Acanthamoeba castellanii Capping Protein: Properties, Mechanism of Action, Immunologic Cross-reactivity, and Localization

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ABSTRACT We report further characterization of the physical and immunologic properties, mechanism of action, and intracellular localization of Acanthamoeba castellanii capping protein, an actin regulatory protein discovered by Isenberg (Isenberg, G., U. Aebi, and T. D. Pollard, 1980, Nature (Lond.) 288:455-459). The native molecular weight calculated from measurements of Stokes’ radius (3.8 nm by gel filtration chromatography) and sedimentation coefficient (4.8 S by sucrose gradient velocity sedimentation) was 74,000 daltons. The subunit molecular weights were 31,000 and 28,000 daltons, so the native molecule is a heterodimer. The two subunits did not immunologically cross-react with each other or with any other proteins from Acanthamoeba or several other organisms.

In studies of the mechanism of action, Isenberg (see above reference) found that capping protein blocked polymerization from the barbed end of actin filaments and sedimented with actin filaments. We confirmed that capping protein binds to actin filaments with a gel filtration assay. Capping protein decreased the length distribution and high shear viscosity of actin filaments. Capping protein did not bundle or cross-link actin filaments. Low concentrations of capping protein increased the critical concentration for muscle and ameba actin polymerization from 0.1 to 0.6 μM in Mg++ and EGTA. Increasing amounts of capping protein did not increase the critical concentration further. In Ca++ capping protein did not change the critical concentration for muscle actin, but did increase the critical concentration for ameba actin. Ca++ had no effect on the ability of capping protein to decrease the low or high shear viscosity of actin filaments.

By indirect fluorescent antibody staining, capping protein was localized to the cell cortex, an area rich in actin filaments. During subcellular fractionation of homogenates, about ⅓ of cellular capping protein banded with a crude membrane fraction. The other ⅔ of cellular capping protein was soluble, with a Stokes’ radius equal to that of the purified protein. The molar ratio of capping protein to actin in the cell was 1:150.

MacLean-Fletcher and Pollard (28) observed a novel activity: a decrease in low shear viscosity of actin filaments in chromatographic profiles of extracts of Acanthamoeba castellanii. Using the falling ball viscometer to measure this activity, Isenberg et al. (23) purified a protein, which was called capping protein because of its ability to inhibit the addition of monomers to the barbed end of actin filaments, and thus capping the ends. Proteins from various sources with similar activity have also been purified (19, 47, 14, 8, 21, 33, 25, 46; for a review see reference 13). Available evidence indicates that all these proteins resemble cytochalasin in their ability to cap the barbed end of actin filaments (9, 7, 27, 29). There are important structural and functional differences among these proteins, however. The molecular weights, subunit composition, and immunologic cross-reactivity are similar for some proteins, especially those from different mammalian tissues, but different for others, especially from evolutionarily distant species. Some are sensitive to calcium, and some have other
activities, such as filament bundling, filament shortening, and nucleation of polymerization, which may or may not be explained by the interaction of a protein with barbed ends of filaments. We report our continued studies of the properties and mechanism of action of capping protein from *Acanthamoeba*.

The functional significance of these proteins in cells is also uncertain. The effect of these proteins on the length of filaments and on the low shear viscosity of filament networks has led to speculation that they may regulate locally the rheological properties of cytoplasm. The binding of the proteins to barbed ends has led to speculation that they may anchor actin filaments to various cell structures, such as membranes. Their ability to promote the nucleation of actin filaments has suggested that they may locally control the number of actin filaments in the cytoplasm, and their ability to bundle actin filaments has suggested a role in the formation and structure of actin filament bundles, such as those found in intestinal microvilli. The exact role of a protein in vivo is a difficult question that we have approached with two experiments. First, we localized capping protein in cells using fluorescent antibodies, and second, we examined the subcellular fractionation of capping protein.

Some of these results were presented in preliminary form at the 1981 and 1982 meetings of the American Society for Cell Biology (11, 41).

**MATERIALS AND METHODS**

Excerpt as noted below, chemicals and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO), and solvents and supplies were purchased from VWR Scientific (Baltimore, MD).

**Buffers**

**Buffer A:** 2 mM Tris/HCl, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl₂, 0.2 mM dithiothreitol. Capping protein buffer: 50 mM KCl, 10 mM imidazole/HCl, pH 7.5, 0.01% (wt/vol) NaN₃. PBS: 150 mM NaCl, 10 mM Na phosphate, pH 7.5, 0.01% (wt/vol) NaN₃. Get-PBS: PBS with 0.2% (wt/vol) gelatin (Knox Gelatin Co., Englewood Cliffs, NJ). TTX-BSA: 0.1% (wt/vol) Triton X-100, 0.1% (wt/vol) BSA (Miles Laboratories, Elkhart, IN), 150 mM NaCl, 10 mM Tris/HCl, pH 7.8, 0.02% (wt/vol) NaN₃.

**Viscometry**

**HIGH SHEAR:** Glass capillary viscometers (100) (Cannon, State College, PA) were suspended in a 25°C water bath. Measurements were made every 2 min and the buffer outflow time was 60 s. The techniques of operation and cleaning and the viscosity calculations have been described (10).

**LOW SHEAR:** Apparent viscosity of actin filament solutions at low shear was measured with a falling ball viscometer (28, 35). The usual conditions were 9.4 A₅₅₀ gel-filtered actin, 50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 10 mM imidazole/HCl, pH 7.0, 0.2 mM ATP. For routine assays, the volume was 200 μl, 5 μl of a column fraction was included, and samples were incubated at 25°C for 10 min.

**Electron Microscopy for Actin Filament Length**

Electron microscopy was performed essentially as described (10). Filament solutions were diluted and applied to glow-discharged, carbon-coated Formvar films on copper grids. Grids were negatively stained with 1% (wt/vol) uranyl acetate, and micrographs at ×16,000-50,000 magnification were taken with a Zeiss 10A electron microscope. The total length of filaments was measured with a map reader, and the number of filament ends was counted. The number of average filament length was calculated as total length divided by one-half the number of ends. (24).

**Purification and Critical Concentration Measurements of Actin**

Actin was prepared from rabbit skeletal muscle by a modification of the procedure of Spudich and Watt (38), with one cycle of polymerization and depolymerization from 0.8 M KCl. Conventional actin was chromatographed on Sephadex G150 in buffer A (30). *Acanthamoeba* actin was prepared by the method of Gordon et al. (20) with two cycles of polymerization and depolymerization before gel filtration.

The preparation and documentation for the use of pyrene-labeled actin has been fully described (26, 12). Pyrene actin had the same polymerization properties as native actin. Fluorescence was measured in a Perkin-Elmer 650-10S fluorometer (Perkin-Elmer Corp., Norwalk, CT) with excitation at 365 nm and emission at 407 nm. Temperature was controlled at 25°C with a circulating water bath.

Critical concentration for actin polymerization was measured as the inflection point of fluorescence vs. total actin concentration. A set of samples was prepared with various actin concentrations and one capping protein concentration. The samples were incubated overnight to allow polymerization to reach steady state, then fluorescence was measured. The mole fraction of pyrene actin to total actin varied from 0.05 to 0.3.

**Antibody Preparation**

Capping protein was reacted with dansyl chloride (Pierce Chemical Co., Rockford, IL) and electrophoresed on 10% SDS polyacrylamide gels. The individual 28,000 and 31,000-dalton bands were sliced from the gel, and the polypeptides were electrophoretically eluted (39). Two New Zealand rabbits were immunized with 28,000-dalton polypeptide and one with 31,000-dalton polypeptide, as described by Fujiwara and Pollard (17). The rabbits were boosted and bled. Immunoglobulins were prepared from serum by ammonium sulfate precipitation at 37% followed by DEAE-cellulose chromatography. To prepare affinity-purified antibodies for staining cells, purified capping protein was conjugated to Sepharose 4B which had been activated by cyanoend bromide (34). Immunoglobulins were applied to the column in PBS, the column was washed with PBS, and the specific antibodies were eluted with 0.1 M glycine pH 2.8.

**Immunoblots**

Sample was electrophoresed on 14% SDS polyacrylamide gels cross-linked with diallyltartardiamide (18). The gel was electrophoretically blotted onto nitrocellulose paper (Schleicher & Schuell, Keene, NH) (40). The paper was incubated with TTX-BSA, antisera or antibodies diluted in TTX-BSA, and finally ¹²⁵I-Protein A (100,000 cpn/ml).

To prepare affinity-purified antibodies for immunoblots, capping protein subunits were separated by SDS PAGE and blotted onto nitrocellulose paper. The paper was stained with amido black to visualize the protein, and capping protein subunit bands were cut out of the paper. Immunoglobulins were applied to the strips of paper, the strips were washed, and specific antibodies were eluted with 0.1 M glycine pH 2.8.

**Imunoassay for Capping Protein**

Dr. Daniel Kiehart developed this solid phase binding assay, based on the procedure of Tsu and Herzenberg (42). Antibodies were diluted with 5 mM imidazole/HCl, pH 7.0, and 50 μl of a 100-fold dilution of rabbit antiprotein serum in TTX-BSA. The wells were rinsed with PBS, and incubated with 0 μl of a 100-fold dilution of rabbit anticapping protein serum in TTX-BSA. The wells were rinsed with TTX-BSA, incubated with 25 μl of ¹²⁵I-Protein A (1,000 cpn/μl) in TTX-BSA, rinsed again, and counted in a Beckman Gamma 4000 (Beckman Instruments, Inc.). In the subcellular fractionation experiments a more quantitative variation of the assay was used. 5 μl of serial dilutions of antigen in PBS with 0.1% (wt/vol) Triton X-100 with or without 0.02% (wt/vol) SDS was applied to a 1 x 1 cm piece of nyloncellulose paper. The paper was treated with antibodies and ¹²⁵I-Protein A as described above.

**Velocity Sedimentation through Sucrose Gradients**

0.1 ml of capping protein alone or with enzymes of known sedimentation coefficient including catalase, yeast alcohol dehydrogenase, and lysosome was sedimented through 5 ml linear 5-20% (wt/vol) sucrose gradients at 4°C (31).
The centrifugation was at 39,000 rpm in a Beckman SW50.1 rotor (Beckman Instruments, Inc.) for 18 h. 0.1-ml fractions were collected and analyzed for enzyme assays (44, 4, 37) and for capping protein with an immunoassay. Plots of sedimentation coefficient vs. fraction number were linear for the standards, and the sedimentation coefficient of capping protein was interpolated from this plot.

Localization of Capping Protein and Actin Filaments with Fluorescence Microscopy

**ADHERENCE OF CELLS TO GLASS COVERSLEIPS:** Glass coverslips were boiled in Alconox and cleaned with ethanol. They were coated with a 0.1% (wt/vol) solution of polylysine, washed with water, and allowed to dry. On the day of the experiment these coverslips were treated with 1% (wt/vol) glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in water for 30 min and washed with water.

*Acanthamoeba castellani* were removed from an aerated suspension culture at early log phase density. Cells were washed from their growth medium into 50 mM NaCl, 20 mM Na phosphate, pH 7.0, and resuspended in a small volume of the same buffer. Cells in suspension were allowed to settle onto the glutaraldehyde-treated, polylysine-coated coverslips for 60 min.

**FIXATION AND PERMEABILIZATION:** Cells adhering to the coverslips were fixed with 2% (wt/vol) formaldehyde (prepared daily from solid paraformaldehyde) in 50 mM NaCl, 20 mM Na phosphate, pH 7.0, for 10 min at room temperature and washed with the same buffer without formaldehyde. To permeabilize the cells, coverslips were dipped into acetone at -20°C for 30 s and immediately transferred to 1 mM ethanolamine in Gel-PBS for 5 min, which quenched the formaldehyde. The permeabilized cells were washed with Gel-PBS.

**ANTIBODY STAINING:** Permeabilized cells were incubated with 50 µg/ml of either affinity-purified rabbit anticaPP protein (28,000 daltons) or immunoglobulins from preimmune serum from the same rabbit in Gel-PBS containing 0.1 mM phenylmethylsulfonylfluoride, and 0.1 mM benzamidine with 10 mM NaCl and homogenized with 1.5 vol of 10% (wt/vol) sucrose, 0.1M KCl, 20 mM imidazole/HCl, pH 7.5, 1 mM ATP, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM benzamidine with 10 vigorous strokes of a tight-fitting Dounce homogenizer on ice. The homogenate was centrifuged at 2,000 g for 5 min to remove whole cells and nuclei. The supernatant was centrifuged at 100,000 g for 90 min in a Beckman Ti 42.1 rotor (Beckman Instruments, Inc.) to pellet membranes. The pellet was suspended in homogenization buffer and layered on top of a 30-ml linear gradient of 30 to 65% (wt/vol) sucrose in 0.1 M KCl, 20 mM imidazole/HCl, pH 7.0, and 0.01% (wt/vol) NaN3. The tubes were centrifuged in a Beckman SW 27 rotor (Beckman Instruments, Inc.) at 25,000 rpm for 18 h. 2-ml fractions were collected and analyzed for protein (6) capping protein with the immunoassay, S'-nucleotidase (45) and β-N-acetyl glucosaminidase (22).

**Protein Determination**

Protein was measured by the method of Bradford (6) with egg albumin as standard.

**Abbreviation used in this paper:** NBD; 7-nitrobenz-2-oxa-1,3-diazole.

### RESULTS

**Purification of Capping Protein**

The purification of capping protein was followed by a functional assay, decrease of the low shear viscosity of actin filaments (Fig. 1), an immunoassay using specific antiserum against the 28,000 and 31,000-dalton polypeptides (Fig. 1), and SDS PAGE (Fig. 2). The functional activity coincided with the immunoassay in the several chromatography steps employed (Fig. 1).

*Acanthamoeba* were grown in aerated suspension cultures, homogenized, and centrifuged, and the supernatant was chromatographed on DEAE-cellulose (Fig. 1A) essentially as described by Maruta et al. (32). Fractions containing activity were pooled, concentrated fivefold by dialysis against dry sucrose, and chromatographed on Sephacryl S-300 (Fig. 1B). The peak fractions were pooled and chromatographed on hydroxylapatite with a concave 0–0.2 M phosphate gradient (Fig. 1C). The peak fractions were pooled and chromatographed on phosphocellulose with a linear 0–0.3 M KCl gradient (Fig. 1D). This procedure was modified in several ways from the previous one (23). Changes included homogenization conditions, DEAE conditions, absence of ammonium sulfate precipitation, Sephacryl S-300 instead of Sephadex G-150, and addition of phosphocellulose chromatography. These changes, notably phosphocellulose chromatography, improved both the purity and yield of the capping protein.

The polypeptide composition of pools from a successful preparation is shown in Fig. 2. In this case, the final material contained only the two capping protein bands and a trace amount of proteolytic fragment of the 28,000-dalton band with a mobility of 30,000 daltons. The final material from a different successful preparation is shown in Fig. 3, lane f. Only the two capping protein bands were present in this preparation. A plot of the log of molecular weight vs. mobility of the standards, excluding the 200,000-dalton standard, was linear. By interpolation from this plot the molecular weights of the capping protein subunits were 28,000 and 31,000 daltons. The error was estimated to be ± 2,000 based on the scatter of the standard points away from the line. No other bands were detected on SDS polyacrylamide gels stained with Coomassie Blue loaded with up to 50 µg of protein. Experiments were performed with material of this quality. The average yield was ~1 mg of pure protein from 500 g of wet packed cells. The pure material had an E280/4 of 8 based on the Bradford protein assay. For unknown reasons, preparations often yielded material of less purity (about ~20 to 80% capping protein), but this material was not used for experiments.

**Physical Properties**

The Stokes’ radius of capping protein was 3.8 nm from the partition coefficient on Sephacryl S-300. Five proteins of known Stokes’ radius were chromatographed, and the Stokes’ radius of capping protein was calculated (1). The sedimentation coefficient of capping protein was 4.8 S by velocity sedimentation through a sucrose gradient (31). From these two measurements, the native molecular weight was calculated using the Svedberg equation, assuming that (a) partial specific volume was 0.72 cm3/g, (b) density was 1.0 g/cm3, and (c) viscosity was 0.01 poise. The calculated native molec-
FIGURE 1 Chromatograms of purification procedure. (A) DEAE cellulose chromatography. Absorbance at 295 nm (solid line), low shear apparent viscosity of actin filaments (○), and KCl concentration (dashed line) are plotted vs. fraction number in the major portion of the graph. In the upper panel counts per minute from the capping protein immunoassay is plotted vs. fraction number. The procedure was essentially that of Maruta et al. (32). Half of the proteose peptone in the culture medium was replaced with yeast extract (Difco Laboratories, Inc., Detroit, MI). About 700 g of wet packed cells was homogenized with 2 vol of 75 mM KCl, 12 mM Na pyrophosphate, 5 mM dithiothreitol, 30 mM imidazole/HCl, pH 7.0, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine in a Dounce homogenizer on ice. The homogenate was spun in a Beckman Ti45 rotor at 40,000 rpm for 70 min. The supernatant solution was adjusted to pH 8.0 with 1 M Tris and dialyzed overnight against 20 vol of 7.5 mM Na pyrophosphate, 0.5 mM dithiothreitol, 10 mM Tris/HCl, pH 8.0. The supernatant solution was then applied to a 5 by 60 cm-column of DEAE-cellulose (DE52, Whatman Chemical Separation, Inc., Clifton, NJ) equilibrated with 10 mM KCl, 1 mM dithiothreitol, 10 mM Tris/HCl, pH 8.0, and eluted with a KCl gradient to 0.4 M, followed by a 1 M wash. 10 peak fractions with 20 ml each were pooled. (B) Gel filtration chromatography. Absorbance at 280 nm (●), low shear apparent viscosity in poise (○), and counts per minute × 10^3 from the capping protein immunoassay (△) are plotted vs. fraction number. The pool from the DEAE column was concentrated and applied to a 4 by 100 cm Sephacryl S-300 column equilibrated with capping protein buffer. For this profile the void volume was fraction 21, and the total volume was fraction 80. Seven peak fractions with 15 ml each were pooled. (C) Hydroxylapatite chromatography. Absorbance at 280 nm (●), low shear apparent viscosity in poise (○), and counts per minute × 10^3 from the capping protein immunoassay (△) on the left ordinate and phosphate concentration (dashed line) on the right ordinate are plotted vs. fraction number. The pool from the gel filtration column was applied to a 1.5 by 15-cm column of hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) equilibrated with capping protein buffer and eluted with a concave phosphate gradient of 400 ml to 0.25 M. Five peak fractions with 6 ml each were pooled. (D) Phosphocellulose chromatography. Absorbance at 280 nm (●) and KCl concentration (dashed line) on the right ordinate and low shear apparent viscosity in poise (○) and counts per minute × 10^3 from the capping protein immunoassay (△) on the left ordinate are plotted vs. fraction number. The pool from the hydroxylapatite column was dialyzed against 10 mM Na succinate, pH 5.5, and applied to a 2 by 10-cm column of phosphocellulose (P1, Whatman Chemical Separation, Inc.) equilibrated with the same buffer. The column was eluted with a 200-ml linear gradient to 0.25 M KCl. Three peak fractions with 4 ml each were pooled.

Antibody Cross-reactivity

In immunoblot experiments on whole cell proteins and pure capping protein, affinity-purified antibodies to the 28,000-dalton polypeptide reacted only with a 28,000-dalton band. Similarly, affinity-purified antibodies to the 31,000-dalton polypeptide reacted only with a 31,000-dalton band (Fig. 3). The mobility of the bands was the same in the whole cell and capping protein samples. Neither antibody recognized any protein of another molecular weight in Acanthamoeba (Fig. 3). Neither antiserum recognized any protein in Astrotynus, Physarum, Drosophila, PTK cells, or chicken heart, gizzard, breast muscle, or anterior latissimus dorsi muscle. Dr. Gerhard Isenberg, working in our laboratory, found that neither antiserum recognized any protein in Dictyostelium, sea urchin eggs, or calf brain.

Capping Protein Binding to Actin Filaments

Binding of capping protein to Acanthamoeba actin filaments was detected by gel filtration (Fig. 4). Actin filaments eluted at the void volume. Capping protein alone eluted at
FIGURE 2 SDS PAGE of capping protein pools at each step in the preparation. The lanes are as follows: (a) high speed supernatant of homogenate; (b) DEAE column pool; (c) S-300 column pool; (d) hydroxylapatite column pool; (e) phosphocellulose column pool. Molecular weight standards in kilodaltons are shown on the left. The gel contained 14% (wt/vol) acrylamide and was stained with Coomassie Blue. DF, dye front.

the back of the column profile, near the total volume. When actin filaments and capping protein were mixed together, some capping protein, measured by immunoassay, eluted at the void volume with the actin filaments. Since actin has a low affinity for immunoglobulins (16), we confirmed that the immunoreactive material in the void volume was capping protein by immunoblots of the fractions. Similar results were obtained with muscle actin.

Effect of Capping Protein on Actin Filament Length and High Shear Viscosity

Muscle actin filaments polymerized in the presence of capping protein were shorter and had a lower high shear viscosity than filaments polymerized in the absence of capping protein (Table I). The activity was observed in the presence and absence of Ca++. No filament bundles were observed by electron microscopy of samples of actin filaments with capping protein.

Effect of Capping Protein on Critical Concentration

In the presence of Mg++ and EGTA, capping protein increased the critical concentration for muscle and ameba actin from 0.1 to 0.6 μM (Table II). With increasing concentrations of capping protein, the critical concentration did not increase further. In the presence of Ca++, capping protein had no effect on the critical concentration of muscle actin but increased the critical concentration of ameba actin from 1.2 to 1.6 μM.

In these experiments critical concentration was determined as the inflection point of a plot of fluorescence from pyrene actin vs. total actin concentration. Capping protein did not change the slope of either the monomer or polymer phases of these plots.
subjected to viscometry. The divalent cation concentrations are concentration minus critical concentration) divided by mean filament length for each sample is listed in the table, but the total length of filaments is not. Filament number concentration was calculated as (total actin concentration - critical concentration) divided by mean filament length. Samples for electron microscopy were not previously subjected to viscometry. The divalent cation concentrations are shown in the table. Other conditions were: 2 mM MgCl₂ and 1 mM EGTA; filled symbols, 0.2 mM CaCl₂, Other conditions, 12.5 μM actin, 0.1 M KCl, 10 mM imidazole/HCl, pH 7.0, 0.2 mM ATP, 50% (vol/vol) buffer A, 25°C.

### Table I

| Divalent cation | Capping protein | Filament-reduced viscosity (nM) | Filament number concentration (nM) | Mean filament length (μm) | Total No. of counted ends |
|----------------|----------------|-------------------------------|-----------------------------------|--------------------------|--------------------------|
| 2 mM MgCl₂     | 0              | 1.09                          | 1.1                               | 10.0                     | 38                       |
| 1 mM EGTA      | 7              | 1.02                          | 5.4                               | 1.6                      | 500                      |
| 40             | 0.65           | 14                            | 0.63                              | 338                      |
| 260            | 0.54           | 27                            | 0.32                              | 321                      |
| 0.2 mM CaCl₂   | 0              | 1.17                          | 0.6                               | 12.5                     | 69                       |
| 7              | 0.87           | 3.2                           | 2.5                               | 295                      |
| 40             | 0.65           | 8.5                           | 0.94                              | 244                      |
| 260            | 0.64           | 7.5                           | 1.07                              | 241                      |

Muscle actin was polymerized overnight in the presence of capping protein. Viscosity was measured in an Ostwald capillary viscometer. Mean filament length was determined by dividing the total length of filaments on micrographs by one-half the number of ends (24). The mean filament length and the total number of ends counted for each sample is listed in the table, but the total length of filaments is not. Filament number concentration was calculated as (total actin concentration minus critical concentration) divided by mean filament length. Samples for electron microscopy were not previously subjected to viscometry. The divalent cation concentrations are shown in the table. Other conditions were: 2 mM MgCl₂ and 1 mM EGTA; filled symbols, 0.2 mM CaCl₂, Other conditions, 12.5 μM actin, 0.1 M KCl, 10 mM imidazole/HCl, pH 7.0, 0.2 mM ATP, 50% (vol/vol) buffer A, 25°C.

### Table II

| Capping protein | Divalent cation | Mg²⁺/ EGTA | Mg²⁺/ EGTA | Mg²⁺/ EGTA | Ca²⁺ | Ca²⁺ | Ca²⁺ |
|-----------------|----------------|------------|------------|------------|------|------|------|
| Actin           | Muscle         | Muscle     | Muscle     | Muscle     |      |      |      |
| 0               | 0.12           | 0.13       | 0.12       | 0.78       | 0.77 | 1.2  |
| 7               | 0.55           | 0.66       | 0.72       | 0.78       | 0.86 | 1.8  |
| 40              | 0.60           | 0.67       | 0.66       | 0.76       | 0.84 | 1.6  |
| 260             | 0.61           | 0.65       | 0.66       | 0.77       | 0.82 | 1.6  |

Critical concentration (listed in micromolar) was measured at steady state using fluorescence of pyrene actin. The conditions were the same as described in Table I. Each column is a separate, single experiment.

### Effect of Capping Protein on Low Shear Viscosity

Capping protein decreased the low shear viscosity of both muscle and ameba actin with a similar but not identical concentration dependence in the presence and absence of Ca²⁺ (Fig. 5). In this experiment capping protein was present at the start of actin polymerization. Capping protein had a similar effect when mixed with preformed filaments.

### Localization of Capping Protein and Actin Filaments in Cells

Actin filaments were localized in fixed and permeabilized Acanthamoeba using NBD-phallacidin, a specific probe for actin filaments (3) (Fig. 6a). Fluorescence was most intense over the hyaline ectoplasm, a peripheral portion of the cytoplasm devoid of granules by phase microscopy and known to be rich in actin filaments (36). Filopodia, which contain actin filament bundles by electron microscopy (36), were also fluorescent.

Indirect fluorescent antibody staining with specific affinity-purified rabbit antibodies against the 28,000-dalton subunit gave a pattern of fluorescence (Fig. 6c) similar to that observed with NBD-phallacidin (Fig. 6a). The hyaline ectoplasm and filopodia were more fluorescent than the rest of the cell. High concentrations of affinity-purified anticapping protein were required to observe specific fluorescent staining. These high concentrations, perhaps necessitated by the low concentration of capping protein present in cells, were responsible for some nonspecific staining of central cytoplasm and certain vacuoles.

### Subcellular Fractionation of Capping Protein

A standard subcellular fractionation procedure was applied to Acanthamoeba to determine if any capping protein was associated with membranes, specifically the plasma membrane. Using a more quantitative variation of the immunoassay for capping protein, we found that about ½ of total cell capping protein pelleted in a high speed centrifugation. Gel filtration of the supernatant on Sephacryl S-300 showed that capping protein, detected by immunoassay, had a partition coefficient of 0.417. Pure capping protein had a partition coefficient of 0.414 on the same column. This centrifugation pelleted membranes and should have pelleted long actin filaments. Sucrose density gradient analysis of the membrane pellet showed that capping protein sedimented to a density identical to that of 5'-nucleotidase, a plasma membrane marker (43). Free protein would not have sedimented to this position. This marker was, however, not well separated from the peak of beta-N-acetyl glucosaminidase, a lysosomal marker, and the major protein peak. Electron microscopy of thin sections of material at this density revealed mitochondria, closed vesicles that were probably lysosomes, and closed...
FIGURE 6 Localization of actin filaments with NBD-phallacidin and capping protein with fluorescent antibodies. Pairs of phase contrast and fluorescence micrographs are shown. (a) NBD-phallacidin. The hyaline ectoplasm, an area of the cell under the plasma membrane which is devoid of granules by phase microscopy, is intensely fluorescent. Filopodia, spikelike projections from the cell surface, are also stained (inset). Fluorescence is also observed throughout open membrane sheets some of which were associated with thin filaments and amorphous material (data not shown).

**Measurement of Total Cell Capping Protein**

Immunoblots were used to estimate the total amount of capping protein in cells. Various dilutions of whole cells and capping protein were electrophoresed in different lanes of one SDS polyacrylamide gel and blotted onto nitrocellulose paper. The paper was treated with anticapping protein antibodies and ¹²⁵I-Protein A. The intensity of the bands on an autoradiogram were compared to determine which dilution of whole cells corresponded to which amount of capping protein. In two separate experiments 1 g of wet packed cells contained 120 µg of capping protein. Since 1 g of wet packed cells contained 100 mg of protein by the Bradford assay, capping protein represented ~0.1% of total cell protein. This measurement shows that the yield of the purification procedure was ~4% of the total capping protein. In data presented by Gordon et al. (20), actin represented ~10% of total cell protein, based on the Coomassie Blue stain associated with the 42,000-dalton peak on an SDS-polyacrylamide gel of a whole cell sample. Therefore we estimate that the molar ratio of actin to capping protein in whole cells was ~150 to 1.

**DISCUSSION**

**Mechanism of Action**

The previous work on *Acanthamoeba* capping protein (23) suggested that capping protein inhibited the addition of actin monomers to the barbed end of actin filaments by binding to that end. The new observations reported here support this mechanism.

First, we asked whether capping protein bound to actin filaments. In rapid gel filtration of a mixture of capping protein with actin, capping protein eluted in the void volume with actin filaments instead of in its usual position near the back of the profile. Therefore, capping protein either bound to the filaments or formed some other large complex with actin. The exclusion limit of the chromatography material was ~22 nm, based on the results of Davis and Bennett (15). In this experiment not all of the capping protein eluted in the void volume, and in our initial experiments with longer columns and lower flow rates, there was no capping protein in the void volume. Our interpretation is that the affinity constant for binding was low enough that the complex fell apart as it moved down the column. Unfortunately, quantitative measurement of binding constants is not possible with this type of experiment (1).

Second, capping protein lowered the length distribution and, as a consequence, the high shear viscosity of actin in the central cytoplasm but not in vacuoles or the nucleus. (b) A control where NBD-phallacidin was incubated with actin filaments, the filaments were sedimented, and cells were stained with the supernatant. No fluorescence was observed. In another control (not shown) excess phalloidin (10 µM) was added to the NBD-phallacidin (0.33 µM) before staining cells. No fluorescence was observed. (c) Anti-capping protein antibodies. The hyaline ectoplasm is intensely fluorescent. The central cytoplasm and certain vacuoles are mildly fluorescent, but these regions are also lightly stained in a control experiment (d) with preimmune immunoglobulins in place of anti-capping protein. The nucleus and most cytoplasmic vacuoles are not fluorescent. Bar, 10 µm.
filaments. At low capping protein concentrations the number of filaments was similar to the concentration of capping protein. The filament length distribution at equilibrium is a complex function of the rates of nucleation, fragmentation, and annealing. Unfortunately, there is no mathematical method to quantitatively relate the filament length distribution with the capping of ends. A shorter filament length distribution might be caused by an increased nucleation rate, increased fragmentation rate, or decreased annealing rate. Although these experiments do not distinguish among these possibilities, a decrease of the annealing rate could be caused by the binding of capping protein to barbed ends of filaments.

Third, capping protein increased the critical concentration for actin polymerization. Two specific characteristics of this increase support the capping mechanism. In Mg"⁺ and EGTA the critical concentration for muscle and ameba actin increased from 0.1 to 0.6 μM but not above 0.6 μM when more capping protein was added. This feature rules out a mechanism where capping protein acts as a monomer-binding protein. The values of the critical concentrations are those predicted by assuming that capping protein prevents monomer association and dissociation at the barbed end of actin filaments. Bonder et al. (5) found that in Mg"⁺ and EGTA the critical concentrations at the barbed and pointed ends were 0.1 and 0.6 μM, respectively. The critical concentration in the absence of capping protein (0.1 μM) should be near that of the barbed end (0.1 μM) since the rate constants are much higher at the barbed than at the pointed end. The critical concentration in the presence of capping protein (0.6 μM) should be that of the pointed end (0.1 μM) if capping protein prevents both monomer addition and loss at the barbed end. Bonder et al. (5) found that the critical concentrations were the same at the two ends for muscle actin in the presence of Ca"⁺, and accordingly in our experiments capping protein did not increase the critical concentration for muscle actin in Ca"⁺. Capping protein did increase the critical concentration for ameba actin in Ca"⁺. In Ca"⁺ the critical concentration and polymerization kinetics of Acanthamoeba actin are different from those of muscle actin, so this difference is not surprising.

In the original report on capping protein (23), capping protein was observed to decrease the amount of actin that sedimented in an ultracentrifugation experiment. This result was interpreted to mean that capping protein increased the critical concentration to 3.2 μM. This experiment gave similar results with the pure material described here, but in light of our critical concentration measurements, capping protein must have caused less actin to sediment by making shorter filaments instead of more monomers. The supernatant from such an experiment did contain short filaments observed by electron microscopy.

Experiments with Cells

These experiments with pure proteins have shown that capping protein can bind to the barbed end of actin filaments and decrease the filament length distribution. These results suggest that capping protein may function in cells to bind actin filaments via their barbed ends to a structure, such as the plasma membrane, and control the number and length of actin filaments that might affect cytoplasmic structure and cell motility. As a first step in the investigation of the role of capping protein in cells, we localized capping protein in cells...
higher molecular weight in Acchanthamoeba. Also, anti-capping protein antibodies did not recognize any protein in whole cell sample of other organisms. Some proteins with activities similar to that of capping protein are sensitive to calcium (19, 47, 14, 8, 21, 33, 46). The activity of capping protein to decrease the low and high shear viscosity of actin filaments was not sensitive to calcium. Capping protein from bovine brain (25) is similar to Acchanthamoeba capping protein in its subunit stoichiometry and molecular weights and its lack of sensitivity to calcium.

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