filament annealing or cross-linking, monomer addition to existing Triton-insoluble or Triton-soluble F-actin filaments, nucleation of monomer to form new filament, or depolymerization of one or both F-actin pools to monomer with subsequent formation of filament. The results obtained in these studies offer direct evidence for and against several of these proposed mechanisms.

FMLP activation of human endotoxin-free PMNs leads to a 1.6-fold increase in total cellular F-actin. However, the increase in F-actin occurs exclusively in the Triton-insoluble F-actin pool. This result limits the potential mechanisms for polymerization to those that result in Triton-insoluble F-actin growth. The concurrent decrease in actin content in the Triton-soluble F-actin pools is consistent either with direct addition of Triton-soluble F-actin to Triton-insoluble F-actin or cycling of Triton-soluble F-actin to G-actin via depolymerization and subsequent G-actin addition to the Triton-insoluble F-actin pool. However, if Triton-soluble F-actin initially depolymerizes to G-actin before its incorporation into Triton-insoluble F-actin, a transient increase in G-actin content would be expected. Experimentally, the opposite result is observed. After FMLP activation, the content of the G-actin pool steadily and consistently decreases in temporal association with the decrease in Triton-soluble F-actin and increase in Triton-insoluble F-actin. Furthermore, these findings are unaffected by the inclusion of phalloidin and/or DNase I in the extraction buffer to stabilize filament and monomer and prevent nonspecific exchange between actin pools during the extraction procedure. The absence of an increase in G-actin argues against a cycling of Triton-soluble F-actin through G-actin to Triton-insoluble F-actin. Taken together, the findings are most consistent with both concurrent monomer addition to Triton-insoluble F-actin and direct binding of Triton-soluble F-actin to Triton-insoluble F-actin.

Further evidence that favors direct Triton-soluble F-actin to Triton-insoluble F-actin addition derives from the observation of a Triton-insoluble F-actin pool increase in excess of the amount of new F-actin filament formation. Total cellular F-actin content increases by 1.6-fold after CTF activation, whereas the Triton-insoluble F-actin pool increases 2.8-fold. This finding is explained by direct addition of one filament pool to another that would not result in an increase in total cellular F-actin content.

Annealing describes the process of one filament combining to the end of another filament. Cross-linking describes side-to-side binding of one filament to another. Evidence suggests that the Triton-soluble F-actin pool combines to the Triton-insoluble F-actin pool predominantly by end-to-end anneal-

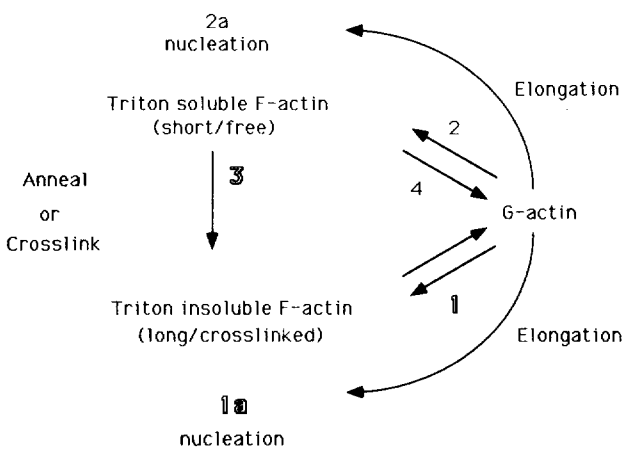

Fig 6. Proposed mechanisms of actin polymerization in CTF-activated PMNs. Shown is a diagrammatic representation of proposed mechanisms of actin polymerization in CTF-activated PMNs. Because actin exists in at least three forms, Triton-insoluble F-actin, Triton-soluble F-actin, and G-actin, multiple mechanisms are possible. Mechanisms resulting in a measurable increase in total F-actin include: direct addition of G-actin monomer to Triton-insoluble F-actin (1) or Triton-soluble F-actin (2), and/or nucleation of G-actin to de novo filament with subsequent addition to Triton-insoluble (1a) or -soluble (2a) F-actin. Direct addition or conversion of Triton-soluble F-actin to Triton-insoluble F-actin (3) could occur without a net change in total F-actin content or the Triton-soluble F-actin pool could initially depolymerize to G-actin with subsequent addition to Triton-insoluble F-actin (4). Because FMLP-activated actin polymerization in PMNs occurs exclusively in the Triton-insoluble F-actin pool with a simultaneous decrease in actin in the Triton-soluble and G-actin pools and because the actin content in the Triton-soluble F-actin and G-actin pools never increases above basal levels, the experimental results support mechanisms 1 or 1a (direct addition of G-actin to Triton-insoluble F-actin) and 3 (direct filament-to-filament addition of Triton-soluble F-actin to Triton-insoluble F-actin).

ing. The majority of Triton-insoluble F-actin growth is inhibited by CD, a barbed end, actin filament capping compound. Therefore, at least 75% of Triton-soluble to Triton-insoluble F-actin conversion as well as more than 75% of new filament growth occurs at the barbed end of the actin filament. Side-to-side cross-linking of one filament to another is not predicted to be CD sensitive. These findings suggest that end-to-end annealing is the primary process of Triton-soluble F-actin to Triton-insoluble F-actin addition. Yet, because 25% of Triton-insoluble F-actin growth is not inhibited by CD, side-to-side filament cross-linking or pointed end filament growth are also possible mechanisms of CTF-induced actin rearrangement. The recent finding of specific association of the actin cross-linking proteins filamin, tropomyosin, and α-actinin with the Triton-insoluble F-actin pool suggests a possible role for filament cross-linkers in CTF-activated actin rearrangements.[18]

G-actin conversion to F-actin predicted by the simple G- to F-actin equilibrium in vitro is likewise supported by the experimental results. The simultaneous decrease in both Triton-soluble F-actin and G-actin content provides evidence that monomer conversion is exclusively to the Triton-insoluble F-actin pool and does not initially cycle through the Triton-soluble F-actin pool. Monomer conversion to filament would result in an increase in total cellular F-actin and thus likely accounts for the 1.6-fold increase in total F-actin content, yet accounts for a minority of the 2.8-fold increase in Triton-insoluble F-actin content. This result may explain the apparent inability of monomer sequestering proteins such as profilin to account for filament growth after CTF activation,[33] because monomer release accounts for a minority of Triton-insoluble F-actin growth. These results do not allow a determination of the exact mechanism of monomer to filament conversion. G-actin could combine directly to preformed Triton-insoluble F-actin filament or could initially generate new nuclei that grow into new filaments that then anneal to existing Triton-insoluble F-actin. Further experiments are required to differentiate these possibilities because no direct assay for filament-to-filament annealing in intact cells is yet described. Figure 6 graphically displays the polymerization mechanisms for which the studies reported here provide experimental evidence.

In summary, these studies represent the first detailed characterization of actin dynamics within and between distinct actin pools in CTF-activated human PMNs. The results confirm and extend the morphologic and functional importance of distinct F-actin pools in living cells and provide direct evidence for filament-to-filament annealing as a major process of microfilamentous cytoskeletal reorganization. Further use of these techniques with other cellular-activating signals should provide an improved understanding of how actin filaments interact with each other and with actin monomers to constantly remodel the PMN cytoskeleton and allow the PMN to perform the motile processes required for normal function.

REFERENCES

1. Stossel TP: The molecular biology of phagocytes and the molecular basis of non-neoplastic phagocyte disorders, in Stamatoyannopoulas G, Nienhuis AW, Leder P, Majerus PW (eds): The Molecular Basis of Blood Diseases. Philadelphia, PA, Saunders, 1987, 499

2. Pollard TD, Cooper JA: Actin and actin binding proteins. A critical evaluation of mechanisms and functions. Ann Rev Biochem 55:987, 1986

3. Watts RG, Crispens MA, Howard TH: A quantitative study of the role of F-actin in producing neutrophil shape. Cell Motil Cytoskeleton 19:159, 1991

4. Southwick FS, Stossel TP: Contractile proteins in leukocyte function. Semin Hematol 20:305, 1983

5. Stossel TP: The mechanical responses of white blood cells, in Gallin JI, Goldstein IM, Snyderman R (eds): Inflammation: Basic Principles and Clinical Correlates. New York, NY, Raven, 1988, p 325

6. Howard TH, Oresajo CO: The kinetics of chemotactic peptide-induced change in F-actin content, F-actin distribution and the shape of neutrophils. J Cell Biol 101:1078, 1985

7. Cassimeris L, McNeill H, Zigmond SH: Chemoattractant stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities. J Cell Biol 110:1067, 1990

8. Bray D, Thomas C: Unpolymerized actin in fibroblasts and brain. J Mol Biol 105:527, 1976

9. Howard TH, Wang D, Berkow RL: Lipolysaccharide modulates chemotactic peptide induced actin polymerization in neutrophils. J Leukoc Biol 47:13, 1990

10. Howard T, Chaponnier C, Yin H, Stossel T: Gelsolin-actin interaction and actin polymerization in human neutrophils. J Cell Biol 110:1983, 1991

11. White JR, Naccache PH, Sha'afi RI: Stimulation by chemotactic factor of actin association with the cytoskeleton in rabbit neutrophils. J Biol Chem 258:14041, 1983

12. Fechheimer M, Zigmond S: Changes in cytoskeletal proteins of PMN induced by chemotactic peptides. Cell Motility 3:349, 1983

13. Howard TH, Meyer WH: Chemotactic peptide modulation of actin assembly and locomotion in neutrophils. J Cell Biol 98: 1265, 1984

14. Wallace PJ, Wersto RP, Packman CH, Lichtman MA: Chemotactic peptide-induced changes in neutrophil actin conformation. J Cell Biol 99:1060, 1984

15. Yassin R, Shefcyk J, White JR, Tao W, Volpi M, Molski TFP, Naccache PH, Feinstein MP, Sha'afi RI: Effects of chemotactic factors and other agents on the amounts of actin and a 65,000 molecular weight protein associated with the cytoskeleton of rabbit and human neutrophils. J Cell Biol 101:182, 1985

16. Watts RG, Howard TH: Evidence for a Gelsolin-rich, labile F-actin pool in human polymorphonuclear leukocytes. Cell Motil Cytoskeleton 21:25, 1992

17. Cano ML, Lauffenburger DA, Zigmond SH: Kinetic analysis of F-actin depolymerization in polymorphonuclear leukocyte lysates indicates that chemoattractant stimulation increases actin filament number without altering the filament length distribution. J Cell Biol 115:677, 1991

18. Watts RG, Howard TH: The role of tropomyosin and actin binding protein in the stable F-actin pool of human neutrophils. Mol Biol Cell 3:39a, 1992

19. Cano ML, Cassimeris L, Fechheimer M, Zigmond SH: Mechanisms responsible for F-actin stabilization after lysis of polymorphonuclear leukocytes. J Cell Biol 116:1123, 1992

20. Phillips DR, Jennings LK, Edwards HH: Identification of membrane proteins mediating the interaction of human platelets. J Cell Biol 86:77, 1980

21. Peterson GL: Determination of total protein. Methods Enzymol 91:95, 1983.

22. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

23. Spudich JA, Watt S: The regulation of rabbit muscle contraction: Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. J Biol Chem 246:4866, 1971

24. Howard TH, Lin S: Specific interaction of cytochalasins with muscle and platelet actin filaments in vitro. J Supramolecular Structure 11:283, 1979

25. Cassella J, Maack DJ: Identification of the inhibitor of low shear viscosity in conventional actin preparations. Biochem Biophys Res Commun 145:625, 1987

26. Coates TD, Watts RG, Hartman R, Howard TH: Relationship of F-actin distribution to development of polar shape in human polymorphonuclear neutrophils. J Cell Biol 117:765, 1992

27. Harvath L: Regulation of neutrophil chemotaxis: Correlations with actin polymerization. Cancer Invest 8:651, 1990

28. Eberle M, Traynor-Kaplan AE, Sklar LA, Norgauer J: Is there a relationship between phosphatidylinositol triphosphate and F-actin polymerization in human neutrophils? J Biol Chem 265:16725, 1990

29. Cooper JA: Effects of cytochalasin and phalloidin on actin. J Cell Biol 105:1473, 1987

30. Fox JEB, Phillips DR: Inhibition of actin polymerization in blood platelets by cytochalasins. Nature 292:650, 1981

31. Cassella JM, Flanagan M, Lin S: Cytochalasin D inhibits actin polymerization and induces depolymerization of actin filaments formed during platelet shape change. Nature 293:302, 1981

32. Hartwig JH: Mechanisms of actin rearrangements mediating platelet activation. J Cell Biol 118:1421, 1992

33. Southwick FS, Young CL: The actin released from profilin-actin complexes is insufficient to account for the increase in F-actin in chemoattractant stimulated polymorphonuclear leukocytes. J Cell Biol 110:1965, 1990