

Dynamics of Capping Protein and Actin Assembly In Vitro: Uncapping Barbed Ends by Polyphosphoinositides

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Abstract. Bursts of actin polymerization in vivo involve the transient appearance of free barbed ends. To determine how rapidly barbed ends might appear and how long they might remain free in vivo, we studied the kinetics of capping protein, the major barbed end capper, binding to barbed ends in vitro. First, the off-rate constant for capping protein leaving a barbed end is slow, predicting a half-life for a capped barbed end of ~30 min. This half-life implies that cells cannot wait for capping protein to spontaneously dissociate from capped barbed ends in order to create free barbed ends. However, we find that phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 4-monophosphate (PIP) cause rapid and efficient dissociation of capping protein from capped filaments. PIP₂ is a

strong candidate for a second messenger regulating actin polymerization; therefore, the ability of PIP₂ to remove capping protein from barbed ends is a potential mechanism for stimulating actin polymerization in vivo. Second, the on-rate constant for capping protein binding to free barbed ends predicts that actin filaments could grow to the length of filaments observed in vivo during one lifetime. Third, capping protein β -subunit isoforms did not differ in their actin binding properties, even in tests with different actin isoforms. A major hypothesis for why capping protein β -subunit isoforms exist is thereby excluded. Fourth, the proposed capping protein regulators, Hsc70 and S100, had no effect on capping protein binding to actin in vitro.

CONTROL of actin assembly is essential for cell locomotion and rapid changes in cell shape. One hypothesis for regulating the location and dynamics of actin polymerization in vivo is to alter the availability of free actin filament barbed ends near the plasma membrane. In vitro, the barbed end of an actin filament is the end favored for actin polymerization and, thus, is a likely site for rapid actin polymerization in cells. Upon stimulation of several types of cells, increased numbers of free barbed ends correlate with increased actin polymerization. The polymerization is sensitive to cytochalasin and, therefore, involves barbed ends (7, 24, 25, 46).

Most actin filament barbed ends are not freely accessible in cells (25, 34) and are likely blocked from elongation by the barbed end binding protein, capping protein (18, 53). Knowing the dynamics of capping protein binding to F-actin and the intracellular concentrations of capping protein and actin would enable us to predict the half-lives of free and capped barbed ends in cells and to predict how capping protein participates in regulating actin assembly. Therefore, we determined the kinetic rate constants for actin binding of capping protein from muscle (CP- β 1) and

vertebrate nonmuscle tissues (CP- β 2). (The name "capping protein," given to the first member of this family of barbed end actin binding proteins [34a], is used here to refer to the vertebrate homologue. To differentiate the vertebrate capping protein isoforms, we use the name CP- β 1, for capping protein containing the β 1-subunit [CP- β 1 is also known as CapZ because of its location at Z-disks [11]], and CP- β 2, for capping protein containing the β 2-subunit.)

We also investigated potential signals proposed to regulate the actin binding activities of capping protein. Regulated uncapping of actin filaments is an attractive mechanism for increasing the number of free barbed ends in response to extracellular signals. Capping protein is released from the actin cytoskeleton upon platelet activation (47), consistent with uncapping. In addition, the maintenance of uncapped filament barbed ends at the rear of *Listeria* has been proposed as one mechanism for the actin assembly that drives movement of the bacterium (44).

Polyphosphoinositides have been proposed to mediate actin assembly (37); in particular, uncapping of barbed ends by phosphatidylinositol 4,5-bisphosphate (PIP₂)¹ has

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1. *Abbreviations used in this paper:* PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate, PIP₃, phosphatidylinositol, 3,4,5-trisphosphate.

been proposed as a mechanism for increasing actin assembly in activated platelets (27). Therefore, we tested the ability of phosphoinositides to promote dissociation of capping protein from F-actin. Hsc70 (21, 28) and S100 proteins (35, 36) also have been reported to interact with capping protein, but their effects on actin binding using purified components are not known. Therefore, we tested whether or not Hsc70 and S100 proteins affect the actin binding properties of capping protein.

Vertebrates have two isoforms of the capping protein β subunit. CP- β 1 is the predominant capping protein of muscle and is found at Z-disks of sarcomeres (11). CP- β 2 is the predominant isoform in nonmuscle tissues. CP- β 2 also is expressed in small amounts in muscle cells, where it is distributed at the cell periphery, but not at Z-disks (54). The two β subunits arise from alternatively spliced mRNA transcripts and only differ in \sim 30 amino acids at their COOH termini. The different COOH-terminal amino acid sequences are presumed to be important because they are highly conserved among vertebrates. Since the COOH terminus of the β subunit is necessary for binding actin (33), we hypothesized that the isoforms differ in their actin binding properties. For example, binding of CP- β 1 to F-actin may be highly stable in order to stabilize the actin filaments of the sarcomere. On the other hand, binding of CP- β 2 to F-actin may be dynamic to regulate the rapid polymerization of cortical actin filaments important for locomotion and changes in shape. A corollary to this hypothesis is that the actin-binding properties of capping protein β subunit isoforms may be differentially modulated by cellular factors such as PIP₂, Hsc70, and S100 proteins.

An alternate hypothesis to account for the existence of two β subunit isoforms is that CP- β 1 and CP- β 2 interact selectively with different isoforms of actin. The distributions of CP- β 1 to sarcomeres and CP- β 2 to the cortex within single cardiomyocytes (54) parallel the distributions of sarcomeric (α -actin) and nonsarcomeric (β - and γ -actins) actins (16, 31, 48). Therefore, CP- β 1 may preferentially bind α -actin and CP- β 2 may preferentially bind β - γ -actin.

To test the hypotheses that (a) the actin binding properties of CP- β 1 and CP- β 2 are different or are differentially regulated, and (b) that CP- β 1 and CP- β 2 interact differentially with actin isoforms, we purified capping protein from chicken kidney (CP- β 2), from chicken brain (CP- β 2), and from chicken pectoral muscle (CP- β 1) and compared their actin binding activities using actin from skeletal muscle (α -actin), from erythrocytes (β -actin), and from brain (β - γ -actin).

Materials and Methods

Proteins, Reagents, and Antibodies

Capping protein of chicken skeletal muscle (CP- β 1) was purified as described (6). α -Actin from chicken skeletal muscle was purified as described (55). Pyrene-labeled actin was prepared as described (13). Non-muscle actins were purified from bovine erythrocytes (β -actin) and from chicken brain (β - γ -actin) according to a method based on DNase I affinity chromatography (40). Spectrin-F-actin seeds were prepared as described (10). Bovine heat-shock cognate protein-70 (Hsc70) and monoclonal anti-Hsc70 were obtained from StressGen Biotechnologies (Victoria, BC, Canada). Cytochalasin D and S-100 proteins (S100A, S100B, and S100aa) were obtained from Sigma Chemical Co. (St. Louis, MO). PIP₂ was purchased from Sigma Chemical Co. (sodium salt) and from Boehringer-Mannheim Corp. (ammonium salt; Indianapolis, IN); both sources of PIP₂

had identical effects in the actin-binding assays. Phosphatidylethanolamine (PE), phosphatidylinositol 4-monophosphate (PIP), phosphatidylinositol (PI), and phosphatidic acid (PA) were obtained from Sigma Chemical Co. Phosphatidylinositol 3,4,5-trisphosphate (PIP₃) was a generous gift from Dr. C.-S. Chen (University of Kentucky, Frankfort, KY) (22). All lipids stock solutions were prepared by sonication at 1 mg/ml in H₂O and stored at -70°C ; aliquots were sonicated for 20 min in a water bath sonicator before use.

mAbs 3F2 and 5B12 were prepared in this laboratory and were purified from ascites fluid using protein A-agarose. mAb 3F2 was elicited in mice by immunization with a fusion protein of glutathione-S-transferase (GST) and the unique 27 COOH-terminal amino acids of chicken capping protein β 2 subunit. mAb 5B12 was elicited in mice by immunization with a fusion protein of GST and the chicken α 1 subunit. mAb 3F2 reacts with the β 2 subunit, but not the β 1 subunit of chicken capping protein (Fig. 1); mAb 5B12 reacts with α 1 and α 2 subunits of chicken capping protein (Fig. 1). mAb 1E5 is specific for chicken capping protein β 1 subunit (33, 54).

Electrophoresis and Immunoblotting

SDS electrophoresis was performed as described (42). Two-dimensional (2-D) gels and immunoblots were performed as described (54); 2-D gels were stained using the Silver Stain Plus reagent (BioRad Labs, Hercules, CA) as described by the manufacturer. Dot blots used to assay column fractions were performed by applying 1–2 μ l of the column fractions to a nitrocellulose filter. The filter was air-dried and reacted with antibodies as for immunoblots.

Purification of Capping Protein (CP- β 2) of Kidney and Brain

CP- β 2 was purified from chicken kidney and from chicken brain using methods similar to the protocol for purification of CP- β 1 from skeletal muscle (6). Tissue was frozen in liquid N₂ (Pel-Freez Biologicals, Rogers, AK) and stored at -70°C until use. Approximately 100 g of brain or kidney was homogenized per 1 liter of 0.6 M KI, 20 mM Na₂S₂O₃, 5 mM 2-mercaptoethanol, 1 mM NaN₃, and the protease inhibitors 0.1 mM PMSF, 0.1 μ M pepstatin A, 0.1 μ M leupeptin, and 0.1 mM benzamide using a polytron. The homogenate was centrifuged at 25,400 g for 30 min, and the resulting supernatant was centrifuged at 100,000 g for 1 h. The supernatant was dialyzed and chromatographed on DEAE-cellulose (DE-53; Whatman, Maidstone, England) as described (6). The capping protein fraction was subjected to (NH₄)₂SO₄ fractionation and chromatographed on Sephacryl S-200 (6). Pooled fractions were dialyzed against buffer H (10 mM KH₂PO₄, pH 7.0, 50 mM KCl, 0.5 mM DTT, and 0.01% NaN₃), and the dialysate was applied to a 2.5 \times 12-cm column of Bio-Gel HT (BioRad Labs) equilibrated in buffer H. The column was washed with \sim 100 ml buffer H and eluted with a 600-ml linear gradient of KH₂PO₄ from 10–400 mM in buffer H. The CP- β 2 was chromatographed on a MonoS column (6), dialyzed against buffer M (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.5 mM DTT, and 0.1 mM PMSF), and applied to a MonoQ column. The column was eluted with a linear gradient from 0–0.35 M KCl

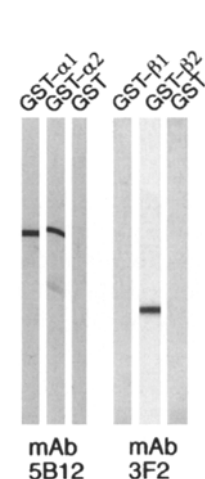


Figure 1. mAb 5B12 and mAb 3F2 are specific for the α -subunits and the β 2 subunit of chicken capping protein, respectively. Western blots containing fusion proteins of glutathione-S-transferase (GST) and the α 1 subunit (GST- α 1), the α 2 subunit (GST- α 2) and GST alone were reacted with mAb 5B12; mAb 5B12 reacts with the α 1- and α 2 subunits. Western blots containing fusion proteins of GST and the β 1 subunit (GST- β 1), GST and 27 COOH-terminal amino acids of the β 2 subunit (GST- β 2), and GST alone were reacted with mAb 3F2; mAb 3F2 reacts with the COOH-terminal region of the β 2 subunit, but not with the β 1 subunit. Neither mAb 5B12 nor mAb 3F2 reacted with GST.

in buffer M; fractions were assayed by falling ball viscometry and immunoblots of SDS gels using mAbs 3F2 and 5B12.

The purity of CP-β2 and a comparison of the subunit compositions of capping protein of skeletal muscle and kidney are documented in silver-stained, 2-D gels (Fig. 2). CP-β1 from skeletal muscle contains the α1- and α2-subunits in a molar ratio of ~1:1 and the β1 subunit (including a modified form of the β1 subunit called β1') (6, 54). CP-β2 from kidney has two α subunits, α1 and α2 in a ratio of ~1:2, and the β2 subunit; CP-β2 from brain had an identical subunit composition. Immunoblots of identical gels probed with antibodies specific for α subunits (mAb 5B12), the β1 subunit (mAb 1E5), and the β2 subunit (mAb 3F2) confirmed the identification of these protein spots (data not shown).

A minor protein component migrating in the 2-D gel with a mobility in the SDS dimension like that of the α subunits but having a more basic isoelectric point was detected in the capping protein preparations from kidney and skeletal muscle (labeled α3? in Fig. 2); this protein reacted on immunoblots probed with mAb 5B12 (data not shown). This minor component may be the chicken homologue of a protein of mouse testicular germ cells that was identified by cDNA cloning and is similar to capping protein α subunits (56); the predicted isoelectric point of the mouse protein (pI = 6.7) is consistent with that of the protein we tentatively designate the α3 subunit on the 2-D gels.

Critical Concentration Measurements and Determination of Dissociation Constants (K_d)

The effect of capping protein on the steady-state actin filament concentra-

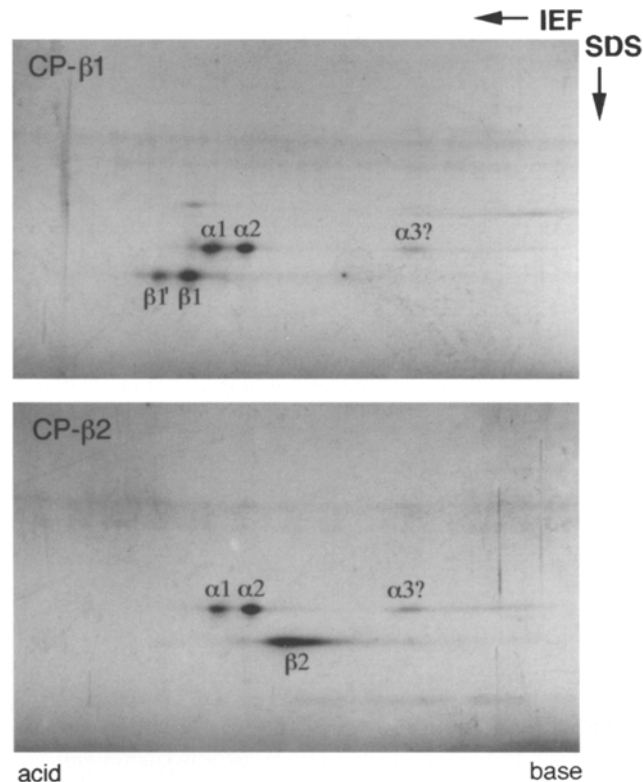


Figure 2. Silver-stained two-dimensional gels of CP-β1 purified from chicken pectoral muscle (*upper gel*) and CP-β2 purified from chicken kidney (*lower gel*). The positions of the individual capping protein subunits were identified by reaction with α- and β-specific antibodies and from the predicted mobilities of the α subunits (15). The major difference in subunit composition between the capping protein isoforms is the β subunit: CP-β1 contains the β1 subunit (β1 and β1') and CP-β2 contains the β2 subunit. Preparations of both capping protein isoforms contained a small amount of a putative, novel α subunit protein we tentatively label "α3?" based on its reaction with mAb 5B12 (data not shown).

tion for skeletal muscle α-actin and erythrocyte β-actin were determined by incubation of 1 μM α- or β-actin (each containing 10% pyrene-labeled α-actin as a tracer for polymerization) in MKEI buffer (0.1 M KCl, 2 mM MgCl₂, 1 mM EGTA and 20 mM imidazole-HCl, pH 7.0) with varying concentrations of CP-β1 and CP-β2. Samples were incubated for 24 h at room temperature with gentle rocking and pyrene fluorescence was measured (13). The K_d for capping protein binding to actin was determined from the amount of capping protein that changes the critical concentration by 50% as described (58) using elongation rate constants for ATP-actin (50).

Actin Polymerization Assays

Actin polymerization from spectrin-F-actin seeds was assayed using 1.5 μM actin (5 or 10% pyrene labeled), 0.054–0.25 nM spectrin-F-actin seeds and varying concentrations of CP-β1 or CP-β2 in MKEI buffer. Monomeric actin was diluted to 1.5 μM in MKEI buffer and preincubated for 160 s to allow exchange of Mg²⁺ for Ca²⁺ on the actin. Spectrin-F-actin seeds and either CP-β1 or CP-β2 were added to the cuvette and pyrene fluorescence (5) was monitored for up to 1,040 s. Assays were performed at 25°C.

Actin Uncapping Assay

The uncapping assay was a modification of the actin polymerization assay using 0.23 nM spectrin-F-actin seeds, 2 μM actin (5% pyrene labeled) and varying concentrations of CP-β1 or CP-β2. The buffer was 0.1 MKEI (0.1 M KCl, 0.2 mM MgCl₂, 0.1 mM EGTA, and 20 mM imidazole-HCl, pH 7.0), which was determined to be optimal for PIP₂-mediated uncapping. The reaction was initiated by the addition of the spectrin-F-actin seeds and capping protein to monomeric actin in 0.1 MKEI. Phospholipids were added to the polymerization reaction at 340 s and the fluorescence was monitored for a total of 1,200 s. Assays were performed at 25°C. Assays were performed using Hsc70 and S-100b in place of phospholipids to test whether these proteins induced uncapping. In some experiments, actin filaments capped by CP-β1 were used in place of spectrin-F-actin seeds. The capped filaments were prepared by mixing 0.11 μM CP-β1 and 11 μM α-actin in 0.1 MKEI buffer overnight at room temperature. To test mixed micelles of PIP₂ and PE, PIP₂ and PE were mixed in molar ratios of 1:3 to 1:100, sonicated in a bath sonicator for 15 min and added to uncapping assays so that the final concentration of phospholipid was 100 μM.

Determination of the Rate Constants for Capping F-actin

The rate constants for capping actin filaments were determined from data collected in actin polymerization assays using the kinetic parameter optimization programs, KINSIM (3) and FITSIM (61). The kinetic mechanism used for the simulation was steps 1 and 2 of the mechanism shown below, where A is actin monomer, AN is the number of actin filaments and also equals the number of spectrin-F-actin seeds, C is capping protein, and PIP₂ is itself.



The concentration of spectrin-F-actin seeds was determined by fitting the experimental data obtained for seeded polymerization of α-actin alone using KINSIM and the on-rate and off-rate constants for muscle α-actin, 11.6 μM⁻¹s⁻¹ and 1.4 s⁻¹, respectively (50). The on-rate and off-rate constants for β-actin were determined using KINSIM and the critical concentration for β-actin (determined experimentally to be 0.16 μM); the on-rate and off-rate constants for nonmuscle actin were 10.0 μM⁻¹s⁻¹ and 1.6 s⁻¹, respectively. This mechanism assumes that there is no pointed end growth from the spectrin-F-actin seeds. To confirm this assumption, we

determined that no actin polymerization was observed during $\sim 1,000$ s after addition of the seeds in the presence of 20 nM cytochalasin D.

For kinetic simulations of the action of PIP₂ on capped filaments, steps 1–5 of the mechanism shown above were used. The on-rate and off-rate constants assigned for step 1 were $11.6 \mu\text{M}^{-1}\text{s}^{-1}$ and 1.4s^{-1} , respectively, and for step 2 were $3.6 \mu\text{M}^{-1}\text{s}^{-1}$ and $4.0 \times 10^{-4} \text{s}^{-1}$, respectively. Rate constants for steps 3–5 were varied over a wide range to obtain simulated progress curves using KINSIM, which were compared with the experimental data for actin polymerization in the presence of capping protein and PIP₂.

Actin Depolymerization Assay

F-actin at $5 \mu\text{M}$ (40–50% pyrene-labeled) was mixed with varying amounts of CP- $\beta 1$ or CP- $\beta 2$, incubated 5 min at room temperature, and diluted 25-fold into G buffer (2 mM Tris-Cl, pH 8.0, 0.2 mM ATP, 0.1 mM dithiothreitol, 0.2 mM CaCl₂, and 0.005% NaN₃) at 25°C in a fluorometer cuvette with a stirring bar (5). The decrease in pyrene fluorescence accompanying actin depolymerization was monitored for 300 s after dilution. To test the effects of PIP₂, phosphatidylinositol 3,4,5-trisphosphate Hsc70, and S-100 proteins on capping activity, 50 nM CP- $\beta 1$ or CP- $\beta 2$ was incubated for 5 min with varying concentrations of the test reagent before addition to F-actin. The effects of Hsc70 were measured in the presence of 5 mM ATP or 5 mM ADP and 5 mM MgCl₂. The effects of S-100 proteins were measured in the presence and absence of CaCl₂ at 0.2–1 mM.

Actin Nucleation Assay

Monomeric actin at $5 \mu\text{M}$ (10% pyrene labeled) was incubated with CP- $\beta 1$ or CP- $\beta 2$ for 5 min in G buffer. Fluorescence of pyrene-actin was monitored after the addition of 1/10 volume of 10 \times MKEI to the cuvette.

Analysis of the Interaction of CP and Hsc70

For gel filtration analysis, CP- $\beta 2$ was mixed in a final volume of 0.2 ml in buffer S (10 mM Tris-HCl, pH 8.0, 0.2 M KCl, 1 mM dithiothreitol, 0.01% NaN₃, 0.5 mM EDTA, 5 mM ADP, and 5 mM MgCl₂) with Hsc70 (molar ratio Hsc70:CP- $\beta 2$ of 2:1). The mixture was applied to a Superose 12 HR column (1 \times 30 cm) equilibrated in buffer S. Fractions were assayed for capping protein and Hsc70 by Western blotting using mAb 5B12, mAb 3F2, and anti-Hsc70. Control runs were of Hsc70 alone and CP- $\beta 2$ alone. For immunoprecipitation analysis, CP- $\beta 1$ or CP- $\beta 2$ was mixed in a molar ratio of 3:1 (Hsc70:CP) with Hsc70 in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin. A final concentration of either 5 mM ATP and 5 mM MgCl₂ or 5 mM ADP and 5 mM MgCl₂ was added to each sample. The mixtures were incubated for 1 h on ice; 10 μg of mAb 5B12, mAb 3F2, mAb 1E5, mouse IgG, or monoclonal anti-Hsc70 was added and incubated for 2 h. Immune complexes were isolated using BioMag goat anti-mouse IgG (PerSeptive Diagnostics, Cambridge, MA), washed eight times in 10 mM Tris-HCl, pH 7.4, 0.45 M NaCl, 1% Triton X-100 and twice in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Triton X-100. The immune complexes were solubilized in SDS sample buffer and analyzed on Western blots probed with a mixture of mAb 5B12, mAb 3F2, mAb 1E5, and anti-Hsc70.

Falling Ball Viscometry Assay

Falling ball viscometry assays were performed at room temperature as described (12).

Results

Kinetic Rate Constants

The rate constants for binding of CP- $\beta 1$ and CP- $\beta 2$ to muscle α -actin, erythrocyte β -actin, and brain $\beta\gamma$ -actin were determined using kinetic simulations of the time course of actin polymerization in the presence of varying concentrations of CP- $\beta 1$ and CP- $\beta 2$ (Fig. 3). The on-rate constants (k_{+}) and the off-rate constants (k_{-}) for CP- $\beta 1$ and CP- $\beta 2$ (Table I) were similar to each other and to those determined for capping activity in supernatant fractions from neutrophils (18). The on-rate constant for bind-

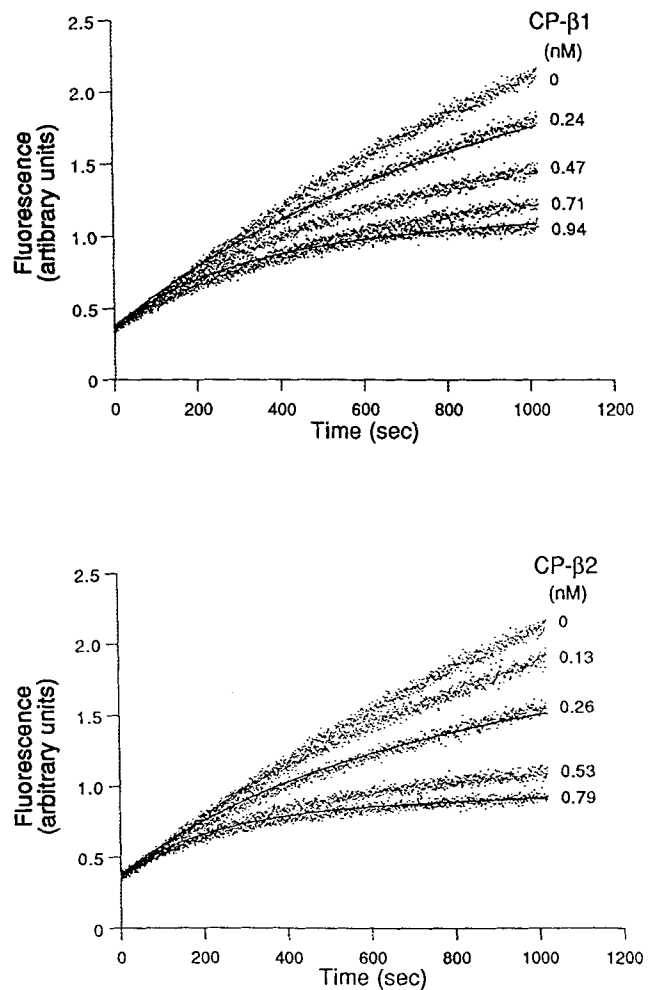


Figure 3. Effect of CP- $\beta 1$ (upper) and CP- $\beta 2$ (lower) on actin polymerization nucleated by spectrin-F-actin seeds. The change in pyrene-actin fluorescence accompanying polymerization is plotted versus time after addition of the capping protein and spectrin-F-actin seeds to a solution of $1.5 \mu\text{M}$ α -actin in MKEI buffer. The solid lines represent the simulated progress curves predicted from kinetic modeling for two concentrations of each capping protein isoform.

ing α -actin was slightly higher for CP- $\beta 2$ (mean value derived from three determinations = $5.7 \mu\text{M}^{-1}\text{s}^{-1}$) than for CP- $\beta 1$ (mean value derived from three determinations = $3.5 \mu\text{M}^{-1}\text{s}^{-1}$). The off-rate constants for dissociation of capping protein from a barbed end were low (mean value derived from six determinations = $4 \times 10^{-4} \text{s}^{-1}$). These rate constants predict that the half-life of a capped barbed end in cells is long (~ 28 min), while the half-life for a free barbed end is short (~ 0.2 s); the implications of these half-lives for control of actin polymerization by capping protein will be considered below (see Discussion).

We also determined the dissociation constant (K_d) for binding CP- $\beta 1$ and CP- $\beta 2$ to muscle and nonmuscle actins by measuring the ability of each isoform to alter the critical concentration for actin polymerization (Fig. 4). The K_d s for actin binding (Table I) were nearly identical for CP- $\beta 1$ and CP- $\beta 2$, ranging from 0.06 ± 0.02 to 0.13 ± 0.02

Table 1. Dissociation Constants and Rate Constants for the Interaction of CP- β 1 and CP- β 2 with α -Actin, β -Actin, and $\beta\gamma$ -Actin

Capping protein isoform	Actin isoform	K_d^* nM	On-rate [‡] constant (k_{+1}) $\mu\text{M}^{-1}\text{s}^{-1}$	Off-rate [‡] constant (k_{-1}) $\text{s}^{-1} \times 10^{-4}$	Off-rate [§] constant (k_{-1}) $\text{s}^{-1} \times 10^{-4}$
CP- β 1 (muscle)	(expt. 1)	0.08 \pm 0.02	3.5 ± 0.2	4.4 ± 0.2	2.7 ± 0.7
	(expt. 2)		3.2 ± 0.03	1.9 ± 0.3	
	(expt. 3)		3.3 ± 0.3	0.0 ± 11.8	
	(expt. 4)		3.9 ± 0.08	2.8 ± 0.8	
CP- β 2 (kidney)	(expt. 1)	0.13 \pm 0.02	5.8 ± 0.03	3.7 ± 0.2	7.5 ± 1.12
	(expt. 2)		5.1 ± 0.04	1.2 ± 0.2	
	(expt. 3)		6.5 ± 0.03	4.9 ± 0.2	
CP- β 2 (brain)	(expt. 5)	0.08 \pm 0.02	5.3 ± 0.09	0.9 ± 0.3	3.1 ± 0.8
CP- β 2 (kidney)	(expt. 4)		3.9 ± 0.04	9.5 ± 0.4	
CP- β 2 (brain)	(expt. 5)		2.3 ± 0.02	8.4 ± 0.4	

*The value for the K_d was calculated from the data in Fig. 4 using the point where the amount of F-actin changed by 50% as described in Materials and Methods. The error in this estimate was determined by estimating the error in the individual data points for each curve and calculation of the upper and lower limits for the K_d .

‡These on-rate and off-rate constants were determined using the simulation program FITSIM; each value is the best fit obtained using 4–5 experimental data sets. The errors are the errors calculated by the algorithm for each independent analysis. The determinations using α -actin were from three independent experiments; the rate constants with β -actin and $\beta\gamma$ -actin were each determined in one experiment.

§This off-rate was calculated from the K_d value indicated in column 3 of this table and the on-rate constant (column 4 of this table) determined using FITSIM. The formula is $K_d = k_{-1}/k_{+1}$.

nM, and there was no difference in the K_d s for binding of CP- β 1 and CP- β 2 to different actin isoforms. Our values for the K_d are consistent with the previously published K_d for CP- β 1 binding to α -actin, which was reported to have an upper limit of 0.5–1 nM (6, 9) and with a K_d of 0.07 nM measured for binding human capping protein prepared by in vitro translation to α -actin (2).

Because the determination of the off-rate constant from kinetic simulations is not as precise as the determination of the on-rate constant, we also calculated the off-rate constants from the on-rate constants and the K_d s ($K_d = k_{-1}/k_{+1}$). The values for these calculated off-rate constants (mean value from four determinations = $4 \times 10^{-4} \text{ s}^{-1}$) are consistent with those obtained using kinetic simulations (Table 1).

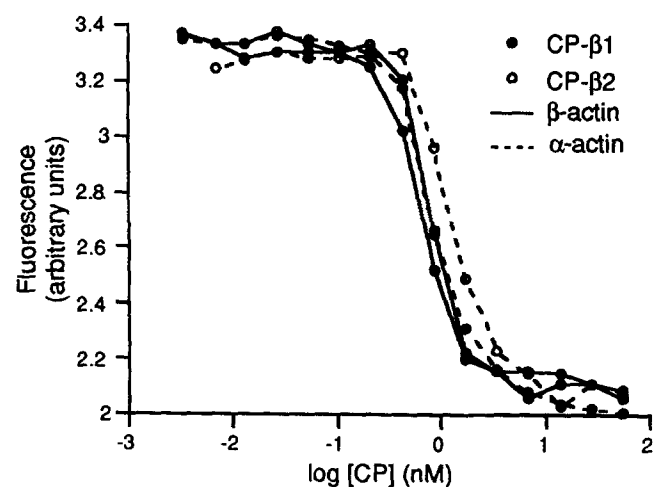


Figure 4. CP- β 1 (●) and CP- β 2 (○) have similar effects on the steady-state critical concentration for polymerization of muscle α -actin (dotted lines) and erythrocyte β -actin (solid lines). Actin at 1 μM was incubated in MKEI buffer with varying concentrations of CP- β 1 or CP- β 2 for 24 h at room temperature. The fluorescence of pyrene-actin is plotted versus the log of the capping protein concentration.

Comparison of the Actin Binding Properties of CP- β 1 and CP- β 2

The rate constants for CP- β 1 and CP- β 2 binding to actin were similar, even in assays with different actin isoforms. To verify these results, we performed three additional assays that test properties different from those tested in the preceding experiments. First, CP- β 1 and CP- β 2 were identical in their concentration dependence for inhibiting depolymerization of F-actin upon dilution (Fig. 5). Second, both CP- β 1 and CP- β 2 promoted nucleation of actin polymerization (Fig. 6); CP- β 1 nucleated actin polymerization slightly better than CP- β 2. Third, CP- β 1 and CP- β 2 had identical effects on the low-shear viscosity of F-actin (data not shown). The results from these assays confirm that the different structures of the β subunit isoforms neither confer a difference in the steady-state equilibrium binding to F-actin nor impart selective binding of CP- β 1 and CP- β 2 to different actin isoforms. The reason for the evolution and conservation of different COOH-terminal β subunit peptides in vertebrates remains unknown. The isoforms are targeted to different locations in the cell (54). At those locations, the isoforms presumably possess some differentiation of function that justifies their targeting.

Regulation of Capping Protein

Regulation of capping activity is one mechanism to alter the availability of free barbed ends in cells. Three potential regulators of capping protein activity have been described based on their interactions with capping protein in vitro: anionic phospholipids, especially PIP_2 (30), heat shock cognate protein 70 (Hsc70) (20, 28) and S100 proteins (35).

Uncapping Actin Filaments by Anionic Phospholipids

PIP_2 rapidly and efficiently promoted the dissociation of capping protein from actin filaments (Fig. 7 A). Thus, PIP_2 could be the signal for uncapping barbed ends during actin remodelling in vivo. A typical uncapping assay using CP- β 1 and α -actin is shown in Fig. 7 A; similar results were ob-

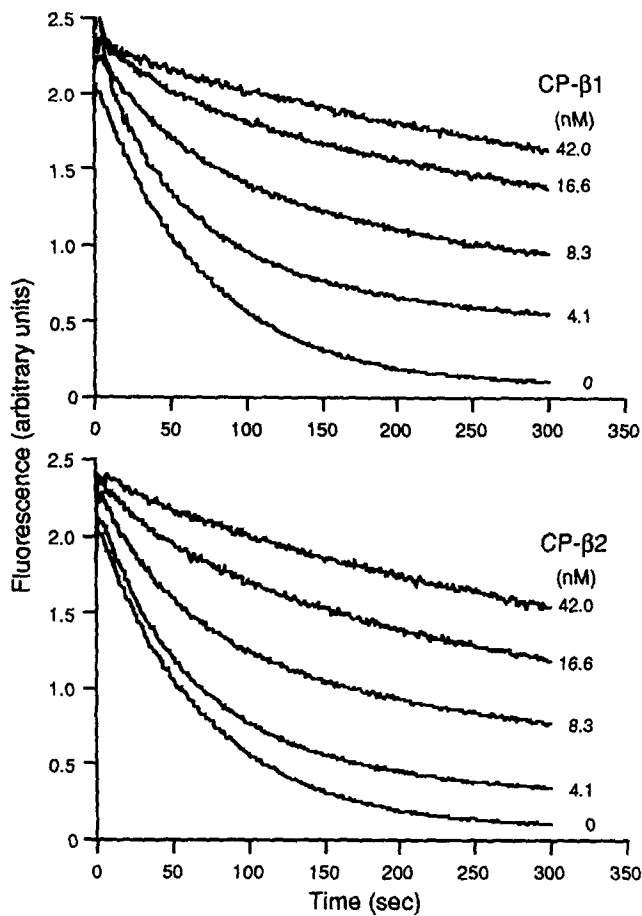


Figure 5. CP- β 1 (upper) and CP- β 2 (lower) have similar effects on depolymerization of actin filaments. Capping proteins were incubated with 5 μ M F-actin for 5 min before dilution of the solution 25-fold into G buffer. The concentration of capping protein (in nM) in the F-actin-capping protein mixture before dilution is indicated beside each curve. Pyrene-actin fluorescence is plotted versus time after dilution.

tained using CP- β 2 and β - γ -actin (data not shown). In this assay designed specifically to look at uncapping, PIP₂ at 36 μ M was added to a polymerization reaction containing capping protein at a time when most of the actin filaments were capped (arrow at 340 s for curve 3 in Fig. 7 A). The rate of actin polymerization increased dramatically upon addition of the PIP₂ (compare curves 2 and 3 in Fig. 7 A), approaching the maximal rate of actin polymerization in the absence of capping protein (compare curves 1 and 3 in Fig. 7 A). Cytochalasin D at 20 nM inhibited the increased rate of polymerization induced by PIP₂ (curve 4 in Fig. 7 A), indicating that free barbed ends had been created. PIP₂ had no effect on the rate of polymerization of actin alone

Figure 7. PIP₂ promotes the dissociation of capping protein from actin filaments. (A) Polymerization of 2 μ M α -actin at barbed ends was nucleated by spectrin-F-actin seeds in the presence (curves 2, 3, and 4) and absence (curve 1) of 12 nM CP- β 1. After 340 s, 36 μ M PIP₂ was added to reactions shown in curves 3 and 4 (arrow). Cytochalasin D at 20 nM also was added at 340 s to the

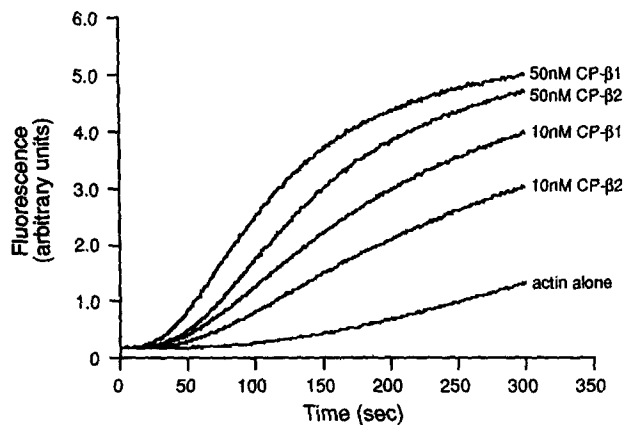
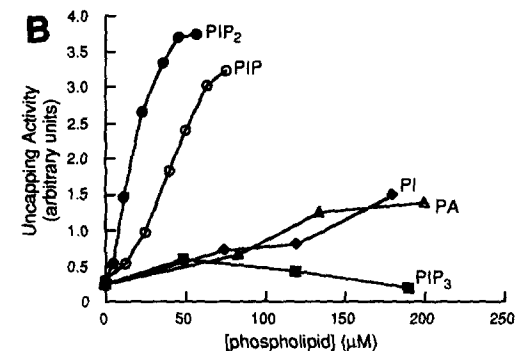
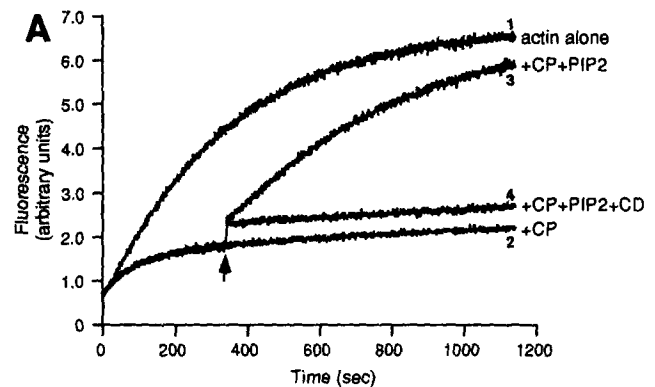


Figure 6. CP- β 1 and CP- β 2 have similar actin filament nucleation activity. CP- β 1 or CP- β 2 at 50 and 10 nM were incubated 5 min with 5 μ M monomeric α -actin. Pyrene-actin fluorescence is plotted versus time after addition of MKEI buffer to initiate polymerization.



reaction in curve 4. The immediate increase in fluorescence observed upon addition of PIP₂ may be due to light scattering by the PIP₂ (39). PIP₂ induced a large increase in the rate of actin polymerization from capped actin filaments (compare curves 2 and 3) that approached the rate of polymerization of actin alone (curve 1). Cytochalasin D inhibited the increase in actin polymerization due to PIP₂ (curve 4). Similar results were obtained in experiments using CP- β 2 with either α -actin or β - γ -actin (data not shown). (B) Comparison of the effects of different phospholipids on uncapping actin filaments. Assays like those in A were performed with 2 μ M α -actin, 4 nM CP- β 1, and varying concentrations of PIP₃, PIP₂, PIP, PI, and PA. Uncapping activity is defined as the rate of actin polymerization, calculated from the slope of the pyrene fluorescence versus time, during the early linear phase after addition of the phospholipid.

(data not shown), thus, PIP₂ did not alter actin monomer interactions and did not sever filaments. Assays using capping protein-capped F-actin seeds in place of spectrin-F-actin seeds gave similar results (data not shown).

Concentrations of PIP₂ > ~40 μM did not further increase the rate of polymerization. The half maximal effect was at ~20 μM PIP₂ (Fig. 7 B). PIP₂ was equally effective in uncapping when present as mixed lipid vesicles with phosphatidylethanolamine (molar ratios of PIP₂/PE of 1:2 and 1:9, data not shown). PIP also uncapped actin filaments but was less efficient than PIP₂ (Fig. 7 B). PI, PA, and, surprisingly, PIP₃ had little or no uncapping activity (Fig. 7 B). PIP₃ has been proposed to control transient actin polymerization (43) since changes in the levels of PIP₃ in platelets (41) and neutrophils (19) parallel the time course of actin polymerization and subsequent depolymerization in stimulated cells. We report the first description of the effect of PIP₃ on the activity of an actin binding protein and find that PIP₃ did not uncap filaments at all and was less potent than PIP₂ in inhibiting capping activity (see below). Thus, the different structural features of polyphosphoinositides, not merely their anionic character, determine how they interact with capping protein.

The uncapping activity of PIP₂ depended critically on the concentration of Mg²⁺; 0.2 mM MgCl₂ supported high activity. At 2 mM MgCl₂, PIP₂ induced a far smaller increase in the rate of actin polymerization in the uncapping assay (data not shown). This slight effect was similar to that seen previously by Heiss et al., using 2 mM MgCl₂ and a different experimental design (30). The dependence of the PIP₂-induced actin polymerization on [Mg²⁺] may be due to aggregation of PIP₂ by divalent cations (38). The free [Mg²⁺] in cells is ~0.5 mM but is heterogeneously distributed. In peripheral regions near the plasma membrane where PIP₂-mediated signaling should occur, the free [Mg²⁺] is significantly lower (17, 52). Thus, the low [Mg²⁺] necessary for optimal PIP₂-mediated uncapping is likely to exist in vivo.

Inhibition of Capping Activity by Phosphoinositides

The capping activity of CP-β2 was inhibited in a concentration-dependent manner by PIP₂ (Fig. 8 B), as previously observed for CP-β1 (Fig. 8 A) (30). PIP₃ also inhibited capping protein but concentrations ~10-fold higher than those required for inhibition by PIP₂, were required for inhibition of capping activity by PIP₃ (Fig. 8 C).

Effects of Hsc70 on Capping Protein Activity

Regulation of *Dictyostelium* capping protein by Hsc70 was suggested by the existence of a stable complex between Hsc70 and capping protein in whole cell extracts (21, 28) and by an Hsc70-induced increase in capping activity of mixtures of bacterially expressed capping protein α and β subunits (28). The availability of purified capping protein and Hsc70 allowed us to test whether or not Hsc70 directly alters capping protein's actin binding activity. Hsc70 from bovine brain in the presence of either 5 mM ATP or 5 mM ADP had no effect on the capping activity of CP-β1 or CP-β2 as measured in actin depolymerization assays (Fig. 9). Hsc70 also did not promote uncapping (data not shown).

We found no evidence for formation of a stable complex

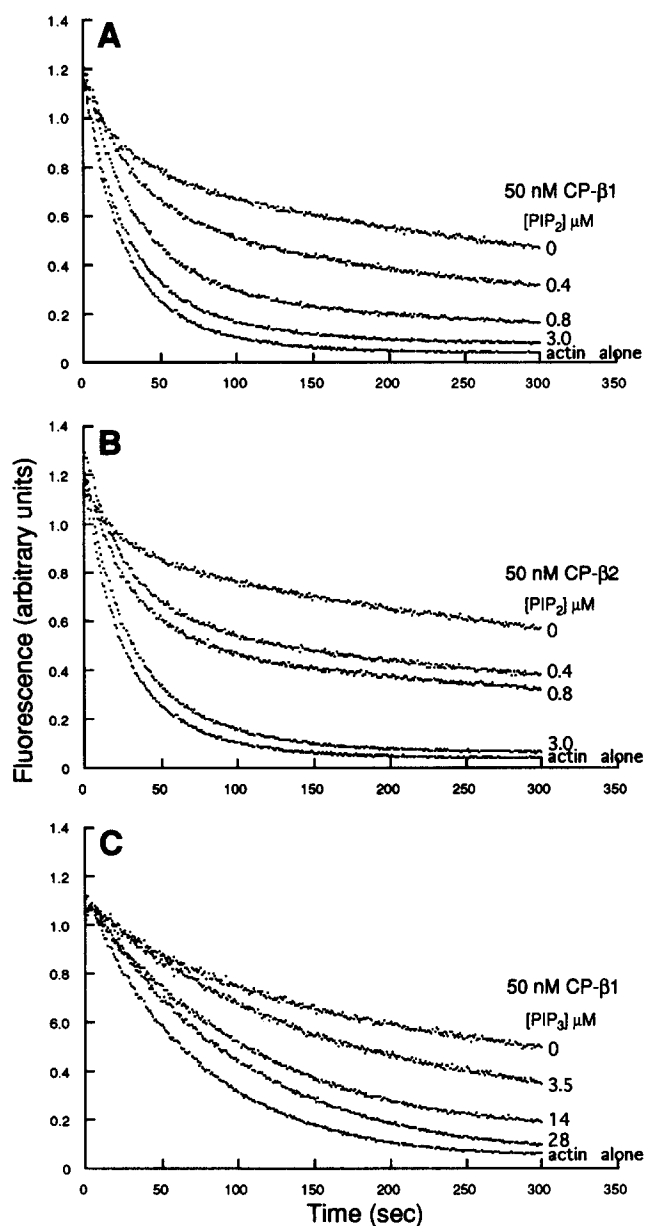


Figure 8. PIP₂ and PIP₃ inhibit the actin capping activity of capping protein. 50 nM CP-β1 (A and C) or 50 nM CP-β2 (B) was incubated with varying amounts of PIP₂ (A and B) or PIP₃ (C) for 5 min at room temperature in G buffer. 5 μM F-α-actin was added to the PIP₂-capping protein mixture and the mixture was diluted in G buffer. Pyrene-actin fluorescence is plotted versus time after dilution. The concentration of PIP₂ or PIP₃ incubated with the capping protein is indicated beside each curve.

of Hsc70 and capping protein. Purified CP-β1 or CP-β2 and Hsc70 were mixed under conditions that stabilize the complex from *Dictyostelium* (21). The mixture was subjected to gel filtration analysis and to immunoprecipitation using antibodies to either capping protein or Hsc70 (28). Results from both sets of experiments indicated that Hsc70 did not associate with capping protein.

Taken together, our results indicate that Hsc70 does not bind to or influence the activity of capping protein, which is in contrast with conclusions of previous studies (28). Recent experiments on purified *Dictyostelium* capping

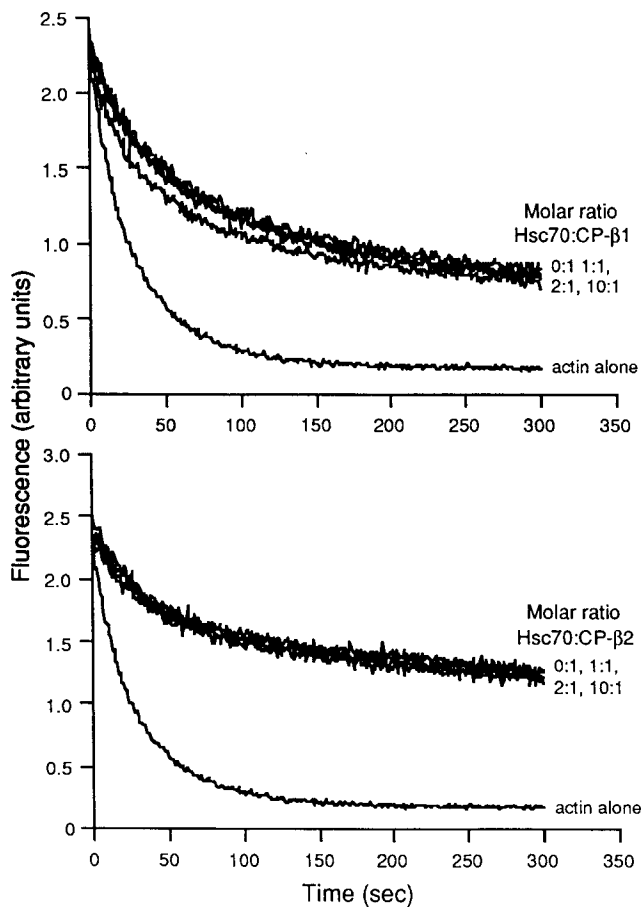


Figure 9. Hsc70 has no effect on the actin capping activity of CP-β1 (upper) and CP-β2 (lower) in actin depolymerization assays. Hsc70, in the presence of 5 mM Mg-ADP, was incubated at varying molar ratios with CP-β1 and CP-β2 for 5 min at room temperature before addition to 5 μ M F- α -actin. The mixture was diluted into G buffer, and the change in pyrene fluorescence was monitored with time after dilution. Experiments performed in the presence of 5 mM Mg-ATP gave identical results (data not shown).

protein also conclude that the isoform of Hsc70 that copurifies with capping protein does not regulate capping protein (21). In the previous studies, which used whole cell extracts, Hsc70 may have acted as a chaperone to promote the folding of capping protein. Alternative explanations for the difference between our results and the earlier studies include that bovine Hsc70 may not interact with chicken capping protein and that a specific isoform of Hsc70, not the one tested here, interacts with capping protein.

Effects of S100 Proteins on Capping Protein Activity

S100 proteins have been proposed to regulate actin filament assembly in vivo via a calcium-dependent interaction with capping protein (35, 36). We tested whether or not S100 proteins alter the interaction of CP-β1 with α -actin in the presence and absence of CaCl_2 . Three types of experiments were performed: falling ball viscometry assays, actin depolymerization assays, and assays for uncapping of

capped actin filaments. We find that S100 protein binding to capping protein does not alter capping protein's interaction with actin (data not shown); therefore, capping protein can simultaneously bind actin and S100 protein. Capping protein may serve to position S100 protein at Z-disks, the location of both capping protein (11) and S100a₀ (23) in muscle. The importance of locating S100 protein in the sarcomere is underscored by the recent finding that S100A1₂ activated in a Ca^{2+} -dependent manner the kinase domain of twitchin, which may be important for organizing muscle structure and regulating contraction (29).

Discussion

Implications of Kinetics of Capping Protein

Knowing the rate constants for the interaction of capping protein with actin filaments in vitro is necessary to understand how capping protein may regulate the dynamics of actin assembly in vivo. Kinetic rate constants allow one to predict the half-lives for free and capped actin filament barbed ends and to predict the amount of actin polymerized during the time a barbed end is free. The rate of uncapping can also be compared with the rate of creation of free barbed ends in cells stimulated by agents that induce rapid changes in the actin cytoskeleton.

Implications of the On-rate Constant

The on-rate constant of $3.0 \mu\text{M}^{-1}\text{s}^{-1}$, together with a cellular concentration of capping protein from 0.2 to 2 μM (14, 18, 34, 47), predicts a half-life for a free barbed end in vivo of 0.1–1 s. Actin polymerization from free barbed ends depends on the concentration of available actin subunits, which includes the pools of profilin-actin and free G-actin monomer (49, 51). At an upper limit for the actin subunit pool in vivo of $\sim 100 \mu\text{M}$ (8), a free barbed end could grow from 0.3 to 3 μm during 0.1 to 1 s. Thus, actin filaments with free barbed ends could elongate to lengths observed for actin filaments in vivo (0.5–0.6 μm in macrophages [26]; 0.18–2.1 μm in neutrophils [7]; $\sim 0.3 \mu\text{m}$ in *Listeria* tails [57]) before being capped.

Implications of the Off-rate Constant

The off-rate constant of $4 \times 10^{-4} \text{s}^{-1}$ for dissociation of capping protein from F-actin predicts a half-life for a capped barbed end of ~ 28 min. This half-life is much longer than the time observed for the increase in number of barbed ends and in F-actin in cells responding to stimuli. For example, within 90 s after neutrophils encountered chemoattractant, the amount of F-actin and the total number of actin filaments more than doubled (7). Also, within 30 s after stimulation of platelets with thrombin, the number of free barbed ends increased from ~ 50 to ~ 500 per platelet (25, 27). This large discrepancy between the rapid rate for actin remodeling in vivo and the slow rate for dissociation of capping protein from barbed ends led us to search for factors that accelerate uncapping of barbed ends. Regulators that cause uncapping also may account for the discrepancy between the apparent K_d of ~ 100 nM for *Dictyostelium* capping protein in whole cell extracts

(34) and the K_d of ~ 1 nM for purified *Dictyostelium* capping protein (21).

Uncapping Actin Filaments by Phosphoinositides

Our tests of several possible regulators of capping protein indicate that the polyphosphoinositides, PIP_2 and, to a lesser extent, PIP , induce the dissociation of capping protein from barbed ends. It is likely that PIP_2 binds directly to capping protein and increases its rate constant for dissociation from F-actin. Kinetic simulations based on such a model predict the observed changes in actin polymerization well (see description of simulations in Materials and Methods). In contrast, simulations of a model in which PIP_2 sequesters free capping protein after it dissociates from barbed ends at the usual slow rate do not fit the experimental data.

Is the PIP_2 -induced actin polymerization from capped filaments observed *in vitro* sufficient to account for increased actin polymerization in stimulated cells? In platelets, ~ 500 new free barbed ends were created from $\sim 2,000$ capped filaments by 30–60 s after stimulation with thrombin (27). At conditions for maximal uncapping *in vitro*, PIP_2 decreased the half-life for a capped barbed end from ~ 28 min to ~ 46 s. We calculate from a $t_{1/2}$ of 46 s that 500 free barbed ends could be created in ~ 19 s. Thus, the rate of PIP_2 -induced uncapping observed *in vitro* is sufficient to account for the number of free barbed ends that appear in stimulated platelets.

Signal transduction pathways in which phosphoinositides function require that the levels of phosphoinositides change in response to the stimulus. However, the total mass of PIP_2 decreases, rather than increases following stimulation of platelets (59). On the other hand, turnover of PIP_2 is increased in platelets upon stimulation (1), raising the possibility that local changes in the PIP_2 concentration occur during signaling. The identification in some cells of specialized membrane domains enriched in PIP_2 , glycosphingolipids, and a polyphosphoinositide phosphatase (4, 32) supports the idea that the PIP_2 concentration may vary spatially.

Model for Actin Assembly In Vivo

We propose a mechanism for remodeling the actin cytoskeleton in stimulated cells that accounts for the increases in the number of actin filaments, number of free barbed ends, and amount of F-actin. Our model is based on three processes: uncapping of barbed ends by the action of PIP_2 , polymerization at the free barbed ends, and severing the long actin filaments (Fig. 10). First, PIP_2 in the plasma membrane (Fig. 10, green) uncaps actin filaments and generates free barbed ends near the membrane. Polymerization at free barbed ends lengthens the filaments and extends lamellae and filopodia. PIP_2 also inhibits capping of free barbed ends, thereby prolonging filament elongation. Next, the elongated filaments break, creating new filaments with free pointed and barbed ends. Finally, when the local PIP_2 level decreases, capping protein caps the free barbed ends to stop polymerization.

We demonstrate here that PIP_2 does uncap actin filament barbed ends. PIP_2 is part of the signaling pathway that induces actin polymerization in permeabilized plate-

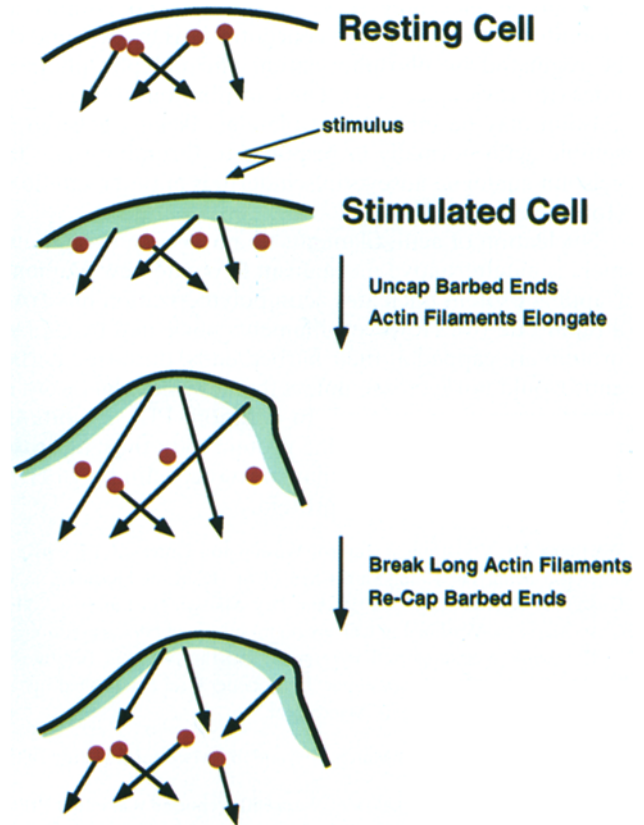


Figure 10. Model for actin assembly in stimulated cells. The PIP_2 concentration increases in a region of the plasma membrane in response to a local extracellular stimulus (green). PIP_2 causes capping protein (red circles) to dissociate from the barbed ends of actin filaments. Actin filaments grow at their free barbed ends and push the plasma membrane outward. Elongated filaments then break, because of either mechanical forces or the action of a severing protein. Finally, new free barbed ends situated far from the membrane where capping protein is active become capped. The net effects are protrusion of the plasma membrane and the creation of new filaments the same length as the existing filaments, of new pointed ends, and of new barbed ends.

lets (27). Furthermore, capping protein is released from platelet cytoskeletons upon stimulation by thrombin (47). These data are consistent with the first steps of our model where signals mediated by PIP_2 induce actin remodeling by uncapping barbed ends.

One aspect of actin remodeling *in vivo* that cannot be explained solely by uncapping is an increase in the number of actin filaments. During platelet activation, the total number of filaments increases from 2,000 to 2,500 per cell (27). More dramatic are the findings in stimulated neutrophils, where the number of actin filaments increases from 1.7×10^5 to 4×10^5 per cell without a change in actin filament length (7). Clearly, additional processes, such as severing or *de novo* nucleation, two processes that create new filaments, are required to explain these data.

We favor a mechanism where long actin filaments are fragmented to create new filaments. One possible mechanism for breaking filaments is via mechanical forces exerted on them by myosins or other factors. Alternatively, actin filament severing proteins of the ADF/cofilin or

gelsolin families may serve this role. ADF/cofilins are ubiquitous, present at high concentrations *in vivo*, and can be regulated by phosphorylation and polyphosphoinositides (for review see [45]). The contribution to severing by gelsolin may be minor since platelets lacking gelsolin assemble actin normally in response to thrombin (60). The gelsolin analogue adseverin/scinderin is another candidate (for review see [53]).

Nucleation of actin filaments *de novo* from actin monomers is an alternative mechanism to create new filaments. Capping protein nucleates actin polymerization *in vitro* at a rapid rate (6). However, filaments nucleated by capping protein are capped at their barbed ends, thus, free barbed ends would not increase unless the new filaments were either uncapped or severed. In addition, PIP₂ inhibits the nucleation activity of capping protein (30); thus, increased PIP₂ would inhibit, rather than promote, actin polymerization nucleated by capping protein.

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Note Added in Proof. Barkalow et al. recently reported that either PIP₂ or a thrombin activating peptide, TRAF, induces the release of capping protein from the cytoskeleton of permeabilized platelets (Fig. 8 in Barkalow, K., W. Witke, D.J. Kwiatkowski, and J.H. Hartwig. 1996. *J. Cell Biol.* 134: 389–399). These data support the initial step of our model (Fig. 10) where polyphosphoinositides release capping protein from actin filaments to create free barbed ends.

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