

# Identification of Actin Nucleation Activity and Polymerization Inhibitor in Ameboid Cells: Their Regulation by Chemotactic Stimulation

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**Abstract.** Actin polymerization occurs in amebae of *Dictyostelium discoideum* after chemotactic stimulation (Hall, A. L., A. Schlein, and J. Condeelis. 1988. *J. Cell. Biochem.* 37:285–299). When cells are lysed with Triton X-100 during stimulation, an actin nucleation activity is detected in lysates by measuring the rate of pyrene-labeled actin polymerization. This stimulated nucleation activity is closely correlated with actin polymerization observed *in vivo* in its kinetics, developmental regulation, and cytochalasin D sensitivity. Actin polymerization is coordinate with pseudopod extension in synchronized populations of cells and is correlated with the accumulation of F actin in pseudopods. The stimulated actin nucleation activity is present in low-speed pellets from Triton lysates (cytoskeletons) within 3 s of stimulation and is stable compared

with the nucleation activity of whole cell lysates. Low-speed supernatants contain a reversible inhibitor of the actin nucleation activity that is itself regulated by chemotactic stimulation. Neither activity requires  $\text{Ca}^{2+}$  and both are fully expressed in 10 mM EGTA. Fractions containing the inhibitor do not sever actin filaments but do inhibit actin polymerization that is seeded by fragments of purified F actin. These results indicate that chemotactic stimulation of *Dictyostelium discoideum* generates both an actin-nucleating activity and an actin-polymerization inhibitor, and suggest that the parallel regulation of these two activities leads to the transient phases of actin polymerization observed *in vivo*. The different compartmentation of these two activities may account for polarized pseudopod extension in gradients of chemoattractant.

**P**SEUDOPOD extension is one of the earliest morphological responses to stimulation with chemotactic hormones in amebae of *Dictyostelium discoideum* (7, 14, 17, 20). The direction of pseudopod extension, in response to hormone challenge, determines the subsequent polarity of cytoplasmic streaming, cell locomotion, and chemotaxis (29, 32). Therefore, understanding ameboid chemotaxis requires analysis of how chemotactic stimulation elicits pseudopod extension.

In the less complex gametes of *Thyone* and *Chlamydomonas*, surface signals elicit pseudopod extension, which is driven by actin polymerization. These signals elicit location-specific pseudopod extension by activating discrete nucleation sites for actin polymerization. Cross-linking of the newly assembled actin generates a rigid structure capable of pushing out the cell membrane during subsequent polymerization and osmotic swelling (11, 31).

It has been shown previously that stimulation of amoeboid cells of *Dictyostelium discoideum* with chemoattractants results in the rapid and reversible polymerization of actin that is correlated with cell shape (8, 20) and cross-linking of filaments into the actin cytoskeleton (12, 24). The amount of F actin in cells increases from 35% in resting to >60% in stimulated cells (20). About half of this F actin in resting cells

is recovered in Triton cytoskeletons, whereas more than two-thirds is recovered in cytoskeletons 10 s after stimulation (12). These results suggest that there may be nucleation and filament cross-linking sites associated with the cell cortex of *Dictyostelium* amebae that are regulated by chemotactic hormones.

Recent work has demonstrated that an actin nucleating activity is present in polymorphonuclear leukocytes that is regulated by chemotactic peptides (6). Using an assay here that is similar to that described for leukocytes we investigate the possibility that *Dictyostelium* amebae contain an actin nucleation activity that is regulated by the chemotactic hormone cAMP.

## Materials and Methods

### Reagents

2' deoxy-cAMP, 8-bromo-cAMP, and DMSO were obtained from Sigma Chemical Co., St. Louis, MO. cAMP was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN and *N*(1-pyrenyl) iodoacetamine was obtained from Molecular Probes, Inc., Eugene, OR. All other materials were reagent grade. Spectrofluorimeter cuvettes were obtained from Thomas Scientific, Philadelphia, PA (cat. no. 2300-240).

## Cells

*Dictyostelium discoideum* strain AX-3 was grown in suspension in HL5 medium as described elsewhere (20). Cells were harvested from growth medium at densities between 2 and  $6 \times 10^6$ /ml and starved in suspension at a density of  $4 \times 10^6$ /ml for 6–8 h in 14.8 mM  $\text{NaH}_2\text{PO}_4$ , 5.2 mM  $\text{K}_2\text{HPO}_4$ , pH 6.6 at 22°C with shaking at 170 rpm. Caffeine was added to 3 mM for 25–40 min before use of the cells, conditions under which *D. discoideum* adenylate cyclase is inhibited and endogenous intercellular signaling is prevented (4). Unless otherwise indicated all manipulations and assays were done at 22°C.

Cells were stimulated with cAMP, one of its analogues, or with the buffer used to prepare the stimulus stock by adding 1 part of stock to 100 parts of cells while shaking continuously at 170 rpm in beakers that had five times the capacity of the cell suspension. Based on experiments with soluble dyes, 90% of the final cAMP concentration was achieved within 1 s of mixing under these conditions (data not shown).

Stocks of cAMP or its analogues were prepared in 14.8 mM  $\text{NaH}_2\text{PO}_4$ , 5.2 mM  $\text{K}_2\text{HPO}_4$ , and 1 mM EGTA, pH 7.0.

## Preparation of Pyrene-labeled Actin and Fluorescence Measurements

Pyrene-labeled actin was prepared by reacting rabbit muscle actin with *N*-(1-pyrenyl)iodoacetamide as described by Cooper et al. (10) except that *N*-(1-pyrenyl)iodoacetamide was dissolved in DMSO to prepare the stock. Pyrene-labeled and unlabeled G actin were chromatographed on Sephadex G-150 (Pharmacia Fine Chemicals, Piscataway, NJ) before use. Both pyrene-labeled and unlabeled G actin were stored by dialysis against buffer A (2 mM Tris-Cl, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM  $\text{CaCl}_2$ ) at an approximate concentration of 20  $\mu\text{M}$  and used within 3 wk as a mixture of ~30% pyrene-labeled and 70% unlabeled G actin. Except where indicated, actin was added to lysates, cytoskeletons and supernatants at 10 s after preparation to a final concentration of 2–3  $\mu\text{M}$ .

Fluorescence was measured in a spectrofluorometer (MPF-3L; Perkin-Elmer Corp., Norwalk, CT) with an excitation wavelength of 365 nm (slit width 3 nm) and an emission wavelength of 407 nm (slit width 10 nm). Samples were exposed to the exciting light only intermittently to avoid photobleaching. Cell lysates and other cell fractions had no measurable autofluorescence at these wavelengths.

The NBD-phalloidin binding assay was performed as detailed by Hall et al. (20).

## Preparation of Cell Lysates, Cytoskeletons, and Supernatants

At various times after stimulation of cells with cAMP (or buffer), one part of the starved cell suspension was added to five parts of lysis buffer (50 mM KCl, 5 mM DTT, 20 mM Pipes, 1 mM ATP, 10 mg/ml BSA, 1  $\mu\text{g}$ /ml chymostatin, leupeptin, and pepstatin, 1–10 mM EGTA, 0.1–2 mM  $\text{MgCl}_2$  and 0.5–0.9% Triton X-100). Variations in the concentrations of EGTA,  $\text{MgCl}_2$ , or Triton X-100 did not result in significant differences in the quantitative results described below except where noted (see Results). If the rate of actin polymerization was to be measured directly in the lysates, cells were lysed in a spectrofluorometer cuvette. If the lysate was to be separated into cytoskeletal pellet and supernatant fractions, cells were lysed in a microfuge tube which was immediately centrifuged at 8,600 *g* for 1–3 min. The supernatant was removed and cytoskeletons were resuspended in a volume of lysis buffer equal to that of the original lysate. Variations in the time of centrifugation did not result in significant quantitative differences in recovery of activities in either the cytoskeleton or supernatant, so 1 min was used routinely. The concentration of cellular protein was 0.18 mg/ml in the total lysate, 0.13 mg/ml in supernatant, and 0.05 mg/ml in resuspended pellets.

## Measurements of the Stability of the Actin Nucleation Activity in Lysates and Cytoskeletons

Cell lysates were prepared at various times after stimulation. Lysates were held at 22°C for the times indicated and then a mixture of pyrene-labeled and -unlabeled G actin was added to 2  $\mu\text{M}$  final concentration. Values obtained by fluorescence emission for the initial rate of actin polymerization in lysates that were held for 10 s before addition of G actin were taken as 100% because 10 s was the briefest interval that could be obtained reproducibly.

Cytoskeletal and supernatant fractions were obtained as described above.

The cytoskeletal pellets were resuspended either in supernatants or lysis buffer. The time after lysis was calculated from the time of resuspension for pellets, to the addition of G actin.

## Severing Assay

An assay similar to one described by Lind et al. (22), for gelsolin, was used to measure the severing activity of *Dictyostelium* severin and supernatants. 60  $\mu\text{l}$  of freshly prepared supernatant derived from cells mixed in lysis buffer containing 0.9% Triton X-100, was incubated with 15  $\mu\text{M}$  F actin containing 30–40% pyrene-labeled actin for 10 s. This mixture was diluted abruptly by 50–100-fold into 20 mM Tris pH 7.8, 0.2 mM ATP, 0.2 mM beta-mercaptoethanol, 2 mM  $\text{MgCl}_2$ , 50 mM KCl, and 0.02%  $\text{NaN}_3$  containing either 1 mM EGTA or 1 mM  $\text{CaCl}_2$ . The rate of depolymerization was measured as the initial rate of fluorescence decrease after dilution of the F actin mixture.

The severing activity of severin was measured as described for the supernatant above except that 60  $\mu\text{l}$  of purified severin solution (kind gift of Alice Brock and Joel Pardee, Cornell University School of Medicine) was substituted for the supernatant.

## Capping Assay

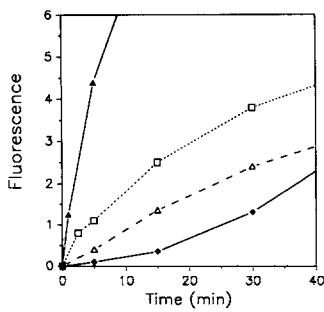
Supernatants were prepared as described above and either diluted with lysis buffer or not and then added to spectrofluorometer cuvettes. A mixture of pyrene-labeled and unlabeled actin was added to a final concentration of 2  $\mu\text{M}$ . Between 30 s and 5 min later, 1  $\mu\text{M}$  unlabeled F actin was added and the initial rate of actin polymerization was recorded.

## Results

### Identification of Actin Nucleation Activity in Lysates from Stimulated Cells

Cells were lysed with Triton X-100 after stimulation with cAMP or its analogues and the amount of nucleation activity was measured as the increase in the initial rate of polymerization of exogenously supplied actin. As shown in Fig. 1, the initial rate of actin polymerization was increased significantly in lysates that were prepared from cells after stimulation with 2'-deoxy-cAMP as compared with lysates from unstimulated cells. Stimulation of cells with the buffer used to prepare the cAMP stocks resulted in lysates that did not cause increases in the initial rate of actin polymerization and data that were indistinguishable from those obtained with lysates from unstimulated cells.

It is clear for several reasons that the ability of lysates from stimulated cells to increase the initial rate of actin polymerization is mediated by the plasma membrane cAMP receptor. First, increases in initial polymerization rate in lysates followed a dose-response relationship (Fig. 2) similar to that seen for increases in F-actin content in whole cells (20) and chemotaxis (3), both of which are mediated by cell surface cAMP receptors. Next, stimulation of cells with 2'-deoxy-cAMP, an analogue of cAMP that activates the cell surface cAMP receptor but neither intracellular cAMP receptors (30) nor cAMP-dependent protein kinase (19, 26), caused increases in the initial polymerization rate that were very similar to changes in F actin content elicited by cAMP in vivo (Fig. 3). Stimulation of cells with 8-bromo-cAMP, an analogue of cAMP that interacts very weakly with the cell surface cAMP receptor but strongly with the intracellular cAMP receptor (30), was ineffective at eliciting increases in the initial rate of actin polymerization in cell lysates. Finally, addition of cAMP or 2'-deoxy-cAMP to lysates after lysis was complete did not elicit increases in the initial rate of actin polymerization (data not shown).

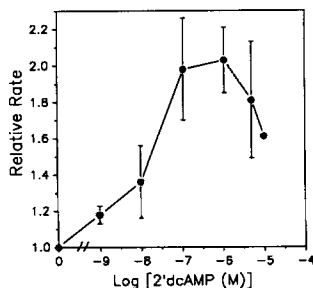


**Figure 1.** Lysates from stimulated cells contain elevated actin nucleation activity. The initial rate of actin polymerization was measured by following the fluorescence of pyrene-labeled rabbit muscle actin over time. Cells were stimulated with  $5 \mu\text{M}$  2'deoxy-cAMP and lysed at the times indicated below. Symbols indicate  $2 \mu\text{M}$  G actin polymerizing in the presence of: (▲)  $0.5 \mu\text{M}$  unlabeled F actin, (◆) no addition of lysate or F actin, (Δ) 0 s lysate, (□) 8 s lysate. The  $0.5 \mu\text{M}$  F actin was used to seed polymerization to demonstrate the rate of polymerization possible in this buffer in response to a known concentration of F actin.

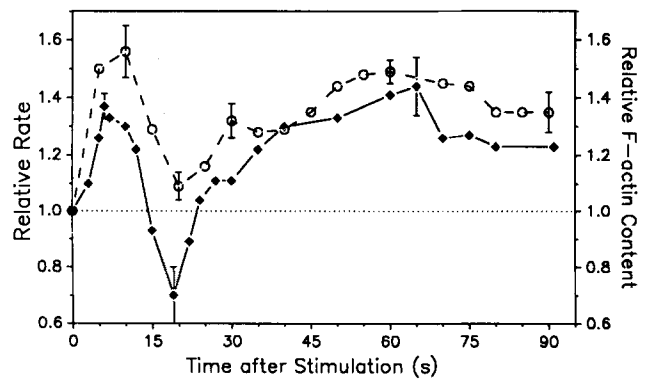
Increases in the rate of nucleation in lysates from stimulated cells are not due to variations in the amount of polymerizable actin because endogenous *Dictyostelium* actin contributes <4% of the total actin in the assay cuvette. Furthermore, the final extent of polymerization is slightly greater in unstimulated lysates as compared with that in stimulated lysates, the opposite result of that expected if stimulation increases the amount of polymerizable actin. Finally, incubation of exogenous G actin with lysates for various times up to several hours had no effect on the polymerizability of actin as measured by fluorescence emission.

Increases in the initial rate of actin polymerization in lysates from stimulated cells varied with time after stimulation, as shown in Fig. 3. The nucleation activity peaked at  $\sim 5$  s and then decreased below prestimulation levels at  $\sim 20$  s. This was followed by a second broad peak of nucleation activity beginning by  $\sim 25$  s and peaking  $\sim 65$  s. Variations in nucleation activity after stimulation were correlated with increases in F actin content measured in situ using the NBD-phalloidin binding assay (Fig. 3). Similar results were obtained when vegetative cells were stimulated with folic acid (8, 21).

Actin polymerization occurring in cell lysates from stimulated cells was effectively inhibited by low concentrations of cytochalasin D (Fig. 4), suggesting that the nucleation activity results in filaments that grow at the preferred, i.e., barbed, end. The amount of inhibition of actin polymerization in cell lysates at the various cytochalasin D concentrations was indistinguishable regardless of the time after chemoattractant stimulation when the lysate was prepared (Fig. 4), indicating



**Figure 2.** Dose-response behavior of actin nucleation activity (measured as described in Materials and Methods) in lysates prepared 8 s after 2'deoxy-cAMP stimulation. Each point represents the average of two to five determinations. Relative rate equals the initial rate of actin polymerization in lysates from stimulated cells divided by that in lysates from unstimulated cells. Error bars indicate SDs.

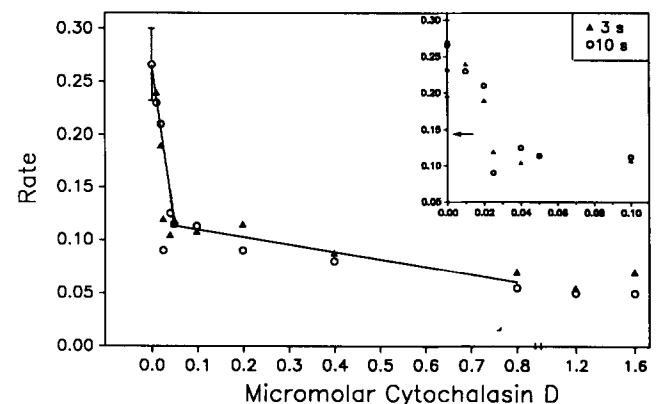


**Figure 3.** The amount of actin nucleation activity recovered in cell lysates varies with time after stimulation. Relative rate equals the initial rate of actin polymerization in lysates from stimulated cells divided by the average of rates in lysates from unstimulated cells over the same duration. The control rate does not change over the interval shown. Error bars are SDs representing at least three separate experiments. (◆) Relative rate after stimulation with  $5 \mu\text{M}$  2'deoxy-cAMP; (○) relative content of F actin in situ measured by NBD-phalloidin binding after stimulation with  $1 \mu\text{M}$  cAMP.

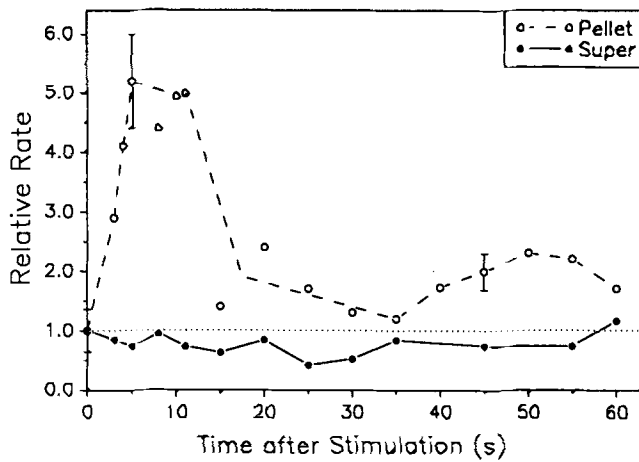
that polymerization was dominated by barbed end growth at all of the times tested. The residual rate of polymerization in the presence of cytochalasin D, which is equal to or below prestimulation rates (arrow in Fig. 4, inset) is probably due to pointed-end assembly, which is only slightly affected by cytochalasins at the concentrations used here (2).

### Actin Nucleation Activity Is Associated with Triton Cytoskeletons

The solubility of the actin nucleation activity was investigated by pelleting the Triton cytoskeletons from lysates using brief, low *g* force centrifugation. The pellet fraction, which is defined here as the triton cytoskeleton, contains  $\sim 30\%$  of the total cellular protein, and  $\sim 20\%$  of the pelleted protein is actin, according to densitometric measurements (12). Cytoskele-



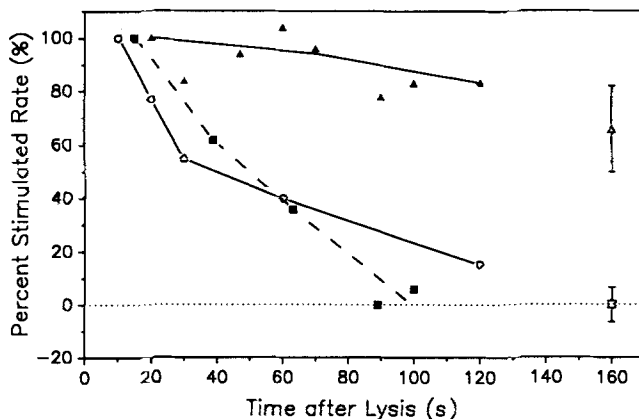
**Figure 4.** Cytochalasin D inhibits the elongation of actin filaments in lysates from stimulated cells. The initial rate (as defined in Fig. 1) of actin polymerization in lysates prepared at 3 and 10 s after stimulation of cells with  $5 \times 10^{-7}$  M 2'deoxy-cAMP is shown. The arrow on the y-axis indicates the initial rate of actin polymerization in unstimulated lysates. Similar results were obtained with lysates at 25 s after stimulation of cells with 2'deoxy-cAMP. Bars are SDs from three experiments.



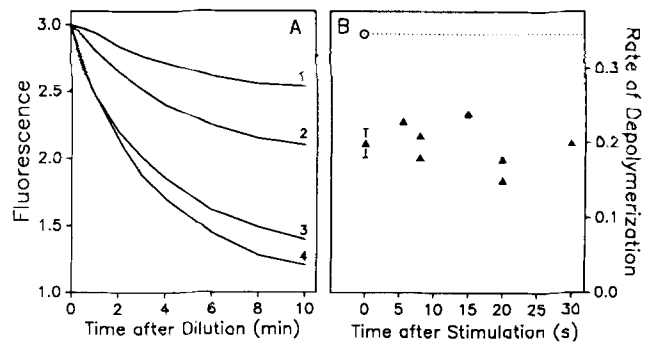
**Figure 5.** The actin nucleation activity of lysates from stimulated cells pellets with the Triton cytoskeleton. The plot shows the initial rate of actin polymerization in association with cytoskeletons from stimulated cells divided by the initial rate of actin polymerization in association with cytoskeletons from unstimulated cells versus time after stimulation.  $5 \mu\text{M}$  2'-deoxy-cAMP was used as the chemoattractant hormone and pellets were formed at  $8,700 g$  for 1 min. Similar results were obtained with  $8,700 g$  for 30 s and 3 min. Error bars show SE of the mean for at least four experiments.

tons prepared by similar procedures to that described here have been shown by electron microscopy to be composed of a network of cortical microfilaments (16, 18).

Measurements of the amount of nucleation activity in cytoskeletal pellets and supernatants by fluorescence emission demonstrates that all of the detectable activity is associated



**Figure 6.** The actin nucleation activity of lysates from stimulated cells decays rapidly back to prestimulation levels, whereas the actin nucleation activity of pelleted cytoskeletons is stable but is inactivated by supernatants. Cells were stimulated with  $0.5 \mu\text{M}$  2'-deoxy-cAMP for 8 s and then lysed. Supernatants and pellets (cytoskeletons) were separated by brief centrifugation and pellets were resuspended in lysis buffer ( $\Delta$ ) or supernatants from unstimulated cells ( $\blacksquare$ ). The plot shows the amount of nucleation activity remaining in lysates ( $\circ$ ) or resuspended cytoskeletons as a function of the time elapsed between cell lysis or resuspension of pellets, respectively, and addition of exogenous G actin. After the nucleation activity of cytoskeletons was allowed to decay to 0% in the presence of unstimulated supernatant, cytoskeletons were pelleted and resuspended in unstimulated supernatant ( $\square$ ,  $n = 4$ ) or lysis buffer ( $\Delta$ ,  $n = 5$ ) and reassayed. Error bars denote SEM.



**Figure 7.** Lysate supernatants slow the rate of depolymerization of F actin. (A) Pyrene-labeled F actin was mixed with (1) supernatants prepared from unstimulated cells; (2) lysis buffer; (3) Purified *Dictyostelium* severin in lysis buffer; (4) purified *Dictyostelium* severin in supernatant from unstimulated cells. 3 and 4 were at a molar ratio of 1 severin/30 actin monomers. F actin was then diluted to  $0.15 \mu\text{M}$  in a buffer containing millimolar calcium. The amount of purified severin added was equal to the amounts of endogenous severin predicted to be present in the supernatant (34). Plot shows typical traces of fluorescence decrease due to depolymerization after dilution. (B) The experiment was repeated with supernatants prepared from cells at various times after stimulation with  $0.5 \mu\text{M}$  2'-deoxy-cAMP. F actin was diluted to  $0.15 \mu\text{M}$  and the rate of depolymerization was recorded as in A. The initial rate of depolymerization (decrease in arbitrary units of fluorescence/min) in the presence of lysate supernatant ( $\blacktriangle$ ) is low compared with that in lysis buffer ( $\circ$ ) at all times sampled. Error bar is the SD from five experiments.

with the cytoskeleton. This is true regardless of the time after stimulation when the measurement is made (Fig. 5). Furthermore, the amount of nucleation activity present in the cytoskeleton varies after stimulation and follows a pattern that is similar to that observed for variations of the nucleation activity in lysates after stimulation.

#### Actin Nucleation Activity in the Cytoskeleton Is Regulated by a Soluble Factor

The stability of the actin nucleation activity in lysates was investigated as summarized in Fig. 6. Lysates prepared from cells at 8 s after stimulation contained nucleation activity that decayed back to prestimulation levels rapidly with a half life of 30 s. Similar results were obtained with lysates from cells that were stimulated for 65 s.

However, nucleation activity associated with cytoskeletons remained stable compared with that in lysates when resuspended in lysis buffer (Fig. 6), suggesting that a soluble factor remaining in the supernatant is responsible for decay of the nucleation activity in lysates.

To study this, pellets were resuspended in supernatants prepared from either stimulated or unstimulated cells. Addition of supernatants to cytoskeletons resulted in rapid decay of the nucleation activity (Fig. 6). Inhibition of nucleation activity was obtained with supernatants prepared from unstimulated cells as well as cells that had been stimulated for various times. Inhibition of the nucleation activity by supernatants was not caused by proteolysis of either the nucleation site or exogenous actin because the addition of the protease inhibitor cocktail described in Materials and Methods actually caused a slight increase in the amount of inhibition ob-

served and incubation of G actin with supernatants for several hours had no effect on the polymerizability of the actin (data not shown). Furthermore, cytoskeletons were mixed with unstimulated supernatant and their nucleation activity was allowed to decay to unstimulated values. When these cytoskeletons were pelleted and resuspended in lysis buffer to remove the supernatant, the nucleation activity was recovered while cytoskeletons resuspended in supernatant remained inactive (Fig. 6).

### Properties of the Inhibitor of Actin Nucleation

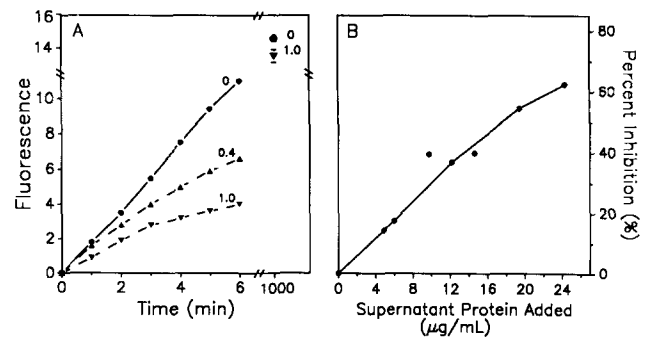
Inhibition of the nucleation activity that is associated with the cytoskeleton might result from severing of filaments. To investigate this possibility, the severing activity of supernatants was measured as described in Materials and Methods. As shown in Fig. 7 A, dilution of pyrene-labeled F actin in buffer leads to loss of fluorescence because of depolymerization. Upon addition of the severing protein severin, to an amount equal to endogenous severin in crude supernatants (34), the rate of depolymerization in buffer is increased due to an increase in the number of filament ends. However, upon dilution of F actin into supernatants capable of inhibiting the actin nucleation activity of cytoskeletons, the rate and extent of actin depolymerization was greatly decreased even relative to that of F actin alone. Addition of purified severin to these supernatants increased the rate of depolymerization to that in buffer containing severin, indicating that an inhibitor of severin was not present in the supernatant. Similar results were obtained with supernatants assayed in the presence of either mM calcium or EGTA, whereas severin required  $\text{Ca}^{2+}$  for severing activity.

Furthermore, supernatants decreased the rate of actin depolymerization regardless of the time after stimulation when lysates were made (Fig. 7 B). Similar results were obtained in the presence of either millimolar calcium or EGTA. These results indicate that supernatants capable of inhibiting the nucleation activity of cytoskeletons do not exhibit severing activity under our assay conditions.

Next, the possibility that inhibition of the nucleation activity might result from capping of filament ends was investigated. The capping activity of supernatants was measured as described in Materials and Methods. Because the assay was done in lysis buffer, the barbed end of the filament is the preferred end for polymerization (25). As a result the capping assay described here is most sensitive to blockade of the barbed end.

As shown in Fig. 8 A, addition of supernatant caused inhibition of the initial rate of seeded actin polymerization. Furthermore, the final extent of polymerization was consistently lower in the presence of supernatant than in buffer alone. The amount of supernatant protein required for inhibition is shown in Fig. 8 B. About 16  $\mu\text{g}/\text{ml}$  of supernatant protein was sufficient for 50% inhibition of the seeded rate of actin polymerization in a solution containing 130  $\mu\text{g}/\text{ml}$  of actin. Calcium was not required for inhibition, because similar results were obtained with concentrations of EGTA in the lysis buffer ranging from 1 mM (free  $\text{Ca}^{2+}$  of  $\sim 0.1 \mu\text{M}$ ) to 10 mM. As the EGTA concentration was increased, the amount of inhibition observed increased by a factor of 1.2 at 10 mM.

The inhibitory activity of the supernatant was not because of alterations in the polymerizability of actin since preincu-



**Figure 8.** Lysate supernatants inhibit the initial rate and decrease the final extent of polymerization from F actin seeds. Lysate supernatants from unstimulated cells were prepared in lysis buffer containing 5 mM EGTA. Polymerization was initiated with 1  $\mu\text{M}$  unlabeled F actin. (A) Polymerization was recorded as fluorescence increase over time in lysis buffer alone (0), 40% supernatant (0.4), or 100% supernatant (1.0). The extent of polymerization after 18 h at 22°C is indicated for 0 and 1.0. Differences in the extent of polymerization were significant at 18 h as indicated by the SDs that fall within the symbols ( $n = 4$  for each). (B) The percent of inhibition of the initial rate of seeded actin polymerization increases with the amount of supernatant protein added. 0% inhibition is the rate of seeded actin polymerization in lysis buffer alone.

bation of G actin with freshly prepared supernatants for 0 or 20 min had no effect on the final extent of polymerization. Aging of supernatants at 22°C before assay caused a slight drop in the amount of inhibition (20% decrease after 30 min) indicating that the inhibitory activity is unstable in the crude supernatant (data not shown).

Finally, the inhibitory activity of supernatants is not likely to be due to *Dictyostelium* profilin that was carried in with the supernatant since, assuming the content of profilin in *Dictyostelium* is similar to that in *Acanthamoeba* (25), there would be only 1 *Dictyostelium* profilin for every 75–80 actin monomers in the assay mixture.

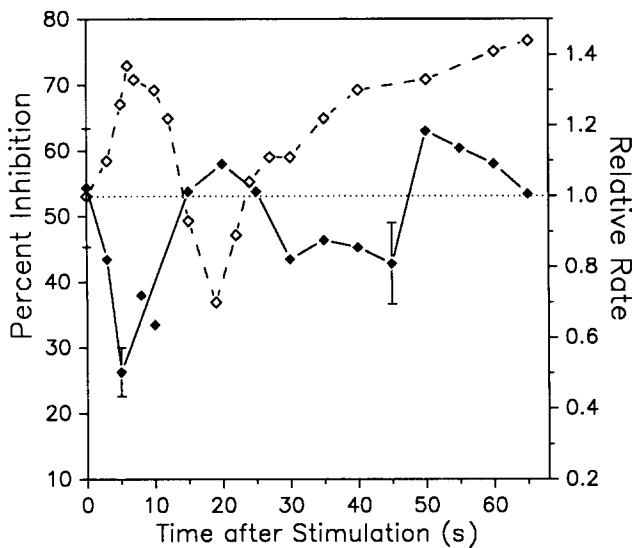
### The Inhibitor of Actin Nucleation Is Regulated after Chemotactic Stimulation

To determine whether the inhibitory activity in the supernatants was regulated by stimulation with cAMP, lysates were prepared from cells at various times before and after stimulation. Supernatants were generated by centrifugation as usual and assayed for their ability to inhibit seeded actin polymerization as described for Fig. 8. As shown in Fig. 9, the amount of inhibition of seeded actin polymerization by supernatants varied markedly after stimulation. Significant decreases in inhibition were seen at 5 s and between 30 and 45 s, and increases above unstimulated levels were seen at 20 and  $\sim 50$  s. The general pattern of regulation of the inhibitor of seeded actin polymerization in supernatants followed the inverse of the pattern of regulation of nucleation activity in lysates for at least the first 45 s after stimulation with 2'deoxy-cAMP (Fig. 9).

## Discussion

### Ameboid Cells Contain a Hormone-stimulated Actin Nucleation Activity

The above results demonstrate that stimulation of aggrega-



**Figure 9.** The inhibitor of seeded actin polymerization in supernatants is regulated after stimulation of cells with cAMP. Inhibition of seeded actin polymerization by supernatants was measured as described in Fig. 8 in 5 mM EGTA (◆). Supernatants were prepared from cells either before (0 s) or after stimulation with 2.8  $\mu$ M 2'deoxy-cAMP at the times indicated. Nucleation activity in cell lysates is shown for comparison (◇).

tion competent *Dictyostelium* amoebae with the chemoattractant cAMP and its analogue 2'deoxy-cAMP causes an increase in the amount of nucleation activity in cell lysates. Similar results have been obtained with feeding amoebae in response to the chemoattractant folic acid (21).

Actin nucleation activity that is stimulated by chemotactic hormones results in actin polymerization that is sensitive to cytochalasin D. Sensitivity to cytochalasin D is observed in lysates prepared even at early times after stimulation, indicating that the contribution of barbed-end assembly to the rate of polymerization does not vary with time across a peak of nucleation activity.

Calcium is not required for expression of the nucleation activity in vitro. In fact, similar amounts of nucleation activity were detected in lysates from stimulated cells in lysis buffer containing either 1 or 10 mM EGTA. Lack of a calcium requirement for nucleation activity in vitro, however, is not informative regarding the situation in vivo because a calcium-dependent step in generating the nucleation activity may have already occurred at a time between cell stimulation and cell lysis. Unfortunately, other studies concerning the involvement of calcium in the rearrangement of the actin cytoskeleton during chemotactic stimulation are not definitive on this point either. Treatment of saponin-permeabilized *Dictyostelium* amoebae with micromolar calcium leads to an increase in the amount of actin in triton cytoskeletons (13). However, the increases in pelletable actin observed in these experiments could result from a calcium-elicited actomyosin contraction as demonstrated in cell extracts (9) and not to de novo assembly of actin filaments. In addition, a cAMP-induced calcium influx, which peaks at 30 s and leads to a calculated increase in cytosolic calcium from 0.3 to 10  $\mu$ M (5), follows kinetics that are temporally correlated with the cringe response (i.e., cell contraction), which peaks at 25–30 s (15, 20, 23) but not nucleation activity. Therefore, the role of calcium in

regulating actin polymerization in general and actin nucleation activity in particular during chemotactic stimulation remains unresolved at present. The development of cell models that can be both stimulated and assayed for actin nucleation and polymerization in vitro will be valuable in determining whether calcium plays a regulatory role in this response.

The parallel change in F actin content of cells and nucleation activity suggests that F actin could be the "nucleus" for assembly. That is, the nucleation activity could be actin filament ends that are uncapped during stimulation. This would explain the rapid appearance of the nucleation activity in the cytoskeleton after stimulation. This hypothesis would emphasize the importance of the inhibitor of nucleation activity that is regulated by stimulation in a way related to the appearance of the nucleation activity.

### **Properties of the Hormone-regulated Inhibitor of Actin Nucleation Activity**

Supernatants prepared from Triton lysates contain an inhibitor of the actin nucleation activity of cytoskeletons. The mechanism of inhibition of the nucleation activity does not involve proteolysis because protease inhibitors do not prevent or slow inhibition and the nucleation activity of cytoskeletons can be recovered after complete decay by washing cytoskeletons in buffer.

Inhibition does not appear to involve a severing activity. This possibility was considered because severing would lead to disruption of the actin cytoskeleton and blockade of the barbed ends of the newly cut filaments. Disruption of the cytoskeleton could result in loss of nucleation activity. However, severing activity was not detected in the supernatants even under assay conditions sufficient to detect the amounts of endogenous severin (discussed below) predicted to be present in the supernatant (34).

The supernatant did inhibit the elongation of actin filaments under assay conditions where the barbed end dominates elongation. This activity was regulated after stimulation of cells with cAMP. This suggests a mechanism of inhibition of actin nucleation activity involving the capping of filaments at their barbed ends. Consistent with this is the finding that the final extent of polymerization was lower in the presence of supernatant as compared with that in buffer. This difference could be explained by the blocking of barbed ends if the concentration of capping protein is  $\sim 5$  nM, i.e.,  $\sim 0.2\%$  of the supernatant protein with an equilibrium constant ranging from  $10^9$  to  $10^{10}$   $M^{-1}$  as measured for brain capping protein and gelsolin, respectively (28, 33).

Inhibition of seeded actin polymerization occurs in vitro in millimolar EGTA, suggesting that, if a capping activity is involved, it does not require calcium. Calcium-insensitive barbed-end capping proteins have been identified in *Dictyostelium*. A small, calcium-insensitive, heterodimeric capper with subunits measuring 34 and 32 kD has been identified (27) which resembles similar capping proteins in brain, *Acanthamoeba*, and skeletal muscle (25).

In addition, a severing protein with significant sequence homology to gelsolin (1) named severin has been isolated from *Dictyostelium* amoebae. It constitutes  $\sim 0.2\%$  of the soluble protein (34). Although this protein has a calcium-regulated severing activity, it could function as a calcium-insensitive capping protein if first bound to G actin in the

presence of calcium. Such a complex is stable in EGTA in vitro and could cap filaments at barbed ends by a process resembling filament reannealing. A capping activity of this kind has been measured for gelsolin by Selve and Wegner (28).

The calcium insensitivity of the inhibitory activity of supernatants does not exclude this latter possible mechanism because the calcium-dependent step may have occurred at a time between cell stimulation and cell lysis. Modulation of the amount of actin-gelsolin complex in platelets after thrombin stimulation (22) suggests that a similar complex involving the related protein severin could form after cAMP stimulation of *Dictyostelium*. Development of cell models that can be both stimulated and assayed for capping activity in vitro will be essential in determining whether calcium plays a role in this process. Further analysis of the types of proteins essential for inhibition will require purification of the inhibitory activity because the supernatant fraction is too crude to resolve the components or mechanisms involved.

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