

Actin Filament Barbed-End Capping Activity in Neutrophil Lysates: The Role of Capping Protein- β_2

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A barbed-end capping activity was found in high speed supernates of neutrophils lysed in submicromolar calcium. In dilute supernate (≥ 100 -fold dilution of cytoplasm), this activity accounted for most of the inhibition of barbed-end elongation of pyrenyl-G-actin from spectrin-F-actin seeds. Pointed-end elongation from gelsolin-capped F-actin seeds was not inhibited at comparable concentrations of supernate, thus excluding actin monomer sequestration as a cause of the observed inhibition. Most of the capping activity was due to capping protein- β_2 (a homologue of cap Z). Thus, while immunoadsorption of $\geq 95\%$ of the gelsolin in the supernate did not decrease capping activity, immunoadsorption of capping protein- β_2 reduced capping activity proportionally to the amount of capping protein- β_2 adsorbed. Depletion of $>90\%$ of capping protein- β_2 from the supernate removed 90% of its capping activity. The functional properties of the capping activity were defined. The dissociation constant for binding to barbed ends (determined by steady state and kinetic analyses) was ~ 1 – 2 nM; the on-rate of capping was between 7×10^5 and 5×10^6 $M^{-1} s^{-1}$; and the off-rate was $\sim 2 \times 10^{-3} s^{-1}$. The concentration of capper free in the intact cell (determined by adsorption of supernate with spectrin-actin seeds) was estimated to be ~ 1 – 2 μM . Thus, there appeared to be enough high affinity capper to cap all the barbed ends in vivo. Nevertheless, immediately after lysis with detergent, neutrophils contained sites that nucleate barbed-end elongation of pyrenyl-G-actin. These barbed ends subsequently become capped with a time course and concentration dependence similar to that of spectrin-F-actin seeds in high speed supernates. These observations suggest that, despite the excess of high affinity capper, some ends either are not capped in vivo or are transiently uncapped upon lysis and dilution.

INTRODUCTION

Cell locomotion involves rapid polymerization of actin (Fuchtbauer *et al.*, 1983; Wang, 1985; Handel *et al.*, 1990; Theriot and Mitchison, 1991; Fechheimer and Zigmond, 1993). This polymerization can be blocked by cytochalasin, suggesting that it occurs at the barbed ends of actin filaments (White *et al.*, 1983; Soll *et al.*,

1988; Cassimeris *et al.*, 1990; Sampath and Pollard, 1991). It is often assumed that in a resting cell, most barbed ends are capped and that the availability of free barbed ends in stimulated cells determines the site and extent of polymerization (Hartwig and Yin, 1989; Stossel, 1989; Cooper, 1991; Symons and Mitchison, 1991; Hartwig, 1992; Fechheimer and Zigmond, 1993; Carlier and Pantaloni, 1994; Redmond *et al.*, 1994). If this model of the cytoskeleton is accurate, it is important to determine the following: (1) What factors cap the barbed ends? (2) What controls the availability of barbed ends: inactivation of the capper? severing of filaments? or creation of de novo nucleation sites?

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The only barbed-end capping protein thus far characterized in neutrophils is gelsolin (Yin and Stossel, 1979; Howard *et al.*, 1990). Regulation of the activity of gelsolin by calcium has been postulated as an essential step in cell movement induced by chemoattractants (Hartwig and Yin, 1989). However, in a mouse line missing gelsolin, neutrophils migrate effectively into inflammatory sites, although their rate of accumulation may be somewhat slower than normal (Witke *et al.*, 1995). This result is consistent with the earlier observation that *Dictyostelium discoideum* mutants missing the gelsolin analogue severin also have normal motility (Andre *et al.*, 1989). Another calcium-dependent capping protein, macrophage-capping protein, or cap 39, is present in macrophages but not in neutrophils (Southwick and DiNubile, 1986; Yu *et al.*, 1990; Weeds and Maciver, 1993). A calcium-independent capping protein, later identified in the Z-lines of striated muscle, appears to be ubiquitous in eukaryotic cells (Isenberg *et al.*, 1980; Cooper *et al.*, 1984; Casella *et al.*, 1987; Hartmann *et al.*, 1989, 1990). Homologues of this heterodimeric-capping protein, here referred to as capping protein- β_2 (on the recommendation of the monitoring editor), are present in a wide variety of organisms from yeast to humans. In chickens, capping protein mRNA and protein are detected in most tissues but the isoform in nonmuscle cells (β_2) is different from that in muscle (β_1) (Schafer *et al.*, 1994). The contribution of capping protein- β_2 to the capping of filament barbed ends in neutrophils is unknown.

In the studies reported here, we determine by immunoadsorption that capping protein- β_2 is the principal capping protein in high speed supernates of neutrophils lysed at submicromolar concentrations of calcium. We define the concentration and affinity of the capping activity in the crude supernate. Finally, we demonstrate that, despite an apparent excess of capper in the cell, some barbed ends are transiently free upon lysis.

MATERIALS AND METHODS

Procurement of Rabbit Neutrophils

Neutrophils were obtained from rabbit peritoneal exudates, as described by Sullivan and Zigmond (1980), except that the animals were sedated with 0.02 mg/kg of acepromazine maleate (Germenta, Kansas City, MO) 20 min before handling.

Preparation of Neutrophil Lysates

Neutrophils were resuspended at 1 or 2×10^8 cells/ml in intracellular physiological buffer (IP buffer), containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 2 mM MgCl₂, 150 mM KCl, 5 mM EGTA, 2 mM potassium phosphate, 10 mM β -glycerol phosphate, 0.2 mM dithiothreitol, 5 mM ATP, brought to pH 7.2 with NaOH. Just before lysis, a mixture of protease inhibitors with final concentrations of 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml benzamide, 10 μ g/ml aprotinin, and 10

μ g/ml TAME-HCl was added. For nitrogen cavitation, cells were incubated on ice in a Parr bomb at 350 psi of N₂ for 15 min before pressure was released. For detergent lysis, cells were resuspended in IP buffer containing the protease inhibitors described above and either 1% Triton X-100 or 1% NP-40. Nitrogen cavitation ("bombing") releases less lysosomal enzymes than detergent lysis (Klempner *et al.*, 1980).

Both bombed and detergent lysates were immediately spun for 20 min at 80,000 rpm in a Beckman L-100 ultracentrifuge and the supernatant was collected. Supernates were either used directly or stored frozen at -80°C . In general, high speed supernates made from cells bombed at 1×10^8 cells/ml had a protein concentration of roughly 2 mg/ml; supernates from detergent-lysed cells at the same cell concentration typically had a protein concentration of approximately 3 mg/ml (presumably because of release of granule proteins into the detergent supernates).

Preparation of Pyrenyl-G-Actin

Actin was purified from acetone powder extracted from the skeletal muscle of New Zealand White rabbits by the method of Spudich and Watt (1971). Pyrenyl-G-actin was prepared from rabbit skeletal muscle actin as described by Murray *et al.* (1981). The pyrenyl-G-actin was applied to a Sephacryl S-200 (Pharmacia, Piscataway, NJ) gel filtration column and then maintained in a storage buffer, containing 5 mM triethanolamine, 0.68 mM ATP, 0.3 mM CaCl₂, 0.1 mM EDTA, 0.02% NaN₃, pH 7.5, at 4°C . The critical actin monomer concentration varied between 0.1–0.2 μM for different preparations.

Actin Elongation Assay

The elongation rate of pyrenyl-G-actin from F-actin seeds (in the presence or absence of neutrophil supernates) was determined from the change in pyrenyl-actin fluorescence (excitation at 370 nm; emission at 410 nm) in a Perkin-Elmer LS5 fluorimeter (Norwalk, CT). The assay buffer contained 25 mM Tris-HCl, 138 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM ATP, and 0.2% NP-40, pH 7.4.

Preparation of Spectrin-F-Actin Seeds

Spectrin-F-actin seeds provide a stable and quantitatively reproducible source of nuclei for actin elongation from both the barbed and pointed ends. Spectrin-F-actin seeds were prepared as described by Casella *et al.* (1986). The number of spectrin-F-actin seeds was determined from the rate of polymerization after converting fluorescence units/time into amount of G-actin polymerizing to F-actin per minute, using as a reference the change in fluorescence occurring upon polymerization of 2 μM G-actin having a critical concentration of 0.12 μM . The on-rates for polymerization under these assay conditions had previously been determined to be $2 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Cano *et al.*, 1991). Our estimate of seed number was compared with an independent determination by Dr. A. Weber (University of Pennsylvania, Philadelphia, PA) using gelsolin-nucleated polymerization rates as a reference. The two estimates agreed within a factor of 2.

Preparation of Gelsolin-F-Actin Seeds

Plasma gelsolin was isolated from rabbit serum (Life Technologies, Gaithersburg, MD) as described by Cooper (1987). Gelsolin-F-actin seeds were prepared by copolymerizing 200 nM gelsolin with 3 μM pyrenyl-G-actin in a modified assay buffer with 0.5 mM CaCl₂ but without EGTA for ≥ 2 h at room temperature. To insure that all the barbed ends were capped by gelsolin, we assessed the ability of cytochalasin to inhibit the rate of elongation from our gelsolin-capped seeds. The presence of 1 μM cytochalasin did not inhibit the elongation rate of 2 μM pyrenyl-G-actin from gelsolin-F-actin seeds, but decreased the initial elongation rate from spectrin-F-actin seeds by $\sim 90\%$.

Determination of the Rate Constants for Capping in Supernates

The rate of pyrenyl-G-actin elongation from spectrin-F-actin seeds was measured without supernate and with various concentrations of supernate. Because elongation from the pointed end is $\leq 10\%$ of the filament elongation rate, the rate of elongation from uncapped seeds was roughly proportional to the product of the concentration of barbed ends and the concentration of G-actin available for polymerization, $[\text{barbed ends}][\text{G-actin}]$. The number of barbed ends free in a sample at any given time could be approximated by the rate of elongation (expressed as the change in fluorescence) divided by the concentration of G-actin available for barbed-end polymerization (expressed as the "steady state" fluorescence end-point of the control actin without supernate minus the fluorescence of the sample at that time). The data were expressed as the fraction of barbed ends free in a sample with supernate relative to the control sample (no supernate). As seen in Figure 7A, the number of barbed ends free in the control (the elongation rate of the control divided by the concentration of G-actin available for polymerization) was essentially constant over the time course of polymerization, as expected. The fraction of barbed ends capped in a sample at any time equaled 1 minus the fraction of barbed ends free at that time.

To determine the rate of approach to steady state binding of the capper to the barbed ends, we plotted $1 - (B_t/B_{\max})$ versus time for various concentration of supernates, where B_t is the fraction of barbed ends capped at any time t and B_{\max} is the maximal fraction of ends capped at steady state. For each concentration of supernate, the rate of elongation per available G-actin, i.e., the number of growing barbed ends, was plotted and a smooth curve was drawn through the data points (see Figure 7A). This curve was used to obtain B_t and B_{\max} for that concentration of supernate.

The slope of the plot of $1 - (B_t/B_{\max})$ versus time was used to determine the $t_{1/2}$ of capping and then the apparent on-rate constant (k_{obs}) for that concentration of supernate (Schonbrunn and Tashjian, 1978). The apparent rate constants were then plotted versus the concentration of supernate present. Assuming that the concentration of the uncapped capper remained essentially constant and the reaction kinetics were thus pseudo-first order, the slope of this plot gave the on-rate of binding (k_{on}) and the intercept gave the off-rate (k_{off}).

Western Blotting

Western blots were used to identify potential capping proteins in the neutrophil supernates. A monoclonal antibody to human plasma gelsolin (GS-2C4; Sigma Chemical, St. Louis, MO) and a variety of antibodies raised against skeletal muscle and nonmuscle capping protein were used as primary antibodies to probe neutrophil extracts for these capping proteins. Secondary antibodies were peroxidase-conjugated monoclonal antibodies against the IgG of the species in which the primary antibodies were made. Detection was by enhanced chemiluminescence (Amersham, Arlington Heights, IL) for capping protein- β_2 and by colorimetric assay for gelsolin.

Immunodepletion of Capping Protein- β_2 and Gelsolin

Monoclonal antibodies directed against the carboxy-terminus of the β_2 -subunit of chicken capping protein were obtained from Dr. Dorothy Schafer working in the laboratory of Dr. John Cooper at Washington University in St. Louis, MO. Monoclonal antibodies against human plasma gelsolin (GS-2C4) were obtained from Sigma. To deplete specific proteins, supernates were incubated in parallel in phosphate-buffered saline with and without antibodies overnight at room temperature. Four percent cross-linked agarose beads coupled to protein A (Sigma) were washed with either phosphate-buffered saline, pH 7.4, (with or without the addition of bovine serum albumin; BSA) or 100 mM Tris-HCl with 150 mM sodium

chloride and 0.1% BSA, pH 8.0. The beads were allowed to settle and the supernatant was removed from the top of the beads. The extracts previously mixed with antibody or buffer were then applied to the beads and the samples were agitated at room temperature or 4°C for at least 12 h. The beads were then centrifuged and the supernate was removed for the elongation assay and Western blots. Quantitation was accomplished by comparing immunodepleted samples to a standard curve generated from twofold dilutions of the parallel buffer-treated samples. The *Image-1* system (Universal Imaging Corporation, Media, PA) was used to measure the "integrated intensity" of the 32-kDa band. In some experiments, samples of antibody against capping protein- β_2 and extract were not applied to beads but used directly in an elongation assay to ascertain whether the antibody inhibited the function of capping protein- β_2 in supernates. There was only a slight reduction in capping activity under these circumstances.

We also used polyclonal anti-capping protein- β_2 antibodies raised in a rabbit against the carboxy-terminus of the β_2 -subunit of chicken nonmuscle capping protein (R25 antibodies, obtained from Drs. Schafer and Cooper) instead of the monoclonal antibodies in a few experiments.

RESULTS

Supernates from Neutrophil Extracts Inhibited Elongation from Exogenous F-Actin Seeds

High speed supernates of neutrophils lysed by nitrogen cavitation inhibited polymerization of pyrenyl-G-actin nucleated by spectrin-F-actin seeds (Figure 1). In the presence of supernate, the elongation rate decreased with time more rapidly than expected from the decrease in G-actin concentration as polymeriza-

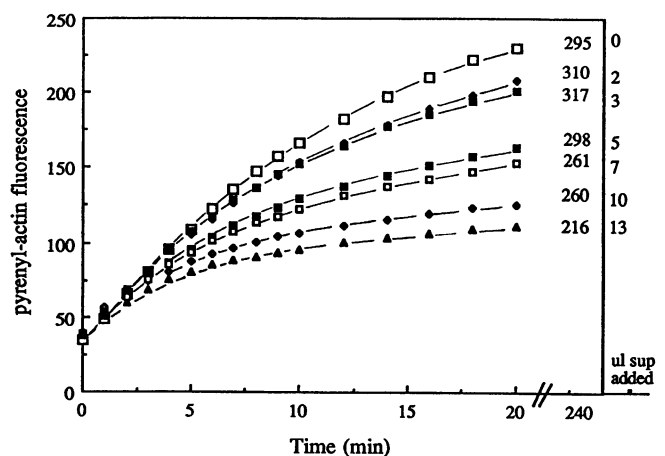


Figure 1. The time course and extent of pyrenyl-G-actin elongation from spectrin-F-actin seeds were dependent on the amount of neutrophil supernate added. Different volumes of high speed supernates from neutrophils lysed at 2×10^8 cells/ml were added to 1 ml of assay buffer containing $1.5 \mu\text{M}$ pyrenyl-G-actin, followed by the addition of 0.4 nM spectrin-F-actin seeds. The pyrenyl-actin fluorescence was followed for 20 min and then measured after 240 min to determine the steady state level of fluorescence. The concentrations of supernate added were 0 (open squares), in duplicate; $2 \mu\text{l}$ (closed diamonds); $3 \mu\text{l}$ (closed squares); $5 \mu\text{l}$ (closed squares); $7 \mu\text{l}$ (open squares); $10 \mu\text{l}$ (closed diamonds); and $13 \mu\text{l}$ (closed triangles), as shown to the right of the figure.

tion proceeded. Both the rate and final magnitude of inhibition were functions of the amount of supernate added. In the experiment shown in Figure 1, approximately 50% inhibition of the elongation rate was achieved with 2 μl of supernate of 2×10^8 cells/ml in 1 ml of assay buffer. This concentration of supernate had little effect on the final amount of pyrenyl-actin polymerized, as seen by the fluorescence at 240 min. This observation suggested that the inhibition was not due to monomer binding.

The Inhibition of Elongation Rate Was Not Due to G-Actin Sequestration

Three lines of evidence confirmed that, at the concentration of supernate used in these experiments, the presence of proteins that sequester G-actin contributed little to the inhibition of the elongation rate. First, as shown in Figure 2A, pre-incubation of pyrenyl-G-actin with cell supernates for 8 min before adding seeds did not reduce the time-dependent inhibition of elongation rate. If the time-dependent inhibition had been due to the slow sequestration of G-actin, pre-incubation of pyrenyl-G-actin with supernate before adding seeds would have decreased the time to reach maximal inhibition. Second, the percent of inhibition by a supernate of 10^7 cells/ml was nearly constant for concentrations of pyrenyl-G-actin between 0.4 and 2.4 μM (Figure 2B), consistent with the inhibition being due to a mechanism other than actin sequestration. Finally, one would not have expected a significant inhibition of elongation based on the amount of monomer binding proteins present in these low concentrations of supernates. A supernate from 10^7 cells/ml contains only 0.9 μM thymosin- β_4 and 0.1 μM profilin associated with 0.6 μM G-actin (Southwick and Young, 1990; Cassimeris *et al.*, 1992).

The Inhibition of Elongation Rate Was Due to Capping of the Barbed Filament Ends

The decrease in the rate of polymerization of pyrenyl-G-actin nucleated by spectrin-F-actin seeds was due to time-dependent capping of barbed ends. This conclusion is supported by three types of experiments. First, in contrast to pre-incubation of supernate with pyrenyl-G-actin, pre-incubation of supernate with spectrin-F-actin seeds largely eliminated the lag before maximal inhibition of the elongation rate (Figure 3A). Thus, the rate-limiting step in the inhibition of the elongation rate by supernates involved the interaction of supernate with F-actin, not G-actin. Second, as shown in Figure 3B, concentrations of supernate that markedly inhibited elongation from spectrin-F-actin seeds did not inhibit pointed-end elongation nucleated by gelsolin-F-actin seeds. When cytochalasin, a drug that rapidly caps barbed ends, was added to a gelsolin-F-actin sample, it caused no inhibition, docu-

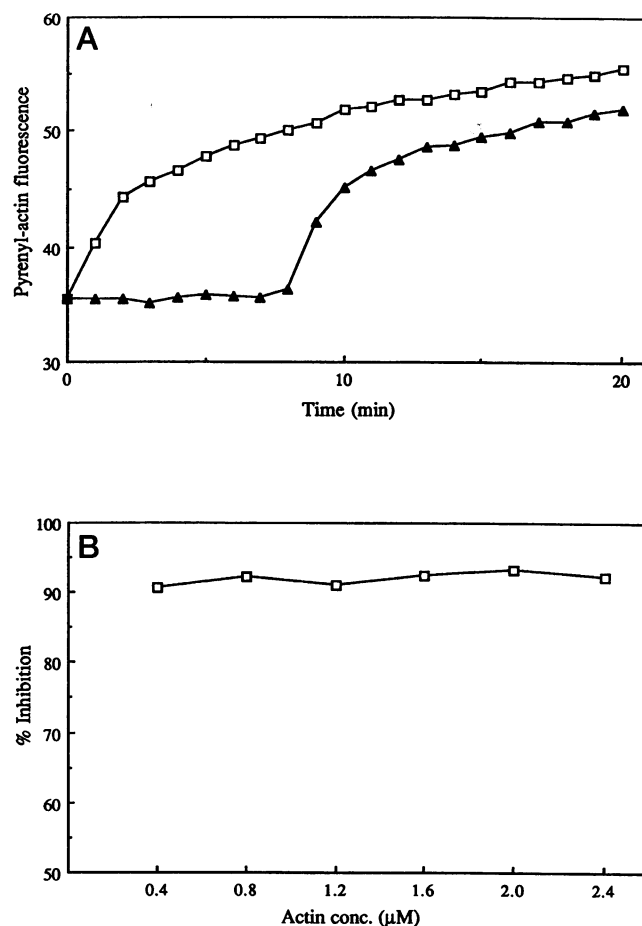


Figure 2. The time course and final magnitude of the inhibition of elongation rate by supernates were not affected by the concentration of pyrenyl-G-actin used or preincubation of the supernate with pyrenyl-G-actin, as would be expected if the inhibitory activity were largely due to actin monomer binders. (A) Preincubation of supernate with pyrenyl-G-actin did not affect the time course of inhibition. Supernate at a final concentration equivalent to 5×10^6 lysed cells/ml was mixed with 1.5 μM pyrenyl-G-actin, and 0.4 nM spectrin-F-actin seeds were added either immediately (open squares) or 8 min later (closed triangles). The fluorescence was followed over time under both conditions. The plots showing the change of fluorescence with time were similar with or without preincubation once the spectrin-F-actin seeds were added. (B) The elongation rate from 2 nM spectrin-F-actin seeds and various concentrations of pyrenyl-G-actin between 0.4 and 2.4 μM in the presence of 50 μl of supernate from cells lysed at 2×10^7 /ml was compared with the rate at each actin concentration without supernate. The final extent of polymerization was proportional to the G-actin concentration, as anticipated. For each sample the elongation rate was determined 15 min after adding seeds, corrected for the G-actin available for polymerization at this time. The data are plotted as the percent inhibition of elongation rate by samples containing supernate relative to samples without supernate at the same actin concentration.

menting that polymerization from these seeds was indeed due to pointed-end elongation. Lastly, the addition of cytochalasin immediately decreased the elongation rate of spectrin-F-actin seeds in the presence of

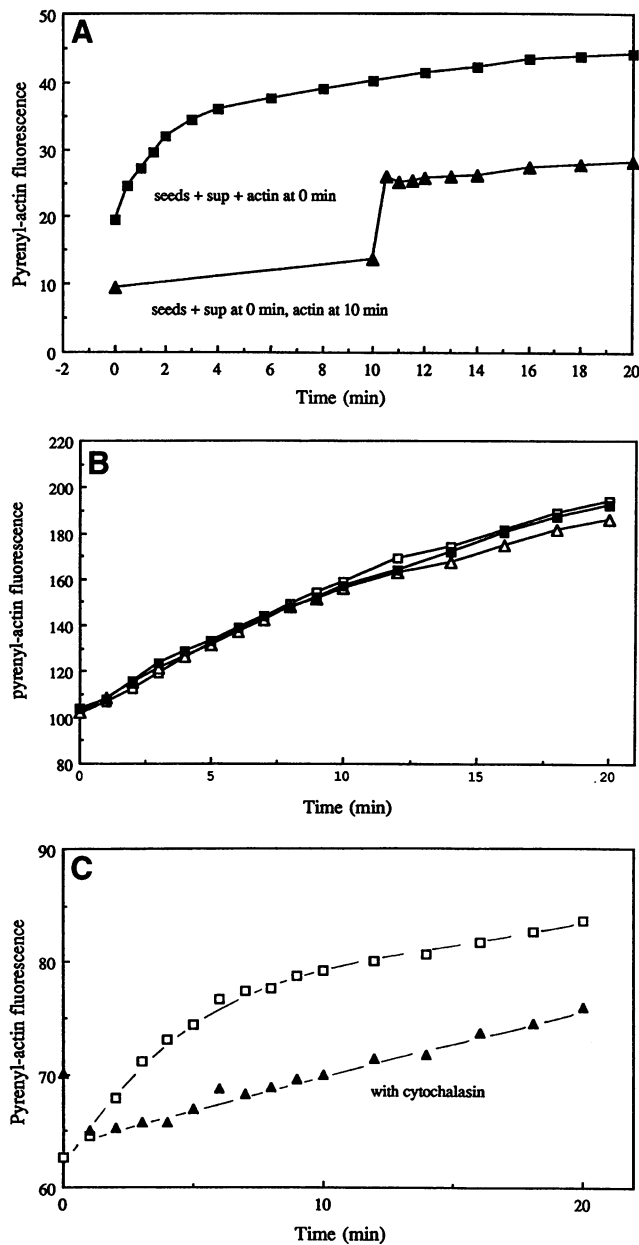


Figure 3. Supernates from neutrophil lysates capped the barbed ends of actin filaments. (A) Pre-incubation of seeds and supernate eliminated the time dependence of capping. Spectrin-F-actin seeds were incubated for 10 min in a cuvette containing $0.3 \mu\text{M}$ pyrenyl-G-actin (to prevent depolymerization of the diluted seeds) in assay buffer with and without supernate. Immediately (upper curve) or after 10 min (lower curve), $1.2 \mu\text{M}$ pyrenyl-G-actin was added to give a final concentration of $1.5 \mu\text{M}$ and the elongation rate was measured. The abrupt increase in fluorescence in the lower curve at 10 min represents the intrinsic fluorescence of the added $1.2 \mu\text{M}$ pyrenyl-G-actin. The initial elongation rate of the sample pre-incubated with seeds approximated the steady state rate in the other sample. (B) Supernates only slightly inhibited pointed-end elongation from gelsolin-capped F-actin seeds at concentrations that caused $>50\%$ inhibition of growth from spectrin-F-actin seeds. Gelsolin-F-actin seeds at a final concentration of 40 nM were added to 1 ml of assay buffer containing $2 \mu\text{M}$ pyrenyl-G-actin with-

$25 \mu\text{l}$ supernate from a lysate of 2×10^8 cells/ml to a rate similar to that achieved after 5–8 min in the absence of cytochalasin (Figure 3C), indicating that most of the barbed ends were capped by this concentration of supernate.

Gelsolin-Actin Complexes Did Not Contribute to the Capping Activity of Supernates

Supernates of neutrophil lysates included the capping proteins gelsolin and capping protein- β_2 , as demonstrated by Western blots in which antibodies to gelsolin (Figure 4A) and capping protein- β_2 (see below) stained single bands with molecular weights of approximately 90 and 32 kDa, respectively. Because the supernates were kept at submicromolar $[\text{Ca}^{++}]$ from the time of lysis, it was unlikely that gelsolin was being activated (Yin *et al.*, 1987; Lamb *et al.*, 1993). However, gelsolin-actin complexes formed before lysis (which only slowly dissociate after chelation of calcium) could contribute to the observed capping activity (Kurth and Bryan, 1984; Lind *et al.*, 1987). To address this possibility, gelsolin was immunoprecipitated from supernates by incubation of supernate with monoclonal antibodies to gelsolin followed by adsorption on protein A-conjugated beads. Removal of $\geq 95\%$ of the gelsolin from the supernate (Figure 4A) did not detectably diminish the capping activity (Figure 4B).

Depletion of Capping Protein- β_2 Correlated with Decreased Capping Activity

Unlike immunoadsorption of gelsolin, immunoadsorption of capping protein- β_2 with a monoclonal antibody markedly diminished the capping activity of supernates. Figure 5 shows that depletion of $\sim 90\%$ of the capping protein- β_2 in the supernate, measured by quantitative Western blotting (Figure 5A), resulted in a loss of $\sim 90\%$ of the capping activity (Figure 5b).

In these immunodepletion experiments, a variable amount of capping protein- β_2 was detectable by Western blotting in the supernate after immunoprecipita-

Figure 3 (cont.) out supernate (open squares and open triangles), or with $25 \mu\text{l}$ (closed squares) or with $10 \mu\text{l}$ (not shown) of the supernate from 2×10^8 lysed cells/ml. The fluorescence was measured at various times up to 20 min, and was unaffected by the presence of supernate. The data shown here are representative of four similar experiments, which exclude monomer binding and pointed-end capping as the mechanism of inhibition. (C) The elongation rate after 10 min in the presence of supernate was similar with or without cytochalasin. Spectrin-F-actin seeds at a final concentration of $\sim 0.4 \text{ nM}$ were added to $2 \mu\text{M}$ pyrenyl-G-actin and $25 \mu\text{l}$ of supernate from 2×10^8 lysed cells/ml (final concentration equivalent to 5×10^6 cells/ml) in the absence (squares) or presence (triangles) of $1 \mu\text{M}$ cytochalasin B. Data are from one experiment representative of four similar experiments. These results imply that essentially all the barbed ends were capped by this concentration of supernate.

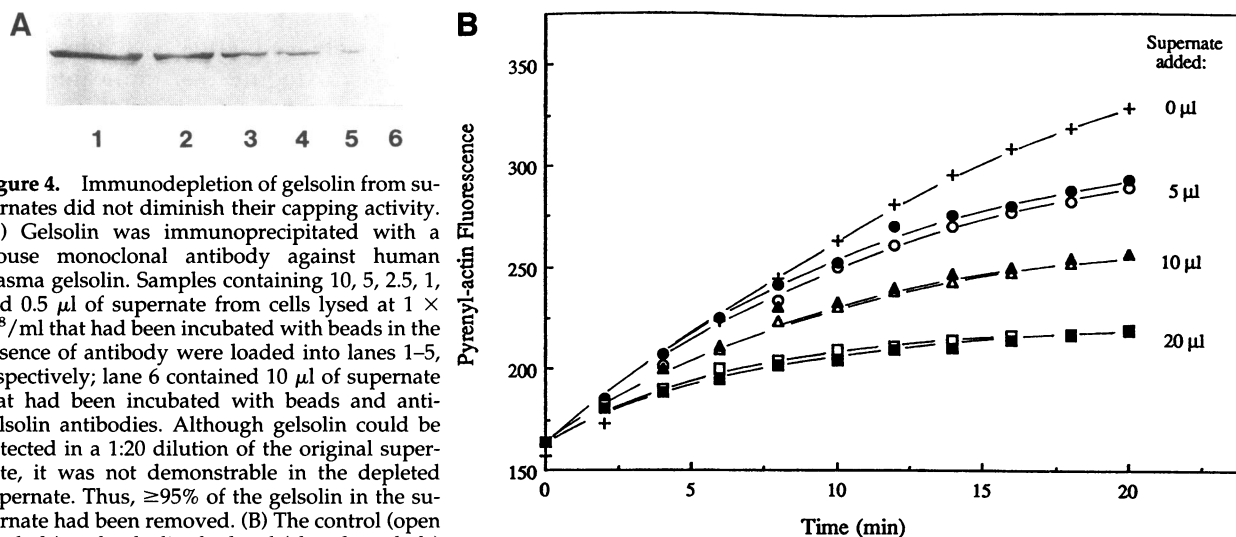


Figure 4. Immunodepletion of gelsolin from supernates did not diminish their capping activity. (A) Gelsolin was immunoprecipitated with a mouse monoclonal antibody against human plasma gelsolin. Samples containing 10, 5, 2.5, 1, and 0.5 μl of supernate from cells lysed at $1 \times 10^8/\text{ml}$ that had been incubated with beads in the absence of antibody were loaded into lanes 1–5, respectively; lane 6 contained 10 μl of supernate that had been incubated with beads and anti-gelsolin antibodies. Although gelsolin could be detected in a 1:20 dilution of the original supernate, it was not demonstrable in the depleted supernate. Thus, $\geq 95\%$ of the gelsolin in the supernate had been removed. (B) The control (open symbols) and gelsolin-depleted (closed symbols) supernates were examined for their ability to inhibit elongation from F-actin seeds. The seeds used in this experiment were 50 μl of 0.5 μM F-actin sheared through a 10 μl Hamilton syringe. The final pyrenyl-G-actin concentration was 1 μM . The amounts of supernate added were 0 (plus signs), 5 μl (circles), 10 μl (triangles), and 20 μl (squares), as indicated at the right of curves. Each data point was the mean of duplicate samples; the error bars were not included for clarity. Depletion of $\geq 95\%$ of the gelsolin in the supernate did not reduce its capping activity.

tion. Figure 6 demonstrates that the diminution of capping activity was proportional to the depletion of capping protein- β_2 over a wide range of concentrations. Immunoabsorption of capping protein- β_2 with a rabbit polyclonal antibody (R25) yielded quantitatively similar results to depletion with the monoclonal antibody; R25 immunoabsorption caused a 44% depletion of capping protein- β_2 on Western blots and a corresponding 42% loss of capping activity (values are the means from two separate experiments).

Analysis of the Rate and Extent of Capping Gave Estimates of the Affinity and Rate Constants for Binding to the Barbed Ends

The fraction of barbed ends capped in resting cells will likely depend on the concentration of capper and its affinity for the barbed filament end. The role of capper in limiting filament growth after stimulation may additionally depend on the rate of capping. Because the inhibition of the elongation rate by dilute supernates was shown to be due principally to barbed-end capping by capping protein- β_2 , we used the cell supernates to characterize the functional properties of neutrophil capping protein. The rate of capping could be determined from the time and concentration dependence of capping, depicted in Figure 1. Figure 7A replots the information in Figure 1 to demonstrate explicitly the time-dependent decrease in the fraction of ends free in various concentration of supernates and illustrates the fitting of smooth curves to these data. At most concentrations of supernate used in the experiments shown in Figure 7A, inhibition of elon-

gation (i.e., capping) had reached a steady state level (a horizontal line on the plot) by ~ 20 min. For the lowest concentrations of supernate, it was necessary to extend the time to 35 min to attain steady state capping. The plateau values from Figure 7A were used to define the extent of capping at steady state for each concentration of supernate; the dose-response curve from this experiment is drawn in Figure 7B and fits a normal binding curve for a $K_D \sim 2.5 \mu\text{l}$ of supernate per ml (equivalent to a final cell concentration of $\sim 5 \times 10^5$ cells/ml). Similar inhibition of elongation rates was observed with supernates from cells lysed at the same cell concentrations by detergent rather than by nitrogen cavitation.

The rate of approach to steady state capping for various concentrations of supernate was analyzed by plotting the approach to "equilibrium" binding given by $1 - (B_t/B_{\text{max}})$, where B_t/B_{max} is the fraction of barbed ends capped at any time t divided by the fraction capped at steady state in that concentration of supernate (see MATERIALS AND METHODS) (Figure 7C). The apparent or observed on-rate k_{obs} was determined from the $t_{1/2}$ (the time to half-maximal capping). The plot of k_{obs} versus the concentration of supernate (Figure 7D) yielded a slope equal to the k_{on} in this and two other experiments the mean (\pm range) for k_{on} was $2.9 \pm 0.7 \times 10^{-4}$ (μl supernate/ml) $^{-1} \text{s}^{-1}$. The y-intercept of these plots yielded the off-rate of capping: $1.9 \pm 0.1 \times 10^{-3} \text{s}^{-1}$. The K_D of binding calculated from the $k_{\text{off}}/k_{\text{on}}$ was about 6.6 μl supernate/ml, approximately three times the K_D estimated from steady state capping

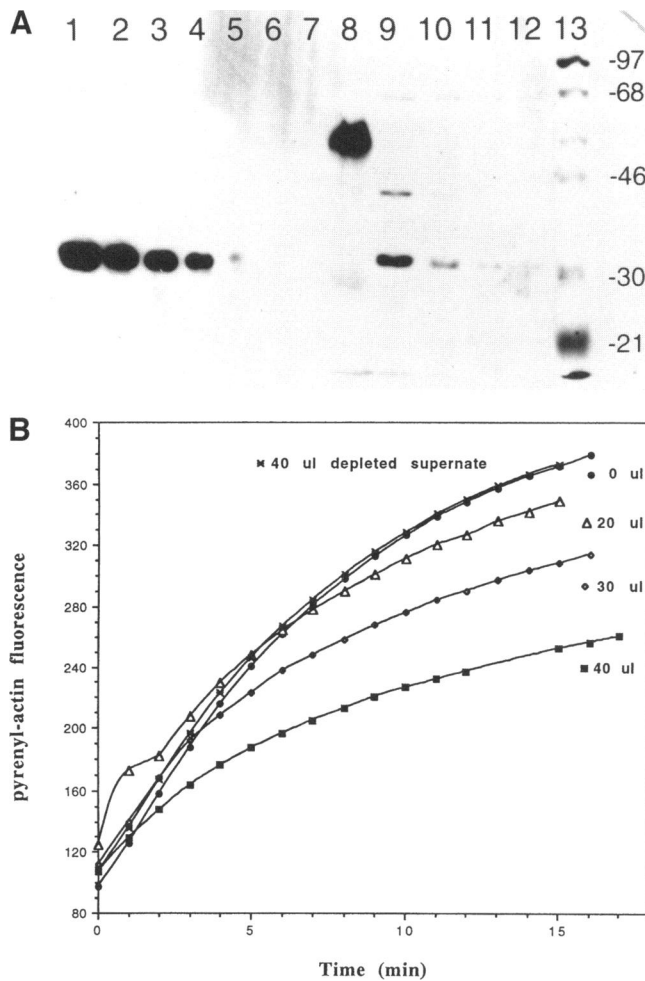


Figure 5. Immunoadsorption of capping protein- β_2 in a supernate almost eliminated the capping activity. (A) The Western blot demonstrates an approximately eightfold decrease in capping protein- β_2 in the supernate of 2×10^8 cells/ml immunodepleted with a monoclonal antibody against the β_2 -subunit of capping protein, compared with supernate treated in parallel with the corresponding buffer, but without antibodies. Lanes 1–6 contain 8, 4, 2, 1, 0.5, and $0.25 \mu\text{l}$ of supernate diluted in buffer without antibodies; lanes 9–12 contain 8, 4, 2, and $1 \mu\text{l}$ of the supernate treated with antibodies to capping protein- β_2 . Lanes 8 and 13 contain molecular weight standards. The primary antibodies used to stain this Western blot were rabbit polyclonal antibodies against capping protein. Similar results were obtained in other immunodepletion experiments in which monoclonal antibodies against capping protein were used as the primary antibody for the Western blots. The faint bands visible in lanes 1, 2, and 9–12 just below the 68-kDa marker were visible after staining with “secondary” antibodies against rabbit, but not mouse IgG, without anti-capping protein- β_2 antibodies. (B) The elongation rates of $1.6 \mu\text{M}$ pyrenyl-G-actin from 4 nM spectrin-F-actin seeds from the same experiment shown above were measured in the presence of 0, 20, 30, and $40 \mu\text{l}$ of control (buffer-treated) supernate and $40 \mu\text{l}$ of depleted (antibody-treated) supernate in a final volume of 1 ml of assay buffer. The depleted sample was compared with a “standard” curve generated by plotting the inhibition of the control samples versus their respective volumes. Forty microliters of the antibody-treated supernate depleted of capping protein- β_2 was required to achieve the equivalent level of inhibition as $\sim 4 \mu\text{l}$ of the buffer-treated control sample. Thus, $\sim 90\%$ of the capping activity

(see Figure 7B). In three additional kinetic analyses using supernates from detergent-lysed cells, the capping parameters per cell were similar to bombed cells.

The Molar Concentration of Capper Could Be Estimated by Exceeding its Binding Capacity

To ascertain the molar concentration of capper present in the supernates, we determined the number of spectrin-F-actin seeds required to deplete the capping activity from supernates. Various concentrations of spectrin-F-actin seeds (between 8 and 32 nM) were incubated with buffer or with cell supernate at a concentration equivalent to 2×10^7 cells/ml for 5 or 10 min before the mixture was diluted to give a final concentration of 0.4 nM seeds. The elongation rates with $1.5 \mu\text{M}$ pyrenyl-G-actin were measured between 1.5 and 3 min after dilution.

As seen in Figure 8, when 8 nM seeds and supernate were pre-incubated, the elongation rate following dilution was inhibited by 80% compared with the rate when 8 nM seeds were pre-incubated with buffer. However, when 32 nM seeds and the same concentration of supernate were pre-incubated, the elongation rate was only inhibited about 50% compared with the buffer control. Because the concentration of capper(s) in a supernate equivalent to 2×10^7 cells/ml was nearly $100 \times$ the K_D of capping (a high enough concentration to cap 90% of the barbed ends), capping could not be limited by the affinity of the capper. Under these conditions, capping was limited by stoichiometry, i.e., the amount of capper present. From this result, we estimated the concentration of capper in the supernate of 2×10^7 unstimulated cells/ml to be $\sim 16 \text{ nM}$. In two additional experiments, the concentration of capper in the same concentration of supernate was estimated to be 12 and 14 nM.

Based on the above estimates, the on-rate of capping [expressed as $\text{M}^{-1} \text{sec}^{-1}$ instead of $(\mu\text{l supernate/ml})^{-1} \text{s}^{-1}$] was between 7×10^5 and $5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$; the off-rate was $\sim 2 \times 10^{-3} \text{ s}^{-1}$. The K_D for the capper (determined from both steady state and kinetic analyses) was $\sim 1\text{--}2 \text{ nM}$. Because the cell volume of 2×10^9 cells is $\sim 1 \text{ ml}$, the concentration of free capper in the intact cell would be $\sim 1.4 \pm 0.2 \mu\text{M}$.

Endogenous Barbed-End Elongation Sites Were Available Immediately after Cell Lysis

The analysis presented so far suggests that neutrophil cytoplasm contains micromolar amounts of a capper having a nanomolar affinity for barbed ends.

Figure 5 (cont). was removed by immunoadsorption, correlating almost exactly with the degree of depletion of the 32-kDa band on the Western blot shown in Figure 5A.

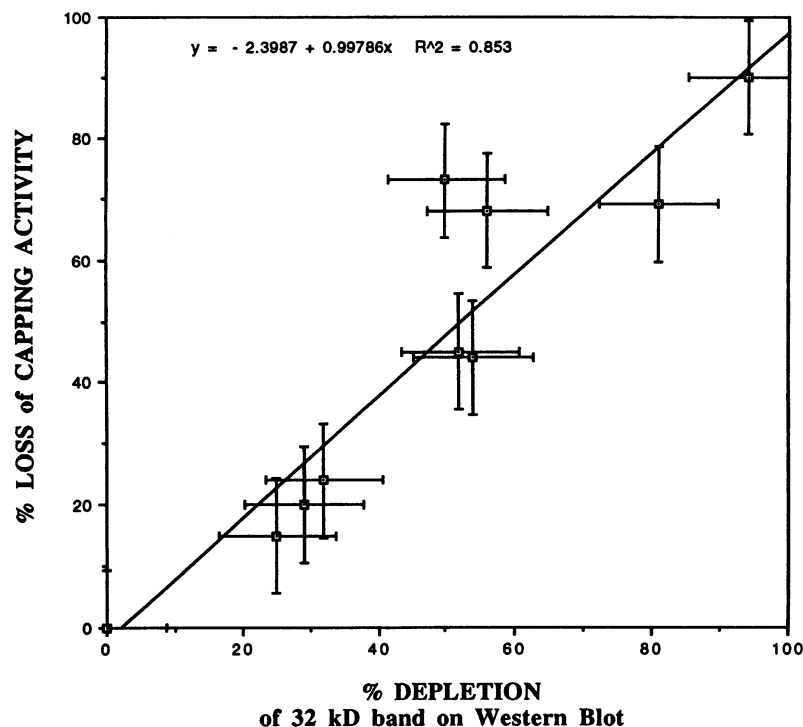


Figure 6. The loss of capping activity in supernates closely correlated with the depletion of capping protein- β_2 . The loss of capping activity in supernates, as determined from comparisons of the elongation rates from spectrin-F-actin seeds in the presence of control or partially depleted supernates, was proportional to the amount of capping protein- β_2 depleted from the supernate with monoclonal antibodies against the β_2 -subunit of capping protein. The error bars represent the standard error of the mean for each parameter.

It is therefore surprising that immediately after lysis, neutrophils contain filaments with free barbed ends that can rapidly nucleate the polymerization of exogenous pyrenyl-G-actin (Carson *et al.*, 1986; Cano *et al.*, 1991, 1992). At low cell concentrations $<10^6$ cells/ml, the number of ends available as nuclei (identified here by the initial rate of elongation) is proportional to the number of cells in the lysate (Carson *et al.*, 1986). However, as shown in Figure 9, as the cell concentration in the lysate was increased, the initial rate of barbed-end elongation no longer increased proportionally with cell number, and the elongation rate decreased with time after lysis. Both the rate and extent of inhibition varied directly with the concentration of lysed cells.

The time and concentration dependence of capping endogenous barbed filament ends in whole cell lysates paralleled that described earlier with high speed supernates and exogenous spectrin-F-actin seeds, suggesting that immediately after lysis and dilution, cells possess free barbed ends that become capped with time. The differences between the plots in Figure 1 (supernates mixed with exogenous nuclei) and Figure 9 (whole cell lysates and endogenous nuclei) are primarily due to the fact that in the experiments with supernates, the number of spectrin-F-actin seeds was held constant as the concentration of capper increased, while with the whole cell lysates, the number of nucleation sites and the concentration of capper increased in parallel.

DISCUSSION

Neutrophil Supernates Contain a Barbed-End Capping Activity

Supernates of lysed neutrophils contain a barbed-end capper that could be depleted with antibodies to capping protein- β_2 . The loss of a 32-kDa immunoreactive protein after adsorption with antibody directed against the β_2 -subunit of capping protein closely correlated with the loss of capping activity. Thus, most of the calcium-independent capping activity in dilute neutrophil supernates observed under our assay conditions is associated with capping protein- β_2 .

It is conceivable that our monoclonal antibody against capping protein- β_2 was cross-reactive with similar epitopes on other capping proteins; if so, we could unwittingly be depleting extracts of other cappers in addition to capping protein- β_2 . This possibility is unlikely for several reasons. First, both our monoclonal and polyclonal antibodies against capping protein- β_2 reliably showed only a single band on Western blotting of neutrophil extracts over >32 -fold range of concentrations. Second, if there were other cappers being depleted, the proportional relationship depicted in Figure 6 between the depletion of capping protein- β_2 and the loss of capping activity would imply that our immunoadsorption process removed any other cappers with an efficiency proportional to the depletion of capping protein- β_2 . Last, immunoadsorption with a rabbit polyclonal antibody (R25) yielded

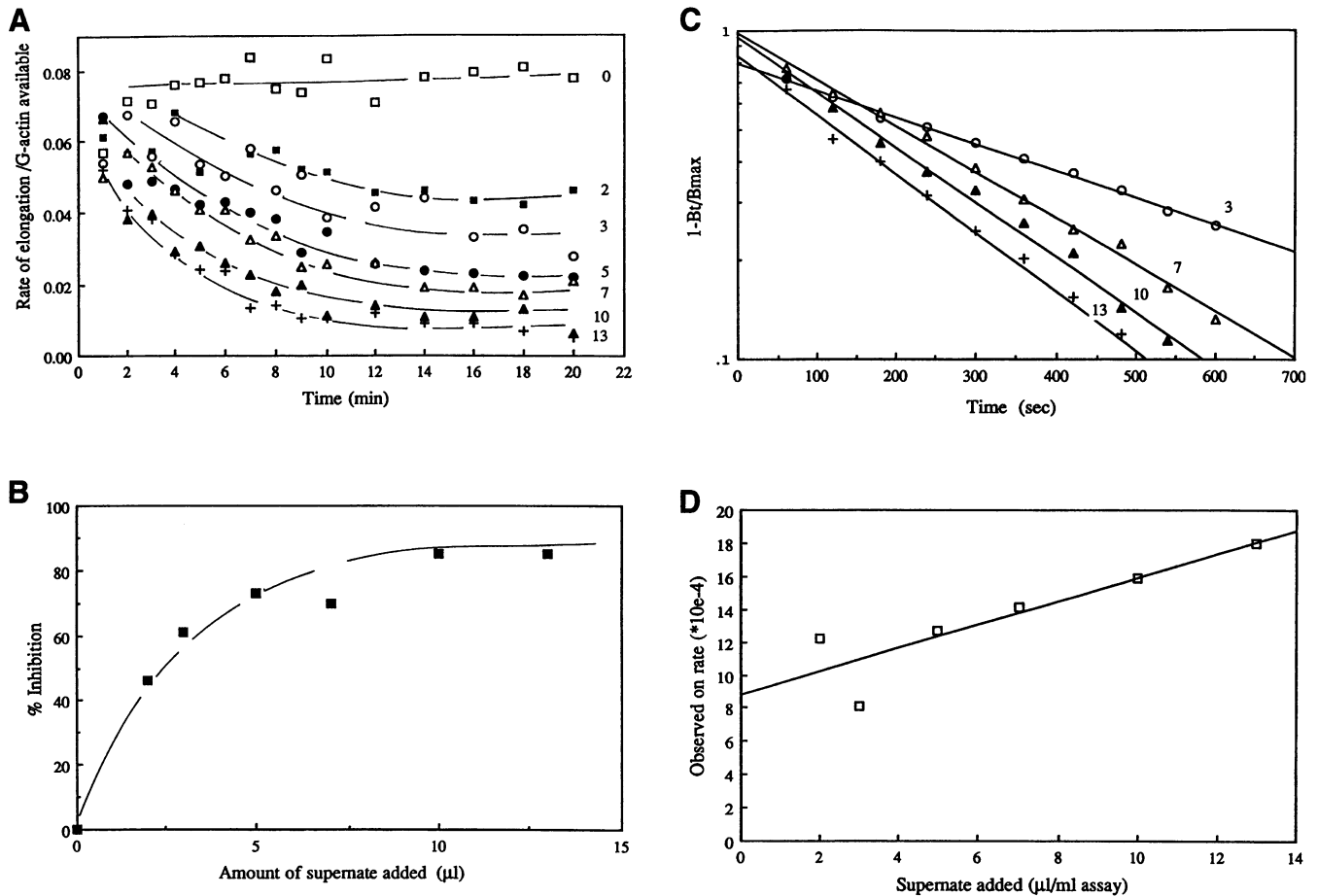


Figure 7. The rate constants for capping and uncapping barbed ends were derived from an analysis of the time course of elongation. (A) The elongation rate corrected for the available G-actin at each time point, i.e., the number of growing ends in arbitrary units, is plotted for different amounts (shown to the right of each curve) of supernate from 2×10^8 lysed cells/ml. This plot was generated from the data shown in Figure 1 (see MATERIALS AND METHODS). (B) The inhibition of the elongation rate by supernate fit a normal binding curve with a K_D of 2.5 μl of supernate/ml. The extent of capping at steady state was determined from the elongation rate between 15 and 20 min after correcting for the G-actin available for polymerization (see MATERIALS AND METHODS). The extent of inhibition was converted to the fraction of barbed ends capped by comparison to the control rate. At the highest concentrations of supernate shown, the fraction of ends capped approached unity. (C) The number of uncapped ends at each time point was determined from the smoothed curves shown in Figure 7A. The decrease in elongation rate reflected the capping of barbed ends. The rate of approach to steady state capping for each concentration of supernate was defined by plotting $1 - (B_t/B_{\max})$ versus time. B_{\max} was the final extent of capping for each supernate and was set at 1; the fraction of capped ends at each time point (B_t/B_{\max}) was then determined. The graph shows data for 3, 7, 10, and 13 μl of supernate/ml, as indicated above the lines in the figure. The slopes of these lines gave the observed on-rate for that concentration of supernate. (D) The slope of each line in Figure 7C was plotted (as well as additional data not presented in Figure 7C) on a semilogarithmic plot versus the concentration of added supernate. The slope of this plot yielded the k_{on} of capping in units of $(\mu\text{l/ml})^{-1} \text{ s}^{-1}$ and the y-intercept gave the k_{off} of capping as s^{-1} . The supernate used in this experiment, derived from the lysis of 2×10^8 cells/ml, had a protein concentration of 3.1 mg/ml. These data fit a straight line with an R^2 value of 0.793. Two other experiments were performed with bombed supernates and yielded similar results.

quantitatively similar results to depletion experiments with the monoclonal antibody.

The Concentration of Capper in the Intact Cell Appears to Be Sufficient to Cap all the Barbed Filament Ends

Because the time required to separate the lysate into supernate and pellet was longer than the time required to reach steady state capping at 2×10^8

cells/ml, any barbed ends free upon lysis would have become capped. Therefore, the capper in the supernate is the fraction that did not bind to endogenous filaments and hence represents only the free capper. We estimate that there are approximately 10^5 filaments in a resting neutrophil (Cano *et al.*, 1991), yielding a concentration in the cytoplasm of $\sim 1 \mu\text{M}$. Our estimates of the free (i.e., excess) capper concentration in the cytoplasm of an intact

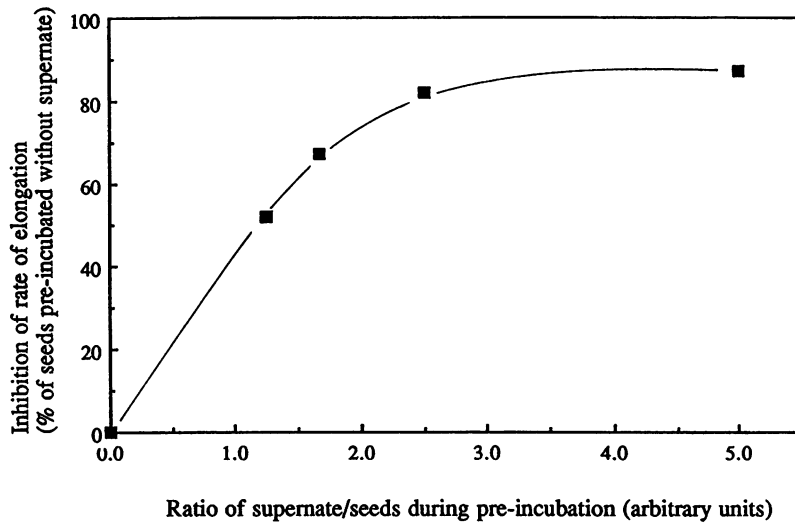


Figure 8. The concentration of capper in a supernate could be estimated by exceeding its binding capacity with excess spectrin-F-actin seeds. Buffer or supernate from a lysate of 2×10^7 cells/ml was incubated with spectrin-F-actin seeds at concentrations of 8, 16, 24, and 32 nM for 5 min. The volume of each sample equivalent to 0.4 nM seeds was then diluted into 1.5 μ M pyrenyl-G-actin. Supernate was added to the assay mixture where appropriate to keep the final supernate concentration in the elongation assay constant. The "initial" rate of elongation was determined between 1.5 and 3 min following the dilution of seeds. The results were plotted as the percent inhibition of the elongation rate observed in the presence relative to the absence of supernate.

cell, determined from our analysis of supernates, were between 1 and 2 μ M. Given that the affinity of the capper for the barbed end was nanomolar, there appeared to be enough capper present in neutrophils to cap all the barbed ends and leave $>1 \mu$ M unbound in the cytoplasm.

The Neutrophil Capper Is Similar to Cappers in Muscle and *D. discoideum*

The properties of the capper from neutrophil lysates were similar to capping protein- β_1 (cap Z) isolated

from skeletal muscle. The affinity of nonmuscle vertebrate capping protein- β_2 has not yet been determined but cap Z has an affinity for the barbed filament end of $\sim 0.5\text{--}1$ nM (Caldwell *et al.*, 1989), similar to the values obtained here for neutrophil capping protein- β_2 . The amount and affinity of neutrophil capping protein are also similar to those previously characterized in supernates of amoebae (*D. discoideum* and *Acanthamoeba castellanii*) and yeast (Cooper *et al.*, 1984; Schleicher *et al.*, 1984; Hall *et al.*, 1989; Hartmann *et al.*, 1989; Sauterer *et al.*, 1991; Amatruda *et al.*, 1992; Eddy *et al.*, 1993).

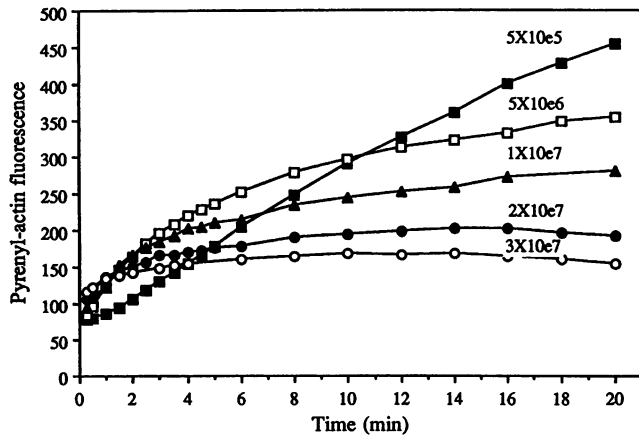


Figure 9. Cell lysates had free barbed-ends immediately after lysis and dilution that slowly became capped. Cells were directly lysed by NP-40 into 2 μ M pyrenyl-G-actin in assay buffer. Final cell concentrations were 5×10^5 , 5×10^6 , 1×10^7 , 2×10^7 , 3×10^7 , and 4×10^7 cells/ml, as noted above the curves. As the cell concentration was increased, the inhibition of the elongation rate also increased. These results, which reflect the assembly of pyrenyl-G-actin onto endogenous free barbed ends, were similar to the results seen with growth from exogenous spectrin-F-actin seeds shown in Figure 1.

Inhibition of Endogenous Filament Growth in Neutrophil Lysates Is Time and Concentration Dependent

From the earlier discussion, lysed cells would not be expected to possess filaments with free barbed ends, yet immediately after lysis barbed ends are free, and in lysates made at sufficiently high cell concentrations, they become capped with a time course similar to spectrin-F-actin seeds. Free barbed ends were also found by Hug *et al.* (1995) after lysis of *Dictyostelium*. Why should cells transiently have free barbed ends after lysis? Several potential explanations exist for this observation, including the following: (1) free barbed ends could be created as an artifact of lysis; (2) barbed ends could be free in vivo; or (3) the affinity and/or concentration of the capper in the cytosol could be less than that found in lysates.

New free barbed ends may be created as an artifact of the procedure if filaments are sheared during lysis or if detergent releases a sequestered nucleation site. However, we get similar estimates of the numbers of free barbed ends in lysates created by mechanical shear (nitrogen bombing or filter lysis) or detergent lysis. Further evidence against random shearing of

microfilaments by lysis is the highly reproducible, time- and concentration-dependent increases in nucleating sites evoked by agonists (Carson *et al.*, 1986; Hall *et al.*, 1989; Hartwig, 1992). These observations either represent a reproducible artifact of shearing (rendering most studies of filament number and length in cells invalid) or imply that lysis itself systematically perturbs steady state capping.

Perhaps the barbed ends free after lysis are also free in the cell and the capper in the lysates is inhibited or sequestered in vivo. Free barbed ends could also exist in vivo if the rate of formation of new filaments exceeded the rate at which these filaments become capped. This might be the case in moving cells where free ends at the cell front support local polymerization (Symons and Mitchison, 1991; Theriot and Mitchison, 1991). However, unstimulated neutrophils are not motile, and the addition of cytochalasin does not decrease the F-actin level (White *et al.*, 1983; Cassimeris *et al.*, 1990). This finding implies that the free G-actin concentration is already near the critical concentration of the pointed ends. Stimulation with chemoattractants causes a doubling of the F-actin level in neutrophils and the addition of cytochalasin after stimulation returns the F-actin to basal levels. Thus, upon lysis of unstimulated neutrophils, as in the studies described here, we would expect most (>90%) of the barbed ends to be capped (Young *et al.*, 1990).

Finally, if all the barbed ends are capped in the cell, lysis may increase the affinity of the capper. This hypothesis requires that the capper on the barbed ends of filaments in the cytosol has a low affinity and rapid off-rate, leading to uncapping upon the dilution coincident with lysis. This low affinity capper could be a modified form of capping protein- β_2 or a different capper present at sufficiently high concentration to compete successfully with capping protein- β_2 despite its lower affinity for the barbed ends. Indeed, the fraction of filaments capped after lysis in *Dictyostelium* suggests that the affinity of the capper in vivo might be only 1/100 of its affinity after lysis (Hug *et al.*, 1995). The affinity of the capping protein analogue in *Dictyostelium* appears to be increased in the presence of a heat shock cognate-70 (HSC-70) protein (Sauterer *et al.*, 1991; Eddy *et al.*, 1993), but other cofactors could have the opposite effect on its affinity. It is important to recognize that a lowered affinity for capping protein- β_2 in vivo could increase the off-rate but need not imply that filaments in the cell have free barbed ends. Because the concentration of capping protein- β_2 in the cell is 1,000 times its in vitro K_D , even if its affinity were decreased by two logs in vivo, there would still be enough to cap essentially all of the barbed ends.

Our data are consistent with the hypothesis that barbed ends are capped in vivo and that cytoplasmic dilution associated with cell lysis either releases an abundant but low affinity second capper with a rapid

off-rate from barbed ends, thus transiently creating free barbed ends until they become capped by the higher affinity capping protein- β_2 , or increases the affinity of capping protein- β_2 by covalent modification or dissociation of a low affinity inhibitor bound in vivo to capping protein- β_2 .

The slow on-rate of capping ($\sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$) could be the critical determinant of actin dynamics in the cytosol. With an initial concentration of free capper in the cytosol of an intact cell of 1.5 μM in the presence of 1 μM free barbed ends, 50% of the filaments would become capped in $\sim 0.6 \text{ s}$ and 90% in $\sim 4 \text{ s}$. This slow rate of capping may obviate the need for chemoattractants to inhibit the capper to allow barbed-end growth. If new filaments arise de novo (Cano *et al.*, 1991), the time course of capping could determine the filament length distribution in the cell cortex.

Summary

Dilute neutrophil lysates contain an abundant capping protein that binds with high affinity to the barbed ends of actin filaments at submicromolar [Ca^{++}]. Immunoadsorption experiments provide strong evidence that the primary calcium-independent capper in neutrophil lysates is capping protein- β_2 . Its affinity and concentration resemble those of analogous cappers in vertebrate-striated muscle and *D. discoideum*. The cellular concentration of this high affinity capper is sufficient to cap all the barbed filament ends in the cell. Nevertheless, cells lysed at high concentration transiently possess free barbed ends that subsequently become capped. These apparently discordant observations could result from effects of cell lysis and cytoplasmic dilution on the concentration and affinity of the capper(s).

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