Capping Protein Terminates but Does Not Initiate Chemoattractant-induced Actin Assembly in *Dictyostelium*

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Abstract. The first step in the directed movement of cells toward a chemotactic source involves the extension of pseudopods initiated by the focal nucleation and polymerization of actin at the leading edge of the cell. We have previously isolated a chemoattractant-regulated barbed-end capping activity from *Dictyostelium* that is uniquely associated with capping protein, also known as cap32/34. Although uncapping of barbed ends by capping protein has been proposed as a mechanism for the generation of free barbed ends after stimulation, in vitro and in situ analysis of the association of capping protein with the actin cytoskeleton after stimulation reveals that capping protein enters, but does not exit, the cytoskeleton during the initiation of actin polymerization. Increased association of capping protein with regions of the cell containing free barbed ends as visualized by exogenous rhodamine-labeled G-actin is also observed after stimulation. An approximate threefold increase in the number of filaments with free barbed ends is accompanied by increases in absolute filament number, whereas the average filament length remains constant. Therefore, a mechanism in which preexisting filaments are uncapped by capping protein, in response to stimulation leading to the generation of free barbed ends and filament elongation, is not supported. A model for actin assembly after stimulation, whereby free barbed ends are generated by either filament severing or de novo nucleation is proposed. In this model, exposure of free barbed ends results in actin assembly, followed by entry of free capping protein into the actin cytoskeleton, which acts to terminate, not initiate, the actin polymerization transient.

During the phenomenon of ameboid chemotaxis, the binding of chemoattractant to external cell surface receptors signals a complex series of intracellular events that ultimately result in changes in cell shape and orientation toward the source of chemoattractant. The extension of pseudopods is one of the primary morphological responses to chemoattractant in *Dictyostelium* amebas. After stimulation of starved *Dictyostelium* cells with the chemoattractant cAMP, a rapid increase in both cytoskeletal actin (McRobbie and Newell, 1983) and actin polymerization is observed (Hall et al., 1988), which correlates with changes in cell shape (Condeelis, 1993). This increase in F-actin after stimulation is reversible and is cotemporal with an increase in actin nucleation activity detected in lysates prepared with Triton X-100 detergent (Hall et al., 1989). All detectable nucleation activity is associated with the Triton-insoluble cytoskeletal fraction and is sensitive to cytochalasin D, suggesting that free barbed ends of filaments associated with the low speed pelletable cytoskeleton are the source of actin nucleation activity in stimulated lysates (Hall et al., 1989).

Characterization of the supernatant fraction revealed a Ca^{2+}-insensitive inhibitor of actin nucleation that is regulated by cAMP with kinetics reciprocal to the actin nucleation activity. This result suggested that a barbed-end capping activity is involved in the generation of free barbed ends that serve as polymerization nuclei after stimulation. Fractionation of cytosolic extracts from resting and stimulated cells confirmed that this inhibitory activity is a barbed-end capping activity that is uniquely regulated during cAMP stimulation (Sauterer et al., 1991). Further isolation of this cAMP-regulated, Ca^{2+}-insensitive, barbed-end capping activity led to the copurification of capping protein and the 70-kD heat shock cognate protein, Hsc70 (Sauterer et al., 1991; Eddy et al., 1993, 1996). *Dictyostelium* capping protein is also known as cap32/34 (Schleicher et al., 1984).

Detailed analysis of the cAMP-regulated capping activity associated with capping protein and Hsc70 (Eddy et al., 1996) demonstrated that although both proteins copurify, capping protein is solely responsible for the capping activity. In addition, Hsc70 does not function as a cofactor in the regulation of the capping activity, since Hsc70 neither

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stimulated nor inhibited the activity of isolated native capping protein (Eddy et al., 1996), contrary to the results observed by Haus et al. (1993) for the interaction of Hsc70 and bacterially expressed capping protein. Studies with the skeletal muscle homologue of capping protein also failed to detect any enhancement in the activity of native capping protein by Hsc70 (Schafer et al., 1996). However, the copurification of Hsc70 and capping protein (Haus et al., 1993; Eddy et al., 1996) suggests that Hsc70, acting as a chaperone, may assist in the proper folding and assembly of nascent capping protein heterodimers in vivo (Eddy et al., 1996) and the expression of fully active recombinant capping protein in vitro (Haus et al., 1993).

Further insight into the function of capping protein in vivo has been obtained through the analysis of cells that under- and overexpress capping protein. Studies by Hug et al. (1995) have shown that capping protein binds to and can determine the number of free barbed ends, consistent with the possibility that capping protein is a buffer of free barbed ends in vivo.

After the identification of capping protein as the protein responsible for the cAMP-regulated capping activity in *Dictyostelium* cytosols (Eddy et al., 1996), we set out to investigate the function of capping protein during cAMP-stimulated actin polymerization transients. To account for the abrupt drop in capping protein-associated capping activity observed in cytosolic extracts from cells lysed after stimulation (Hall et al., 1989), either (*a*) the activity of capping protein is switched off by a mechanism not involving Hsc70, or (*b*) capping protein remains active but is recompartmentalized from the cytosol into the cytoskeleton in response to the appearance of free barbed ends elicited independently by cAMP, resulting in a loss of capping activity from the cytosol.

Studies in platelets have addressed the recompartmentalization of capping protein during stimulated actin polymerization. Stimulation of platelets with thrombin releases a small amount (10–15%) of capping protein from the Triton-insoluble cytoskeleton (Nachmias et al., 1996). In addition, treatment of *n*-octyl β-D glucopyranoside permeabilized platelets with the thrombin receptor activating fragment also releases a similar fraction of capping protein from the cytoskeleton (Barkalow et al., 1996), suggesting the capping activity of a small fraction of capping protein is either switched off, or the on rate of capping protein for the barbed ends is reduced after stimulation (Schafer et al., 1996). These results are consistent with a model where an uncapping mechanism is responsible for the generation of free barbed ends. Paradoxically, the vast majority of capping protein was found to associate with the Triton-insoluble cytoskeleton upon thrombin activation of intact platelets (Barkalow et al., 1996), also consistent with a model in which free barbed ends, generated by a mechanism that does not involve uncapping by capping protein, are subsequently capped by capping protein. These results leave unclear the precise mechanism of chemoattractant-induced actin nucleation and the function of capping protein during stimulated actin assembly in excitable cells.

In this paper, we present a kinetic and spatial localization analysis on the cytoskeletal association of capping protein in *Dictyostelium* after cAMP stimulation, and compare these results to the appearance of free barbed ends with high temporal resolution. These results define a role for capping protein in the early actin polymerization transients after stimulation of motile cells, and suggest a mechanism for generating free barbed ends during this event.

Materials and Methods

Reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. *Escherichia coli* DNA, assistance with the DNase I assay, and polyclonal antisera against *Dictyostelium* capping protein were provided by the laboratory of J.A. Cooper (Washington University Medical School, St. Louis, MO).

Triton-insoluble Cytoskeletons

Triton-insoluble cytoskeletons were prepared as described by Dharmawardhane et al. (1989) with some modifications. *Dictyostelium* strain (AX3) cells were starved at 10^7 cells/ml in suspension for 5.5 h at 22°C and then transferred to a 10° or 22°C shaking water bath for 30 min as indicated in the presence of 3 mM caffeine. At this caffeine concentration, *Dictyostelium* adenylate cyclase is inhibited and exogenous intercellular cAMP signaling is prevented (Brenner and Thomas, 1980). At various times after stimulation with 10 μM 2′ deoxy-cAMP, 2×10^6 cells/ml (final) were lysed in L buffer (20 mM Pipes, pH 7, 50 mM KCl, 5 mM EGTA, 0.2 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.5% Triton X-100, 5 μg/ml each of leupeptin, chymotrypsin, and pepstatin A) with or without 20 μM phalloidin. Cells were also mechanically lysed by forced passage through a 3-μm nucleopore filter (Millipore Corp., Waters Chromatography, Milford, MA) according to Das and Henderson (1983). Lysates were vortexed for 5 s and immediately microfuged at 4°C for 3 min at 8,700 *g* (low speed cytoskeleton), or centrifuged for 1 h at 415,000 *g* in a rotator (high speed cytoskeleton; TLA-100.2; Beckman Instrs., Fullerton, CA). The low or high speed Triton-insoluble cytoskeleton pellets were resuspended on ice in 20% lysate volume of TBS, pH 7.6, plus protease inhibitors, and Western blotted using anti–capping protein-α antibodies followed by ^{125}I–protein A (DuPont-NEN, Boston, MA) as previously described (Eddy et al., 1996). Actin levels in cytoskeleton pellets were determined by Coomassie blue staining of the 42-kD actin band after SDS-PAGE as described (Laemmli, 1970). Densitometry analysis of Coomassie blue–stained SDS–polyacrylamide gels and autoradiograms were performed on a computing desitometer (ImageQuant Software, version 3.3; Molecular Dynamics, Inc., Sunnyvale, CA). The percent total actin and capping protein in low speed Triton-insoluble cytoskeletons was calculated as: 100 × (lysate value − supernatant value)/lysate value, or directly from densitometry of pellet fractions. Both methods led to the same conclusions.

In Vitro Actin Nucleation Assay

Dictyostelium AX3 cells were starved at $4–6 \times 10^6$ cells/ml for 5.5 h at 22°C, transferred to 10° or 22°C for 30 min in the presence of 3 mM caffeine. At various times after stimulation with 10 μM 2′ deoxy-cAMP, 10^6 cells/ml (final) were lysed in L buffer plus 10 mg/ml bovine serum albumin. 2 μM G-actin (10% pyrene-labeled) was immediately added to the lysate, and actin polymerization was monitored by an increase in pyrene fluorescence with a fluorescence spectrophotometer (F-2000; Hitachi, Ltd., Tokyo, Japan) using 365 and 407 nm as the excitation and emission wavelengths, respectively. Relative nucleation rate is defined as the ratio of initial rate in stimulated lysates to initial rate in resting lysates.

Quantitation of Free Pointed Ends

The number of free pointed filament ends was determined using the DNase I inhibition assay as previously described (Podolski and Steck, 1990). *Dictyostelium* AX3 cells were starved at 10^7 cells/ml for 5.5 h at 22°C, concentrated to 2×10^7, and transferred to 10°C for 30 min in the presence of 3 mM caffeine. Cells were lysed at various times after stimulation with 10 μM 2′-deoxy cAMP. The number of filament pointed ends was calculated by fitting experimental Cpm values to a *Dictyostelium* G-actin standard curve at fixed DNase I concentration.

Cellular Protein Concentration

Total protein concentration in *Dictyostelium* AX3 cells harvested at 4–12 × 10^6 cells/ml was determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard curve. Capping protein was identified by Western blotting of SDS–polyacrylamide gels of cell lysates. Coomassie blue–stained gels of cell lysates were analysed by densitometry. Cellular concentrations of actin and capping protein were calculated based on percent of total density of the lysate.

Immunofluorescence Microscopy

Dictyostelium AX3 cells were harvested as previously described (Hall et al., 1989), and starved for 5 h in 14.8 mM NaH_2PO_4, 5.2 mM K_2PO_4, pH 6.6, at 10^6 cells/ml. Cells were allowed to settle onto 12-mm circular glass coverslips (Fisher Scientific Co., Pittsburgh, PA) for 30 min at 22°C and then transferred to 10°C for 30 min in the presence of 3 mM caffeine. Control cells or cells stimulated with 10 μM 2′ deoxy-cAMP for 20 s were fixed for 3.5 min in 1% glutaraldehyde (Ted Pella, Inc., Redding, CA), 0.1% Triton X-100 in general buffer (10 mM Pipes, pH 6.8, 20 mM KPO_4, 5 mM EGTA, 2 mM $MgCl_2$). For preparation of cytoskeletons, cells were treated for 1 min in buffer B (15 mM Pipes, pH 6.9, 6.25 mM Hepes, 10 mM EGTA, 0.5 mM $MgCl_2$) containing 0.75% Triton X-100, 10 μM phalloidin, 5 μg/ml each of leupeptin, chymotrypsin, and pepstatin A after cAMP stimulation and then fixed for 3.5 min in 1% glutaraldehyde with 5 μM phalloidin in buffer B. Autofluorescence was quenched with 1 mg/ml $NaBH_4$ in general buffer. Coverslips were blocked in 1% bovine serum albumin, 1% fetal calf serum in TBS, pH 7.6, plus 0.1 μM rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) for 15 min and incubated with 50 μg/ml of a polyclonal antibody against *Dictyostelium* capping protein-α (Hug at al., 1995), for 1 h. Fluorescein-labeled goat anti–rabbit IgG (Cappel Laboratories, Malvern, PA) preabsorbed to fixed *Dictyostelium* AX3 cells was used at 10 μg/ml. Cells were mounted in gelvatol plus 6 mg/ml of *n*-propyl gallate as an antibleaching agent and examined with a scanning confocal microscope (MRC600, equipped with a Kr/Ar laser; Bio-Rad Laboratories, Hercules, CA) to ensure complete separation of the fluorescein and rhodamine channels. 0.8–1.0-μM optical sections of stained cells were imaged with a microscope (Diaphot with 60× flat field objective [NA = 1.4]; Nikon, Inc., Tokyo, Japan). Images were prepared using Adobe Photoshop software, version 3.0.5.

In Situ Actin Nucleation Assay

For visualization of actin nucleation sites microscopically using rhodamine-labeled G-actin, AX3 cells were starved and caffeine treated for immunofluorescence microscopy as described. Control cells or cells stimulated with 10 μM 2′ deoxy-cAMP at 10°C were treated with C buffer (10 mM Pipes, pH 7, 20 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 20 μM phalloidin, 0.1% Triton X-100, 5 μg/ml each of chymostatin, leupeptin, and pepstatin A) for 30 s and then washed for 30 s in C buffer minus Triton X-100 and phalloidin. Rhodamine-labeled rabbit skeletal muscle actin, prepared as described in Chan et al. (1997), was diluted to 12 μM in 1 mM Hepes, pH 7.5, 0.2 mM $MgCl_2$, 0.2 mM ATP, sonicated for 5 s, and clarified by ultracentrifugation in an airfuge (Beckman Instrs.) at 22 psi for 20 min, and held on ice for 30 min. Immediately before use, rhodamine-labeled G-actin was diluted to 0.45 μM in 10 mM Pipes, pH 7, 30 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM ATP, and incubated with cells for 5 min at 10°C. Polymerization was terminated by fixation in C buffer plus 1% glutaraldehyde, 5 μM phalloidin for 3.5 min, and coverslips costained for capping protein as described. Images were collected with a camera (KAF 1400 cooled CCD with 1 × 1 binning; Photometrics Ltd., Tucson, AZ) and microscope (IX70 with an infinity corrected 60× objective [NA = 1.4]; Olympus, Tokyo, Japan).

Results

Capping Protein Rapidly Associates with the Cytoskeleton After cAMP Stimulation

It has been shown previously that the chemoattractant cAMP elicits a transient increase in actin nucleation activity 5 s after stimulation in Triton X-100 lysates prepared from starved *Dictyostelium* amebas (Hall et al., 1989). This increase in actin nucleation activity is associated with the low speed Triton-insoluble cytoskeleton and corresponds to an increase in actin polymer content therein. Coincident with the increase in actin nucleation and polymerization, a drop in the level of activity of a single capping activity in cell lysate supernatants is observed (Sauterer et al., 1991). This cAMP-regulated capping activity has been identified as capping protein (Eddy et al., 1996).

One possible interpretation of this drop in capping activity in lysate supernatants after chemotactic stimulation is that the capping activity of capping protein is switched off globally, resulting in the uncapping of capping protein from barbed filament ends. Free barbed ends in the cytoskeleton can serve as nuclei for actin polymerization. In this event, capping protein will be lost from the cytoskeleton after stimulation at times when cytoskeleton-associated actin nucleation activity is increased.

To test whether the uncapping of actin filaments by capping protein is a viable mechanism for the exposure of free barbed ends, low speed cytoskeletons were prepared from Triton X-100 lysates prepared at various times after cAMP stimulation of 6-h starved *Dictyostelium* at 22°C. The low speed Triton-insoluble fraction has been shown previously to contain all of the nucleation activity present in stimulated cells (Hall et al., 1989). The levels of both F-actin and capping protein in the low speed cytoskeleton were analyzed by densitometry of Coomassie blue–stained gels and immunoblots, respectively. At ~5 s after stimulation with cAMP, the levels of F-actin increased ~2.3-fold relative to unstimulated control levels, whereas the level of capping protein associated with the low speed cytoskeleton increased ~2.2-fold (Fig. 1 *A*). To eliminate the possibility that the association of capping protein with Triton-insoluble cytoskeletons may be nonspecific due to the presence of detergent, cells were lysed by forced passage through 3-μm nucleopore membranes (Millipore Corp.) in the absence of Triton X-100. Low speed pellet fractions prepared from nucleopore lysates of *Dictyostelium* cells are enriched in F-actin and membrane sheets (Das and Henderson, 1983). The low speed cytoskeleton fraction prepared by nucleopore lysis displayed a peak increase of ~2.1- and ~2.35-fold in F-actin and capping protein, respectively, relative to prestimulatory levels at ~5 s following cAMP stimulation. These results suggest that capping protein is not lost from the cross-linked cytoskeleton in response to stimulation as predicted in an uncapping mechanism, but rather shows an increased association with the cross-linked cytoskeleton during the peak of actin nucleation.

Lysates Prepared from Cells Cooled to 10°C before cAMP Stimulation Display a Prolonged Actin Nucleation Response

Under the normal growing temperature of 22°C (Fig. 1 *A*), our initial findings demonstrate that capping protein enters rather than exits the cross-linked cytoskeletal fraction at the 5-s peak of actin nucleation. However, due to the rapidity of this reaction, it is unclear from the data collected at 22°C whether a small fraction of capping protein exits the cytoskeletal fraction before the peak of cAMP-stimulated actin nucleation. Therefore, to investigate whether

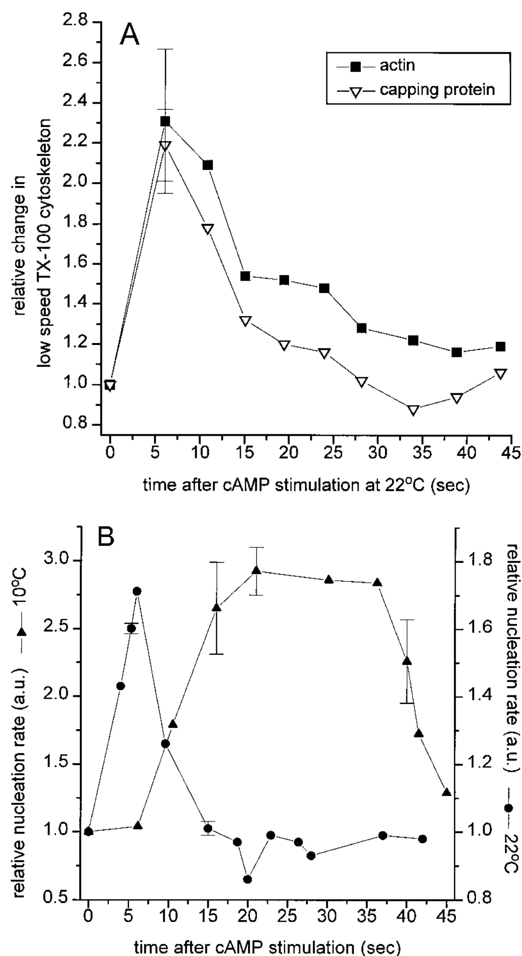

Figure 1. (*A*) Association of capping protein with the low speed Triton-insoluble cytoskeleton after cAMP stimulation at 22°C. *Dictyostelium* AX3 cells (10^7 cells/ml) in 20 mM phosphate buffer, pH 6.6, were starved in suspension for 5.5 h at 22°C, and then treated with 3 mM caffeine for 30 min. At various times after stimulation with 10 μM 2′ deoxy-cAMP, 2×10^6 cells/ml (final) were lysed in L buffer containing 0.5% Triton X-100. Lysates were immediately microfuged for 3 min at 8,700 *g* and the low speed Triton-insoluble cytoskeleton pellets were resuspended to 20% of lysate volume and Western blotted using anti–capping protein-α antibodies followed by densitometry. Actin levels in cytoskeleton pellets were determined by densitometry of Coomassie blue staining of the 42-kD actin band after SDS-PAGE. The 5-s time point value represents data from three separate determinations. (*B*) Relative actin nucleation rate of *Dictyostelium* lysates after stimulation at 22°C and 10°C. AX3 cells were starved at 4–6 × 10^6 cells/ml at 22°C and transferred to either 22° or 10°C as described in Materials and Methods. At various times after stimulation with 10 μM 2′ deoxy-cAMP, 10^6 cells/ml (final) were lysed in L buffer containing 0.5% Triton X-100. 2 μM G-actin (30% pyrene labeled) was immediately added to the lysate, and the initial rate of actin polymerization was monitored as an increase in pyrene fluorescence. Relative nucleation rate is defined as the ratio of initial rate in stimulated lysates to initial rate in resting lysates. Points represent data from three separate experiments ± the standard deviation.

capping protein may undergo transient uncapping from barbed filament ends before the 5-s peak, the temporal resolution of the actin nucleation response was enhanced by equilibrating the cells to 10°C before stimulation with cAMP. Equilibrating *Dictyostelium* amebas to 10°C slows responses to chemotactic stimuli by a factor of four and has been used successfully to study a variety of responses without disruption of any known cellular functions (Gerisch et al., 1979), including the extent of myosin II–mediated contractility (Kuczmarski et al., 1991). In addition, motility in resting and cAMP-stimulated *Dictyostelium* still occurs at 10°C, as expected for an organism that naturally occurs in forest soil where temperatures are ∼10°C. A slowing of the actin nucleation response by a factor of four was observed after cAMP stimulation at 10°C, shifting the peak to ∼20 s poststimulation (Fig. 1 *B*). In addition, the amplitude of the relative rate of actin nucleation was increased slightly as compared to that observed at 22°C.

Capping Protein Enters Both the Cross-linked and Total Cytoskeletal Fraction after cAMP Stimulation

Equilibrating starved *Dictyostelium* amebas at 10°C before cAMP stimulation allowed changes in the association of capping protein with the low speed Triton-insoluble cytoskeleton during the actin nucleation response to be monitored with higher temporal resolution. At ∼20 s after stimulation, the levels of F-actin increased ∼2.8-fold, relative to unstimulated control levels, whereas the level of capping protein associated with the low speed cytoskeleton increased ∼2.5-fold (Fig. 2 *A*; Table I). At no time during the initial phase of actin nucleation was capping protein observed to exit the low speed cytoskeleton.

The accumulation of actin filaments in the low speed cytoskeletal fraction requires the cross-linking of filaments into a structure pelletable under low *g*-force conditions (Cox et al., 1995). To address the concern that the cross-linked actin filament fraction may represent a special subset of the total F-actin in cells, the analysis was repeated under high *g*-force spin conditions (392,500 *g* for 1 h). Under these conditions, ∼100% of the F-actin present in vivo is pelleted (Hall et al., 1988). Upon stimulation, capping protein was found to enter, but not exit, the high speed cytoskeletal fraction (Fig. 2 *B*), with kinetics similar to that observed in the low speed cytoskeleton (Fig. 2 *A*).

After lysis of cells with Triton X-100, a fraction of actin filaments may undergo depolymerization during centrifugation. To address the possibility that the high and low speed cytoskeletons may underrepresent the total amount of F-actin due to depolymerization, 20 μM phalloidin was included in the lysis buffer to stabilize the F-actin. In the presence of 20 μM phalloidin, resting levels of total actin recovered in the low speed pellet were increased ∼1.8-fold (Table I). In agreement with Fig. 2, *A* and *B*, capping protein was observed to enter, but not exit, the low and high speed cytoskeletons upon stimulation when the cytoskeletal content was measured in the presence of 20 μM phalloidin (Fig. 2, *C* and *D*). Taken together, these results demonstrate that capping protein enters but never exits the actin cytoskeleton at all times during the initiation of actin polymerization following cAMP stimulation.

Capping Protein Enters the Actin-rich Cell Cortex upon cAMP Stimulation

Previous studies by Wessels et al. (1989) showed that before cAMP treatment, polarized *Dictyostelium* amebas ex-

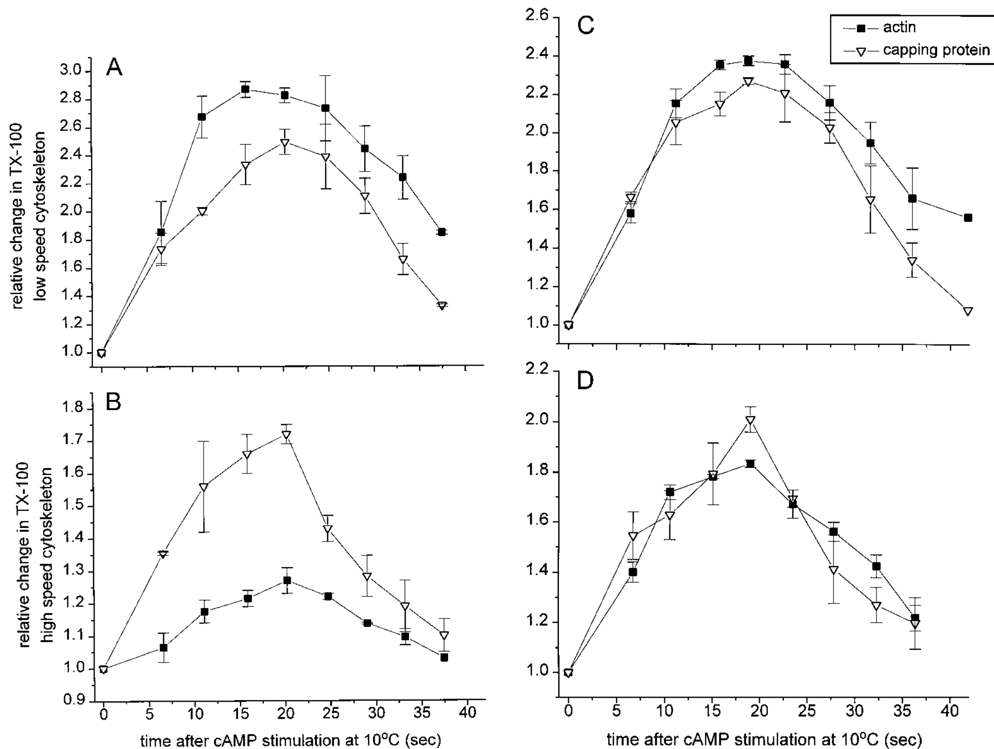

Figure 2. Association of capping protein with the low and high speed Triton-insoluble cytoskeleton after stimulation at 10°C in the presence and absence of phalloidin. AX3 cells were starved at 22°C and transferred to 10°C as described in Materials and Methods. At various times after stimulation with 10 μM 2′ deoxy-cAMP, 2 × 10^6 cells/ml (final) were lysed in L buffer containing 0.5% Triton X-100 alone (*A* and *B*), or L buffer containing 20 μM phalloidin (*C* and *D*). Low speed Triton-insoluble cytoskeleton pellets (*A* and *C*) were recovered by centrifugation in a microfuge for 3 min at 8,700 *g*. High speed Triton-insoluble cytoskeleton pellets (*B* and *D*) were recovered by centrifugation for 1 h at 415,000 *g* in a Beckman TLA-100.2 rotator, a force sufficient to pellet individual actin filaments. The low speed or high speed cytoskeleton pellets were resuspended on ice in 20% lysate volume and Western blotted using anti–capping protein-α antibodies followed by densitometry. Actin levels in cytoskeleton pellets were determined by densitometry of Coomassie blue staining of the 42-kD actin band after SDS-PAGE. Values represent data from three separate experiments ± the standard deviation.

hibit an intense accumulation of F-actin in anterior pseudopodia as shown by fluorescein-phalloidin staining. However, 5 s after stimulation at 22°C and concomitant with the peak of actin nucleation/polymerization detected in vitro, the F-actin staining is lost from the pseudopodia and becomes relocalized almost globally throughout the cell cortex, just below the plasma membrane. To determine the in situ localization of capping protein in response to cAMP stimulation, we performed indirect immunofluorescence using capping protein antibodies on glutaraldehyde-fixed whole cells and Triton-insoluble cytoskeletons. F-actin localization by rhodamine-phalloidin staining of both unstimulated whole cells and Triton-insoluble cytoskeletons was primarily in pseudopodia of highly polarized cells, in addition to circumferential staining of the cell cortex in cells displaying a flattened, rounded morphology (Figs. 3 *c* and 4 *c*). Capping protein displayed a punctate cytosolic localization in both unstimulated whole cells (Fig. 3 *e*) and Triton-insoluble cytoskeletons (Fig. 4 *e*) with a more pronounced staining in areas of F-actin localization, particularly in the cell cortex (Figs. 3 *c* and 4 *c*).

Table I. Percent Total Actin and Capping Protein Recovered in Low Speed Triton-insoluble Cytoskeletons After cAMP Stimulation

	0 s + phalloidin	20 s + phalloidin	0 s − phalloidin	20 s − phalloidin
Actin	35.78 ± 4.44	74.88 ± 1.66	20.15 ± 5.96	52.54 ± 1.69
Capping protein	25.65 ± 1.50	50.33 ± 2.76	18.07 ± 3.26	44.72 ± 4.79

The percent total actin and capping protein in low speed Triton-insoluble cytoskeletons was calculated by densitometry as (lysate value − supernatant value)/lysate value from four separate experiments.

At 20 s after cAMP stimulation at 10°C, both whole cells and Triton-insoluble cytoskeletons showed an increase in F-actin (Figs. 3 *d* and 4 *d*) and capping protein (Figs. 3 *f* and 4 *f*) staining of the cell cortex with a redistribution from pseudopods to the cell cortex in polarized cells. Therefore, capping protein colocalizes with F-actin and undergoes a redistribution to the cell cortex along with F-actin in response to cAMP stimulation.

Capping Protein Localizes to Regions of Actin Nucleation Visualized by Exogenous Rhodamine-labeled G-actin

The localization of capping protein mirrors changes in F-actin localization in response to stimulation, suggesting that capping protein binds to the barbed ends of actin filaments. To investigate this further, the localization of capping protein relative to regions of the cell containing barbed ends after stimulation was determined using a rhodamine-labeled G-actin polymerization assay. To visualize sites of actin nucleation and, therefore, free barbed filament ends in situ, we incubated permeabilized, phalloidin-stabilized *Dictyostelium* cells with exogenous rhodamine-labeled G-actin under polymerizing conditions. This technique has been used to visualize the distribution of free barbed ends in permeabilized fibroblasts (Symons and Mitchison, 1991), polymorphonuclear leukocytes (Redmond and Zigmond, 1993), and mammary adenocarcinoma cells (Chan et al.,

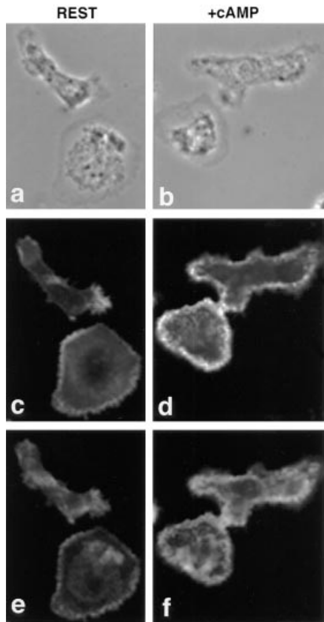

Figure 3. Immunofluorescence of capping protein in resting and stimulated *Dictyostelium* cells at 10°C. AX3 cells were starved for 5 h and allowed to settle onto coverslips for 30 min at 22°C, and then transferred to 10°C for 30 min. For whole cell preparations, cells were fixed at 0 (*Rest*) and 20 s (+*cAMP*) after stimulation with 10 μM 2′ deoxy-cAMP. Phase contrast: (*a* and *b*); rhodamine-phalloidin (*c* and *d*); anti–capping protein-α (*e* and *f*). Images shown are representative of typical cells most commonly observed in these experiments. Confocal images represent 0.8–1.0-μm-thick optical sections of the stained cells.

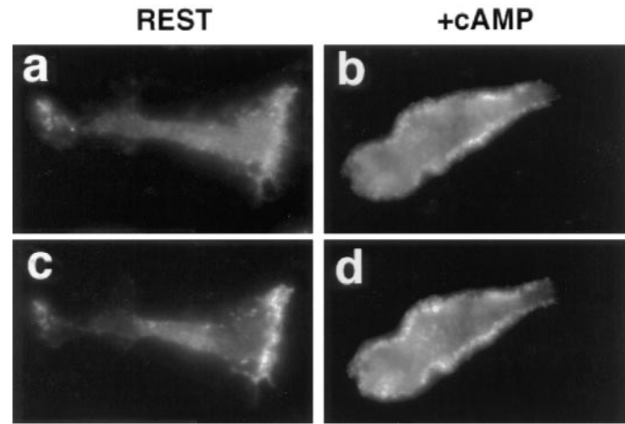

Figure 5. Incorporation of exogenous rhodamine-labeled G-actin and localization of capping protein in resting and stimulated *Dictyostelium* at 10°C. AX3 cells were starved as described in Fig. 3 and stimulated with 10 μM 2′ deoxy-cAMP at 10°C for 0 (*rest*) and 20 s (+*cAMP*). Cells were then permeablized with 0.1% Triton X-100 in the presence of 20 μM phalloidin, rinsed, and incubated with 0.45 μM rhodamine-labeled G-actin, prepared as described (Chan et al., 1997), for 5 min. Polymerization was terminated by fixation and stained for capping protein as described. Rhodamine-labeled G-actin (*a* and *b*); anti–capping protein-α (*c* and *d*). Images shown are representative of typical cells most commonly observed in these experiments.

1997). Unstimulated cells that possessed a polarized morphology contained sites of rhodamine-labeled G-actin incorporation primarily in the anterior pseudopod (Fig. 5 *a*). No incorporation of rhodamine-labeled G-actin was observed in the presence cytochalasin D (data not shown) indicating that sites of rhodamine-labeled G-actin incorporation represent free barbed ends. The pattern of rhodamine-labeled G-actin incorporation colocalized with capping protein staining (Fig. 5, *a* and *c*). At 20 s after cAMP stimulation at 10°C, there was a redistribution of the incorporation of rhodamine-labeled G-actin and capping protein staining to the entire cell cortex (Fig. 5, *b* and *d*), consistent with the redistribution of F-actin globally throughout the cell cortex after stimulation (Figs. 3 *c* and *d*, and 4 *c* and *d*), and as observed by Wessels et al. (1989). These results demonstrate that sites of increased free barbed ends, F-actin, and capping protein are all colocalized to the cell cortex in response to cAMP stimulation.

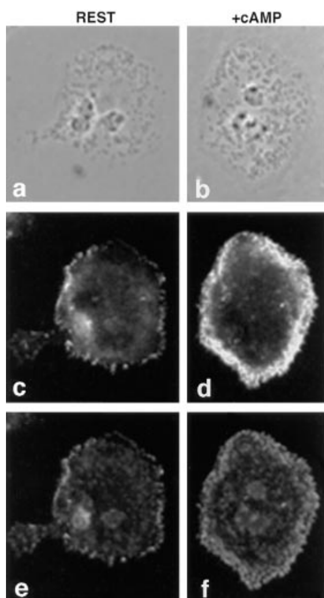

Figure 4. Immunofluorescence of capping protein in resting and stimulated *Dictyostelium* Triton cytoskeletons at 10°C. For preparation of Triton cytoskeletons, cells were starved as described in Fig. 3, and treated with 0.75% Triton X-100 for 1 min and fixed at 0 (*rest*) and 20 s (+*cAMP*) after 10 μM 2′ deoxy-cAMP stimulation. Phase contrast (*a* and *b*); rhodamine-phalloidin (*c* and *d*); anti–capping protein-α (*e* and *f*). Images shown are representative of typical cells most commonly observed in these experiments. Confocal images represent 0.8–1.0-μm-thick optical sections of the stained cells.

The Number of Actin Filaments Increases during cAMP-stimulated Actin Nucleation

The results of the Triton cytoskeleton assays (Figs. 1 *A* and 2), combined with the in situ colocalization of capping protein with regions of increased F-actin staining and free barbed ends (Figs. 3–5), indicate that capping protein is associating with free barbed ends as they appear after stimulation. These in vitro and in situ results strongly suggest that free barbed ends are not generated by an uncapping mechanism involving capping protein.

In a pure uncapping mechanism, a capping protein would be released from the cytoskeleton as the barbed ends of preexisiting actin filaments become exposed in response to stimulation and the number of actin filaments before and after cAMP stimulation would remain constant. We investigated this prediction of the uncapping mechanism by quantitating the number of pointed ends of actin filaments in the cytoskeleton after stimulation and therefore the number of actin filaments, using a DNase I binding assay, originally developed to score the number of actin filaments in resting *Dictyostelium* (Podolski and Steck, 1990). As shown in Fig. 6, the number of filaments increased by ~2.4-fold, an increase of 2.24×10^5 per cell within 20 s after cAMP stimulation at 10°C.

The facts that all the nucleation activity in stimulated cells is recovered in the low speed Triton-insoluble cytoskeleton (Hall et al., 1989), and the amount of F-actin recovered in the cytoskeleton is close to that measured in the intact cell (Hall et al., 1988), led us to use the low speed Triton-insoluble cytoskeleton as the appropriate compart-

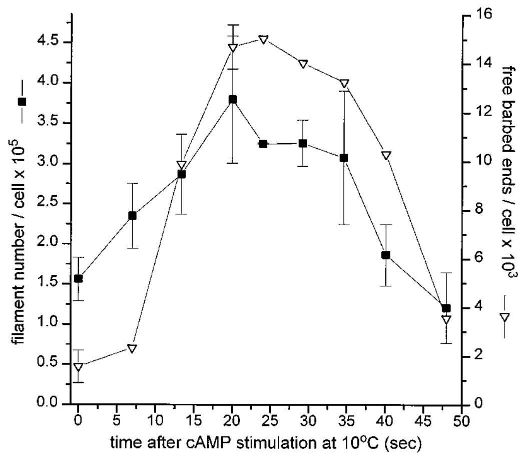

Figure 6. Quantitation of actin filaments and free barbed ends in *Dictyostelium* Triton-insoluble cytoskeletons after stimulation at 10°C. The number of actin filaments was determined using the DNase I inhibition assay (Podolski and Steck, 1990). AX3 cells were starved at 10^7 cells/ml for 5.5 h at 22°C, concentrated to 2×10^7 in buffer, and transferred to 10°C. At various times after stimulation with 10 μM 2′ deoxy-cAMP, cells were lysed into buffer B containing 2% Triton X-100 and 20 μM phalloidin and the low speed Triton-insoluble cytoskeleton was processed as described. The number of filament pointed ends were calculated by fitting experimental CPM values to a *Dictyostelium* G-actin standard curve at 4 nM DNase I. Free barbed ends were calculated as described for Table II. 0 and 20 s time points represent data collected from three separate experiments; all other points represent data collected from two separate experiments.

ment to compare filament number, concentration of barbed ends, capping protein and F-actin, and filament length (Table II).

After stimulation, the number of free barbed ends in the cytoskeleton shows an increase from 1.5×10^3 in a resting cell to a peak of 1.47×10^4 in a stimulated cell (Fig. 6). Therefore, at 20 s poststimulation, the vast majority (~96%) of filaments are capped (Table II). This result is consistent with the large incorporation of capping protein into the cytoskeleton after stimulation as shown in Fig. 2 and Table II. This increased capping protein incorporation would be necessary to cap the large number of barbed ends created by stimulation.

Discussion

The goal of this work was to determine the compartmentation of capping protein relative to free barbed ends in such a way as to test the proposal that a simple uncapping model involving capping protein can explain the timing of appearance and number of free barbed ends, and amount of F-actin assembled after stimulation of a chemotactic cell, *Dictyostelium.* This is the first comprehensive study in which all of these measurements were made on the same cell cultures, and the number of filaments present in cells before and after stimulation were measured directly using the DNase binding assay.

Predictions of a Simple Uncapping Model

Previous studies document that the capping activity associated with capping protein is lost from the cytosol after stimulation of *Dictyostelium* amebas with chemoattractant (Hall et al., 1989; Sauterer et al., 1991; Eddy et al., 1996). A simple uncapping model would explain this as a switching off of the capping activity of capping protein and would predict the following (Fig. 7 *A*): (*a*) As the capping activity of capping protein is inhibited after stimulation, capping protein would dissociate from barbed ends of preexisting filaments; (*b*) the average filament length would increase as G-actin assembles onto the preexisting filaments; and (*c*) the number of filaments would remain constant.

The Behavior of Capping Protein Does Not Conform to a Simple Uncapping Model

To test the prediction that capping protein should exit the cytoskeleton in response to stimulation, we undertook a kinetic analysis of the association of capping protein with the actin cytoskeleton at various times and conditions following cAMP stimulation of *Dictyostelium* amebas. Under all conditions tested, capping protein was observed to enter, but not exit, the cytoskeleton after cAMP stimulation. Neither the steady state increase in the number of free barbed ends (Fig. 6) nor the uncapping of amounts of cap-

Table II. cAMP-stimulated Actin Polymerization in Low Speed Triton-insoluble Cytoskeletons

Time	Filament number	Barbed ends		Capping protein	F-actin	Avg. filament length
		Total	Free			
s		*nM*	*nM (n)*	*nM (n)*	*μM*	*μm*
0	$1.56 \times 10^5 \pm 0.27 \times 10^5$	518 ± 89	5 ± 2.4 (1,505 ± 722)	540 ± 97 ($1.6 \times 10^5 \pm 0.29 \times 10^5$)	33.6 ± 10	0.33 ± 0.1
20	$3.80 \times 10^5 \pm 0.79 \times 10^5$	1,262 ± 262	49 ± 4 (14,749 ± 1,204)	1,320 ± 141 ($3.9 \times 10^5 \pm 0.42 \times 10^5$)	89.0 ± 3	0.36 ± 0.01

Values were measured and/or calculated from low speed Triton-insoluble cytoskeletons for experiments conducted at 10°C as described in Fig. 2 *A* using a cell volume of 0.5 pl for log phase cells. Filament number was measured using the DNase I inhibition assay from cells harvested at 10^7 cells/ml (Podolski and Steck, 1990). The mean concentration values of total actin and capping protein were determined as 167.8 ± 79.8 and 3.0 ± 1.4 μM, respectively. Total barbed ends (nM) were calculated from the filament number, assuming one barbed end per actin filament. Free barbed ends (nM) and number of molecules/cytoskeleton were measured from data in Fig. 1 *B* using a standard curve relating the rate of increase in pyrene-fluorescence to actin polymer content and published barbed-end rate constants of $K_+ = 11.6/\mu M$ s and $K_- = 1.4/s$ (Pollard, 1986), after subtracting the contribution of pointed ends. Similar numbers of barbed ends were calculated from rates obtained by polymerization in 0.5 μM pyrene actin where assembly occurs from barbed ends only. The amounts of F-actin (*μM*) and capping protein (*nM*), and number of capping protein molecules/cytoskeleton in the low speed Triton-insoluble cytoskeletons (− *phalloidin*) were determined from percentages summarized in Table I. Cellular concentrations of capping protein and actin were measured as described in Materials and Methods. Average filament length (*μm*) was calculated from the filament number per cell and F-actin content, assuming a value of 2.7 nm per actin monomer in an actin filament (Huxley, 1963; Egelman et al., 1982; Holmes et al., 1990).

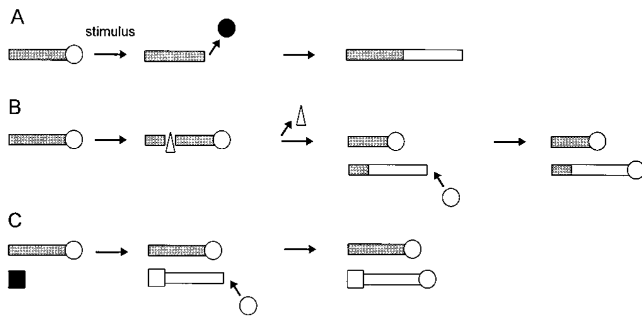

Figure 7. Models for free barbed-end generation after chemotactic stimulation. In all models, filament elongation occurs from the barbed or preferred growing end. Preexisting actin filaments are shaded. Newly polymerized actin filaments are open. (*A*) Uncapping model. *Open circle*, active capping protein; *closed circle*, inactive capping protein. After stimulation, the capping activity of capping protein is switched off, exposing free barbed ends for assembly on preexisting filaments. (*B*) Severing model. *Open triangle*, severing protein; *open circle*, capping protein. After stimulation, actin filaments are severed, exposing free barbed ends for assembly. Capping protein binds to free barbed ends, terminating the polymerization transient. (*C*) De novo nucleation model. *Closed square*, inactive actin nucleation template; *open square*, active actin nucleation template; *open circle*, capping protein. After stimulation, an actin nucleation template is switched on, creating new barbed ends. After assembly, capping protein binds to free barbed ends, terminating the polymerization transient.

ping protein at the detection limit of the assays used here (~30 nM or 1.5×10^{-20} mol/cell) can account for the amount of F-actin assembled in the 20 s after stimulation. This is true because ~1.7×10^7 actin subunits are polymerized into filaments during the 20-s interval after stimulation. The nonfilamentous actin concentration required to polymerize this much F-actin, based on published rate constants (Pollard, 1986) from an undetected 30 nM of barbed ends or from the number of free barbed ends actually measured (Fig. 6), would be ⩾100 μM. This value is 1.5-fold above that measured for unpolymerized actin by Hall et al. (1989) during this interval.

The in vitro association of capping protein with the actin cytoskeleton after cAMP stimulation was also analyzed by in situ fluorescence. The location of free barbed ends in situ was identified by incorporation of exogenous rhodamine-labeled G-actin. Colocalization of capping protein, free barbed ends as rhodamine-actin, and F-actin were observed in the actin cytoskeleton at 0 and 20 s after cAMP stimulation at 10°C. A redistribution of capping protein, free barbed ends, and F-actin staining from the leading edge of anterior pseudopods in unstimulated cells to the submembraneous cortex were observed after stimulation. Therefore, capping protein colocalizes with F-actin and free barbed ends in resting and stimulated cells, which is consistent with the interpretation that capping protein in the cytoskeleton as defined here is associated with the barbed ends of actin filaments. Since the amount of capping protein is equimolar with the barbed ends in the cytoskeleton both before and after stimulation (Table II), it is improbable that recruitment of capping protein to the cytoskeleton is barbed end independent. Taken together, these in vitro and in situ results are inconsistent with the loss of capping protein from the actin cytoskeleton as free barbed ends appear as predicted by an uncapping mechanism.

Since uncapping by capping protein as a mechanism for the generation of free barbed ends in response to stimulation is not supported by our data, uncapping by other barbed-end capping proteins must be considered. However, uncapping by gelsolin family members such as severin (Brown et al., 1982; Andre et al., 1989), and cap100 (protovilin) (Hofmann et al., 1992) has been investigated and seems unlikely since, after cAMP stimulation, only the capping activity associated with capping protein shows a change in cell lysates, whereas the other capping activities present do not (Sauterer et al., 1991). Although this does not completely rule out the existence of a capping activity not detectable in lysate supernatants that is regulated during stimulation, the simplest explanation of this result is that capping protein is the dominant capping activity in *Dictyostelium* as in neutrophils (DiNubile et al., 1995) and platelets (Barkalow et al., 1996), and is responsible for terminating the actin polymerization transient.

An Increase in Actin Filament Number Occurs without a Change in Filament Length After Stimulation

This study reports the first direct measurement of the number of filament pointed ends in chemotactic cells after stimulation using a direct DNase binding assay developed previously (Poldolski and Steck, 1990). As shown in Table II and Fig. 6, the number of filaments increases by ~2.4-fold, an increase of 2.24×10^5 per cell within 20 s after cAMP stimulation at 10°C. The free barbed ends per *Dictyostelium* cell increases from ~1,500 to 15,000 during stimulation. This means that the free barbed ends increase from ~1 to 3.9% of the total barbed ends in the cell upon stimulation (Table II), indicating that the vast majority of filaments are capped in both resting and stimulated cells (Fig. 6).

The amount of capping protein measured in the low speed cytoskeleton is approximately equimolar with the total number of barbed ends in this compartment, indicating that capping protein is predominantly responsible for the capping of these filaments (Table II). Furthermore, since the number of barbed ends has increased dramatically after stimulation, the amount of capping protein entering the cytoskeleton to cap these new filaments is significant, and accounts for the large increase in capping protein entering the cytoskeleton after stimulation (Fig. 2), and the large drop in capping protein-associated capping activity in the cytosol (Hall et al., 1989). Specifically, 27% of the capping activity is lost from the cytosol after stimulation, which corresponds closely with the 23% recompartmentalization of capping protein from the cytosol measured in this study. Therefore, the loss of capping activity from the cytosol after stimulation can be most easily explained as a redistribution of capping protein, and not the switching off of its capping activity.

Another prediction of a simple uncapping mechanism is that after uncapping of barbed ends, the average length of filaments should increase as the preexisting filaments polymerize. Using the value of 2.7 nm/monomer reported for the structure of the actin filament (Huxley, 1963; Egelman et al., 1982; Holmes et al., 1990), and the filament number

and F-actin concentration (Table II), an average length was calculated for the filaments recovered in the low speed cytoskeleton before and after stimulation. The average filament length remained unchanged, from 0.33 ± 0.1 μm in unstimulated cells, to 0.36 ± 0.01 μm in cells 20 s after cAMP stimulation at 10°C (Table II). The filament length for resting *Dictyostelium*, using the DNase I binding assay as reported by Podolski and Steck (1990), averaged 0.2 μm, whereas filament lengths measured by electron microscopy were ~1 μm (Cox et al., 1995). The filament length values reported in this study, an average of ~0.35 μm, fall closer to the former estimate. We believe that the difference between these studies is due to a difference in the filament populations measured and a difference in the attachment state of the cells used for these measurements. In the current study, the length was determined for all filaments in suspended cells, whereas in Cox et al. (1995), the length was determined for filaments running parallel to the surface in adherent cells within pseudopods only. However, this difference does not affect the conclusion from the current study since the filament length distribution does not change after stimulation. Therefore, an uncapping mechanism for the generation of free barbed ends is not supported by these results, since the average filament number increases while remaining constant in length after stimulation (Table II).

In general, the results reported in this study of *Dictyostelium* closely correspond with those reported for neutrophils (Cano et al., 1991; DiNubile et al., 1995), i.e., that capping protein dominates the capping of barbed ends, and the number of filaments increases by more than 2×10^5 after stimulation. Correspondence of the increase in filament number is interesting in particular because estimates of the number of filaments in neutrophils were calculated from depolymerization rate experiments assuming that the off rate of actin monomers is invariant in situ. However, since the off rate in vitro can be altered by actin binding proteins (Weber et al., 1994; Carlier et al., 1997), a more direct measurement of filament number after stimulation was required. Hence, we used the DNase I assay as a direct measurement of filament number in this study. The closeness of the values measured in neutrophils and *Dictyostelium* before and after stimulation by these two distinct methods argues compellingly that the increase in filament number is real. These results also suggest that the mechanism underlying stimulated actin polymerization may be common in both cell types and may not involve any increase in monomer off rate.

Finally, *Dictyostelium* mutants that overexpress capping protein polymerized less actin than wild-type cells, whereas mutants that underexpress capping protein polymerized more actin than wild type (Hug et al., 1995). These observations further support the role of capping protein as a terminator, not initiator, of free barbed ends after chemotactic stimulation.

Model for Chemoattractant-stimulated Actin Polymerization

The following model for chemoattractant-induced actin assembly accounts for the observed data including increases in filament number without increase in length, free barbed ends, and F-actin content and entry of capping protein into the cytoskeleton after stimulation. First, chemoattractant stimulation activates an increase in the number of free barbed ends in the cell cortex by a mechanism that does not involve the uncapping of preexisting barbed filament ends. Such a mechanism may include the nucleation of actin filaments de novo or the exposure of free barbed ends by filament severing (Fig. 7, *B* and *C*). Next, polymerization of actin filaments occurs from these newly exposed free barbed ends. Finally, as filaments polymerize, free capping protein binds to the polymerizing barbed ends, terminating their growth.

A similar model has been proposed by Schafer et al. (1996), to explain chemoattractant-induced actin polymerization. In the Schafer et al. model, the sequence of events after stimulation is uncapping, polymerization, severing, and finally, capping. By replacing uncapping with severing, the Schafer et al. model is consistent with our results.

If the model shown in either Fig. 7, *B* and *C* is correct, it must account for the increase of F-actin content observed in the cytoskeleton of ~2.6-fold (from 34 to 89 μM) within 20 s. During this 55 μM increase in F-actin within 20 s (2.75 μM/s), the rate of increase of new filaments is 1.1×10^4/s after stimulation. Using the on and off rates previously determined for the pointed and barbed ends of filaments (Pollard, 1986), ~6 μM G-actin would be sufficient to support this rate of F-actin assembly if the half-life of the barbed end of each of these new filaments is 1 s. This value for G-actin is consistent with the low end of the range reported for nonfilamentous actin in vivo. Under these conditions, the average filament would add ~77 subunits or 0.2 μm of filament length. This last point is most consistent with a severing reaction (Fig. 7 *b*), in which each preexisting filament is severed only once (Table II).

Since our results indicate that capping protein is predominantly responsible for the capping of filaments after stimulation, the on rate of capping protein must be 0.35 μM/s in the presence of ~2 μM free capping protein to achieve a barbed end half-life of 1 s. This on-rate value is predicted from the dissociation constant (K_d) of 0.8 nM measured for purified *Dictyostelium* capping protein (Eddy et al., 1996), and from the average off rate of 4×10^{-4}/s measured for various vertebrate isoforms of capping protein (Schafer et al., 1996). This value is also similar to the lower estimate for capping protein on rate in neutrophils (DiNubile et al., 1995), but slower than that measured for purified vertebrate capping protein of 3 μM/s (Schafer et al., 1996). In *Dictyostelium* cells, the K_d of capping protein for actin barbed ends was estimated to be >100-fold of the K_d measured for purified capping protein (compare Hug et al., 1995, and Eddy et al., 1996), indicating that if the off rate of 4×10^{-4}/s applies, the on rate in vivo would be as low as 0.004 μM/s. Thus, the on rate of capping protein may be slowed considerably in vivo by regulatory factors such as PIP_2, to increase the half-life of the barbed end and thereby increase the polymerization time (Schafer et al., 1996). However, based on our results, the intrinsic on rate of purified capping protein may be consistent with the extent of actin polymerization observed after stimulation in *Dictyostelium.*

Alternative Mechanisms for the Generation of Free Barbed Ends

The results presented here require consideration of mech-

anisms for the generation of free barbed ends that do not involve uncapping. An increase in the number of barbed ends might be accomplished by severing preexisting filaments, thereby creating new barbed ends (Fig. 7 *B*). In such a model, preexisting actin filaments are broken via tractional forces generated by myosins, or severed by gelsolin-related proteins (Witke et al., 1995), such as severin (Andre et al., 1989), or members of the actin depolymerizing factor (ADF)[1]/cofilin protein family. Filament severing by severin may be minor, however, since *Dictyostelium* cells deficient in severin show normal motility and chemotaxis to cAMP (Andre et al., 1989). ADF/cofilin proteins that include ADF, cofilin, actophorin, and destrin have been described to sever actin filaments in vitro without capping of the barbed ends in a pH and phosphorylation-dependent manner (Agnew et al., 1995; Moon and Drubin, 1995). The increase in the level of F-actin detected in *Dictyostelium* mutants that overexpress cofilin is consistent with a role for cofilin in increasing barbed-end number in vivo (Aizawa et al., 1996).

De novo nucleation has also been proposed as a potential mechanism of actin polymerization (Fig. 7 *C*). In this model, free barbed ends do not result from uncapping or severing of preexisting actin filaments, but rather multiprotein complexes can serve as templates from which actin monomers can elongate to form filaments. Candidate proteins include the Arp2/3 complex, reported to induce F-actin cloud formation around *Listeria* in platelet extracts (Welch et al., 1997), and talin, a membrane-associated protein that has been shown to nucleate actin polymerization in vitro (Kaufmann et al., 1991; Goldman et al., 1992). The talin homologue filopodin (Kreitmeier at el., 1995), accumulates at the leading edge at 30 s after stimulation in *Dictyostelium*, but the role of filopodin during the 5-s actin nucleation transient remains undetermined.

It is possible that both mechanisms operate in cells, with de novo nucleation predominating in regions of the cytoplasm where there are few preexisting filaments and weak severing predominating in regions where there are numerous preexisting filaments. Future studies will be required to distinguish between severing and de novo nucleation as potential mechanisms of actin nucleation in response to chemoattractant stimulation.

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1. *Abbreviation used in this paper*: ADF, actin depolymerizing factor.

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