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Effects of CapZ, an Actin Capping Protein of Muscle, on the Polymerization of Actin†

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ABSTRACT: We have studied the interaction of CapZ, a barbed-end actin capping protein from the Z line of skeletal muscle, with actin. CapZ blocks actin polymerization and depolymerization (i.e., it "caps") at the barbed end with a K_d of approximately 0.5–1 nM or less, measured by three different assays. CapZ inhibits the polymerization of ATP–actin onto filament ends with ATP subunits slightly less than onto ends with ADP subunits, and onto ends with ADP–BeF_3^- subunits about as much as ends with ADP subunits. No effect of CapZ is seen at the pointed end by measurements either of polymerization from acrosomal processes or of the critical concentration for polymerization at steady state. CapZ has no measureable ability to sever actin filaments in a filament dilution assay. CapZ nucleates actin polymerization at a rate proportional to the first power of the CapZ concentration and the 2.5 power of the actin concentration. No significant binding is observed between CapZ and rhodamine-labeled actin monomers by fluorescence photobleaching recovery. These new experiments are consistent with but do not distinguish between three models for nucleation proposed previously (Cooper & Pollard, 1985). As a prelude to the functional studies, the purification protocol for CapZ was refined to yield 2 mg/kg of chicken breast muscle in 1 week. The activity is stable in solution and can be lyophilized. The native molecular weight is 59 600 ± 2000 by equilibrium ultracentrifugation, and the extinction coefficient is 1.25 mL mg^{-1} cm^{-1} by interference optics. Polymorphism of the α and β subunits has been detected by isoelectric focusing and reverse-phase chromatography. CapZ contains no phosphate (<0.1 mol/mol).

CapZ is a heterodimeric protein with subunits of M_r 36 000 (α subunit) and 32 000 (β subunit) (Casella et al., 1986). CapZ binds to the barbed end of actin filaments in vitro and is located at the Z line in skeletal muscle (Casella et al., 1987). Since the barbed ends of actin filaments are also located at the Z line, CapZ may attach the actin filaments to the Z line. CapZ is part of a family of capping proteins (Stossel et al., 1985; Pollard & Cooper, 1986) that are heterodimers, bind the barbed ends of actin filaments, and do not require Ca^{2+} for activity. These proteins are widely distributed, having been purified from *Acanthamoeba* (Isenberg et al., 1980; Cooper et al., 1984), *Dictyostelium* (Schleicher et al., 1984), bovine brain (Kiliman & Isenberg, 1982), and chicken skeletal muscle (Casella et al., 1986). The α and β subunits are not similar to each other by antibody cross-reactivity, peptide maps (Casella et al., 1986; Cooper et al., 1984, 1986), or primary structure (Casella et al., 1989; Caldwell et al., 1989). The primary structures of CapZ α and β show no similarity to those of other actin binding proteins or actin itself (Casella et al., 1989; Caldwell et al., 1989).

Understanding the mechanism of action of CapZ on actin in vitro is important because it will allow us to make and test predictions about the role of CapZ in cells. In muscle, actin filaments in sarcomeres have a defined location, polarity, and length. In nonmuscle cells, actin filaments often have a well-defined spatial arrangement that can change over time. Actin assembly itself may provide the motive force for cell movement. CapZ and its nonmuscle analogues may regulate actin assembly in cells.

Actin contains a bound nucleotide that can be ATP, ADP–P_i, or ADP. If capping proteins bind differently to actin with different nucleotides, this might be a mechanism for regulating actin polymerization in cells. Actin monomers contain ATP, which is converted to ADP–P_i and then ADP after the monomer adds to a filament end (Korn et al., 1987). Previous work on the mechanism of action of capping proteins with actin has shown that they bind tightly to the barbed end of actin filaments (Kiliman & Isenberg, 1982; Cooper et al., 1984; Casella et al., 1986), with a K_d of 0.5 nM for brain capping protein (Wanger & Wegner, 1985). In this report, we extend these studies by determining the K_d with different assays using filaments that are either polymerizing, at steady state, or depolymerizing. One of the assays is used to directly compare barbed ends with subunits that contain ATP or ADP or ADP–BeF_3^-. BeF_3^- binds to actin filaments as a phosphate analogue and induces a state resembling ADP–P_i* (Combeau & Carlier, 1988). We find little or no difference in the ability of CapZ to bind to barbed ends with different nucleotides.

Capping proteins accelerate the polymerization of actin from monomers. The time course of actin polymerization induced by *Acanthamoeba* capping protein, analyzed by kinetic modeling, showed that capping protein accelerates but does not

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bypass the process of nucleation (Cooper & Pollard, 1985). Models in which capping protein bypassed the nucleation phase either by severing actin filaments or by resembling the pointed end of an actin filament fit the data poorly, and three models in which capping protein participated in the nucleation phase of actin polymerization fit the data well. In this report, we have extended these results in an effort to choose among the three models with new experiments that measure the direct binding of CapZ to actin as well as the kinetics of actin polymerization. We find that CapZ also accelerates but does not bypass the nucleation process but that the new data do not distinguish between the three models that predicted the data well previously.

The gelsolin family of actin binding proteins is capable of severing actin filaments as well as capping barbed ends and nucleating actin polymerization (Stossel et al., 1985; Pollard & Cooper, 1986). The capping protein family causes actin filaments to shorten (Kilimann & Isenberg, 1982; Cooper, et al., 1984) for which severing is a possible mechanism. In this report, we measure the severing activity of CapZ in a quantitative assay, with a comparison to gelsolin, and find that CapZ has no severing activity.

Materials and Methods

Unless stated otherwise, chemicals and chromatography resins were from Sigma Chemical Co. (St. Louis, MO), and solvents and supplies were from Fisher Scientific (St. Louis, MO).

Purification and Properties of CapZ. The starting material was chicken breasts obtained from a local abattoir (Levin Poultry, St. Louis, MO). The chickens had been sacrificed by exsanguination 1 to several hours before we received them. Acetone powder was prepared from the pectoralis major and minor muscles (typically 1 kg) as described (Spudich & Watt, 1971) and stored at −20 °C. Acetone powder (typically 100 g) was extracted with 20 mL/g Ca^{2+} buffer G (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM DTT, and 1 mM NaN_3, with the protease inhibitors 0.1 mM PMSF, 0.1 μM pepstatin A, 0.1 μM leupeptin, and 0.1 mM benzamidine) with stirring at 4 °C for 30 min. The mixture was filtered through cheesecloth. The extract was used to prepare actin (below), and the muscle residue was extracted again with Ca^{2+} buffer G. Next the residue was extracted with 15 mL/g of 1 M KCl, 0.1 mM EDTA, and protease inhibitors (listed above) for 60 min, and then twice again for 30 min each. The residue was extracted twice with 0.6 M KI, 20 mM $Na_2S_2O_3$, 5 mM 2-mercaptoethanol, 1 mM NaN_3, and protease inhibitors (listed above) for 30 min, first with 15 mL/g and then with 7.5 mL/g. The combined KI extracts were dialyzed against 4 volumes of H_2O for 4 h and then against 4 volumes of buffer B (50 mM KCl, 1 mM 2-mercaptoethanol, 1 mM NaN_3, 10 mM Tris-HCl, pH 8.0, and 0.1 mM PMSF) overnight.

The dialyzed extract was centrifuged at 10000*g* for 10 min to remove a flocculent white precipitate and applied to a 5 × 100 cm column of DEAE-cellulose (Whatman DE52) preequilibrated with buffer B with 100 mM KCl. After the sample was applied, the column was immediately eluted with a 4-L KCl gradient from 100 to 400 mM KCl. Twenty-milliliter fractions were collected and assayed for filament shortening activity by either falling-ball viscometry or a tube-tipping assay (Pollard & Cooper, 1982). CapZ eluted at 100–150 mM KCl. The pooled fractions were subjected to $(NH_4)_2SO_4$ fractionation; the 50–75% saturation pellet was collected, suspended in a minimal volume (about 10 mL) of buffer B, and dialyzed against buffer B for 2 h. The sample was clarified and applied to a 2.5 × 100 cm Sephacryl S-200 column in buffer B. Eight-milliliter fractions were collected and assayed as above; the CapZ peak was generally identified by A_{280} as the left shoulder of the main peak.

The pooled fractions were dialyzed against 20 mM MES, pH 6.0, and 1 mM NaN_3 overnight, clarified, and 0.22-μm-filtered. The sample was applied at 2 mL/min to a 1.6 × 10 cm MonoS column on an FPLC system (Pharmacia, Piscataway, NJ) equilibrated in the same buffer. The column was eluted with a linear gradient of KCl at 3 mM/min to 150 mM, and CapZ eluted at 100 mM KCl. Figure 1A shows SDS–polyacrylamide gel electrophoresis of the material through the stages of purification. The average yield was 2 mg/kg of muscle. The pooled fractions were dialyzed against 5 mM Tris-HCl, pH 8.0, 0.1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, 0.22-μm-filtered, and stored at 4 °C.

The activity of CapZ is stable in 5 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 mM PMSF for several weeks at 4 °C. When lyophilized in 10% (w/v) sucrose and 50 mM NH_4HCO_3 and stored at −20 °C, about half of the CapZ can be redissolved. The soluble CapZ has full activity, measured by the ability of CapZ to increase the rate of polymerization of actin monomers. Solutions of CapZ at 35 nM (2 μg/mL) showed no measurable (<10%) adsorption to glass, quartz, polyethylene, and polypropylene. One milliliter of CapZ solution was incubated for 30 min in a cuvette or tube of the appropriate material; then the solution was removed and added to 0.2 mL of Bradford concentrated protein reagent (Bradford, 1976).

The native molecular weight, measured by sedimentation equilibrium as described (Cooper et al., 1987), is 59 600 ± 2000, which is the mean of three determinations ± error estimated from the scatter of the data about a straight line. The CapZ concentration was at 0.8–1.0 mg/mL in 0.1 M KCl, 10 mM Tris-HCl, pH 8.0, and 1 mM NaN_3. The speed was 24 000 rpm, and scans were recorded at 24, 28, and 48 h. No trend was noted over time. The value for the native molecular weight agrees with the previous value of 61 000 calculated from the Stokes' radius and sedimentation coefficient (Casella et al., 1986) and the value of 64 310 calculated from the cDNA sequences (Caldwell et al., 1989; Casella et al., 1989). The υ is 0.7253 cm^3/g, calculated from the amino acid composition (Caldwell et al., 1989; Casella et al., 1989) by the method for Cohn and Edsall (1943). The extinction coefficient at 280 nm is 1.25 ± 0.02 mL mg^{-1} cm^{-1}, as determined by a protein measurement using the interference optical system on a Beckman Model E analytical ultracentrifuge (Babul & Stellwagon, 1969). The ultraviolet/visible absorption spectrum, recorded on a Cary double-beam spectrophotometer, has a λ_{max} at 279 nm and shoulders at 284 and 290 nm, which is consistent with a combination of tryptophan and tyrosine (data not shown).

By SDS–polyacrylamide gel electrophoresis with DTT, the subunits have molecular weights of 36 000 ± 2000 (α) and 32 000 ± 2000 (β), confirming the previous values (Casella et al., 1986). The β subunit often shows closely spaced bands of slightly lower molecular weight, which may represent proteolysis, posttranslational modification, or isoforms. The number and intensity of these bands vary with the preparation. In the absence of DTT, the subunits are still separated (Figure 1B), indicating that they are not linked by disulfide bonds.

Two-dimensional gel electrophoresis (O'Farrell, 1975) with isoelectric focusing [8 M urea, 2% (w/v) Triton X-100, 1 mM DTT, and 2% (w/v) ampholines (3.5–10)] followed by SDS–polyacrylamide gel electrophoresis [14% (w/v) acrylamide]

(Gibson, 1974) shows that both α and β subunits have two major isoforms (Figure 1C). The α subunit has a third minor form. Additional minor spots appear below each of the β isoforms, which suggests that they are the result of electrically neutral modifications of β. The α subunit is more basic than the β subunit. By reverse-phase HPLC on a C_4 column in 0.1% (v/v) TFA eluted with a gradient of acetonitrile, the α subunit elutes at one position, and the β subunit elutes at three different positions (data not shown). The mobility of the β subunit in the three positions is 32 kDa by SDS–polyacrylamide gel electrophoresis.

The total phosphate content of CapZ was measured with a malachite green based assay (Selden & Pollard, 1983). No phosphate was detected, and the detection limit was 0.1 mol/mol in the assay.

Preparation of Actin. Actin was prepared as described (Spudich & Watt, 1971), gel-filtered (MacLean-Fletcher & Pollard, 1980), and lyophilized in Ca^{2+} buffer G with 2 mg of sucrose/mg of actin. Lyophilized actin was stored at −20 °C, dialyzed overnight against Ca^{2+} buffer G, and centrifuged at 100000*g* for 30 min before use. In control experiments measuring the critical concentration at steady state and the kinetics of polymerization from monomers, lyophilized actin was identical with actin that had never been lyophilized.

Actin Polymerization Assays. Pyrene-labeled actin (Kouyama & Mihashi, 1981) was prepared and used in fluorometer-based polymerization assays as described (Cooper et al., 1983b). The depolymerization assay used to measure severing activity was a modification of the method of Bryan (1986). Pyrene-actin (70% labeled) was polymerized in Ca^{2+} buffer G with 0.1 M KCl and 2 mM $MgCl_2$. A 5 μM stock of filaments was diluted to 0.1 μM in a fluorometer cuvette in the same buffer at time zero. The material to be assayed was added before or after the filaments as noted. Fluorescence was monitored over time. Rabbit plasma gelsolin, used as a control in these experiments, was prepared as described (Cooper et al., 1987).

To measure capping activity at the barbed and pointed ends, acrosomal processes from *Limulus* sperm were prepared and used in capping assays as described (Pollard, 1986). *Limulus* were obtained from the Marine Biological Laboratory (Woods Hole, MA) and stored in room temperature seawater tanks at the St. Louis Zoo. Bundle lengths were measured on a Zeiss 10A electron microscope using a series of rulers that compared the bundles to physical landmarks on the screen.

Measurement of CapZ–Actin Binding by Fluorescence Photobleaching Recovery. Fluorescence photobleaching recovery (FPR) was performed in the laboratory of Dr. Elliot Elson (Department of Biochemistry and Molecular Biophysics) as described (Cooper et al., 1988). The sample chamber, developed by David J. Loftus, was a quartz slide and coverslip separated by a rubber O-ring, and held 80 μL. The chamber components were treated with 5 mg/mL BSA and baked before use, to minimize the possibility of adsorption. Actin was labeled with tetramethylrhodamine iodoacetamide (Molecular Probes, Eugene, OR) as described (Tait & Frieden, 1982). SDS–polyacrylamide gel electrophoresis showed no free dye. To ensure that the labeling reaction was complete, actin was labeled in the conventional manner and with 5 times the amount of dye. The two samples were found to be labeled with rhodamine to the same extent. In a control experiment to test whether CapZ can nucleate polymerization of TMR-actin, CapZ increased the rate of polymerization of fully labeled TMR-actin monomers. A trace (5%) of pyrene-actin was added to monitor polymerization.

In the FPR assays, TMR-actin was present in 2 mM $MgCl_2$, 1 mM EGTA, 0.1 M KCl, and 20 mM imidazole hydrochloride, pH 7.0 (MKEI buffer), at 1 μM, which is above the critical concentration of 0.1 μM for this buffer; however, nucleation is so slow at this actin concentration that polymerization should not occur even over several hours (Cooper et al., 1983a). The absence of polymerization was checked by a control experiment in which the recovery time for actin in the polymerizing buffer was observed to be the same as that for actin in depolymerizing buffer (Ca^{2+} buffer G).

Each run consisted of 1024 scans with 1000 channels per scan and 2 ms per channel. The bleach time was 7 ms. The recovery curves were fit to a one-component recovery with a new program written by Qian Hong in Dr. Elson's laboratory. In each experiment, six runs were performed on each sample. In experiment 1, all the runs were performed on one sample, followed by all the runs on the second sample. In experiment 2, runs were performed alternately on the two samples.

Results

Purification and Properties of CapZ. Modifications of the published purification protocol (Casella et al., 1986) led to improvements in time and yield. Figure 1A shows the material through the stages of purification. The protocol, described in detail under Materials and Methods, yields 2 mg of CapZ/kg of muscle in 1 week. The activity of CapZ is relatively stable. The native molecular weight, measured by sedimentation equilibrium, is 59 600 ± 2000. The extinction coefficient at 280 nm is 1.25 ± 0.02 mL mg^{-1} cm^{-1}. Two-dimensional gel electrophoresis with isoelectric focusing followed by SDS–polyacrylamide gel electrophoresis shows that both α and β subunits have two major isoforms (Figure 1C).

Capping of Barbed Ends. The affinity of CapZ for barbed ends of actin filaments was similar in three different functional assays. First, the ability of CapZ to alter the apparent critical concentration at steady state was measured. Since the critical concentration of the barbed and pointed ends is different in Mg^{2+}, one can calculate the fraction of capped barbed ends from a measurement of the apparent critical concentration. From four experiments like the one in Figure 2, the K_d is 0.5 ± 0.1 nM. In this calculation, the K_d is lower than the concentration that changes the critical concentration by 50% because the rate constants for actin at the barbed end are larger than those at the pointed ends (Walsh et al., 1984). The calculation uses published elongation rate constants for ATP–actin at the two ends (Pollard, 1986). In this calculation, the number of filament ends is unknown and assumed to be small relative to the K_d; therefore, the K_d is less than 0.5 nM if the assumption is incorrect. The weight concentration of actin was 800 nM in this experiment, and filament lengths of 1–12 μm have been observed by fluorescence microscopy (Yanagida et al., 1984), so the number concentration of actin filaments may have been 0.2–2 nM.

Second, the ability of CapZ to inhibit the growth of actin filaments from acrosomal processes was measured. This assay allows the examination of the barbed and pointed ends separately. CapZ was mixed with the processes for several minutes before the actin monomers were added to begin the polymerization. Each process contains many (10–50) actin filaments (Bonder et al., 1983, which are bundled together at the end of the assay to permit the most accurate length measurement (Pollard, 1986). Because of this bundling, however, one cannot determine how many filaments on each process grew. In fact, the most reliable observation in this experiment is that CapZ did not affect the length of the bundles but it did increase the fraction of bundles that showed no growth. At high concen-

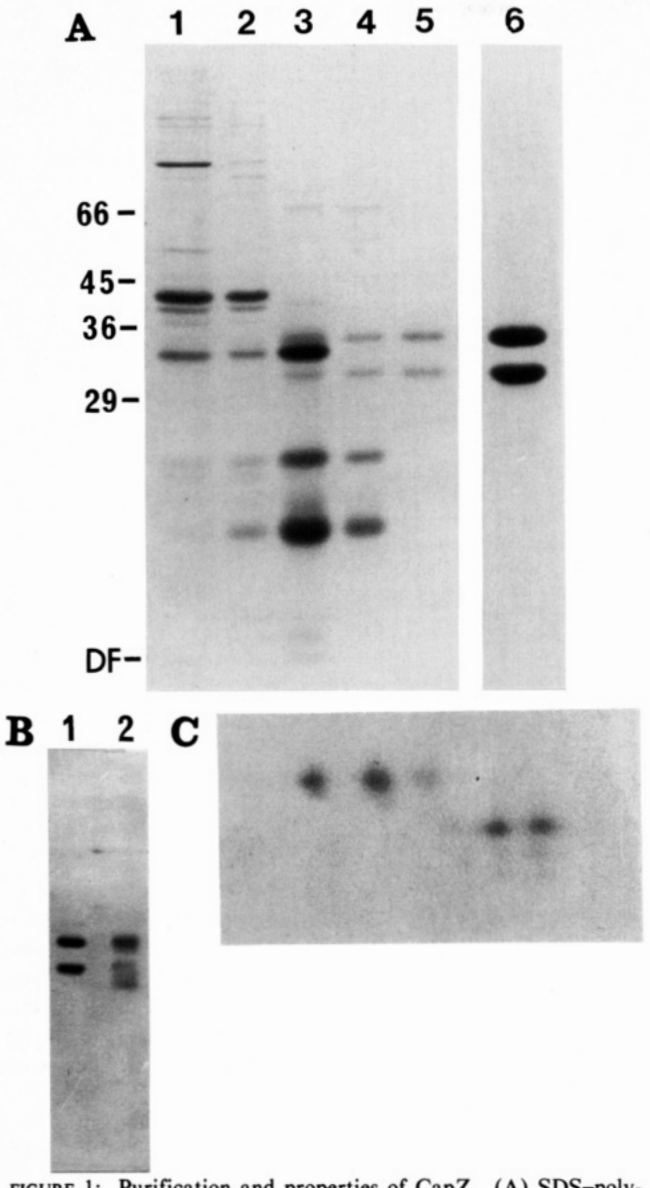

FIGURE 1: Purification and properties of CapZ. (A) SDS–polyacrylamide gel electrophoresis of CapZ pools through purification. The lanes are (1) KI extract of acetone powder, (2) pool after DEAE column, (3) $(NH_4)_2SO_4$ fraction (50–75% saturation pellets), (4) pool after S-200 column, and (5) purified CapZ pool after MonoS column. Lane 6 is from a different gel and shows a heavier loading of the material in lane 5. (B) Separation of CapZ subunits without a reducing agent. CapZ was electrophoresed on an SDS–polyacrylamide gel with 40 mM DTT (lane 1) and without DTT (lane 2). (C) Two-dimensional electrophoresis of CapZ. Isoelectric focusing was performed in the horizontal dimension; the acidic side (anode) is on the right. SDS–polyacrylamide gel electrophoresis was then performed in the vertical dimension. Only a small region of the gel is shown here. No other spots were observed.

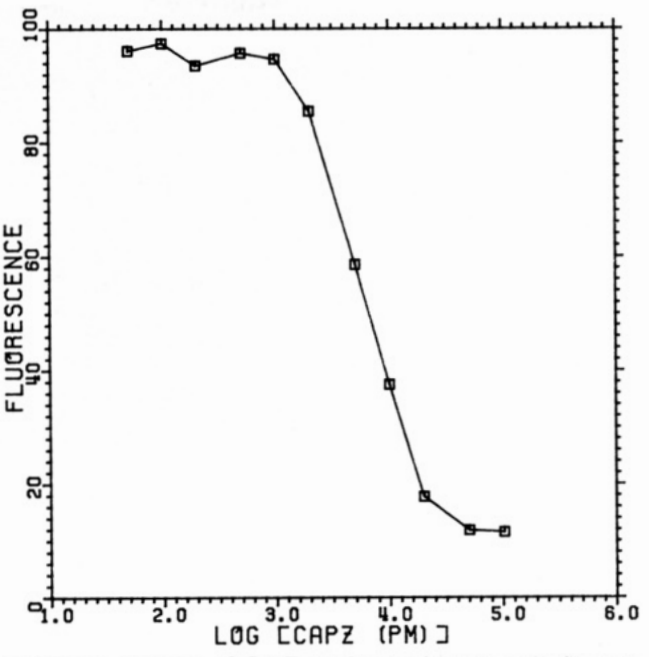

FIGURE 2: Titration of CapZ on the steady-state actin filament concentration. This experiment measures the effect of CapZ on the critical concentration. The fluorescence of pyrene-actin (arbitrary units) is plotted vs the log of the CapZ concentration in picomolar. Fluorescence is proportional to actin filament concentration. Actin monomers were added to the samples, but similar results were obtained when the actin was prepolymerized. The samples were incubated at room temperature for 24 h, and similar readings were obtained at 48 h. The actin concentration was 0.8 μM with 20% pyrene labeled, and similar results were obtained with 1.6 μM actin and 10% and 60% pyrene label. The buffer was MKEI.

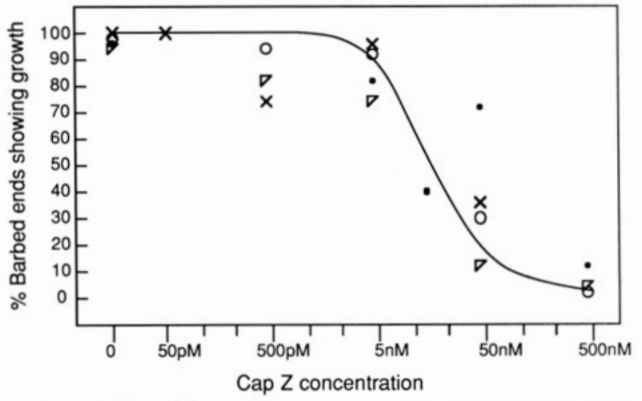

FIGURE 3: Effect of CapZ concentration on actin polymerization from the barbed end of acrosomal processes. The percentage of processes that showed growth is plotted against the concentration of CapZ on a log scale. CapZ was incubated with the processes for 15 min in MKEI buffer. Actin monomers were added at 1.4 μM for 30 s. In a control experiment without CapZ, polymerization was linear with respect to time under these conditions. The four symbols represent experiments on different days.

trations of CapZ, no bundles showed growth (Figure 3). We reasoned that this phenomenon could be explained if the dissociation rate for CapZ binding to barbed ends is slow enough to prevent the system from reaching equilibrium during the time of the experiment, which is 5–30 s. That is, a given actin filament in the acrosomal process might be either capped or uncapped at the start of the experiment and remain in that state through the experiment. Given a K_d of 5×10^{-10} M (measured above) and a diffusion-limited association rate constant of 10^7 M^{-1} s^{-1}, the dissociation rate constant would be 5×10^{-3} s^{-1}. For gelsolin, another barbed-end capping protein, the measured dissociation rate constant is approximately 10^{-4} s^{-1} (Selve & Wegner, 1986). Therefore, we assumed that the CapZ, which is added to the bundles before the actin, binds to a certain fraction of the filaments and that during the experiment the bound CapZ does not dissociate from the filament ends. With these assumptions and a K_d of 0.5 nM, the fraction of bundles showing growth was calculated and is plotted as the smooth curve in Figure 3 and agrees well with the data. This calculation includes the number of filaments in the bundles. We calculated the theoretical curve for 10 and 50 filaments, which are the extremes based on our observations without the bundling chemical spermine, and found only an insignificant difference in the curve.

As in the previous experiment, the concentration of filaments is unknown, so the K_d may be less than 0.5 nM. An upper

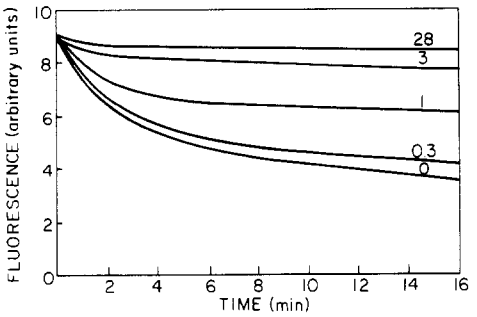

FIGURE 4: Effect of CapZ concentration on depolymerization of actin filaments. The fluorescence of pyrene-actin, which is proportional to the filament weight concentration, is plotted vs time. Actin filaments at 5 μM were diluted to 0.1 μM at $t = -0.5$ min in the fluorometer cuvette with stirring. At time zero, CapZ was added. The concentration of CapZ (nanomolar) is indicated next to each curve. An additional curve, with 0.1 nM CapZ, was indistinguishable from the control with no CapZ and is not shown.

limit for the concentration of filaments can be estimated from the observation that the plot of barbed-end growth vs time is linear for 90 s at 1.4 μM. The assay could definitely have measured a 20% drop in growth rate; therefore, the monomer concentration dropped by less than 20%, and, given the elongation rate constants (Pollard, 1986), the filament number concentration was less than 0.2 nM.

Third, the ability of CapZ to bind to ADP-containing ends of actin filaments was measured with a depolymerization assay. In this assay, actin filaments are diluted to the critical concentration, so that they depolymerize from their ends. After a short time, these ends should only contain actin subunits with ADP. CapZ is then added, and barbed ends are capped, which decreases the rate of depolymerization. The data show that concentrations of CapZ above 0.1 nM decrease the depolymerization rate (Figure 4); 50% inhibition occurs at approximately 1 nM. The number of filament ends is also unknown in this experiment, so the value of 1 nM represents an upper limit for the K_d. The number concentration of filaments may be estimated from the initial rate of depolymerization of the actin control without CapZ. That rate was 0.4 nM/s, which, given the dissociation rate constant of 7.5 s^{-1} (Pollard, 1986), implies a filament number concentration of 0.05 nM. On the other hand, the time course of depolymerization of actin filaments is biphasic, which is not understood (Walsh et al., 1984), so the initial rate may not reflect depolymerization of filaments at 7.5 s^{-1}.

Therefore, the ability of CapZ to bind to barbed ends is similar in these three functional assays, in which actin filaments are polymerizing, are at steady state, or are depolymerizing. We are interested in whether CapZ binds differently to barbed ends in which the actin subunits contain ATP, ADP-P_i, or ADP. In the assays above, the ends in the depolymerization assay should contain ADP, but the state of the nucleotide in the other two assays is problematic. The acrosomal processes are at steady state before the CapZ is added, and in the critical concentration experiment, the system is allowed to reach steady state. In these cases, the free barbed ends may have subunits with ATP or ADP, and the ends bound to CapZ probably have ADP, because monomer exchange is prevented.

To address the issue directly, we designed two experiments in which the nucleotide content of the actin was different, and those different actins were tested for their ability to bind CapZ in the same functional assay. First, to compare the ability of CapZ to bind to barbed ends with ATP subunits vs ones with ADP subunits, CapZ was added to filaments that were either

Table I: Inhibition of Growth of Filaments by CapZ: Comparison of Filament Ends with ADP, ATP, and ADP-BeF_3^-[a]

[CapZ] (nM)	Experiment 1[b] growth rate (% of control) ADP	ATP
0.3	81, 81	91, 102
1.0	52, 50	73, 84
3.0	27, 36	65, 66

[CapZ] (nM)	Experiment 2[c] growth rate (% of control) ADP	ADP-BeF_3^-
1	49	35
3	20	18
10	7	10

[a] The initial rate of polymerization, measured by fluorescence of pyrene-actin, is listed. Ten percent of the actin was labeled with pyrene. The initial rate was determined from recordings of fluorescence vs time. The conditions were 2 mM $MgCl_2$, Ca^{2+} buffer G, 20 °C. [b] Growing filaments should have ATP-containing ends, and depolymerizing filaments should have ADP-containing ends. In this experiment, we added CapZ to these two types of filaments and measured the ability of CapZ to inhibit subsequent growth. Five micromolar filaments in ATP was diluted to 0.25 μM to induce depolymerization. For ADP-filaments, CapZ was then added, followed by 5 μM ATP-actin monomers. For ATP-filaments, 5 μM ATP-actin monomers were added first, followed by CapZ. [c] This experiment was identical with experiment 1, except that for the right column (ADP-BeF_3^-) the actin filament seeds were polymerized in and diluted into 0.1 mM $BeSO_4$, 2 mM NaF, 2 mM $MgCl_2$ and Ca^{2+} buffer G instead of 2 mM $MgCl_2$ and Ca^{2+} buffer G.

polymerizing or depolymerizing. The polymerizing filaments should contain ATP subunits, and the depolymerizing filaments should contain ADP subunits. To determine whether CapZ had bound to the filaments, we then added actin monomers and measured the rate of polymerization. The data show a small but reproducible difference, with CapZ binding to ADP-filaments better than to ATP-filaments (Table I, experiment 1).

The ATP in actin filaments is unstable and hydrolyzes to ADP. Therefore, to check that the ATP-filaments in this experiment do contain ATP at their ends, we compared the relative rates of polymerization and ATP hydrolysis. The net polymerization rate is $k_+[A_1] - k_- = (11.6\ \mu M^{-1}\ s^{-1})(5\ \mu M) - 1.4\ s^{-1} = 57\ s^{-1}$ [rate constants from Pollard (1986)]. The ATP hydrolysis rate is less, at 13 s^{-1} (Korn et al., 1987). The rate of P_i release (conversion of ADP-P_i to ADP) is far less, at 0.006 s^{-1} (Korn et al., 1987). Therefore, the barbed ends should contain predominantly subunits with ATP and fewer subunits with ADP-P_i. Of course, if the binding of CapZ increases the rate of ATP hydrolysis, then this reasoning may be incorrect, and the actin subunits may contain ADP shortly after CapZ binds.

In the second experiment involving a direct comparison of nucleotides, we used BeF_3^-, which binds to actin filaments as a phosphate analogue and induces an ADP-BeF_3^- state that functionally resembles ADP-P_i* (Combeau & Carlier, 1988). We compared the ability of CapZ to bind to ADP-BeF_3^- ends vs ADP ends in growth experiments similar those above. The data show no consistent difference between the two nucleotides (Table I, experiment 2).

Absence of Effect of CapZ on the Pointed End. In experiments with acrosomal processes, where the barbed and pointed ends are morphologically distinguishable, CapZ had no effect on growth at the pointed end at high CapZ concentrations that completely inhibited barbed-end growth. The CapZ concentration was 0.47 μM, the actin monomer con-

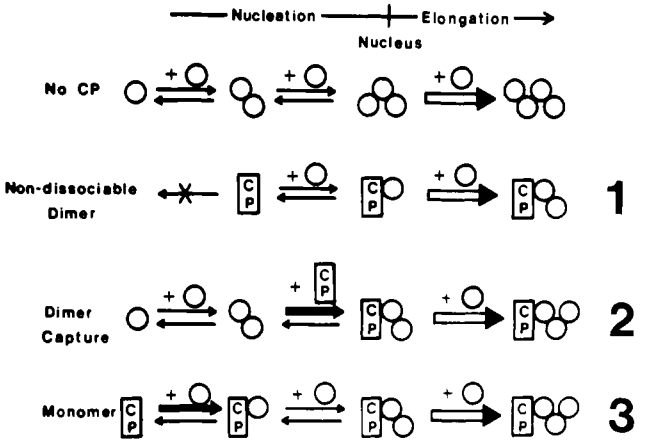

FIGURE 5: Diagram of three models in which CapZ accelerates the nucleation of actin [reprinted from Cooper and Pollard (1985)]. In model 1, CapZ resembles a nondissociable actin dimer, and the nucleus is a CapZ–actin monomer complex. In models 2 and 3, the nucleus is a CapZ–actin dimer complex. In model 2, CapZ binds poorly to actin monomers and well to actin dimers. In model 3, CapZ first binds relatively well to one actin monomer, and then another actin monomer binds with the same equilibrium constant as for actin trimer formation.

centration was 1.4 μM, and the incubation time was 2 min. The fraction of pointed ends of bundles with growth was 65% (10/15) with CapZ and 73% (15/21) without CapZ. The length of the new filament growth on the bundles with growth was 0.24 ± 0.08 μm (N = 15) with CapZ and 0.24 ± 0.08 μm (N = 21) without CapZ.

Also, the critical concentration in the presence of high concentrations of CapZ approaches but does not exceed the critical concentration for the pointed end (0.6 μM, Figure 2) (Bonder et al., 1983), which implies that CapZ does not cap the pointed end.

Nucleation. Our previous work with *Acanthamoeba* capping protein showed that capping protein increased the rate of polymerization of actin monomers in a manner consistent with an increased rate of nucleation (Cooper & Pollard, 1985). Two models in which capping protein bypassed the normal nucleation process of actin polymerization were excluded. These models were one in which capping protein resembled the pointed end of an actin filament and one in which capping protein severed actin filaments. On the other hand, three models in which capping protein accelerated but did not bypass nucleation were all consistent with the experimental data. Those models are diagrammed in Figure 5. Using CapZ, we have been able to extend these studies with new types of experiments and larger amounts of pure protein. First, we find that CapZ, like *Acanthamoeba* capping protein, accelerates the rate of actin polymerization from monomers. When actin monomers in Ca^{2+} (Ca^{2+} buffer G) are polymerized by the addition of 2 mM Mg^{2+}, the polymerization rate is increased by CapZ, but a lag phase persists (Figure 6A). Preincubation of CapZ with actin in Ca^{2+} buffer G has no effect on the lag phase, indicating that CapZ does not nucleate in Ca^{2+} buffer G. However, the lag phase is nearly eliminated when CapZ is added to Ca^{2+}–actin that has been primed with Mg^{2+} and EGTA (Figure 6A, curve 4) or to Mg^{2+}–actin that has been primed with 250 μM Mg^{2+}, which induces the conformational change described by Frieden (1982) (Figure 6B). Preincubation of CapZ with Mg^{2+}-primed actin does cause a slight increase in the initial rate of polymerization (Figure 6B). Since Mg^{2+}–actin spontaneously nucleates much more effectively than does Ca^{2+}–actin (Cooper et al., 1983a), the finding that CapZ nucleates Mg^{2+}–actin more effectively than Ca^{2+}–actin is consistent with the idea that CapZ acts through, rather than

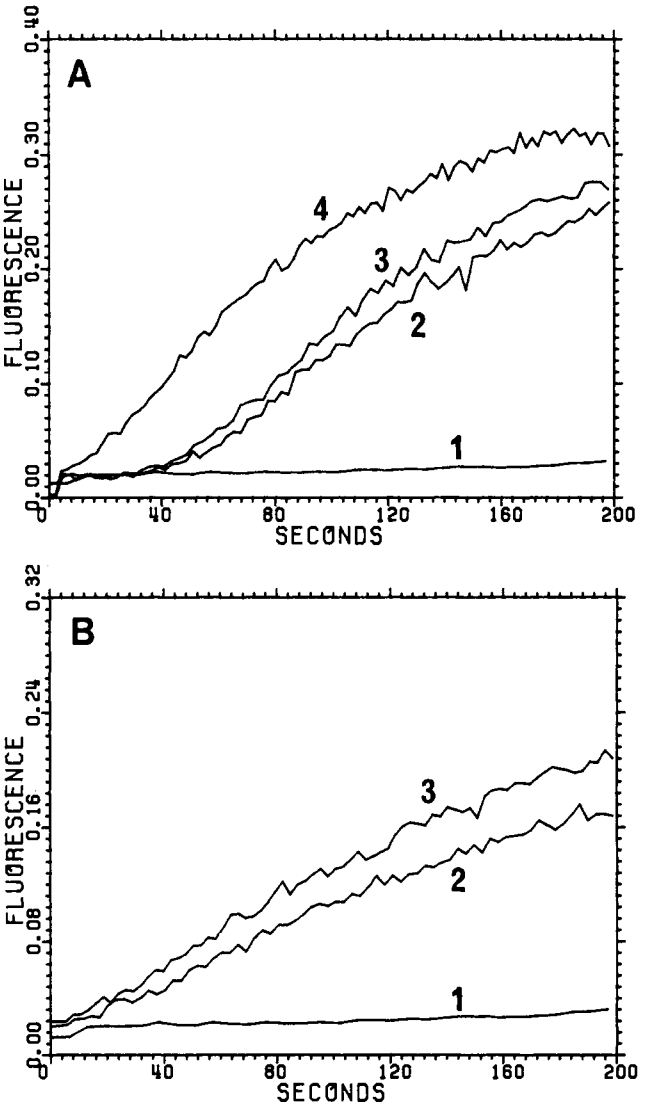

FIGURE 6: (A) Effect of CapZ concentration on polymerization of Ca^{2+}–actin. Fluorescence of pyrene-actin (arbitrary units) is plotted vs time. The curves are (1) actin alone with addition of MKEI buffer at t = 0, (2) addition of CapZ at t = −5 min and MKEI buffer at t = 0, (3) addition of CapZ and MKEI buffer at t = 0, and (4) addition of MKEI buffer at t = −2 min and CapZ at t = 0. The actin concentration was 5 μM (10% labeled with pyrene), and the CapZ concentration was 50 nM. (B) Effect of CapZ concentration on polymerization of Mg^{2+}–actin. Fluorescence of pyrene-actin (arbitrary units) is plotted vs time. The curves are (1) control, actin alone without CapZ, (2) Mg^{2+}-primed actin, to which CapZ was added at time zero, and (3) Mg^{2+}-primed actin, to which CapZ was added at −5 min. The actin in this experiment was prepared in Mg^{2+} buffer G, which has 50 μM $MgCl_2$ in place of $CaCl_2$, and then primed with an additional 200 μM $MgCl_2$ for 5 min before use. At time zero, an additional 1.8 mM $MgCl_2$ was added to induce polymerization. The actin concentration was 5 μM (10% labeled with pyrene), and the CapZ concentration was 50 nM.

bypassing, the actin nucleation mechanism.

We examined how the initial rate of CapZ-induced polymerization of Mg^{2+}-primed actin depends on the concentration of actin and CapZ. Since the polymerization rate is directly proportional to filament number, the initial rate should be proportional to the number of nuclei created by CapZ. The rate depends on the CapZ concentrtion to the first power (Figure 7A) and on the actin concentration to the 2.5 power (Figure 7B). With Ca^{2+}–actin, the rate depends on the actin concentration to the 2.4 power (Figure 7B). These observations suggest that the nucleus created by the addition of CapZ includes one molecule of CapZ and one or two molecules of

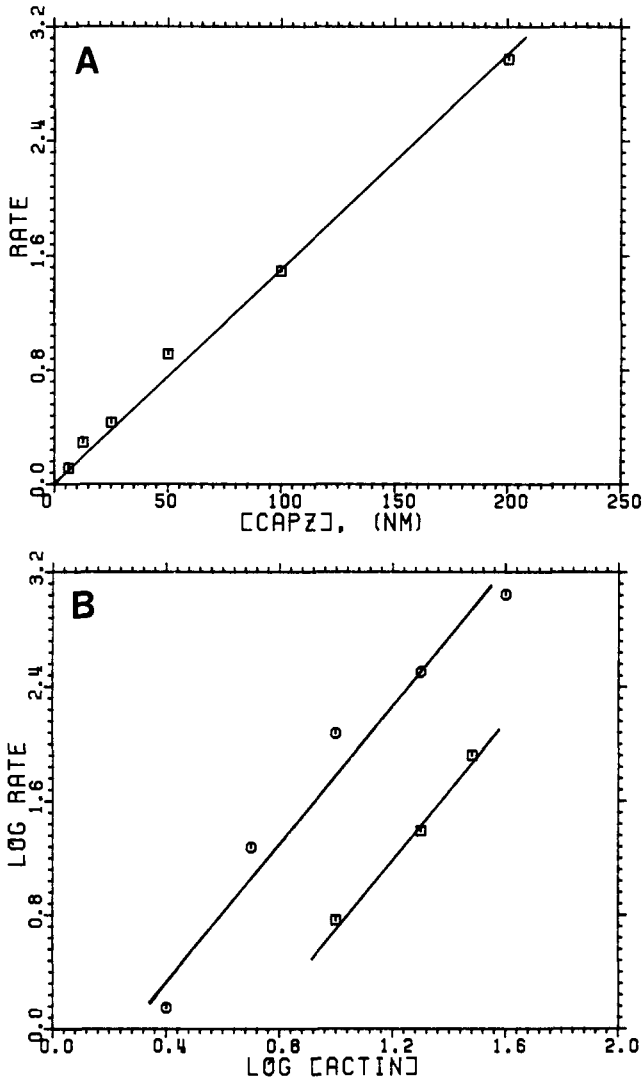

FIGURE 7: (A) Titration of CapZ on the initial rate of polymerization of Mg^{2+}-primed actin. The initial rate (arbitrary units) from curves such as in the previous figure is plotted vs CapZ concentration. CapZ was added at $t = 0$. (B) Titration of actin on the initial rate of polymerization induced by CapZ. The log of the maximal rate of polymerization (taken from data like those in Figure 6) is plotted vs the log of the actin concentration. The circles are Mg^{2+}–actin, primed with 200 μM $MgCl_2$, and polymerized by the addition of 50 nM CapZ and 1.8 mM $MgCl_2$ at $t = 0$. The squares are Ca^{2+}–actin, polymerized by the addition of 50 nM CapZ and 0.1 M KCl at $t = 0$.

actin. (If CapZ itself were a nucleus, resembling a pointed end of a filament, the rate would depend on the first power of the actin concentration.) This observation is consistent with models 1–3 and does not distinguish between them, because the proposed nucleus in the models contain one or two molecules of actin.

Binding of CapZ to Actin Monomers by FPR. To further test these three models, we measured the binding of CapZ to actin monomers in an FPR experiment. The assay was performed in the same polymerizing buffer used for the other experiments above to allow us to compare this binding to the binding of CapZ to barbed ends of filaments. Using a polymerizing buffer implies that the actin concentration must be low; otherwise, the actin would polymerize, and CapZ would bind tightly to filament ends.

In this experiment, the mobility (diffusion coefficient) of a low concentration (1 μM) of TMR-actin was measured in the presence and absence of a higher concentration (5.1 μM) of CapZ. In two different experiments, the recovery times for actin plus Cap Z were slightly greater than those for actin alone. In one experiment, the recovery times (τ_D, in milliseconds) were 75 ± 6 for actin alone and 85 ± 7 for actin with CapZ, and in another experiment 94 ± 8 for actin alone and 100 ± 9 for actin with CapZ. These data are the mean ± standard deviation with $N = 6$ in each case. The two experiments were performed on different days, but all the data in each experiment were collected within 1 day. In a Student's t test with two samples (actin alone and actin plus CapZ) and a one-tailed hypothesis (actin plus CapZ is greater than actin alone), the P values are between 0.01 and 0.025 for experiment 1 and between 0.1 and 0.25 for experiment 2. The difference between experiments 1 and 2 is most likely due to a difference in beam size, which varies slightly with the daily alignment of the laser. A 13% difference in beam radius would account for the observed difference in the recovery times.

Even though these differences were not statistically significant, the method would have been able to detect complex formation. A complex of CapZ with one actin (models 1 and 3) would have a molecular weight of 102 000 (42 500 ± 59 500). The diffusion coefficient is inversely proportional to the Stokes' radius, which depends on the shape of the complex. A minimum estimate for the Stokes' radius assumes a spherical shape. In this case, the ratio of the radius of the complex to that of free actin would be 1.34 (the cube root of the ratio of the molecular weight). Therefore, one would expect the FPR recovery time to increase by 34%. Such an increase would have been detected given the error in this experiment. Formation of a complex of one CapZ with two actin molecules (model 2) would lead to an increase of 50%, by a similar calculation.

To place a lower limit on the K_d for each model, we calculated the maximum bound fraction, based on the predicted diffusion coefficient for the spherical complex (which is a minimum estimate), using the maximum recovery time increase of 13% (Table II, third column).

Comparison of Binding Predicted by Polymerization with That Measured by FPR. Given the observed rates of CapZ-induced polymerization (from the data of Figure 7A), the elongation rate constants of the pointed end (Pollard, 1986), and the equilibrium constants for actin dimer and trimer formation (Frieden, 1983), one can calculate equilibrium constants for CapZ binding to actin in models 1–3 (Table II, second column). Comparing these values with the lower limits for the K_d from FPR (Table II, third column) shows that the data are consistent with all three models.

Table II: Comparison of the Equilibrium Constant (K_d) for the Binding of CapZ to Actin Calculated from the CapZ-Induced Polymerization Rate with That Calculated from the Negative FPR Binding Measurements[a]

model	K_D calcd from polymerization rate	K_D limit from FPR expt
(1) nondissociable dimer[b]	205 μM	>16 μM
(2) dimer capture[c]	100 pM	>20 pM
(3) monomer[b]	20 μM	>16 μM

[a] For the second column, using the rates measured in Figure 7A, the number of pointed ends created by CapZ was calculated by using the pointed-end elongation rate constants (Pollard, 1986). Then the K_d of CapZ for actin was calculated for each model, as required to produce that number of pointed ends. The third column is the limit on the equilibrium constant (K_d) for binding of CapZ to actin, based on the maximum possible effect detected by FPR. Given the maximum effect, the comparison does not preclude any of the three models. All the calculations assume that the nucleus size is a trimer (Cooper & Pollard, 1985), the K_d for dimer formation is 1 M, and the K_d for trimer formation is 5 μM (Frieden, 1983). [b] Values for binding of CapZ to actin monomer. [c] Values for binding of CapZ to actin dimer.

Table III: Assay of Severing Activity of CapZ by a Depolymerization Assay

addition	depolymerization rate (pM/s)
none (actin control)	34
gelsolin (10 nM)	934
gelsolin (5 nM)	97
CapZ (50 nM)	2.5
Gelsolin (10 nM) plus CapZ (50 nM)	988

Absence of Severing Activity of CapZ. To test whether CapZ can sever actin filaments, we performed a dilution assay, in which the rate of filament depolymerization is proportional to the number of filament ends. Proteins with severing activity create new filament ends and cause an increased depolymerization rate. Plasma gelsolin was used as a positive control. The data show no severing activity on the part of CapZ; 50 nM CapZ showed no increase in depolymerization rate, while 5 nM gelsolin had a measurable increase in rate (Table III). The depolymerization rate only decreases with CapZ, due to the capping of barbed ends. Gelsolin also caps barbed ends; the increased depolymerization rate is presumably due to an increased number of pointed ends.

To test whether gelsolin in fact remains bound to the barbed end of filaments that it creates by severing or dissociates to create a free barbed end, we added CapZ along with gelsolin in a depolymerization assay (Table III). Cap Z had no effect; the depolymerization rate was the same as that for gelsolin alone, which indicates that gelsolin does not dissociate from the barbed ends of actin filaments after it severs them.

Discussion

Interaction of CapZ with Actin in Vitro. CapZ binds tightly to barbed ends of actin filaments, with a K_d of approximately 0.5–1 nM or less measured in assays with filaments that are polymerizing, at steady state, or depolymerizing. The nucleotide bound to actin changes on polymerization, undergoing transitions from ATP to ADP-P_i to ADP (Korn et al., 1987). CapZ inhibits the growth of ends with ATP subunits slightly less well than it does for ends with ADP subunits.

Our preparation of CapZ conains no activity that caps pointed ends, as measured by growth from acrosomal processes and the critical concentration at steady state. Our preparation contains isoforms for both the α and β subunits, as also observed for brain capping protein (Kilimann & Isenberg, 1982). Although preparations of β-actinin, a proposed pointed-end capping protein, contain CapZ and isoforms (Oosawa et al., 1987; Funatsu et al., 1988), we find no evidence that our preparation of CapZ contains pointed-end capping activity.

The experiments on nucleation show that CapZ accelerates but does not bypass the nucleation process for actin polymerization, which agrees with our previous work with *Acanthamoeba* capping protein & Pollard, 1985). The use of CapZ has allowed us to perform additional types of experiments requiring larger amounts of pure protein. Two models in which CapZ bypasses the nucleation process are excluded. The model in which capping protein resembles the pointed end of a filament is excluded by the dependence of polymerization rate on actin concentration (Figure 7B), and the model in which capping protein severs actin filaments is excluded by the absence of severing activity in a direct assay (Table III). Three other models, each consistent with the *Acanthamoeba* capping protein data (Cooper & Pollard, 1985), are also consistent with the CapZ data in this report. These models involve CapZ binding relatively weakly to actin monomers or dimers and forming a nucleation site for actin polymerization. The CapZ data here do not exclude any of these models in which the normal process of actin nucleation is accelerated. The FPR experiment to measure CapZ binding to actin monomers or dimers was not able to choose between the models; however, a similar experiment with a more sensitive method might be successful.

Comparison of Muscle and Nonmuscle Capping Protein. In skeletal muscle, CapZ is associated with a stable array of actin filaments, but actin filaments in nonmuscle cells are dynamic, polymerizing and depolymerizing at various times and places. To understand the role of capping proteins in nonmuscle cells, especially to use the analog of the role of CapZ in muscle, a comparison of CapZ with nonmuscle capping protein is important. The results here with CapZ are similar to those published previously with brain and *Acanthamoeba* capping protein. The K_d for binding to the barbed end is 0.5 nM for brain capping protein (Wanger & Wegner, 1985) and for CapZ. The nucleating ability of CapZ is similar to that of *Acanthamoeba* capping protein by a rough comparison of these data with the previous data (Cooper & Pollard, 1985) although the experiments were not identical. Brain capping protein also nucleates actin polymerization (Kilimann & Isenberg, 1982). Kinetic modeling of the effect of *Acanthamoeba* capping protein on the time course of actin polymerization excluded a model in which capping protein severed actin filaments (Cooper & Pollard, 1985), and CapZ has no severing activity in a direct assay in this report. Both CapZ and brain capping proteins have isoforms of both the α and β subunits (Kilimann & Isenberg, 1982).

Implications of the in Vitro Mechanisms for Cells. These experiments on the mechanism of action of CapZ in vitro suggest interesting speculations about the role of capping protein in muscle and nonmuscle cells. In muscle cells, CapZ should cap the barbed ends of the actin filaments at the Z disks since the in vitro binding is tight. Capping the barbed ends ensures that the actin filaments in the sarcomere have the correct polarity for interacting with myosin to produce force. Since CapZ can nucleate filament formation in vitro, it may also do so in vivo during myofibrillogenesis. If CapZ is bound to the nascent Z disk, it could nucleate actin filament assembly, ensuring the proper location and polarity of the filaments in the sarcomere.

In nonmuscle cells, the observation that capping protein binds tightly to barbed ends in vitro implies that all the barbed ends would be capped if the concentration of capping protein is sufficient. If the capping protein concentration is greater than the concentration of barbed ends, the additional capping protein would be free in the cytoplasm. On the basis of the in vitro experiments, free capping protein would not sever actin filaments. One interesting question is whether free capping protein would nucleate the formation of new filaments, to which it would remain bound at the barbed end. The common feature of the nucleation models is that capping protein does not bind actin monmers tightly, as does gelsolin; therefore, the excess capping protein may remain free. The key unknown variable is the actin monomer concentration. The actin monomer concentration should equal the critical concentration of the pointed end, if all the barbed ends are capped and the pointed ends are not. However, if the pointed ends are also capped, by some currently undescribed mechanism, then the actin mononer concentration could be much higher. Since actin monomers are probably stored in pools bound to profilin or the depactin/ADF/actophorin family, release of the stored actin could temporarily raise the actin monomer concentration and promote nucleation. If nucleation is promoted, by raising

the monomer concentration, it should occur in association with capping protein since self-nucleation of actin is very unfavorable.

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