SINGLE MOLECULE KINETIC ANALYSIS OF ACTIN FILAMENT CAPPING: POLYPHOSPHOINOSITIDES DO NOT DISSOCIATE CAPPING PROTEINS

Jeffrey R. Kuhn† and Thomas D. Pollard*

Department of Molecular, Cellular, and Developmental Biology, *Molecular Biophysics & Biochemistry and *Cell Biology, Yale University, New Haven, CT 06520, USA

†Present address: Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0406, USA.

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Address correspondence to: Thomas Pollard, MCDB, KBT-548, Yale University, P.O. Box 208103, New Haven, CT 06520-8103, E-mail: thomas.pollard@yale.edu, Fax: 203-432-6161

We investigated how heterodimeric capping proteins bind to and dissociate from the barbed ends of actin filaments by observing single muscle actin filaments by total internal reflection fluorescence (TIRF) microscopy. The barbed end rate constants for mouse capping protein association of 2.6×10^6 M^{-1}s^{-1} and dissociation of 0.0003 s^{-1} agree with published values measured in bulk assays. The polyphosphoinositides (PPIs) PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 prevent CP from binding to barbed ends, but three different assays showed that none of these lipids dissociate CP from filaments at concentrations that block CP binding to barbed ends. The affinity of fission yeast CP for barbed ends is a thousand-fold less than mouse CP, owing to a slower association rate constant (1.1×10^5 M^{-1}s^{-1}) and a faster dissociation rate constant (0.004 s^{-1}). PPIs do not inhibit binding of fission yeast CP to filament ends. Comparison of homology models revealed that fission yeast CP lacks a large patch of basic residues along the actin-binding surface on mouse CP. PPIs binding to this site might interfere sterically with capping, but this site would be inaccessible when CP is bound to the end of a filament.

The motility of animal cells depends on the coordinated assembly of actin filaments at the leading edge. Arp2/3 complex promotes actin polymerization by nucleating filaments that grow as branches from a mother filament (1,2). Capping protein (CP, called CapZ in muscle) appears to play an important role in generating the zone of short, highly branched actin filaments at the leading edge. CP is a heterodimer of structurally similar α and β subunits that form a mushroom-like structure with one flexible and one immobile COOH-terminal “tentacle” that bind the two terminal subunits of an actin filament (3). CP binds the barbed end of actin filaments tightly with a K_d of 0.1 to 1 nM. Given an association rate constant of 3 to 4 µM^{-1}s^{-1} and a cytoplasmic concentration of 1 µM (4), CP should terminate the growth of actin filaments with a half-time of about 0.2 s after their nucleation. Terminating growth quickly keeps the filaments short, so that they do not buckle under the force of polymerization as they push the membrane forward.

Vertebrate CP dissociates from barbed ends with a half-time of about 30 min (4), far too slow for simple, spontaneous dissociation to be involved with the conversion of the network of short, branched filaments at the leading edge to longer, unbranched filaments toward the cell body. As CP prevents both...
dissociation of subunits from the barbed end and end-to-end filament annealing, some mechanism must accelerate the removal of CP from filament ends.

Both lipids and proteins, such as Ena/VASP (5-7) and CARMIL (8), can inhibit the interaction of CP with actin filaments. Polyphosphatidylinositides (PPIs) bind CP and block its interaction with filaments both in vivo (9) and in vitro (10). Addition of PIP₂ to mixtures of capped filaments, CP and actin monomers in vitro increases the rate of polymerization (4). These experiments led to the widely accepted view that PPIs not only inhibit association of CP with barbed ends but also dissociate CP from actin filaments. These two effects are postulated to bias the growth of actin filament branches toward the inside of the plasma membrane at the leading edge (11,12). Steric interference would be the simplest way for PPIs to block interaction of CP with filament ends. Mutation of three residues surrounding a putative PIP₂ binding site reduces the affinity of CP for PIP₂ 5- to 60-fold and barbed ends 400-fold (13). Although the binding sites on CP for the barbed end and PIP₂ overlap, dissociation of CP from a barbed end by PIP₂ would presumably require an allosteric mechanism.

To examine the question of uncapping from a new perspective, we used total internal reflection fluorescence (TIRF) microscopy to observe capping and uncapping of individual filaments in vitro. We obtained association and dissociation rate constants for vertebrate CP in good agreement with those previously measured in bulk assays. We also confirmed that PI(3,4)P₂, PI(4,5)P₂, and PI(3,4,5)P₂ sequester CP and prevent its binding to barbed ends. However, none of these PPIs accelerated the dissociation of CP from filaments. Although PIP₂ inhibits budding yeast CP (14), we discovered that PPIs do not inhibit binding of fission yeast CP to filament ends, in spite of other similarities to budding yeast and vertebrate CPs. This experiment of nature gave us an opportunity to identify an excellent candidate for an alternate PPI binding site by comparing the electrostatic surface potentials of homology models of CPs that differ in their ability to bind PPIs.

**EXPERIMENTAL PROCEDURES**

**Proteins**

We purified actin from rabbit skeletal muscle acetone powder through one round of polymerization, depolymerization, and gel filtration (15). Actin was labeled with pyrenyl iodoacetamide (Molecular Probes, Eugene, OR) (16) or with Oregon green 488 (OG) iodoacetamide (Molecular Probes, Eugene, OR) and further purified with DEAE Sepharose ion exchange chromatography followed by depolymerization and gel filtration (15). Before use, labeled and unlabeled acts were dialyzed overnight against fresh buffer G (2 mM Tris-Cl pH 8, 0.2 mM ATP, 1 mM Na₃, 0.1 mM CaCl₂, 0.5 mM dithiothreitol, DTT) and centrifuged at 100,000×g for 2 hr at 4°C.

Recombinant mouse capping protein (MmCP) α1 and β2 subunits were expressed on a single plasmid (pET3d[m-α1/β2]) and purified from bacterial lysates (17). We purified recombinant fission yeast capping protein (SpCP) subunits Acap1 and Acap2 expressed on a single plasmid (pET3-acp1acp2) (18). Capping proteins were dialyzed into Ca²⁺/Mg²⁺ free CP buffer (10 mM imidazole pH 7.5, 50 mM KCl, 1 mM DTT, 0.1 mM EGTA, 2 mM NaN₃) containing 50% glycerol, flash frozen in liquid N₂, and stored at -80°C. We determined the activity of a frozen MmCP stock by equilibrating a range of MmCP concentrations with actin seeds and then measuring the rate of pyrene actin assembly from capped seeds (10). Frozen stocks were 2- to 4-fold less active than freshly prepared MmCP.

Myosin from rabbit skeletal muscle (19) was treated with N-ethylmaleimide (NEM, Sigma, St. Louis, MO) (20) and stored...
at -20°C in 10 mM imidazole pH 7, 0.5 M KCl, 1 mM DTT, and 50% glycerol.

We estimated protein concentrations from the following extinction coefficients:
unlabeled actin, $A_{290} = 26,600 \text{ M}^{-1}\text{cm}^{-1}$ (21); $MmCP$, $A_{280} = 76,300 \text{ M}^{-1}\text{cm}^{-1}$ (17); $SpCP$, $A_{280} = 72,000 \text{ M}^{-1}\text{cm}^{-1}$ (18). Myosin, $A_{280} = 260,000 \text{ M}^{-1}\text{cm}^{-1}$ (22) For OG labeled actin, total actin concentration was estimated from the corrected extinction coefficient, $A_{290} - 0.17 \times A_{491} = A^{*}_{290} = 26,600 \text{ M}^{-1}\text{cm}^{-1}$ and labeled actin fraction from $A_{491} = 77,800 \text{ M}^{-1}\text{cm}^{-1}$ (15). For pyrene labeled actin, total actin concentration was estimated from the corrected extinction coefficient, $A_{290} - 0.127 \times A_{344} = A^{*}_{290} = 26,600 \text{ M}^{-1}\text{cm}^{-1}$ and labeled actin fraction from $A_{344} = 22,000 \text{ M}^{-1}\text{cm}^{-1}$ (23).

Synthetic D-myo-phosphatidylinositols (diC16) from Echelon Biosciences (Salt Lake City, UT) were resuspended in chloroform, aliquotted into glass vials, dried under vacuum or nitrogen gas, and stored at -20°C. Before use, we added Ca$^{2+}$/Mg$^{2+}$ free CP buffer to an aliquot of phospholipid to a final concentration of 200 µM, incubated at 4°C for 4 to 16 hours to suspend, and sonicated for 5 min in a water bath sonicator and an additional 1 min before each experiment to form micelles.

**TIRF Polymerization Experiments**

Crown glass slides were cleaned and prepared as flow cells (15). We preincubated each flow cell for 2 minutes with 0.2 µM NEM-inactivated myosin in high-salt Tris buffered saline (HS-TBS, 50 mM Tris-Cl pH 7.6, 600 mM NaCl) containing a $10^4$ dilution of 200 nm crimson fluorescent microspheres (Molecular Probes, Eugene, OR) followed for 1 min each with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) in HS-TBS and then 1% BSA in low salt TBS (LS-TBS, 50 mM Tris-Cl pH 7.6, 150 mM NaCl).

We mixed labeled and unlabeled Ca-ATP actin to the desired labeled fraction at 4.44× the final concentration, mixed 9:1 with 10× Mg exchange buffer (10× ME: 10 mM EGTA, 1 mM MgCl$_2$) and incubated on ice for 2 minutes to form 4× final concentration of Mg-ATP actin. We placed 8 µL of Mg-ATP at the bottom of a 1.5 mL Eppendorf tube and on the side, we mixed 4 µL of 8× final concentrations of Ca$^{2+}$/Mg$^{2+}$ free CP or buffer and 4 µL of 8× final concentrations of phospholipid or buffer and incubated at room temperature for 5 minutes. To start the reaction, we washed both drops together with 16 µL of 2× TIR buffer (2×: 100 mM KCl, 2 mM MgCl$_2$, 2 mM EGTA, 20 mM imidazole, pH 7.0, 200 mM DTT, 0.4 mM ATP, 30 mM glucose, 1% methylcellulose, 40 µg/ml catalase, 200µg/ml glucose oxidase) and wicked 16 µL through a prepared flow chamber. All subsequent buffer exchanges were similar except that Mg-buffer G (9:1 mixture of Buffer G:10× ME) was substituted for Mg-ATP actin when preexisting filaments were incubated with CP alone before dissociation experiments. For longer uncapping experiments, we sealed the chamber ends with VALAP (1:1:1 Vaseline:Lanolin:Parafin) to reduce filament breakage due to evaporative loss induced flow. Images were captured once every 6 to 60 seconds, depending on conditions, for a total of 100 to 200 frames per experiment.

**Bulk Pyrenyl-actin Spectroscopy Assay of Growth from Capped Seeds.**

**Long seeds:** We mixed Ca-ATP actin 8:1 with 10× ME and incubated on ice for 2 minutes followed by 9:1 mix with 10× KMEI (10×: 500 mM KCl, 10 mM MgCl$_2$, 10 mM EGTA, 100 mM imidazole, pH 7) for a final actin concentration of 6 µM. Filaments were polymerized at 20°C for over 2 hours and used without dilution. **Short seeds:** 6 µM Mg-ATP actin was mixed as above, polymerized for 10 minutes, diluted 1:9 in Buffer F (8:1:1 mixture of Buffer G:10× ME:10× KMEI), and vortexed for 10 sec on maximum speed (Vortex Genie 2, VWR Scientific Products, West Chester, PA). We used short seeds 1 minute after vortexing. Average seed length

3
was calculated from [filament] / [ends] after dilution.

**Capped seeds:** We gently added undiluted long seeds or freshly prepared short seeds 1:1 to a drop of 150 nM MmCP and incubated for 5 minutes. Wide-bore pipette tips (USA Scientific, Ocala, FL) and gentle pipetting were used in all subsequent mixing steps. **Elongation from capped seeds:** 2.5 µM (30% pyrenyl) Ca-ATP-actin monomers were converted to Mg-ATP-actin by mixing 10:1 with 10× ME on ice for 2 minutes. Separate drops of capped seeds, pyrenyl-actin, and PPI were mixed together with Buffer F for a final 15-fold dilution of seeds and a final concentration of 10 nM MmCP, 1.2 µM (30% pyrenyl) Mg-ATP-actin, and 24 µM PPI. A Gemini XPS fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) monitored the fluorescence increase over time with excitation at 365 nm and emission at 407 nm.

**Calculation of [Filamentous Actin] and [Ends]:** Scatter from PPIs micelles increased the fluorescent signal by a constant amount (4). Assuming that the concentration of filamentous pyrenyl-actin was zero at the beginning of the reaction and the polymerization rate was constant for the first 4 minutes, we used a linear fit of the first 250 seconds of each polymerization trace to estimate fluorescence intensity at 0 seconds and subtracted this value. Seeded actin polymerization curves that reached a plateau were used to estimate the final fluorescence intensity for [Filament] = 1.2 – 0.17 = 1.03 µM, assuming a critical concentration of $K_C = 0.17$ µM, and for [Actin] = 1.2 – [Filament]. We applied these scaling factors to all curves and obtained polymerization rates from the slope of a 250 second window of data. Using polymerization rate constants of $k_+ = 12.9$ µM$^{-1}$s$^{-1}$ and $k_- = 2.2$ s$^{-1}$ (24), we estimated the concentration of ends as [Ends] = rate/(12.9[Actin] - 2.2).

**Bulk Rhodamine-phalloidin Fixation of Filaments Grown from Capped Seeds.**

**Avidin-coated coverslips:** Coverslips were cleaned for 30 minutes with a 1:9 mixture of H$_2$O saturated KOH:ethanol, rinsed thoroughly with ddH$_2$O, cleaned for 30 minutes in 2 M HCl, and rinsed with ddH$_2$O. Coverslips were coated with 5% γ-amino propyl triethoxy-silane (Aldrich) in 95% ethanol, 5% H$_2$O, pH 5 with acetic acid, for 1 hour and rinsed thoroughly with ethanol. Coverslips were dried with N$_2$ gas, coated for 1 hour with avidin (Sigma) dissolved to 1 mg/mL in 10 mM Tris pH 8, 2.5% ethanol, rinsed in ddH$_2$O, and dried with N$_2$ gas (25).

**Marked capped seeds:** We converted 60% OG labeled Ca-ATP-actin monomers to 2 µM Mg-ATP-actin by mixing 9:1 with 10× ME on ice for 2 minutes. Short seeds (see above) were diluted an additional 2-fold before mixing 1:1 with 60% OG Mg-ATP actin monomers for 1 minute to mark barbed ends. We then mixed the products 2:1 with 60 nM MmCP for 5 minutes to cap barbed ends.

**Marked elongation from capped seeds:** 20% OG Ca-ATP-actin monomers were mixed 9:1 to a final concentration of 8 µM with 1× ME on ice for 2 minutes. To 25 µL of 2× Buffer F, we added 5 µL of 8 µM (20% OG) actin monomers and 5 µL of 200 µM PPI. 15 µL of capped seeds were slowly added using a large orifice tip and the reaction was incubated for 5 minutes before the addition of 5 µL of 10 µM (40% rhodamine-, 60% biotin-labeled) phalloidin to label and stabilize filaments. After 5 minutes, stabilized filaments were slowly diluted 20- to 40-fold in 1× TIR Buffer, 6 µL was added to a slide and covered with an avidin-coated coverslip.

**Data Collection and Analysis**

We modified an Olympus (Melville, NY) IX-71 microscope for prism-based TIRF microscopy (15). We captured and processed all images with custom-designed Java plugins for ImageJ (National Institutes of Health, [http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)). Crimson fluorescent
beads at the slide surface acted as autofocus targets (15).

We traced filaments and created linear kymographs using a custom plugin for ImageJ (15). A maximum intensity search algorithm “snapped” a rough hand trace to the filament center to create a refined filament trace and measure total length. For linear kymographs, the plugin sub-sampled filament intensities at 1 pixel increments along this refined trace and past both ends and replotted intensities as a single column with horizontal position dictated by frame number. We collected length measurements for each filament over several frames and analyzed growth in a standard Excel (Microsoft Corp, Redmond, WA) spreadsheet. Filament length was converted to subunits using 370 subunits/µm (26).

To measure CP association times, we added CP to preexisting filaments concomitantly with a higher labeled fraction of actin. Once a filament’s barbed end growth halted, we measured the length from the jump in filament brightness to the end of the filament through several frames. The average of this length, \( \overline{L} \), divided by the separately measured linear growth rate, \( R \), gave the time between addition and capping, \( t_c = \overline{L} / R \).

To measure CP dissociation rates, we washed CP from preexisting filaments with two chamber volumes of buffer concomitantly with a higher labeled fraction of actin. Upon barbed end growth resumption, we traced length of the bright filament segment through several frames and estimated the uncapping time, \( t_u \), from the average measured time and length and the separately measured growth rate as \( t_u = \overline{L} / R \).

We estimated the affinity of \( Mm \)CP for PPI from the decrease in the apparent association rate constant in the presence of PPI. Assuming that PPI binding decreased available CP by mass action, the ratio of available to total CP, \( r = [C_{\text{avail}}] / [C_{\text{total}}] \), was determined from the decrease in the apparent association rate in PPI compared to the native association rate, \( k_+ \), as \( r = k_{+,\text{app},\text{PPI}} / k_+ \). The affinity of CP for PI was estimated from \( K_{d,\text{PPI}} = [C_{\text{avail}}] \times [\text{PPI}] / [C \cdot \text{PPI}] \), or \( K_{d,\text{PPI}} = r \times [\text{PPI}] / (1 - r) \).

For hidden Markov modeling (HMM) of \( SpCP \) association/dissociation, we measured lengths of 10 to 15 filaments per experiment through every frame. A Savitzky-Golay smoothing derivative filter (27) transformed each length trace \( L_i \) into smoothed instantaneous growth rates at each time point. These instantaneous growth rates were uniformly high during obvious uncapped states and near zero during capped states. With these instantaneous growth rates as reporters of the true hidden barbed-end state (capped or uncapped), we used QuB kinetic analysis software, available from http://www.qub.buffalo.edu, (28,29) for Baum-Welch idealization and to estimate rate constants for capping and uncapping. We assumed a simple, second order model for capping protein (C) binding to Mg-ATP actin filament barbed ends \( (A_n) \) of

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A_n + C \leftrightarrow A_nC
\]

Maximum interval likelihood (MIL) analysis of the frequency of durations of all capped or uncapped states yielded a rate, \( k_1 \), for the transition from an uncapped to a capped state, and a transition rate constant, \( k_2 \), for the reverse. At high CP concentrations, the durations of uncapped states decreased towards the acquisition frequency and were more likely to be hidden, increasing error in the estimation of \( k_2 \). At low CP concentrations, the frequency of capping and subsequent uncapping events decreased, increasing error in both \( k_1 \) and \( k_2 \). For comparison across all concentrations, we summed the two transition rates to form a single apparent equilibrium rate \( (k_{\text{app}} = k_1 + k_2) \), similar to the rate for a bulk assay to reach equilibrium. This apparent rate was less sensitive to concentration-dependent error than the either transition rate alone. The association and dissociation rate constants, \( k_+ \) and

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K_{d,\text{PPI}} = [C_{\text{avail}}] \times [\text{PPI}] / [C \cdot \text{PPI}]
\]
and \( k_\text{c} \), were obtained either from the slope and y-intercept of the apparent rate versus concentration, or in single [CP] experiments from average over three experiments as \( k_\text{c} = k_1 / [\text{CP}] \) and \( k_\text{c} = k_2 \).

**Homology Modeling**

We aligned multiple CP sequences using the MUSCLE algorithm (30) and submitted aligned sequences to SWISS-MODEL (31) for construction and refinement of homology models based on the structure of chicken \( \alpha_1 \beta_1 \) CapZ, PDB\# 1IZN (3). We calculated electrostatic surface potentials with the APBS Package (32) at pH 7 and 50 mM ion concentration. Molecular surfaces were calculated and displayed using UCSF Chimera (33) and colored for iso-potential values of -15, 0, and +15 kBT/ec.

**RESULTS**

**Kinetics of capping and capping by mouse capping protein**

We observed the growth and capping of individual actin filaments by total internal reflection fluorescence (TIRF) microscopy using muscle actin labeled with Oregon Green (OG-actin). For example, Fig. 1A shows a time course of filaments polymerizing in 2 \( \mu \)M Mg-ATP actin monomer before and after the addition of 4 nM murine capping protein (\( Mm \)\text{CP}) to the reaction buffer. Straightened kymographs of individual filaments (Fig. 1B) show linear barbed-end growth that was terminated by capping protein. The fraction of capped filaments in a sample followed an exponential time course, consistent with pseudo first-order conditions (Fig. 1C). As slow mixing occasionally caused a lag in capping during the first minute of observation, we discarded the first few capping events in these experiments to maximize the "goodness of fit" to an exponential (\( R^2 \) value). The exponential association rate depended linearly on \( Mm \)\text{CP} concentration over a 20-fold range (Fig. 1D), giving an association rate constant of 2.6 \( \pm \) 0.1 \( \mu \)M\(^{-1}\)s\(^{-1}\) for \( Mm \)\text{CP} binding to Mg-ATP actin filament barbed ends (Table I). This value is similar to previous measurements in bulk samples of 3.5 \( \mu \)M\(^{-1}\)s\(^{-1}\) for purified chicken CP \( \alpha_1 \beta_1 \) (4) and 6.3 \( \mu \)M\(^{-1}\)s\(^{-1}\) for recombinant chicken CP \( \alpha_1 \beta_1 \) (34).

Occasionally in experiments without capping protein, one or two growth pauses occurred during observation of a field containing many filaments due to surface binding effects. The rarity of these events precluded analysis of this pseudo-capping.

Murine capping protein dissociated slowly from filament barbed ends when we washed out free CP (Fig. 2A). We interpret the onset of growth as dissociation of \( Mm \)\text{CP}. Most filaments grew at a constant rate (Fig. 2B), but a few were re-capped and stopped elongating. The fraction of capped filaments declined exponentially over time (Fig. 2C), giving a dissociation rate constant of 2.7 \( \pm \) 0.1 \( \times \)10\(^{-4}\) s\(^{-1}\) (half time of 43 min). The ratio of the dissociation and association rate constants gave a \( K_d \) of 103 \( \pm \) 7 pM (Table I), similar to previous measurements with bulk samples of 80 pM for chicken capping protein (4,34).

**Effects of polyphosphoinositides on capping and uncapping by mouse capping proteins**

Polyphosphoinositides bind capping proteins and inhibit capping of barbed ends in bulk samples (10). Our observations of individual actin filaments confirmed that 25 \( \mu \)M concentrations of micelles of three different PPIs inhibit the rate of capping over a range of capping protein concentrations (Fig. 3A, Table II). The dependence of the capping rate on \( Mm \)\text{CP} concentration was the same in controls and with 25 \( \mu \)M phosphatidylinositol (PI), but was 3.5 fold lower in PI(3,4)\( P_2 \), 6.7 fold lower in PI(4,5)\( P_2 \), and 6.2 fold lower in PI(3,4,5)\( P_3 \). Assuming that \( Mm \)\text{CP} bound to micelles cannot bind filaments and that these rates were lower because of mass action, \( Mm \)\text{CP} would have dissociation equilibrium constants for PPIs of 4.4 to 10 \( \mu \)M (Table II).
Divalent cations such as Mg\(^{2+}\) are thought to inhibit PPI binding to CP either through micelle aggregation (35) or charge masking. At low MgCl\(_2\) concentrations (0.1 mM), the association of CP with barbed ends (Fig. 3B, Table II) was ten-fold slower in PI(4,5)P\(_2\) and PI(3,4,5)P\(_3\) than at high MgCl\(_2\) concentrations and five-fold slower in PI(3,4)P\(_2\). Lowering Mg\(^{2+}\) concentrations increased the apparent affinity of CP for PPIs to 0.4 to 1.5 µM. CP association with filaments was also slower in PI than in buffer under low Mg\(^{2+}\) conditions, indicating that the phosphate linking the inositol ring to the lipid moiety may play some role in PPI binding.

We used bulk samples to evaluate the concentration of barbed ends available for elongation by pyrenyl actin in 0.1 mM Mg\(^{2+}\). Under conditions where the polymerization rate depended on the concentration of ends, we confirmed (Fig. 4A) that addition of PIP\(_2\) to bulk samples of capped actin filaments increased the concentration of ends (10,36). Polymerization was faster in PIP\(_2\) and PIP\(_3\) than in PI or control capped filaments (Fig. 4A). The most straightforward interpretation of the high elongation rates is that PIP\(_2\) and PIP\(_3\) promote dissociation of capping protein from barbed ends. However, dissociation would have to be fast, given the absence of a lag after adding the PPIs to capped filaments in both the published work and in our experiments. In contrast, none of the PPIs uncapped filaments in the microscopic assay (Fig. 3C-D). Neither PI, PI(3,4)P\(_2\), PI(4,5)P\(_2\), nor PI(3,4,5)P\(_3\) significantly altered the time course of \(Mm\)CP dissociation in either 0.1 mM or 1.0 mM Mg\(^{2+}\) in these microscopic assays.

Given this difference between the bulk and microscopic assays, we considered the possibility that filaments break during mixing bulk samples and that PPIs prevent recapping by sequestering free CP. Although we mixed capped seeds with PPIs in bulk experiments slowly and with large orifice pipette tips, any shearing force would potentially break filaments of moderate length or entangled in a network (37).

To test this hypothesis we compared the effect of PPIs on elongation of long and short capped seeds diluted to a similar concentration of ends (Fig. 4A-B). The mean length of long seeds was 21 µm based on the rate of elongation measured with pyrene actin in bulk samples (Fig. 4E). These long filaments tended to be entangled in bundles typically longer than 10 µm when viewed by fluorescence microscopy (Fig. 4F). The free filaments in these samples measured 7 ± 7 µm long (Fig. 4F). The mean length of the short filament seeds made by vortexing polymerized actin was 1.6 ± 1.4 µm by microscopy (Fig. 4G) and 2.7 µm by the elongation assay (Fig. 4E).

We used elongation experiments with 1.2 µM pyrenyl actin monomers (Fig. 4 C-D) to measure the concentrations of uncapped barbed ends over time (Fig. 4C-E). Mouse capping protein reduced the rate of elongation of both long and short seeds and thus the concentrations of free ends ten-fold. Mixing 25 µM PI with either long or short capped seeds had no effect on the rate of elongation. Mixing long capped seeds with PPIs increased the elongation rate (Fig. 4A) and thus the concentration of free barbed ends (Fig. 4E). These increases were 6-fold by PI(3,4)P\(_2\), 6-fold by PI(4,5)P\(_2\), and 4-fold by PI(3,4,5)P\(_3\), a significant portion of the 8-fold difference in free ends between uncapped and capped long seeds. In contrast, mixing short capped seeds with PI(3,4)P\(_2\), PI(4,5)P\(_2\) or PI(3,4,5)P\(_3\) increased the elongation rate only 1.4- to 2.8-fold (Fig. 4D), a small fraction of the average 11-fold difference in free ends between uncapped and capped short seeds. As these experiments were done side-by-side, we conclude that longer, entangled seeds are more likely to break during mixing than short seeds, and that much of generation of free barbed ends is due to filament breakage and sequestration of CP by PPIs rather than...
uncapping. Since the results in bulk polymerization assays were similar in buffers containing 0.1 and 1.0 mM Mg\(^{2+}\) (not shown) and since the Mg\(^{2+}\) concentration did not affect uncapping in microscopic assays, we performed all subsequent experiments at 1 mM MgCl\(_2\) concentrations.

We also tested the uncapping hypothesis by microscopic observations of filaments stabilized with phalloidin. We elongated unlabeled filament seeds with a high mole fraction of OG-actin followed by capping by MmCP. We diluted these seeds into polymerization buffer with a low mole fraction of OG-actin to learn if any barbed end elongated. After 5 minutes we stabilized and labeled the filaments with rhodamine-phalloidin. Uncapped filaments (Fig. 5A) had a characteristic staining pattern of red pointed ends, bright green middles, and dim green barbed ends, while capped filaments (Fig. 5B) had red pointed ends and bright green barbed ends. Using these criteria, we counted the number of capped and uncapped filaments in each condition and ignored broken filaments. In control experiments without capping protein, 88 ± 5% of filaments had an uncapped staining pattern. With capping protein, the fraction of uncapped filaments dropped to 6 ± 3%. At concentrations of 20 µM, neither PI (10 ± 8% uncapped, not shown), PI(3,4)P\(_2\) (11 ± 4% uncapped, not shown), PI(4,5)P\(_2\) (15 ± 4% uncapped, Fig. 5C), nor PI(3,4,5)P\(_3\) (14 ± 5% uncapped, not shown) uncapped the filaments during 5 minutes of incubation (Fig. 5D). Thus, the increase in free barbed ends seen in bulk uncapping experiments is unlikely to be due to uncapping.

*Interactions of fission yeast capping protein with actin filament barbed ends*

Filaments growing from skeletal muscle Mg-ATP-actin monomers in the presence of fission yeast capping protein fluctuated between phases of steady elongation and pauses (Fig. 6A, B). The pauses presumably represent capping events, which end by dissociation of SpCP from barbed ends followed by elongation. Short pauses are difficult to detect by time lapse TIRF microscopy, so we could not measure SpCP association directly. Rather, since capping protein binding to filaments is a stochastic, Markov process, we used growth traces of individual filaments and hidden Markov modeling (HMM) to estimate the “hidden” capped or uncapped state of each filament at each time point along with the association and dissociation rates of SpCP at filament barbed ends.

We tracked the lengths of ten to fifteen filaments per experiment over time to obtain individual growth curves (Fig. 6B) and their derivatives. Growth rates were linear, but individual length measurements varied by 200 to 400 subunits (S.E.) from the expected value due to filament motion. We took the derivative of each filament’s growth data to obtain the instantaneous growth rate at each frame (Fig. 6C). Although growth rates were uniform throughout the field for the uncapped state and essentially zero during the capped state (Fig. 6B-C), noise in the rate data hindered manual assignment of state transitions. We therefore used the Baum-Welch algorithm (38) to assign capped or uncapped state to rate traces based on the local amplitude and noise of each segment. We combined sections of obvious linear growth or capping from four filaments to obtain the mean (amplitude) and standard deviation (noise) of growth rates for each experiment. The Baum-Welch interval detection algorithm used these values, along with an initial estimate of the association and dissociation rate constants to estimate the hidden capping state of each filament (Fig. 6C, bold lines). A maximum interval likelihood (MIL) algorithm then used the fractional occupancy of each state and frequencies of transitions from capped to uncapped or *vice versa* to estimate the rates of transition between these two states. Using these new rate constants, we reapplied the Baum-Welch
interval detection to refine the state occupancies followed by the MIL algorithm to refine rate constants. We repeated this process until the rate constants stabilized. The apparent rate estimated by HMM (Fig. 6D) was proportional to the concentration of SpCP, giving an association rate constant of 0.11 µM⁻¹s⁻¹ and a dissociation rate constant of 3.1×10⁻³ s⁻¹ for a binding affinity of 29 nM.

To measure SpCP dissociation rates directly, we grew Mg-ATP actin seeds in flow cells, equilibrated them for 5 minutes with SpCP, and removed capping protein by washing with higher fraction-labeled Mg-ATP actin. We estimated SpCP dissociation times from the resumption of filament growth as in Figure 2 (not shown) and observed a single exponential dissociation rate constant of 5.7 ± 0.1 ×10⁻³ s⁻¹ (half time of 2 min).

Unlike murine capping protein, 25 µM phosphoinositides did not significantly affect the rate of association of 20 nM SpCP with actin filaments (Fig. 6E, Table III). We observed the effects of phosphoinositides on SpCP dissociation by equilibrating filaments with SpCP and then washing SpCP out of the reaction buffer with higher fraction-labeled actin and 25 µM phosphoinositide or buffer. The time course of the initiation of regrowth (Fig. 7A) was best fit by the sum of two exponentials: a fast dissociation half-life of 10 to 50 s with an amplitude of 0.6 (Fig. 7B); and a slow dissociation half-life of 2 to 4 min with an amplitude of 0.4 (Fig. 7C). Our analysis assumed that every filament was capped at the start of the experiment, but SpCP rapidly associated and dissociated from filaments. The fast dissociation therefore likely represents filaments that were not capped during the final buffer exchange. As we observed a slow single exponential dissociation in other experiments (not shown), the slow dissociation likely represents the true dissociation rate constant for SpCP in the presence of phosphoinositides. Like murine capping protein, phosphoinositides did not significantly affect fission yeast capping protein dissociation when measured directly (Fig. 7C) or via HMM estimation (Fig. 6F, Table III).

Homology models to identify potential PPI binding sites in CP

The lack of effect of PPIs on S. pombe CP offers an experiment of nature to provide clues regarding PPI binding sites on other CPs. We constructed homology models of capping proteins from several species based on the crystal structure of chicken CP (3) (Supplemental Fig. 1). Vertebrate, insect, and plant CPs have a large area of positive potential on the β subunit mushroom “head” (Fig. 8A, Lys142, Lys143, and Lys181) that is absent in fission yeast (Fig. 8B, large arrows) and reduced in budding yeast (not shown) CPs. This large basic patch was proposed as a potential PPI binding sites based on hydrophobic scanning of the CP sequence (39) and lies on the surface that interacts with the barbed end of an actin filament (3,34). It is thus a strong candidate to contribute to PPI binding. This basic patch is adjacent to, but distinct from a cluster of basic residues in the α tentacle hinge region of many CPs including fission yeast CP. Mutation of three of the basic residues in the hinge region reduce the affinity of chicken CP for PIP₂ about 4 fold (13).

DISCUSSION

By directly observing capping and uncapping of actin filaments with TIRF microscopy, we obtained association and dissociation rate constants for mouse CP very similar to those previously obtained through bulk polymerization assays (4). These previous measurements fitted polymerization curves of bulk samples to a simple model of capping without contributions from filament nucleation, breakage, annealing, and pointed end growth, so these side reactions must be negligible in carefully executed bulk capping experiments.

We also agree with Schafer et al. (4) that PPIs appear to uncap filaments in a bulk
polymerization assay. On the other hand, PPIs did not uncap actin filaments in either of our microscopy assays. Control experiments provided evidence for an hypothesis to reconcile these apparently contradictory observations. We found that mixing can break long capped filaments in bulk samples, thus exposing new barbed ends. In the presence of CP, these barbed ends are rapidly recapped unless PPIs sequester the capping protein. This gives the appearance of uncapping by PPIs. Filament breakage and annealing were not considered in the previous bulk uncapping experiments (4), perhaps because they utilized spectrin-actin seeds with filaments of only 13 to 14 subunits (40). However, we calculate from the actin polymer concentration and exponential capping rate that half of the filaments were at least 4.1 µm long, 25% were at least 6.2 µm long, and 2% of filaments were uncapped and 16.9 µm long at the time that Schafer et al (4) added PIP2 in their uncapping experiment. Filaments this long were subjected to breakage in our control experiments.

Any bulk assay designed to test for uncapping activity must distinguish filament breakage combined with CP sequestration from noncompetitive removal of CP from filament ends. Similar assays using spectrin-actin seeds led to the conclusion that Ena/VASP (5,7) and CARMIL (8) compete capping protein from filaments, so the uncapping activities of these important CP inhibitors should be verified at a single filament level.

We confirmed earlier observations that PI(3,4)P2 and PI(4,5)P2 inhibit capping in vitro (4,10) at the single filament level, but unlike Schafer (4), we also found a substantial inhibition of capping by PI(3,4,5)P3. Assuming a bimolecular reaction of MmCP with PPI and that only free MmCP participates in capping, the dissociation equilibrium constants for MmCP binding PIP2 and PIP3 are in the range of 5 to 10 µM in 1 mM MgCl2 and about ten-fold lower in 0.1 mM MgCl2.

Kim et al (13) also investigated uncapping by PPIs using TIRF microscopy. They introduced capped filaments into flow-cells, then flowed PIP2 and fresh actin into the chambers and counted the fraction of growing filaments at five-minute intervals. With 50 µM PIP2, twice the concentration that appeared to uncap filaments rapidly in their bulk assay, only 10% of filaments were growing after 40 minutes, the half-time for CP dissociation. Even in the absence of PIP2, only 12% of filaments were growing 60 minutes after CP washout. Based on their estimated dissociation rate constant of 5.7×10^-4 s^-1, 87% of filaments should have been uncapped within 60 minutes. This low fraction of growing filaments might be explained if a substantial amount of free CP remained in the chamber to recap barbed ends. Apparent uncapping half-times faster than the natural CP dissociation half time of 40 minutes were only seen at 150 – 500 µM PIP2. As filament breakage due to Brownian motion of filaments and evaporative flow are problematic in TIRF assays (for example, see filaments b-d in Fig. 1B), the life history of every filament must be observed to rule out growth due to filament breakage rather than uncapping. Although we cannot rule out allosteric uncapping at very high concentrations of PIP2, another explanation is that PIP2 sequestered the remaining free CP and prevented re-capping of uncapped or broken filaments in their assay. Alternatively, insufficient slide blocking during the BSA wash step can lead to an overly adherent surface which could block either filament growth or CP dissociation due to nonspecific binding of capped or uncapped filament ends to the glass surface. High concentrations of negatively charged lipids could act as an efficient slide blocking agent, allowing barbed ends to grow as they broke or uncapped. As filaments were capped prior to their introduction into flow cells in the study of Kim et al., only uncapping would be affected by nonspecific adherence. In either case, insuring
that TIRF microscopy can accurately measure the same CP dissociation rates seen in bulk assays without PPIs is essential for determining the dissociation rates in the presence of PPIs.

Uncapping is a possible mechanism to explain how PPIs stimulate the creation of actin filament barbed ends in cells and permeabilized cells (9,41). However, given our evidence that PPIs sequester CP but do not uncap filaments, PPIs are more likely to generate barbed ends by acting on activators of Arp2/3 complex or other mechanisms. These alternatives are consistent with the observation that stimulation of Dictyostelium with cAMP generates free barbed ends while increasing, rather than decreasing, CP association with the actin cytoskeleton (11). Similarly, mechanisms other than uncapping must be considered to explain the strong bias in the orientation of barbed ends toward the leading edge of motile cells (12).

Gelsolin can simultaneously sever and cap actin filaments (42), but severing activity has never been reported for CP. Filaments can break spontaneously while being observed with TIRF illumination. Without CP the new barbed end grows. In the presence of CP some broken filaments grew (Fig. 1B filament “d”) but others did not (Fig. 1B filament “b”). Thus capping protein may have a low level of severing activity, although these events may be due to spontaneous breakage followed by rapid capping.

Observations on fission yeast capping protein

*S. pombe* CP has the most divergent biochemical properties among the various CPs studied to date. *SpCP* binds the barbed end of vertebrate actin filaments 30 to 50 times slower and dissociates 10 times faster than mouse CP and is the only characterized CP that is not inhibited by PPIs. These differences provide a window to examine the structural basis of these binding reactions. *S. pombe* CP has a greater overall negative charge (-26.5) at pH 7.0 than mouse CP (-13.2), so electrostatic repulsion from negatively charged actin and PPIs may contribute to the lower affinities. More importantly, both the positive charge along the actin binding (43) surface (Fig. 8B, large arrow) and on the flexible, actin-binding β-tentacle (Fig. 8B, arrowhead) are substantially greater on *Mm*CP than *SpCP*.

The larger of these positive patches is also an attractive potential PPI binding site. First, it is one of amphipathic sites predicted as a potential PIP₂ binding site by a theoretical analysis of CP structure (39). Second, the absence of this basic patch in *S. pombe* CP can explain the lack of PPI binding. Third, binding of PIP₂ or PIP₃ to this basic patch would sterically hinder actin binding. Fourth, binding to the barbed end of an actin filament would preclude binding of PIP₂ or PIP₃ micelles to this site, accounting for their inability to dissociate *Mm*CP from actin filaments.

Mutational analysis of mouse CP (13) identified an inhibitory PIP₂ binding site flanked by three residues, α₁(Lys256), α₁(Arg260), and β₁(Arg225), near the α-C-terminal. Although triple mutation of all three residues reduced the affinity of CP for PIP₂ 5-fold in a tryptophan fluorescence assay and 60-fold in an activity-based assay, the affinity of the CP triple mutant for filament barbed ends was reduced 450-fold, complicating any interpretation of competition between PIP₂ and barbed ends for CP at this site. Furthermore, these sites are well conserved in fission yeast, and are less likely to explain the lack of PPI binding seen here. Further mutational analysis of the large basic patch proposed here and by Smith et al (39) would be instructive.

Yamashita et al (3) proposed another candidate PPI binding site where residues H209, R163, and K95 of the β subunit bind two nitrates (Fig. 8B) in the crystal structure of chicken CP. Given the location of these residues below the α-tentacle, away from the actin binding region (43), PPI binding at this site would have to act allosterically to affect CP activity. Furthermore, two of these three
residues are also present in both yeast species and consequently cannot account for the lack of PPI binding in *pombe* CP. Although mutational analysis of one of these residues, β1(Lys95) failed to show any effect on PIP2 binding (13), the nitrate binding site cannot yet be ruled out as a PPI binding site.

Little is known about the links between phosphatidylinositol signaling and the actin cytoskeleton in yeast. Both fission and budding yeast contain functional PI(4)P 5-kinases (its3 in fission yeast and mss4 in budding yeast). At the restrictive temperature, temperature sensitive mutations of these PI(4)P 5-kinases lead to reduced in actin cables and disruption in actin patch polarity but not patch number. In contrast, cytokinetic actin rings are normal in its3-1 mutations at the restrictive temperature. Given that neither PIP2 nor PIP3 affect the interaction of SpCP with actin and that PIP2 reduction alters the localization but not the maturation of actin patches, it is likely that PIP2 acts upstream rather than directly on CP in yeast.

The association rate constant for SpCP fits well with the time course of actin patch formation. Actin patches assemble and disassemble within 20 seconds (44) and contain about one CP dimer for every two Arp2/3 complexes (45). Thus, about half of all barbed ends are capped at any point in time. With a cytoplasmic concentration of 1.2 μM (45) and an association rate constant of 0.04 μM⁻¹s⁻¹ the half time for capping would be 14 seconds. This is a bit too slow to cap all of the filaments during the 10 second growth phase, so macromolecular crowding may speed up the reaction in the cell. The two minute half-time for dissociation of SpCP from filaments is long enough to protect barbed ends from further polymerization during the 10 second disassembly phase and some other mechanism must account for the rapid depolymerization.

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FIGURE LEGENDS

Figure 1. Association of murine CP with the barbed ends of growing actin filaments. Conditions: 2 µM (20% or 40% Oregon Green 488 labeled) Mg-ATP actin, 0 to 10 nM MmCP, 10 mM imidazole, pH 7.0, 50 mM KCl, 1 mM MgCl2, 1 mM EGTA, 100 mM DTT, 0.2 mM ATP, 50 µM CaCl2, 15 mM glucose, 20 µg/ml catalase, 100 µg/ml glucose oxidase, 0.5% methylcellulose at 25°C. (A-C) Filaments grown in 4 nM MmCP. (A) Time course of filament growth. Initially 20% labeled Mg-ATP actin was polymerized, and growing filament barbed ends (open arrowheads) were observed by TIRF microscopy. The buffer was exchanged with 40% labeled Mg-ATP actin and 4 nM MmCP (plus symbols). Wedges mark the transition to brighter filament sections growing in the presence of MmCP. Filled arrowheads mark the times when individual barbed ends ceased growth. Numbers (min:sec) indicate frame times. Scale bar is 10 µm. (B) Linear kymographs of individual filaments a-d from (A) with time on the horizontal axis and length on the vertical axis. Filament barbed ends are oriented at top. The steps in the dashed lines indicate when MmCP was added. (C) Time course of capping of 24 filaments in 4 nM MmCP (●). An exponential fit (line) gave k_{obs} of 0.01 s^{-1}. (D) Dependence of k_{obs} on the concentration of MmCP (322 total events). The slope gave an association rate constant of 2.6 ± 0.1 ×10^6 M^{-1}s^{-1}.

Figure 2. Dissociation of murine CP from filament barbed ends. Conditions: buffers as in Figure 1. (A) Time course of filament elongation observed by TIRF microscopy. Initially 2 µM (25% labeled) Mg-ATP actin was polymerized in a flow cell for 6 to 8 min (not shown) and exchanged with a solution of 20 nM MmCP without free actin for 5 min (22× association half-times), followed by a solution of 0.4 µM (50% labeled) Mg-ATP actin without capping protein and observed for 3 hours at 1 minute intervals. Capped filament barbed ends (closed arrowheads) grew as brighter segments upon CP dissociation (wedge to open arrowhead). Representative filaments marked a, b are highlighted in B (bold lines). Numbers (min:sec) indicate frame times. Scale bar is 10 µm. (B) Time course of actin filament lengths after the transient pulse of 20 nM MmCP (dashed line). (C) Time course of uncapping of all 99 filaments observed in three experiments (●). An exponential fit (line) gave a dissociation rate constant of 2.7 ± 0.1 ×10^{-4} s^{-1}.

Figure 3. Effects of phosphoinositides on association and dissociation of MmCP on filament barbed ends in high and low concentrations of Mg^{2+}. Conditions: general buffers as in Figure 1; the buffer in A and C contained 1 mM MgCl2, 1 mM EGTA; the buffer in B and D contained 0.1 mM MgCl2, 1 mM EGTA. Symbols are defined in D. (A-B) Dependence of the observed rate of capping on the concentration of MmCP in the presence of 25 µM phosphoinositides. Filaments were polymerized from 1 µM (25% labeled) Mg-ATP actin for 5 to 10 min in a flow cell. Capping was initiated by exchanging the flow cell buffer with a solution containing 0.5 µM (50% labeled) ATP actin and a range of concentrations of MmCP preincubated in Ca^{2+}/Mg^{2+} free buffer with 25 µM phosphoinositide for 5 minutes. We analyzed the time courses of approximately 190 capping events (total of all MmCP concentrations) per PPI condition (in A) or 120 total capping events per PPI condition (in B) as in Figure 1 to obtain association rates at each concentration of MmCP. (C-D) Time course of uncapping filaments in the presence of 25 µM phosphoinositides. Filaments were grown in flow cells from 1 µM (25% labeled) Mg-ATP actin for 5 to 10 min and capped by exchanging with buffer containing 20 nM MmCP without free actin for 5 min. Uncapping was initiated by exchanging the buffer with 0.25 µM (50% labeled)
Mg-ATP actin and 25 µM of the indicated phosphoinositide or buffer. We analyzed an average of 74 MmCP dissociation events (in C) or 50 dissociation events (in D) from three different experiments per condition as in Figure 2 to obtain dissociation rate constants.

**Figure 4. Influence of seed length on PPI-mediated “uncapping” of filaments.** Conditions: 1.2 µM (30% pyrene labeled) Mg-ATP actin, 10 nM final MmCP, 25 µM PPI in polymerization buffer (10 mM imidazole, pH 7.0, 50 mM KCl, 0.1 mM MgCl₂, 1 mM EGTA, 0.5 mM DTT, 0.2 mM ATP, 0.1 mM CaCl₂) at 25°C. (A-D) Effect of MmCP and PPIs on the time course of elongation of actin filament from seeds measured by the fluorescence of pyrene-labeled actin. Curves are averages from at least three experiments. Symbols marking fluorescent traces are defined in D. (A) Elongation from long seeds alone or long seeds preincubated with 75 nM MmCP and further diluted in polymerization buffer with pyrene actin with or without 25 µM of various PPIs. Long seeds were assembled from 6 µM Mg-ATP actin over 2 hours. (B) Elongation from short seeds in the same conditions as A. Short seeds were prepared immediately before use by polymerizing 6 µM Mg-ATP actin for 10 min, diluting ten-fold into polymerization buffer, and shearing on a lab vortex mixer for 10 seconds. (C-D) Concentration of filament ends over time calculated from A-B. End concentration shown for (C) long seeds or (D) short seeds are an average of three experiments. (E) Concentration of ends at 500 sec for long (gray bars) and short (white bars) seeds. Values shown are the average and S.D. of three experiments. (F) Fluorescence micrograph of long seeds stained with rhodamine-phalloidin. Lengths of entangled filaments could not be measured, but the average length of isolated filaments (arrowheads) is indicated below (mean ± S.D.). Entangled filament bundles were typically much longer than 10 µm. (G) Fluorescence micrograph of short seeds. Staining with rhodamine-phalloidin showed that these seeds were short (1.6 µm) and not entangled. Scale bar is 10 µm.

**Figure 5. Alternative microscopic assay to evaluate uncapping by phosphoinositides.** Conditions: buffers as in Figure 4 except 1 mM MgCl₂ and 1 mM EGTA. (A-C) Fluorescence micrographs of actin filaments. Short unlabeled actin seeds were incubated for 1 minute in 1 µM, 60% Oregon Green-labeled Mg-ATP actin followed by reaction with 20 nM MmCP for 5 minutes in 60% Oregon Green-labeled actin diluted to 0.7 µM. Capped seeds were diluted in 1 µM, 20% Oregon Green labeled Mg-ATP actin and Ca²⁺/Mg²⁺ free PPI to a final concentration of 6 nM MmCP and 20 µM PPI, polymerized for 5 min, and stained with rhodamine-phalloidin (40% labeled). (A) Elongation of marked filaments prepared without MmCP or PPI showing uncapped filaments (see inset). (B) Incubation of marked filaments capped with MmCP and followed with actin monomers without PPI showing no barbed end growth (see inset in A). (C) Capped filaments incubated with PI(4,5)P₂ for 5 minutes showing mostly capped filaments and a few filaments that elongated from broken ends. Scale bar is 10 µm. (D) Bar chart of the fraction of uncapped filaments, excluding broken filaments (average and S.D. of 3 experiments). N is the number of filaments scored for each condition.

**Figure 6. Association and dissociation of fission yeast capping protein (SpCP) on filament barbed ends analyzed by a Hidden Markov Model (HMM) method.** Conditions: buffers as in Figure 1. (A) Time series of fluorescence micrographs of actin filaments growing in the presence 1 µM (20% labeled) Mg-ATP actin and 64 nM SpCP (added at 12 minutes, plus symbols in each frame). Filament barbed ends alternated between growing at a constant rate (open arrowheads)
and halting (closed arrowheads). Representative filaments marked a, b are highlighted in B-C. Numbers (min:sec) indicate frame times. Scale bar is 10 µm. (B) Time course of the change in length of representative filaments. The dashed line indicates the step increase in MmCP. (C) Derivative of length versus time data from B showing instantaneous growth rates of representative filaments. The dashed line indicates the step increase in MmCP. Filaments either grew uniformly at 7.4 subunits·s⁻¹ (uncapped state) or zero subunits·s⁻¹ (capped state). Baum-Welch estimations (bold lines) were used to “idealize” the capped (down) or uncapped (up) state of a filament at each time point. (D) Dependence of the apparent rate (sum of association and dissociation rates as described in experimental procedures) on the concentration of SpCP. We used Maximum Interval Likelihood (MIL) optimizations to estimate an apparent equilibrium rate, \( k_{\text{app}} \), for a range of SpCP concentrations. Linear regression (bold line) provided an association rate constant of \( 1.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \), a dissociation rate constant of \( 3.1 \times 10^{-3} \text{ s}^{-1} \), and a \( K_d \) of 29 nM. (E-F) Effect of PPIs on the association (E) and dissociation (F) rate constants for SpCP binding to filament barbed ends. Both the association and dissociation rate constants were estimated at a single SpCP concentration by the HMM method from movies of filaments growing intermittently in 0.5 µM (50% labeled) Mg-ATP actin, 20 nM SpCP (concentration adjusted for active fraction of frozen stock), and 25 µM of the indicated phosphoinositide or buffer. Values shown are the mean and S.D. of three experiments (average of 199 total events) per condition. The mean association and dissociation rate constants obtained in D are shown for comparison in E-F (No PI, dark bars).

Figure 7. Dissociation of fission yeast capping protein from actin filament barbed ends. Conditions: buffers as in Figure 1. (A) Time course of the uncapping of actin filament barbed ends. Actin filament seeds were grown in flow chambers from 1 µM (25% labeled) Mg-ATP and incubated with 40 nM SpCP for 5 minutes. Uncapping was initiated by exchanging the chamber buffer with 1 µM (50% labeled) Mg-ATP actin alone or with 25 µM of PPI. These data come from three experiments with an average of 75 dissociation events per condition. These data were best fit by the sum of two exponentials (not shown) as explained in the text. (B) Fast exponential dissociation rates from A. (C) Slow exponential dissociation rates from A. Values represent means and standard error of estimation of the fit. The single exponential rate constant from a separate experiment (No PI, dark bar) is shown for comparison.

Figure 8. Homology models of MmCP and SpCP reveal a potential PPI binding site. Homology models based on the crystal structure of chicken CP (3) showing the actin binding surface. (A) Ribbon diagram of the homology model of mouse CP with a yellow \( \alpha_1 \) subunit and a red \( \beta_2 \) subunit. The arrows mark potential PPI binding sites: (○) residues flanking two nitrate binding sites proposed by Yamashita et al (3) from their crystal structure; (●) site found by Kim et al (13); (★) large positively charged site missing in S. pombe and proposed by Smith et al (39). The carboxyl terminal \( \alpha^- \) (cyan) and \( \beta^- \) (green) tentacles are highlighted along with proposed PPI binding residues. (B) Surface representations of mouse (left) and S. pombe (right) CP homology models. Surfaces are colored for iso-potential values of +15 (blue), 0 (white) and -15 (red) \( k_B T/\varepsilon_c \). Arrows indicate regions of positive charge that are present on the actin binding surface (large arrow) and \( \beta^-\)tentacle (arrowhead) of mouse CP but absent in pombe CP. Large circles indicate the PIP2 binding site found by Kim et al.
### Table I. Mouse Capping Protein Rate and Equilibrium Constants

| Experiment | \(k_+\) \(\times 10^6\) M\(^{-1}\) s\(^{-1}\) | \(k_-\) \(\times 10^{-4}\) s\(^{-1}\) | \(K_{d,\text{BE}}\) nM |
|------------|--------------------------------|--------------------------------|-----------------|
| Control    | 2.6 ± 0.1                       | 2.7 ± 0.1                       | 0.10            |
| 1 mM Mg\(^{2+}\), PI Buffer | 2.6 ± 0.2                       | 2.2 ± 0.1                       | 0.08            |
| 0.1 mM Mg\(^{2+}\), PI Buffer | 2.6 ± 0.3                       | 3.1 ± 0.2                       | 0.12            |
| Chicken CP, Schafer et al, 1996 | 3.5                             | 4                              | 0.08            |

\(^1\)Dissociation of CP from filament barbed ends, estimated from \(k_- / k_+\).

### Table II. Mouse Capping Protein Association and Dissociation Rates with PPIs

| Experiment                  | \(k_{+\text{app}}\) \(\times 10^6\) M\(^{-1}\) s\(^{-1}\) | \(k_{-\text{app}}\) \(\times 10^{-4}\) s\(^{-1}\) | \(K_{d,\text{PPI}}\) µM |
|-----------------------------|----------------------------------------------------------|--------------------------------|-----------------|
| 1 mM Mg\(^{2+}\), 25 µM PI | 2.7 ± 0.3                                                 | 3.4 ± 0.2                       |                |
| 1 mM Mg\(^{2+}\), 25 µM PI(3,4)P\(_2\) | 0.74 ± 0.05                                             | 3.0 ± 0.1                       | 9.9             |
| 1 mM Mg\(^{2+}\), 25 µM PI(4,5)P\(_2\) | 0.39 ± 0.04                                             | 1.9 ± 0.1                       | 4.4             |
| 1 mM Mg\(^{2+}\), 25 µM PI(3,4,5)P\(_3\) | 0.42 ± 0.03                                             | 3.7 ± 0.2                       | 4.8             |
| 0.1 mM Mg\(^{2+}\), 25 µM PI | 0.58 ± 0.08                                              | 2.4 ± 0.1                       | 7.1             |
| 0.1 mM Mg\(^{2+}\), 25 µM PI(3,4)P\(_2\) | 0.15 ± 0.02                                             | 1.8 ± 0.1                       | 1.5             |
| 0.1 mM Mg\(^{2+}\), 25 µM PI(4,5)P\(_2\) | 0.04 ± 0.01                                             | 1.9 ± 0.1                       | 0.42            |
| 0.1 mM Mg\(^{2+}\), 25 µM PI(3,4,5)P\(_3\) | 0.04 ± 0.01                                             | 2.1 ± 0.2                       | 0.40            |

\(^1\)Apparent association and dissociation rates

\(^2\)Dissociation of CP from polyphosphoinositide, estimated from the decrease in \(k_{+\text{app}}\) by mass action (see Experimental Procedures).
### Table III. *S. pombe* Capping Protein Rate and Equilibrium Constants

| Experiment                      | $k_+ \text{ Markov}$ | $k_- \text{ Markov}$ | $k_- \text{ direct}^1$ | $K_d^2$ | $K_d^3$ |
|---------------------------------|----------------------|----------------------|------------------------|---------|---------|
|                                 | $\times 10^6 M^{-1} s^{-1}$ | $\times 10^3 s^{-1}$ | $\times 10^3 s^{-1}$ | nM      | nM      |
| Apparent rate vs. conc.         | 1.1 ± 0.2            | 3.1 ± 3.1            | 5.7 ± 0.1              | 29      | 52      |
| Average with PI Buffer          | 0.9 ± 0.3            | 5.1 ± 2.1            | 3.8 ± 0.4              | 60      | 45      |
| with 25 µM PI                   | 1.2 ± 0.1            | 5.7 ± 0.5            | 4.4 ± 0.2              | 49      | 38      |
| with 25 µM PI(3,4)P₂            | 0.9 ± 0.3            | 4.8 ± 0.8            | 4.1 ± 1.0              | 51      | 44      |
| with 25 µM PI(4,5)P₂            | 0.9 ± 0.1            | 5.7 ± 2.0            | 2.9 ± 0.1              | 64      | 33      |
| with 25 µM PI(3,4,5)P₃          | 1.0 ± 0.4            | 5.8 ± 2.1            | 2.6 ± 1.2              | 56      | 26      |

1Direct dissociation rate experiment. Slow component of double exponential fit.
2Average Markov dissociation rate / Average Markov association rate
3Direct dissociation rate / Average Markov association rate
Figure 2, Kuhn JR and Pollard TD

A

0:50:00
1:30:00
2:10:00
2:50:00

10 µm

B

ΔLength, ×10^3 subunits

Time, ×10^3 s

C

Fraction Capped

Time, ×10^3 s
Figure 3, Kuhn JR and Pollard TD
Figure 4, Kuhn JR and Pollard TD
Figure 5, Kuhn JR and Pollard TD
Figure 6, Kuhn JR and Pollard TD
Figure 7, Kuhn JR and Pollard TD
Figure 8, Kuhn JR and Pollard TD
Single molecule kinetic analysis of actin filament capping: Polyphosphoinositides do not dissociate capping proteins
Jeffrey R. Kuhn and Thomas D. Pollard

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