Actin Polymerization Induced by GTPγS in Permeabilized Neutrophils Is Induced and Maintained by Free Barbed Ends*

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To address the mechanisms through which agonists stimulate actin polymerization, we examined the roles of monomer sequestering proteins and free barbed ends on actin polymerization induced by guanosine 5'-3-O-(thio)triphosphate (GTPγS) in neutrophils permeabilized with streptolysin O. Addition of profilin (without GTPγS) caused a net decrease in F-actin. Thus, merely making profilin available in the cell was not sufficient to induce actin polymerization. On the other hand, addition of profilin hardly affected the polymerization induced by GTPγS, while thymosin β4 or DNase I decreased this polymerization. These data suggested that GTPγS induced polymerization by increasing the availability of barbed ends. In the presence of cytochalasin B, profilin did inhibit polymerization induced by GTPγS, demonstrating that GTPγS did not inhibit profilin’s monomer sequestering ability.

The F-actin induced by GTPγS was not limited by a time-dependent loss of G-actin or G-proteins from permeabilized cells since, following stimulation with suboptimal concentrations of GTPγS, addition of more GTPγS induced further polymerization. Barbed ends remained free after F-actin reached plateau since (a) cytochalasin B caused depolymerization of induced F-actin and (b) profilin did not depolymerize induced F-actin unless the cells were first treated with cytochalasin to cap barbed ends. The data indicate that GTPγS maintains an increased level of F-actin by keeping at least a few barbed ends available for polymerization.

Neutrophils treated with inflammatory mediators increase their F-actin level by shifting the steady state from G- to F-actin. However, which of the many factors that cause this shift in vitro account for it in vivo are unknown. Possible factors include: (a) a shift in the nucleotide bound to G-actin from ADP to ATP or (b) inhibition of the major monomer sequestering proteins. These factors now seem unlikely (Rosenblatt et al., 1995; Carlier et al., 1993; Redmond et al., 1994; Safer et al., 1990; Cassimeris et al., 1992; Nachmias et al., 1993), so interest is focused on (c) the availability of profilin and (d) the availability of free barbed ends.

Profilin, first identified from its ability to inhibit actin polymerization (Carlsson et al., 1977), is now also known to promote polymerization (Pantaloni and Carlier, 1993). When profilin is added in vitro to a mixture of thymosin β4 (Tβ4), G-actin, and F-actin, the F-actin decreases or increases depending on whether the filament barbed ends are capped or free (Pantaloni and Carlier, 1993; Carlier and Pantaloni, 1994). Cells contain Tβ4, G-actin, and F-actin. Thus, if, in the resting cell some barbed ends are free but profilin is sequestered, an agonist could induce polymerization merely by releasing profilin from a sequestered pool. Profilin might well be sequestered in cells because it is distributed nonhomogeneously in the cell (Buss et al., 1992) and because it binds strongly to inositol bisphosphate (Lassing and Lindberg, 1985). Furthermore, some agonists may release profilin bound to inositol bisphosphate, thus freeing it to promote polymerization (Sohn and Goldschmidt-Clermont, 1994; Machesky and Pollard, 1993). The hypothesis that profilin release induces actin polymerization has not been tested.

Free barbed ends are of interest because in vitro increasing the fraction of filaments with free (uncapped) barbed ends induces polymerization (Yin and Stossel, 1979; Pollard, 1986). However, even in cells, evidence that free barbed ends regulate the actin steady state is equivocal. On one hand, cells lysed after stimulation with agonist have an increased number of sites that nucleate barbed end elongation of exogenous actin (Carson et al., 1986; Condeelis et al., 1988; Hall et al., 1989; Hartwig, 1992; Nachmias et al., 1993). This increase in nucleation sites occurs even when net polymerization is blocked by cytochalasin, suggesting these sites may be the cause rather than the effect of polymerization (Hartwig, 1992). On the other hand, most of the filaments in the lysate of resting neutrophils appear to have free barbed ends, and the increase in number of barbed ends upon stimulation is matched by an equal increase in pointed ends (Cano et al., 1991; Carson et al., 1986). Merely doubling the number of filaments should not shift the critical concentration. Furthermore, it is unclear if these barbed ends are available in the intact cell or freed only upon lysis and dilution. Indeed, at high cell concentrations, lysates contain sufficient capping activity to cap all the barbed ends present in both control and stimulated cells (Cassimeris et al., 1992; Southwick and Dinubile, 1986). Thus, it is unclear what fraction of filaments are free in the intact cell and whether this fraction is altered by stimulation. Attempts to determine whether free barbed ends stimulate actin polymerization in intact cells by injection of free barbed ends (small actin filaments) gives negative or ambiguous results (Sanders and Wang, 1990; Handel et al., 1990).

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¶ The abbreviations used are Tβ4, thymosin β4; SLO, streptolysin O; TRITC, tetramethylrhodamine isothiocyanate; GTPγS, guanosine 5'-3-O-(thio)triphosphosphate; 1 S. H. Zigmond, unpublished results.
barbed ends that is based on experiments with cytochalasin, a barbed end capper. Cytochalasin inhibits agonist-induced polymerization in neutrophils, suggesting that polymerization occurs primarily at barbed ends (MacLean-Fletcher and Pollard, 1980; White et al., 1983). However, this does not indicate that the freeing of barbed ends is the regulated event; a similar inhibition by cytochalasin would be expected were agonist releasing profilin to facilitate polymerization on existing free ends. Furthermore, cytochalasin has many effects such as stabilizing actin dimers and increasing the rate of ATP hydrolysis (Sampath and Pollard, 1991). Thus, cytochalasin could exert its effects in cells by shifting the nucleotide bound to G-actin from ATP to ADP. So overall, the evidence supporting regulation of F-actin by freeing of barbed ends is weak.

One cannot study how an agonist causes actin polymerization in vitro because after cell lysis both natural agonists and GTP-γS cease to stimulate an increase in F-actin. However, permeabilized neutrophils, like intact cells, double their F-actin level upon addition of appropriate agonist (Downey et al., 1989; Therrien and Naccache, 1989; Bengtsson et al., 1990; Redmond et al., 1994). Neutrophils permeabilized with streptolysin O (SLO) have large pores, allowing entry of exogenous proteins up to 120,000 Da (Redmond et al., 1994; Bhakdi et al., 1993). This permitted us to modulate the cytoplasmic G-actin by means of exogenous monomer binding proteins. We utilized the different properties of monomeric actin binding proteins to dissect the changes that lead to actin polymerization. In particular, we utilized the unusual properties of G-actin profilin complex, which can contribute G-actin to the barbed but not pointed end of an actin filament (Tilney et al., 1983; Pollard and Cooper, 1984; Pring et al., 1992; Pantaloni and Carlier, 1993; Giuliano and Taylor, 1994). The results put on firmer ground the idea that increase in F-actin by chemotaxants is mediated through an increase in the availability of free barbed ends.

**EXPERIMENTAL PROCEDURES**

**Materials**—GTP-γS, TRITC-phalloidin, phallacidin, and DNase I were obtained from Sigma. The G-actin binding by DNase I was calibrated by ability of purified G-actin to inhibit DNase I activity; this was 50% of the DNase I present based on protein concentration.

**Cells**—Rabbit peritoneal exudate neutrophils, prepared as described previously (Sullivan and Zigmond, 1980), were washed 3 times in saline (0.9% NaCl) and resuspended to $5 \times 10^7$ cells/ml in cell buffer (Hanks’ without bicarbonate, calcium, or magnesium and with 10 mM Hepes, pH 7.2).

**Streptolysin O Permeabilization**—One part of the cell suspension at $2 \times 5 \times 10^7$ cells/ml was mixed with three parts of streptococcal cytotoxin streptolysin O (Murex, Norcross, GA) in phosphate-buffered saline at 2.0 international units (IU)/ml, and incubated on ice for 3 min. Thus, cells were permeabilized with 2 or $0.9 \times 10^{-6}$ IU of SLO/cell (the higher ratio of SLO/cell resulted in faster exchange of proteins between the cell and the medium). The excess of SLO was removed by pelleting in a microcentrifuge for 15 at 15,000 rpm. The cell pellet was resuspended at $4 \times 10^6$ cells/ml in intracellular physiological (IP) buffer (135 mM KCl, 10 mM NaCl, 2 mM MgCl$_2$, 2 mM EGTA, 10 mM Hepes, pH 7.0) at room temperature. Cells were incubated at room temperature for 2 min (warming time) before stimulus was added. The pores induced by streptolysin O do not form in the cold, so the cells only become permeable after warming. The time courses plotted in the figures begin after warming at room temperature for 2 min.

**Saturable Staining with TRITC-Phalloidin** as Assay of F-actin Levels—F-actin levels were determined by phalloidin staining as described by Howard and Cano (1985). Changes in the state of actin were stopped by addition of 1% glutaraldehyde and TRITC-phalloidin (final concentration, $2 \times 10^{-7}$ M). To determine the nonsaturable staining, unlabeled phalloidin (final concentration, $2 \times 10^{-6}$ M) was added with TRITC-phalloidin to some samples. Samples were stained for at least 1 h, pelleted for 7 min at 15,000 rpm in a microcentrifuge and drained. From each pellet, corresponding to $2 \times 10^6$ cells, the TRITC-phalloidin was extracted with 0.8 ml of methanol for at least 48 h before the rhodamine fluorescence (540 nm; 575 nm; Slits: 3, 20) was read in a Perkin Elmer LS5 fluorimeter. TRITC-phalloidin staining values reported are the saturable staining; the total fluorescence extracted minus the nonsaturable fluorescence, i.e., the fluorescence extracted from cells stained in the presence of unlabeled phalloidin.

Nucleation Sites Assay—Permeabilized cells with or without stimulus were processed as described above. The nucleation sites assay was repeated as described previously using $4 \times 10^5$ cells/ml in assay buffer (Cano et al., 1991).

**Profilin Purification**—Profilin was isolated from calf spleen according to the method of Kaiser et al. (1989) and J anmy (1991). The actin and profilin were eluted with extraction buffer containing 3 mM and 7 mM urea, respectively. The profilin peak, identified by $A_{280}$, was imme diately lyzed against 20 mM Tris·HCl, pH 7.4, containing 0.1% sodium azide. Profilin sample was run on 12.5% SDS gel to check for purity. In one profilin preparation contaminated with actin, we repeated the polyacrylamide column to obtain a clean profilin sample. Yield was approximately 50 mg of profilin/300 g of spleen.

Thymosin β4 (Tβ4) was isolated from calf spleen by the method of Cassimeris et al. (1992) with minor modifications.

**Modelling of Data**—To determine if the amount of polymerization induced by GTP-γS could be caused by (a) a shift in the affinity of the filament from 0.5 μM to 0.1 μM (as might happen if barbed ends became available), we calculated the amount of G-actin that would be released from a Tβ4-bound pool after such a shift. The amount released depended on the amount of Tβ4-actin complexes present in the SLO-permeabilized cells and chemoattractant-induced F-actin. Using the evidence of exogenous monomer binding proteins (Cassimeris et al., 1992; Fechheimer and Zigmond, 1993; Nachmias et al., 1993; Pantaloni and Carlier, 1993). For these calculations, we assumed that (a) PMNs contain about 250 μM Tβ4 and about 115 μM G-actin (Cassimeris et al., 1992) (for simplicity, the contribution of profilin was ignored); (b) that the $K_a$ of the actin binding of cellular G-actin to DNase I was 1 nm and to Tβ4 is 0.6 μM (Weber et al., 1992); (c) that the rate of equilibration of G-actin with Tβ4 was very rapid, thus the DNase I and exogenous Tβ4 in the permeabilized cells were considered to be at steady state with the endogenous G-actin/Tβ4 (Pantaloni and Carlier, 1993).

The amount of G-actin, Tβ4, and profilin in a permeabilized cell is now a non-equilibrium because exogenous proteins are continually leaving. Analysis of the G-actin released into the medium (assayed by DNase I inhibition) indicated that at the time of stimulation with GTP-γS (i.e. 2 min) about half of the G-actin pool has left the cell (Redmond et al., 1994). Since Tβ4 is in rapid equilibrium with the G-actin, we assumed that half of the Tβ4 has also left the cell. Indeed, slightly more than 50% of the profilin released by Tβ4 lysis was released into the medium after warming SLO-permeabilized cells for 2 min. Profilin released from permeabilized cells or after Triton lysis was collected on a polyacrylamide column, eluted with urea, and quantified by comparison to profilin standards on Coomassie Blue-stained SDS gels (Kaiser et al., 1989).

Thus after 2 min, with about 125 μM Tβ4 and 57.5 μM G-actin remaining in the cell, about 57 μM Tβ4 would be bound to G-actin and the G-actin in the G-actin/Tβ4 complex would be ~0.2 μM (the free pointed end). We then calculated the amount of G-actin that would be released from Tβ4 if the affinity of F-actin were decreased to 0.1 μM, allowing actin to polymerize until the free G-actin decreased to 0.1 μM. This would allow G-actin bound to Tβ4 to be released until the complex was in equilibrium with 0.1 μM G-actin. The amount of G-actin that would polymerize in the absence of exogenous monomer binding proteins (~40 μM) was set at 100%.

Since the concentration of free G-actin in the medium outside the SLO-permeabilized cells was extremely low, it was unlikely that G-actin binding proteins in the external medium would increase the rate of G-actin exit from permeabilized cells. Thus, the effects must be mediated by the proteins that enter the cell. To simulate the effects of exogenous monomer binders on polymerization induced by GTP-γS stimulation, we assumed the concentration of monomer binder inside the cell (bound or free) during the polymerization (i.e. between 3 and 5 min) was equal to the concentration added to the medium. While this is an overestimate, the actual value would be greater than half of this concentration.

To evaluate the effects of exogenous monomer binders on the pool of Tβ4-actin present inside the cell and responding to the change in free G-actin concentration, we assumed that essentially all of the DNase I, which has an affinity for G-actin of about 1 nm (Weber et al., 1992), that entered the cell would bind G-actin. This would decrease the pool of Tβ4-actin complex present, lower the concentration of free G-actin, and cause F-actin depolymerization, as observed (Fig. 1). We assumed that a fraction of the Tβ4 that entered the cell would bind G-actin (the fraction determined by its affinity, which was assumed to be the same...
Fig. 1. Concentration dependence of DNase I, Tβ4, and profilin on basal and GTPγS-induced F-actin levels. Cells permeabilized with 2 × 10^7 IU SLO/cell were resuspended in IP buffer containing various concentrations of DNase I (panel A), Tβ4 (panel B), or profilin (panel C). After incubating for 3 min at room temperature, 100 

\[
\frac{[P]}{[P]} = \frac{[P]}{[P]} \cdot K_p + A \cdot K_p + A \approx \frac{[P]}{[P]} = \frac{[P]}{[P]} \cdot A \quad (k_p + A) \approx \frac{[P]}{[P]}
\]

where \([P]_0\) is free profilin, \([PA]_0\) is the profilin-G-actin complex, \(f_p\) is the fraction of barbed filament ends with profilin bound, and \(f_a\) is the fraction free to elongate. \(K_p\) and \(K_p\) are the affinity constants of profilin for free G-actin and barbed filament ends, respectively. \(A\) is then obtained by setting the net filament elongation rate to zero

\[
f_k = f_k \cdot A + k_p \cdot (k_p - f_k) - f_k \cdot k_p + \frac{[PA]}{[PA]} = k_p \cdot A - k_p = 0
\]

where \(k_p\), \(k_p\), and \(k_4\) are the rate constants of filament elongation at the barbed and pointed ends by G-actin, and at the barbed end by the profilin-G-actin complex respectively; and \(k_p\), \(k_p\), and \(k_4\) are the corresponding depolymerization rate constants. This equation, which yields a cubic equation in \(A\), was conveniently solved numerically by binary search with an initial range from zero to the pointed end critical concentration.

RESULTS

Exogenous G-actin Binding Proteins, Including Profilin, Lower Basal F-actin Levels—Inclusion of G-actin sequestering proteins, DNase I or Tβ4, in the buffer at the time of permeabilization (warming) caused a decrease in basal F-actin of SLO-permeabilized cells as indicated by TRITC-phalloidin staining (lower curves in Fig. 1, A and B). The magnitude of the decrease depended on the concentration of sequestering protein added. It was unlikely that these effects were mediated by sequestering G-actin that had left the permeabilized cell, since the cell concentration was such that concentration of G-actin in the medium was always exceedingly low (see “Experimental Procedures”). Rather, the proteins presumably acted after entering the permeabilized cell and sequestering the free G-actin there. In order to affect the high concentrations of buffered G-actin present in the cytoplasm, high concentrations of the monomer binding protein were needed. With DNase, a 50% decrease in basal F-actin staining was seen after warming for 4 min in 15 μM and a maximal decrease of about 60% in 30 μM. With Tβ4, a 50% decrease was seen with about 50 μM. As expected, the molar concentrations of Tβ4 required to cause a given amount of depolymerization was greater than that of DNase I since the affinity of Tβ4 for cellular G-actin is much lower than that of DNase I (K_d = 0.6 μM) due to G-actin interaction with the cytoplasm, high concentrations of the monomer binding protein were needed. With DNase, a 50% decrease in basal F-actin staining was seen after warming for 4 min in 15 μM and a maximal decrease of about 60% in 30 μM. With Tβ4, a 50% decrease was seen with about 50 μM. As expected, the molar concentrations of Tβ4 required to cause a given amount of depolymerization was greater than that of DNase I since the affinity of Tβ4 for cellular G-actin is much lower than that of DNase I (K_d = 0.6 μM) due to G-actin interaction with the cytoplasm. The data from three (A) or two (B and C) different experiments were pooled by setting as 100% the basal level of saturable TRITC-phalloidin (basal fluorescence minus the fluorescence in the presence of excess unlabeled phalloidin). Error bars indicate the S.D. (A) or range of values (B and C).

as endogenous Tβ4, 0.6 μM. This would increase the amount of Tβ4-actin complex present and cause depolymerization. While some of the G-actin released by depolymerization would leave the cell, some would bind to the monomer binders present; for this calculation, the contribution of G-actin released by depolymerization was ignored.

We then calculated for each concentration of monomer binder in the cell the change in the amount of G-actin that would be bound to Tβ4 (endogenous and exogenous) if stimulation changed the critical concentration as described above. This change was expressed as a percentage of the change occurring in the absence of exogenous monomer-binding proteins.

Calculation of Critical Concentration—The apparent actin critical concentration A (the free G-actin at which no net filament elongation occurs) was calculated as a function of the fraction of filaments whose barbed ends are not blocked by capping proteins other than profilin, \(f_b\), and at various total profilin concentrations, \([P]\), as follows. Following Pantaloni and Carlier (1993), we assumed that profilin binding to G-actin and barbed filament ends was at equilibrium, yielding

\[
P = \frac{[PA]}{[PA]} + \frac{[PA]}{[PA]} + \frac{[PA]}{[PA]} = \frac{[PA]}{[PA]} \quad (k_p + A) \approx \frac{[PA]}{[PA]}
\]
ends were made available by GTP above that of stimulated F-actin. This might occur if barbed was below the critical concentration of the basal F-actin but filaments for G-actin such that the free G-actin concentration

Fig. 2. Time course of polymerization in the presence of profilin or Tβ4. Cells were permeabilized and resuspended in buffer with (triangles) or without (squares) 40 μM profilin (panel A) or 40 μM Tβ4 (panel B). After warming for 2 min, 100 μM GTPγS was added to half of the samples (filled symbols), which were then fixed and stained with TRITC-phalloidin at various times. In each case, the data are from one experiment representative of two.

filaments for G-actin such that the free G-actin concentration was below the critical concentration of the basal F-actin but above that of stimulated F-actin. This might occur if barbed ends were made available by GTPγS stimulation. Interestingly, profilin at concentrations up to 100 μM caused little decrease (9% ± 8%) in the level of F-actin achieved after GTPγS addition (Fig. 1C). The presence of profilin had little effect on the rate of polymerization; rather the increase in the magnitude of the change in F-actin seen in the presence of profilin resulted from polymerization continuing until it reached the same level (or occasionally greater) in the presence as in the absence of exogenous profilin (Fig. 2A). In contrast, in the presence of exogenous Tβ4, polymerization never reached the level achieved in its absence (Fig. 2B). The ability of profilin to decrease basal F-actin without significantly inhibiting GTPγS induced polymerization is consistent with the hypothesis that barbed ends were capped in the resting cell and freed upon stimulation.

The freeing of barbed ends, by shifting the affinity of F-actin (for G-actin), could account for the concentration dependence of both DNase I and thymosin β4 on the amount of GTPγS-induced actin polymerization. The data from these experiments were compared with those predicted from a simple model (see “Experimental Procedures”) in which the F-actin increase equalled the amount of G-actin released from a Tβ4 complex and polymerizing when the affinity of the F-actin shifted from 0.5 to 0.1 μM. When we assumed that the concentration of monomer binder in the cell by the time of fixation (5 min) equalled the concentration added to the medium, the predicted increases in F-actin were similar to those observed (Fig. 3). The

Fig. 3. Model of the effects of Tβ4 and DNase I on the polymerization induced by GTPγS. Data on the amount of F-actin induced by GTPγS in the presence of different concentrations of Tβ4 (filled squares) or DNase I (filled triangles) from Fig. 1, A and B, are compared with predictions from a model of monomer sequestering activity (open squares) as described under “Experimental Procedures.”

fact that the model fits both the DNase I and Tβ4 data suggested that stimulation was not selectively inactivating Tβ4, since this would have made Tβ4 a less effective inhibitor than DNase I. The model did not replicate the slight increase in the magnitude of the F-actin induced by GTPγS in the presence of low concentrations of Tβ4. An increase in the magnitude of F-actin induced by GTPγS was also seen with profilin at all concentrations but not with DNase I. This increase was probably due to polymerization of the profilin and Tβ4-actin complexes that arose as a result of the depolymerization of basal F-actin. These complexes were ignored in the model, as noted under “Experimental Procedures,” but could contribute to the increase in F-actin after stimulation. However, the DNase I-actin complexes are of too high affinity to contribute.

The hypothesis that GTPγS acts by making free barbed ends available is supported by experiments with cytochalasin. Cytochalasin B inhibited much of the GTPγS-induced polymerization. Maximal inhibition was achieved with concentrations of cytochalasin B between 2 and 10 μM. At these concentrations, the polymerization induced by 100 μM GTPγS was inhibited by 64 ± 3% (Fig. 4A). Since in intact cells cytochalasin B completely inhibits the polymerization induced by chemoattractant, it is not clear why the inhibition in SLO-permeabilized cells was not complete. In the presence of cytochalasin, the magnitude of the decrease in F-actin caused by either profilin (Fig. 4B) or 40 μM Tβ4 (not shown) was similar before and after GTPγS stimulation. This ruled out the possibility that the failure of profilin to decrease the GTPγS-induced F-actin in the absence of cytochalasin was due to GTPγS inhibiting its sequestering ability. The presence of cytochalasin B had little effect on basal F-actin level or on the ability of 40 μM profilin to lower the basal F-actin levels (Fig. 4B). This is consistent with the hypothesis that the steady state was already determined primarily by the pointed ends.

The Rate and Level of Polymerization Depended on the Concentration of GTPγS Added—If GTPγS induces actin polymerization by making barbed ends available for polymerization, one might expect that the rate of polymerization would depend on the number of barbed ends free and thus on the GTPγS concentration. Indeed, the rate of polymerization was a function of the concentration of GTPγS added. A plateau level of staining was achieved about 45 s after the addition of 100 μM GTPγS, about 1 min after addition of 1 μM GTPγS and about 4 min after the addition of 100 nM GTPγS (Fig. 5).

To determine if the cessation of polymerization at the plateau was due to depletion of GTPγS, we examined whether GTPγS was still available at the time the plateau level of F-actin was reached. Supernatants of permeabilized cells that
concentration of G-actin, which was continually being lost from GTP-gTPS stimulation and was limited eventually by depletion of the cellular reservoir of G-actin. The data from four different experiments were normalized as in Fig. 1. Error bars indicate the S.D. B, the effect of cytochalasin on polymerization induced in the presence of profilin. Cells were incubated in buffer with or without 40 μM profilin and with or without 2 mM cytochalasin B. After 2 min, 100 μM GTP-gTPS was added, and the samples were fixed and stained 2 min later. Data plotted are duplicates from a single experiment representative of two experiments.

had been incubated 15 min with or without a suboptimal concentration of GTP-gTPS (300 nM) were ultrafiltered (cut off 10,000 daltons) to remove proteins such as G-actin and monomer binding proteins and then tested for their ability to stimulate polymerization in freshly permeabilized cells. The supernatant from control cells had no effect on F-actin levels, but the supernatant from stimulated cells induced polymerization to approximately the same level as that observed with the original cytoskeleton (data not shown). Thus, the termination of net polymerization at plateau was not limited by depletion of GTP-gTPS.

Cells Stimulated with Suboptimal Concentrations of GTP-gTPS Were Able to Increase F-actin Level Upon Addition of More GTP-gTPS—It was interesting that the plateau level of polymerization depended on the concentration of GTP-gTPS added. If a significant number of barbed ends were freed and remained free upon stimulation, then even at low concentrations of GTP-gTPS, polymerization might continue until the free G-actin was decreased nearly to the critical concentration of the barbed end (Young et al., 1990). It was possible that the plateau level of polymerization achieved depended on the rate of polymerization and was limited eventually by depletion of the cellular concentration of G-actin, which was continually being lost from the permeabilized cells. However, cells stimulated with a suboptimal dose of GTP-gTPS were able to further polymerize actin when stimulated again with a higher concentration of GTP-gTPS, indicating that after the F-actin had reached a plateau, a reservoir of G-actin and G-proteins remained and was capable of responding (Fig. 6). Cells incubated for 4 min with 0.1 μM GTP-gTPS increased their F-actin level further when 100 μM GTP-gTPS was added. The amount of polymerization achieved after the sequential additions of GTP-gTPS was greater than that induced in cells warmed for 6 min before addition of 100 μM GTP-gTPS. Cell responsiveness does decrease with the duration of warming, probably because G-actin and other components are leaving the cell (Redmond et al., 1994); the higher level of F-actin achieved after sequential additions of GTP-gTPS may be due to the fact that the initial polymerization decreased the amount of G-actin that left the cell during this period.3

Barbed Ends Remain Free after the F-actin Has Reached Its Plateau Level—The sustained level of polymerization seen following GTP-gTPS stimulation may indicate that GTP-gTPS inhibited depolymerization or that the F-actin in permeabilized cells, like lysed cells, was inhibited from depolymerizing (Cano et al., 1992b). However, addition of various concentrations of T4 (10, 20, or 40 μM) at 6 min after permeabilization (4 min after GTP-gTPS stimulation) caused equal decreases in stimulated and basal F-actin (not shown). Alternatively, the sustained F-actin level may indicate that a fraction of filaments still have their barbed ends free. This possibility is supported by the fact that addition of cytochalasin lowered the plateau level of F-actin. 3 min after addition of cytochalasin B, there was a loss of 35 ± 7%, n = 3, of the F-actin induced by GTP-gTPS (Fig. 7). The addition of cytochalasin B to unstimulated cells had little effect on basal F-actin.

This conclusion that barbed ends remained free was further supported by the observation that addition of profilin to stimulated cells did not decrease the plateau level of F-actin. The TRITC-phalloidin staining remained unchanged or was increased slightly (Fig. 8A). Profilin did cause depolymerization of basal F-actin in unstimulated cells that had been permeabilized for the same period of time, indicating that prolonged

3 M. Tardif, S. Huang, T. Redmond, D. Safer, M. Pring, and S. H. Zigmond, unpublished observation.
Different experiments were pooled as described in Fig. 1. Stained with TRITC-phalloidin at various times. The data from 3 different experiments were pooled as described in Fig. 1.

Fig. 6. Effect of sequential addition of an increased dose of GTPγS on induced F-actin levels. Permeabilized cells were warmed in IP buffer for 2 min, then incubated (at time = 0) without (open circles) or with 100 nM GTPγS (open squares). At 4 min, 100 μM GTPγS was added to some samples of resting (filled circles) and stimulated (filled squares). Samples were fixed at various times. The data from two different experiments were pooled as described in Fig. 1.

Fig. 7. Time course of change in basal and GTPγS-induced F-actin levels caused by addition of cytochalasin B. Cells, permeabilized and warmed in IP buffer for 2 min, were incubated at (time = 0) without (open circles) or with 300 nM GTPγS (open squares). At 3 min, 4 μM cytochalasin B was added on a fraction of both resting (filled circles) and stimulated (filled squares) cells. Samples were fixed and stained with TRITC-phalloidin at various times. The data from 3 different experiments were pooled as described in Fig. 1. Error bars indicate the S.D.

permeabilization did not on its own make cells insensitive to profilin (Fig. 8B). However, when profilin was added to cells that had been stimulated with GTPγS and then treated with cytochalasin B to block all barbed ends, it did cause depolymerization (Fig. 8A). Thus, when the barbed ends were capped with cytochalasin, profilin did cause depolymerization even in the presence of GTPγS. This indicated that the differential effects of profilin on basal and stimulated cells were due to differences in the availability of free barbed ends and reinforced the conclusion that GTPγS did not inhibit the G-actin sequestering ability of profilin.

The Number of Free Barbed Ends Available at Plateau Appears to be Low—The effects of profilin, cytochalasin, and the differential effects of DNase I and Tp40 on basal and stimulated F-actin all supported the conclusion that GTPγS induced actin polymerization by increasing the availability of free barbed ends. However, the fraction of filaments in the permeabilized cells having free barbed ends was not known. The presence of barbed ends would be expected to increase the rate of depolymerization when the free G-actin concentration was below the critical concentration. We compared the rate of depolymerization of resting and stimulated cells after addition of DNase I to decrease the free G-actin concentration. Addition of DNase I (25 μM) decreased the GTPγS-induced and basal F-actin levels (Fig. 9). The initial rate of depolymerization of GTPγS-induced F-actin was only slightly greater (about 2-fold) than that of basal F-actin. With time, DNase I reduced the F-actin in both control and stimulated cells to ≈20% of the original basal level. Thus, the stimulated F-actin did depolymerize, and the failure to detect differences in the rates of depolymerization between control and stimulated cells could not be due to the fact that only basal F-actin was depolymerizing.

To determine if this slight increase in rate was due to barbed-end depolymerization, we compared the rate of depolymerization induced by DNase I in the presence and absence of cytochalasin, which blocks barbed end depolymerization. The presence of cytochalasin did not detectably decrease the rate of depolymerization over that seen with DNase alone (data not shown). Nor did the presence of cytochalasin decrease the rate of depolymerization observed when (a) the DNase concentration was increased to 100 μM to insure that the G-actin concentration was below the critical concentration of the barbed end or (b) the DNase was added soon after GTPγS when the F-actin level was still rising.

GTPγS increased the number of sites able to nucleate actin polymerization after cell lysis—To determine if permeabilized cells, like intact cells, did increase the number of nucleation sites available after lysis, we examined the ability of permeabilized cells lysed with Triton to nucleate barbed-end elongation of exogenous pyrenyl actin (Cano et al., 1991). GTPγS
stimulation of SLO-permeabilized cells increased the number of filament ends that were free following cell lysis. The time course of increase in nucleation sites following stimulation with 100 μM GTPγS paralleled the F-actin polymerization; a maximum increase occurred at about 45 s and then was maintained for at least 10 min (Fig. 10A). The number of sites induced within 3 min of stimulation with GTPγS depended on the concentration of GTPγS added. With 100 μM GTPγS, the number was 2.04 ± 0.43 times that of unstimulated cells (n = 5); with 1 μM GTPγS, the number was 1.52 ± 0.30 times the basal level (n = 5) (Fig. 10B). It was not possible to detect an increase with concentrations less than 0.3 μM GTPγS.

It is important to note that unstimulated cells also nucleated barbed-end elongation after lysis. The presence of 2 μM cytochalasin reduced the rate of polymerization of 1.5 μM pyrenyl-actin more than 80%, suggesting that after lysis, greater than 20% of the filaments had free barbed ends (Korn et al., 1987). However, as noted above, the effects of adding cytochalasin and profilin to unstimulated cells suggested that most barbed ends were capped. Thus, sites that nucleate barbed end polymerization after lysis may not have been free in the permeabilized cell before lysis.

In the Permeabilized Cell, the Fraction of Filaments with Free Barbed Ends May Be Small—The similar rate of DNase I induced depolymerization of resting and stimulated F-actin and the lack of effect by cytochalasin suggested that in stimulated cells a relatively small fraction of the filaments had free barbed ends. This is compatible with the fact that freeing a small fraction of the total number of barbed ends is sufficient to shift the actin critical concentration (Young et al., 1990). With pure actin, using parameters described under "Experimental Procedures," the transition from all barbed ends capped to all barbed ends uncapped would shift the critical concentration from 0.5 to 0.115 μM; uncapping only 10% of the filaments could decrease the critical concentration from 0.5 to 0.2 μM. Since profilin has been shown to alter the level of polymerization, we asked whether profilin would affect this change in critical concentration as a function of free barbed ends. Indeed, the presence of profilin exaggerates the effects of uncapping. Using rate constants estimated by Pantaloni and Carlier (1993) in the presence of 5 μM profilin and sufficient G-actin, freeing 10% of the barbed end would increase the critical concentration to 0.046 μM; freeing only 1% of the ends would decrease the critical concentration to 0.226 μM (Fig. 11; Pring et al., 1992). In the presence of 30 μM profilin, the critical concentrations with 10 and 1% of the barbed ends free would be 0.025 and 0.14 μM, respectively. We estimated from preliminary data (see "Experimental Procedures") that about 10 μM profilin would be present in the cells 2 min after permeabilization. Thus, the freeing of less than 10% barbed ends could significantly shift the critical concentration and induce polymerization.

DISCUSSION

Permeabilized Cells Are Useful for Studies of the Regulation of Actin Polymerization—Since permeabilized neutrophils respond to agonists by polymerizing actin, they allow investigation, not possible after cell lysis, of the mechanisms regulating the actin steady state. Permeabilized neutrophils mimic the behavior of intact neutrophils in that they can be stimulated through chemoattractant receptor or downstream of the receptor by GTPγS (Redmond et al., 1994). The basal F-actin level as well as that induced by stimulation are similar to those in intact cells. Finally, as demonstrated here, like intact cells, the increase in F-actin is associated with an increase in the number of free barbed ends available after lysis to nucleate actin elongation of exogenous pyrenyl actin. For the studies described here we have chosen to stimulate polymerization with GTPγS. GTPγS presumably acts, at least in part, through the pertussis toxin-sensitive trimeric G-protein through which chemoattractants act, although it may also activate downstream small G-proteins (Redmond et al., 1994).
Stimulation with GTPγS has the advantages that (a) it induces a large and stable polymerization of F-actin and (b) the response is not limited by receptor dynamics, e.g. phosphorylation and internalization, or by GTP hydrolysis. Thus the magnitude of the F-actin increase may closely reflect the properties of actin steady state.

While the cytoplasm of a permeabilized cell is changing over time as soluble components diffuse out, it is possible to systematically investigate the effects of exogenous factors within a limited time window. Because the experiments are very rapid, one can alter the composition of the cytoplasm without the limited time window. Because the experiments are very rapid, one can alter the composition of the cytoplasm without the secondary effects that can result from chronic alterations, for example, following transfection of intact cells.

Filament Barbed Ends Are Mostly Capped in Unstimulated Cells but Are Available following GTPγS Addition—The effect of exogenous profilin on permeabilized cells has provided strong evidence that (a) the presence of profilin alone was not sufficient to stimulate polymerization and (b) GTPγS-induced polymerization and maintenance of actin polymerization occurs through a shift in the actin steady state toward that of the barbed ends. Thus, while addition of exogenous DNase I and Tβ4 caused depolymerization of basal F-actin and inhibited the increase in F-actin induced by GTPγS, addition of profilin decreased the basal F-actin level but had little or no effect on the GTPγS-induced increase in F-actin. Since the profilin-G-actin complex, unlike complexes with the other G-actin binding proteins, does not block polymerization at the filament barbed ends, these data suggested that in the resting cell most barbed ends were capped but after stimulation at least some were free. This conclusion was supported by the observation that profilin decreased the F-actin after stimulation only if cytochalasin was also present. This observation ruled out the possibility that GTPγS inactivated profilin. While these studies do not rule out the possibility that stimulation regulates other factors, the regulation of the availability of free barbed ends may account for many other agonist-induced changes in steady state.

A 5-fold decrease in the critical concentration caused by freeing barbed ends could account for the greater inhibition of basal than stimulated F-actin by DNase I and Tβ4. The fact that both DNase I and Tβ4 were equally well fit by a simple model suggests that the properties of Tβ4 were not modified, even transiently, by stimulation with GTPγS. Furthermore, in the presence of cytochalasin, both profilin and Tβ4 caused similar decreases in F-actin in the presence or absence of GTPγS, indicating that neither protein was inhibited by GTPγS. This extends previous studies that had shown that there was no stable modification of Tβ4 following stimulation (Safer et al., 1990; Cassimeris et al., 1992; Nachmias et al., 1993), but had left open the possibility that it might be transiently modified at the time of stimulation. Local modification of Tβ4 or profilin is not ruled out by the studies presented here.

The fraction of filaments that have free barbed ends before and after polymerization in the permeabilized cell is not known. In the resting cell, the fraction appears to be low as profilin acts as an effective monomer-sequestering protein. However, the fact that profilin did not decrease resting F-actin levels as well as Tβ4 may indicate that in the resting cell some barbed ends are free. Indeed, in some experiments cytochalasin did decrease the basal F-actin slightly, and profilin had a slightly greater effect on basal F-actin when cytochalasin was present (see Fig. 4b). Although cell lysates made after stimulation showed a large increase in the number of free barbed ends, in the permeabilized cell, it was not possible to detect the presence of free barbed ends by studies of the rate of depolymerization. The 2-fold increase in rate of depolymerization induced by DNase I in stimulated versus control cells may result from a 2-fold increase in filament number associated with the increase in F-actin, i.e. 2 times more pointed ends (Cano et al., 1991). Furthermore, the presence of cytochalasin did not detectably slow the DNase-induced depolymerization of stimulated F-actin.

One can imagine various factors including the presence of cross-linking proteins that might limit our ability to detect barbed ends from the kinetics of depolymerization. We therefore also examine the rate of polymerization induced by phalloidin. Addition of phalloidin to permeabilized cells stimulated actin polymerization in both resting and stimulated cells. The phalloidin presumably acts by inhibiting depolymerization while allowing addition of monomer at either filament end. However, the rate of polymerization was no greater in stimulated than in control cells, even when the cells were stimulated with suboptimal concentrations of GTPγS to insure that a reservoir of G-actin remained. Nor could we detect an effect of cytochalasin on this rate, even though in vitro cytochalasin effectively inhibited polymerization at the barbed end in the presence of phalloidin.

The discrepancy between the data showing that free barbed ends determine the stimulated F-actin steady state and the inability to actually measure free barbed ends in stimulated permeabilized cells is reconciled by a calculation showing that in the presence of profilin, a very small fraction of filaments with free barbed ends can shift the critical concentration. Thus, the number available at any time may be well below current detection limits. These studies shed no light on the mechanism through which barbed ends become available upon GTPγS stimulation. They may arise from uncapping, cutting of filaments or de novo nucleation of new filaments. These mechanisms remain to be defined.

Conclusions—Studies using neutrophils permeable to profilin and other G-actin sequestering proteins provide strong evidence that GTPγS increases and maintains elevated F-actin levels by increasing the fraction of actin filaments with barbed ends available for polymerization. The number of free barbed ends appears to be a small fraction of the total filaments present.

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