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# Kinetics of the Interaction of a 41-Kilodalton Macrophage Capping Protein with Actin: Promotion of Nucleation during Prolongation of the Lag Period†

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ABSTRACT: A 41-kilodalton macrophage capping protein (MCP) has been isolated which is capable of forming complexes with actin monomers in addition to capping the barbed ends of actin filaments (Southwick & DiNubile, 1986). The protein is calcium activated in a fully reversible manner. Using kinetic assays, we determined a capping constant, defined here as a modified $K_d$, of 1 nM and a $K_d$ of 3-4 $\mu$M for MCP-actin monomer complex formation. MCP weakly nucleates actin polymerization: more than 0.5 $\mu$M MCP is necessary to shorten the lag period, and 1 $\mu$M MCP at an actin/MCP ratio of 10 reduces the average length of actin filaments to about 200 molecules per filament. We determined that the actin nucleus that survives MCP inactivation contains a minimum number of five actin molecules. These experiments also make a point with respect to the interpretation of the prolongation of the lag period. We directly demonstrate that in the presence of an actin binding protein a prolongation of the lag period can be associated with increased nucleation, contrary to the usual interpretation in the literature that it indicates no or decreased nucleation by the actin binding protein.

Control of actin filament assembly plays an important role in such diverse physiological processes in nonmuscle cells as platelet activation, chemotaxis, phagocytosis, granule secretion, and cytokinesis [cf. Fox and Phillips (1983) and Korn (1978)]. A number of actin binding proteins are thought to participate in the regulation of filament assembly, and substantial progress has been made in recent years in elucidating the details of the interaction of some of these proteins with actin in vitro [cf. Pollard and Cooper (1986) and Stossel et al. (1985)]. Two classes of actin binding proteins may be of particular importance in regulating actin filament assembly: the monomer binding proteins, such as profilin, and the barbed end capping proteins. Among the latter, villin, which is found in the epithelial microvilli of intestines and kidney proximal tubules, and gelsolin, which is present in most cells and in mammalian blood, have been best characterized (Mooseker, 1985; Janmey et al., 1985; Kwiatkowski et al., 1988).

Recently, Southwick and DiNubile (1986) isolated a new capping protein from macrophage extracts, called here macrophage capping protein (MCP).[1] MCP is activated by calcium; in contrast to gelsolin and similar to villin, the calcium activation is rapidly and completely reversible. In contrast to both gelsolin and villin, this protein does not sever actin filaments. In order to better evaluate the biological potential

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[1] Abbreviations: ATP, adenosine 5'-triphosphate; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MCP, macrophage capping protein.

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of this protein, we studied the kinetics of the interaction of MCP with actin.

Overall, MCP interacts with actin much more weakly than does gelsolin, the other calcium-regulated capping protein in macrophages. Thus, the capping constant, defined here as a modified $K_d$, is 1 nM as compared to less than 1 pm for gelsolin, and the monomer binding $K_d$ is between 3 and 4 $\mu$M as compared to a $K_d$ well below nanomolar for gelsolin. Nucleation by MCP requires relatively high MCP concentrations, and only 1 in 20 MCP molecules initiates an actin filament under conditions where each gelsolin molecule nucleates 1 actin filament (Walsh et al., 1984).

## Materials and Methods

*Protein Preparations.* Actin was prepared from acetone powder as previously described (Murray et al., 1981). Chromatography was carried out by upward flow through Bio-Gel 150 because the resolving power of the column for the removal of nucleating activity lasted longer than during downward flow. Pyrenylactin was prepared according to Kouyama and Mihashi (1981) with the modifications described previously (Northrop et al., 1986). The critical concentrations of different native actin preparations containing 10% pyrenylactin varied between 0.08 and 0.2 $\mu$M for uncapped filaments and between 0.4 and 0.6 $\mu$M (usually 0.5 $\mu$M) for barbed end capped filaments.

Actin was stored as monomer in liquid nitrogen after vitrification in 50-$\mu$L aliquots. Except for the occasional small amount of precipitate, activity was fully maintained after one cycle of vitrification and defrosting. We have not succeeded in refreezing actin without significant loss in activity. Actin was quickly defrosted in a room temperature water bath under continuous stirring, sonicated (5 s), allowed to depolymerize for 2 h on ice, and centrifuged for 2 h at 120000$g$ to remove actin nuclei. Defrosted actin was used for up to 1 week.

The macrophage capping protein (MCP) was prepared from rabbit alveolar macrophages as previously described (Southwick & DiNubile, 1986); 40 mL of packed volume of macrophages yielded 5.0 mg of MCP of greater than 90% purity as assessed by scanning densitometry.

Acrosomes were isolated from *Limulus* sperm according to Tilney (1975, 1983) with slight modifications such as more intensive mechanical dispersion of the sperm material, increasing the number of low-speed centrifugations for the removal of nuclear material, omitting the intermediate centrifugation, and keeping the acrosomes in actin polymerizing medium. The acrosomes usually contained a certain amount of DNA which had no effect other than raising the light-scattering background during fluorescence measurements. Complete avoidance of the DNA contamination lowered the acrosome yield to intolerably low levels. Acrosomes remained active over a period of 2–3 days.

Villin was prepared according to Coleman and Mooseker (1985) and stored for several weeks at 4 °C.

Protein concentrations were calculated for actin using $E_{290}$ = 24.9 $mM^{-1}$ with the correction for pyrenyl absorption according to Selden and colleagues (1983), for villin using $E_{280}$ = 123.5 $mM^{-1}$ $cm^{-1}$, and for MCP according to Bradford (1976) using a rabbit immunoglobulin standard.

*Fluorescence and Kinetic Measurements.* Changes in G- and F-actin were calculated from the fluorescence measurements as previously described (Weber et al., 1987a).

All experiments were carried out with magnesium actin (Mg-actin), the physiological form of actin (Weber et al., 1969). With Mg-actin, elongation starts with a maximal rate, whereas with calcium actin the rate slowly increases after initiation of elongation in parallel with the conversion of Ca- to Mg-actin on exposure to the polymerizing medium. The conversion to Mg-actin was carried out as previously described with the following modifications: [EGTA][1] was in 10–20 $\mu$M excess over [calcium], [Mg] was in 50 $\mu$M excess over [ATP], and [ATP] was usually between 5 and 50 $\mu$M. All polymerization measurements were carried out at 20 °C in a polymerizing medium consisting of 10 mM imidazole hydrochloride, pH 7.4, 0.5 mM ATP, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM $NaN_3$, and calcium or EGTA as indicated in the figure legends.

Stock solutions of actin, villin, or MCP used for experimental assays were never lower than 1 $\mu$M in order to avoid loss of a significant fraction of the protein by unspecific binding to vessel walls (Weeds et al., 1986). Furthermore, at dilutions to 1 $\mu$M we maximized the ratio of volume to vessel wall surface area by using only full vessels. Villin also has a marked tendency to be absorbed by the walls of Eppendorf tubes as indicated by a comparison between activities at the same final villin concentration after dilution from stock solutions of high and of low concentrations.

Nuclei for measurements of elongation at the barbed ends were either acrosomes (limited to about 0.1–0.3 nM barbed ends per assay in order to minimize the light-scattering background) or short villin-capped actin polymers prepared as previously described [5–10 nM villin, actin/villin ratio usually 25 since lower ratios result in the presence of free villin (Weber et al., 1987a)]. Villin was removed from the polymer ends by dilution into a polymerizing medium containing 5.0 mM EGTA (Northrop et al., 1986). Nuclei for measurements of elongation at the pointed filament ends were villin-capped actin polymers prepared as described above; elongation was measured in a calcium-containing polymerizing medium.

Elongation rates were measured as previously described. Whether the time course was exponential was determined graphically by plotting log [(fluorescence$_{t_\infty}$ − fluorescence$_t$)/fluorescence$_{t_\infty}$] versus time, whereby $t_\infty$ indicates the time of the end-point reading.

For measurements of depolymerization rates, F-actin was diluted to 50 nM with precautions to minimize shearing during the dilution. The degree of capping on the basis of depolymerization measurements was calculated according to

$$[b_{(+MCP)}]/[b_{total}] = 1 - \frac{rate_{(+MCP)} - rate_p}{rate_{(-MCP)} - rate_p}$$

where b represents barbed ends, $b_{(+MCP)}$ capped barbed ends, and $rate_p$ the depolymerization rate of villin-capped filaments.

End points of polymerization used for analysis were taken after overnight incubation at 20 °C because usually the last assays of a series were begun too late to have reached the end point on the same day. In addition, at low MCP concentrations which prolonged the lag period substantially, end points of polymerization were not reached in less than 12 h. When comparisons between end points after 12 and 24 h were possible, we obtained variable results: frequently, there was no change in polymerization; sometimes a small amount of depolymerization occurred overnight, especially with aged proteins. In most of the experiments described here, the actin was completely stable. When some depolymerization occurred overnight, we assumed that the extent of denaturation was uniform for all assays. This assumption appeared justified because a graph of the 24-h end points versus the total actin concentration gave a straight line.

*Calibration of the Relationship between the Concentration of Stable Nuclei and Rates of Elongation at the Barbed*

*Filament End.* In order to calculate the concentration of stable nuclei from elongation rates in 5 mM EGTA, calibration curves were constructed for each actin preparation by plotting elongation rates as a function of known number concentrations of stable nuclei, using the short villin-capped actin polymers described above. At constant G-actin concentrations, elongation rates (in 5.0 mM EGTA and in calcium) have been shown to be a linear function of the concentration of added villin-capped actin polymers (Northrop et al., 1986; Pollard, 1986). For a given concentration of villin-capped actin polymers, the elongation rate in 5 mM EGTA is about 10 times faster than in calcium because, in EGTA, villin is rapidly inactivated and elongation occurs primarily at the barbed end [Pollard & Mooseker, 1981; Walsh et al., 1984; cf. Pollard and Cooper (1986)]. At a constant concentration of nuclei, elongation rates at barbed filament ends increase proportionally with increasing $c - c_\infty$ ([G-actin] − critical concentration), whereas at the pointed filament end, the relationship between $c - c_\infty$ and elongation rate is somewhat curvilinear (Weber et al., 1987b). Whereas data collection is accurate within 10%, the absolute value for the nuclei concentration calculated on the basis of the villin concentration is inaccurate to the extent that villin, which was used over a period of several months after preparation, had been partially denatured. However, the absolute nuclei concentration was irrelevant for our analyses as long as the concentration did not change within one series of experiments. This is the case since the experimental series presented here were each carried out within a short time span.

*Estimation of the MCP-Bound Actin Concentrations from the Degree of Inhibition of Elongation Rates at the Pointed Ends of Villin-Capped Actin Filaments.* We compared the rate of fluorescence increase in the presence and absence of MCP after the near-simultaneous addition of salt, villin-capped actin filaments (actin/villin = 25), and MCP (in this order) to G-actin. The elongation rates in the presence of various MCP concentrations were not corrected for the fluorescence difference between free and bound actin since the maximal correction amounted to less than 10%. The initial free G-actin concentration in the presence of MCP was obtained from the curvilinear calibration curves (determined for each actin preparation used) showing initial elongation rates as a function of initial G-actin concentrations. The fraction of MCP-sequestered G-actin was calculated according to MCP–G-actin = added G-actin − free G-actin. The implicit assumption that the nuclei concentration remained constant during the measurement was based on the relative slowness of MCP-induced nucleation compared to the short duration of the initial rate measurements (2 min) and appears justified by the $K_d$ value (see Results).

*Estimation of the MCP-Bound Actin from the Decrease in Final Actin Incorporation into Villin-Capped Actin Filaments.* Under the assumption that MCP had no effect on the critical concentration of villin-capped actin filaments, the decrease in the amount of polymerized actin in the presence of MCP was taken to represent the amount of MCP-bound actin ([MCP-actin] = $[F_{-MCP}] - [F_{+MCP}]$; F = polymerized actin). In order to obtain the correct F-actin concentration from the end-point fluorescence, a relatively small correction had to be made for the fluorescence difference between free and MCP-bound G-actin (the correction lowered the $K_d$ value for the single-site binding model from 1.85 to 1.35 μM).

## RESULTS

*Validation of the Use of Pyrenylactin for the Study of Macrophage Capping Protein.* In order to use pyrenylactin for the study of MCP kinetics, it was necessary to determine whether actin labeling significantly altered its interaction with MCP as has been shown for profilin (Malm, 1984; Lal & Korn, 1985). We used the effect of MCP on the lag period of polymerization as a function test. We compared the effect of MCP on the lag period of 100% pyrenyl-labeled actin with that on the lag period of native actin, containing only 5% pyrenylactin (cf. below and Discussion). MCP prolonged the lag period of both 5 and 100% labeled actin to a similar extent, 2–3-fold (15 nM MCP, 1–2 μM total actin; data not shown), suggesting that MCP capping of actin filaments is not significantly affected by pyrenyl labeling of the actin. Binding of MCP to monomeric actin was indicated by an increase in pyrenyl fluorescence (Southwick & DiNubile, 1986; see below).

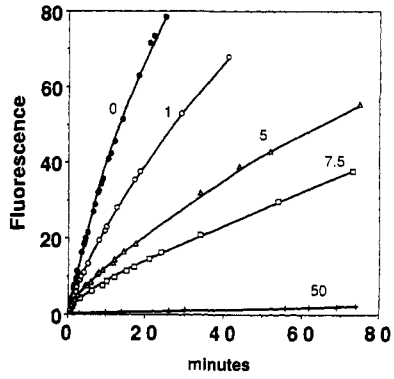


FIGURE 1: Inhibition of the rate of elongation of acrosomes with increasing concentrations of MCP. The increase of fluorescence with time was followed after the near-simultaneous addition of a salt mixture (see Materials and Methods for the composition of the polymerization medium), MCP, and *Limulus* acrosomes (0.12 nM final barbed end concentration) to 0.5 μM G-actin. The mixing period was 20–30 s. The numbers on the curves indicate MCP concentrations in nanomolar.

*Evidence That the 41K Protein Is the Macrophage Capping Protein.* It was difficult to completely separate two contaminating proteins of 29 and 61 kDa from the bulk of the 41-kDa fraction, and only relatively small amounts of completely pure 41-kDa protein were obtained. Most preparations contained about 5% of each of the two contaminants. Although this amount of contaminating protein was insufficient to account for the monomer binding, it could have accounted for capping (see below) if the contaminating proteins had a very high affinity for the barbed filament end. This possibility was ruled out by comparing the capping activity of fractions enriched in the 29- and 61-kDa bands with that of a fraction containing nothing but the 41-kDa protein and its 36-kDa degradation product. The capping activity of the proteins was evaluated on the basis of their ability, in substoichiometric amounts, to inhibit the rate of filament elongation of acrosomes at the barbed end (described in Figure 1); 0.3 μg/mL (about 8 nM) of a mixture of 41 + 36 kDa inhibited the rate by about 80%. By contrast, the same concentration of pure 61-kDa protein had no effect on the elongation rate of acrosomes. The 25–40% inhibition by the fraction enriched in 29-kDa protein was probably consistent with its content of the 41-kDa protein.

*Determination of the Capping Constant of Macrophage Capping Protein for Barbed Filament Ends by Filament Elongation Measurements.* The extent of capping was evaluated from the degree of inhibition of the elongation rates at the barbed ends of *Limulus* acrosomal false discharges. *Limulus* acrosomes consist of highly cross-linked actin filament bundles which do not depolymerize in the absence of monomeric actin (Tilney, 1975). Thus, in the range of monomer concentrations used in our experiments (below the critical concentration for the pointed end), the net rate of elongation

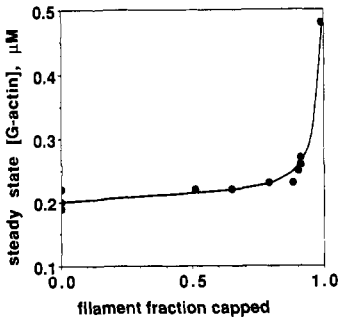


FIGURE 2: Change in critical concentration with increasing extent of capping, calculated from overnight end points of the elongation experiments in Figure 1. 92 and 99% capping occurred at 10 and 50 nM total MCP.

reflected events only at the barbed filament ends.

The control rates in the absence of MCP were initially linear and later decreased gradually with progressive removal of G-actin into actin filaments (Figure 1). The time course of the control was described by a single exponential. In the presence of MCP, the elongation rate decreased rapidly with time and was not linear in the beginning; in addition, only the later part of the time course was described by a single exponential. Presumably, capping of the acrosomes by MCP had not been completed during the mixing period, and thus the number of free barbed acrosome ends continued to decrease during the first part of the elongation rate measurements. We assumed that capping had reached equilibrium when the time course of elongation started to follow a single exponential. Depending on the concentration of added MCP, this took between 1 and 20 min. When capping had reached equilibrium, the degree of inhibition of elongation by MCP was evaluated from a comparison with control rates at the same G-actin (fluorescence) levels. The inhibition of the rate of elongation is related to capping by the expression:

$$\text{capped filaments/total filaments} = 1 - [\text{rate}_{(+\text{MCP})}/\text{rate}_{(-\text{MCP})}]$$

The ratio of uncapped to capped filaments as a function of increasing [MCP] is described by the equation:

$$[b]/[b_{(+\text{MCP})}] = \{k_{-(\text{MCP})} + k_{-(\text{MCP-G})}\}/\{k_{+(\text{MCP})}[\text{MCP}] + k_{+(\text{MCP-G})}[\text{MCP-G}] + k_{+(\text{MCP-G2})}[\text{MCP-G}_2]\}$$

where b indicates free barbed ends and $b_{(+\text{MCP})}$ the capped barbed ends, MCP, MCP–G, and MCP–$G_2$ indicate free MCP and MCP complexed with actin monomers and dimers, respectively (see below), and $k_+$ and $k_-$ indicate the rate constants for association and dissociation of the various MCP species indicated by the subscripts. Ignoring MCP–G and MCP–$G_2$, which probably contributed less than 25% of the total MCP under the conditions of the experiments described here, the expression may be simplified to

$$[b][\text{MCP}]/[b_{(+\text{MCP})}] = \frac{k_{-(\text{MCP})} + k_{-(\text{MCP-G})}}{k_{+(\text{MCP})}} = K_d$$

A double-reciprocal plot of the fraction of capped filaments versus the total concentration of MCP was linear, and the slope indicated a value of 0.8 nM for the $K_d$ (data not shown).

The steady-state G-actin concentration increased sharply between 91 and 99% capping as expected when the association rate constant at the barbed end ($k_{+b}$) is reduced from a value equal to approximately 10 times $k_{+p}$ to near 0 and when $k_{-p}$ is similar to $k_{-b}$ (Figure 2) (Walsh et al., 1984; Wanger & Wegner, 1985).

*Capping Constant Determined from Depolymerization Measurements and Effect of Macrophage Capping Protein on the Dissociation Rate Constant of the Terminal Actin Monomer at the Barbed End.* Previous measurements of the effect of MCP on the rate of actin filament depolymerization at subcritical concentrations of total actin have shown that, at saturating concentrations, MCP lowers the rate of depolymerization about 10-fold (Southwick & DiNubile, 1986); i.e., the total depolymerization rate is close to that for the pointed end alone [Walsh et al., 1984; cf. review by Pollard and Cooper (1986)]. Our measurements of depolymerization rates with increasing MCP concentrations showed that 1 nM MCP inhibited the rate of depolymerization by more than 50% and the double-reciprocal plot of the fraction of capped filaments versus free MCP concentration gave a value of 0.63 nM for the capping constant (data not shown). In this case, the capping constant was defined as $K_d = [k_{-(\text{MCP})} + k_{-(\text{MCP-G})}]/k_{+(\text{MCP})}$ since, at the very low G-actin levels present during the initial phase of depolymerization (from 2.5 to 10 nM), the concentration of MCP complexed with actin monomers was insignificant. Similar results were obtained when depolymerization rate measurements were made using actin copolymerized with MCP at a ratio 10/1; in this case, the lowest concentration of MCP used, 5 nM, gave about 90% inhibition of the rate of depolymerization (data not shown).

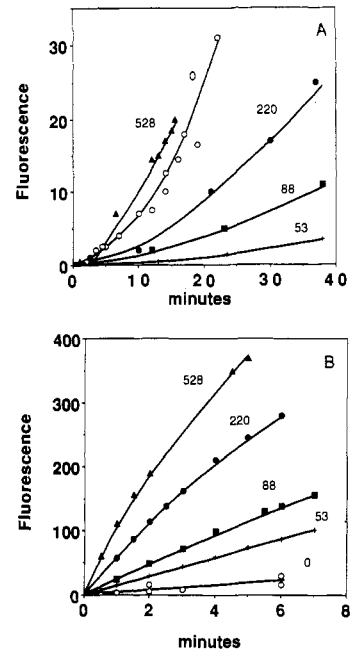


FIGURE 3: Prolongation of the lag period (A) and promotion of nuclei formation (B) by MCP. (A) and (B) represent the first and the second part of the same experiment. (A) 1.4 μM Mg–G-actin was incubated with varying concentrations of MCP (53–528 nM) in polymerizing buffer containing 200 μM $CaCl_2$. After polymerization of about 55 nM actin, at time points varying from about 20 (control) to 100 min (53 nM MCP), EGTA was added to a final concentration of 5 mM (at time 0 in panel B). (B) Initial rates of elongation after EGTA addition. The numbers on the curves indicate MCP concentrations in nanomolar. Note the difference in the scale of the coordinates between (A) and (B).

*Nucleation by Macrophage Capping Protein.* Nucleation by an actin binding protein is easily recognizable if it is associated with a decrease in the lag period. By contrast, prolongation of the lag period by a capping protein is usually interpreted to mean that the capping protein is not able to form nuclei (Wegner & Aktories, 1988). MCP, over a concentration range of 1–300 nM, prolonged the lag period (Figure 3A). However, the extent of the prolongation became less with increasing concentrations of MCP, suggesting that MCP weakly nucleated actin polymerization (Figure 3A). Therefore, we measured nucleation directly according to the following

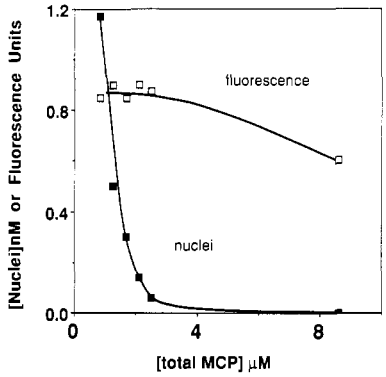


FIGURE 4: Formation of stable nuclei and the instantaneous fluorescence increment with increasing MCP concentrations. The ordinate gives both the fluorescence values in arbitrary units and the concentration of nuclei in nanomolar. A constant concentration of G-actin (0.84 μM) was incubated with increasing MCP concentrations (0.84–8.4 μM) in the presence of calcium. Mixing of G-actin with MCP was associated with an instantaneous fluorescence increment which increased G-actin fluorescence by a factor of 2–3. The nuclei concentration was obtained after MCP inactivation as described in the text.

rationale. Increased nucleation by MCP would result in an increase in the polymerization rate if it were not masked by the nearly 10-fold decrease in elongation rate resulting from blockage of the barbed ends. The effect of nucleation on the polymerization rate will be revealed if the MCP cap is removed by EGTA addition after MCP had a chance to form MCP-actin nuclei (Southwick & DiNubile, 1986). An increased polymerization rate after incubation of monomeric actin with MCP would indicate that MCP caused the formation of actin nuclei capable of surviving MCP inactivation.

The measurements were carried out in two different ways: in the first set of experiments, incubation in the presence of calcium was continued until a predetermined amount of F-actin had been formed (Figure 3). This means that the incubation periods were relatively short for controls and for assays containing MCP in very high concentrations and that the incubation periods were very long for low concentrations of MCP. Here, the rate of elongation after EGTA addition was directly proportional to the concentration of stable nuclei since the G-actin concentrations (total actin − F-actin) were the same in all assays.

In the second set of experiments (data not shown), the incubation period was held constant, and, consequently, the amount of actin that had polymerized at the time of EGTA addition was not the same after incubation with different MCP concentrations. This means that elongation rates in EGTA were measured at different G-actin concentrations. Therefore, the polymerization rates were corrected for differences in $c - c_\infty$ on the basis of the observation that elongation rates of noncapped actin filaments are directly proportional to $c - c_\infty$ [cf. Korn et al. (1987) and Pollard (1986)].

Following EGTA addition, elongation rates of actin incubated with MCP were always higher than control rates (with the exception of two MCP concentrations below saturation for capping; data not shown), indicating that MCP nucleated actin polymerization.

Nuclei sufficiently large to be stable after MCP inactivation by EGTA were also formed at free G-actin concentrations at or below the critical concentration for the pointed filament end (Figure 4). In this experiment, fluorescence remained constant during 2–3-h incubation periods in the presence of calcium at the level attained immediately after mixing actin and MCP (see below). After EGTA addition, the critical concentration was lowered to the value for uncapped actin filaments, actin monomers complexed to MCP were released, and, as a result, actin was polymerized. The rates of polymerization were greater than those of controls containing the same G-actin concentrations, indicating that MCP had formed actin nuclei during the incubation period. The nuclei concentration was obtained from calibration curves (Materials and Methods).

The nuclei concentration fell off sharply with increasing concentrations of MCP in limiting actin (Figure 4). Under these conditions, monomer binding to MCP increased (see below), and the concentration of free G-actin decreased. Assuming that the MCP-induced nucleation was described by the reaction MCP + $nG \leftrightarrow$ MCP–G$n$(Nuc), the minimal size ($n$) of a stable nucleus (Nuc) should be obtained by measuring changes in the steady-state nuclei concentration as a function of the free actin concentration, according to the equation:

$$[\text{Nuc}] = [\text{G}]^n[\text{MCP}]/K$$

In order to combine all data obtained at different concentrations of MCP and actin, we plotted [Nuc]/[free MCP] in a double-log plot versus the free G-actin concentration. A straight line was obtained with a value of 5.3 for $n$ (data not shown). This result suggests that the stable nucleus contains a minimum number of five actin monomers.

The nucleation rate is one of the determinants of the average filament length [cf. Oosawa and Asakura (1975)]. Binding proteins capable of nucleating actin assembly shorten the average filament length. We estimated the effect of MCP on the average filament length by comparing the depolymerization rate of 50 nM MCP-copolymerized actin filaments with the depolymerization rate of villin-copolymerized actin filaments of known average length [average length = moles of actin/villin-capped filament = moles of actin/moles of villin (Walsh et al., 1984; Coleman & Mooseker, 1985)]. In the presence of MCP, at a ratio of MCP to actin of 1/10 (1.0 μM MCP), the average filament length was decreased to about 200 actin molecules per filament as compared to a value of 600–1800 for controls (the wide variation in the control reflecting different degrees of shearing during transfer and dilution).

*Monomer Binding by Macrophage Capping Protein.* To better understand the process of nucleation by MCP, we studied its interaction with monomeric actin. It had been observed previously that MCP increased pyrenylactin fluorescence instantaneously when added to subcritical actin concentrations (Southwick & DiNubile, 1986). The fluorescence increase reached a plateau value at a ratio of 1 monomer per MCP, suggesting the formation of an MCP–actin monomer complex. In order to obtain an adequate fluorescence signal, these measurements had been carried out under conditions of very high values for $c_\infty$ in the absence of KCl and at low Mg concentrations.

For physiological salt concentrations (0.1 M KCl and 2 mM $MgCl_2$), we evaluated monomer binding by MCP on the basis of two measurements: first, the effect of MCP on the elongation rate at the pointed ends of villin-capped actin filaments; second, the effect of MCP on the final extent of actin incorporation into barbed end capped filaments. The control elongation rate of villin-capped actin filaments is a nonlinear function of the initial G-actin concentration (Figure 5A) (Weber et al., 1987b). MCP in increasing concentrations progressively decreased the elongation rate (Figure 5B) and in high concentrations caused the complete depolymerization of the filaments (data not shown). Consistent with monomer binding by MCP, increasing concentrations of MCP also decreased the total amount of actin incorporated into the villin-capped filaments (Figure 5C). These measurements were

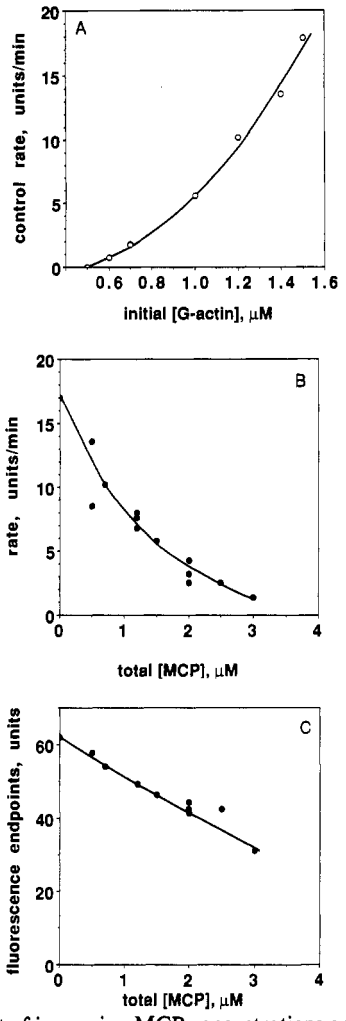


FIGURE 5: Effect of increasing MCP concentrations on the elongation rates and on the total actin incorporation into villin-capped actin filaments. Initial rates of elongation of villin-capped actin filaments (8 nM villin, 200 nM F-actin) with (A) increasing concentrations of G-actin in the absence of MCP and (B) a constant concentration of 1.5 $\mu$M G-actin and increasing concentrations of MCP; (C) overnight end points of the elongation assays in (B) corrected for the fluorescence of MCP-bound actin (Materials and Methods); 0.38 $\mu$M critical concentration, obtained from overnight end points of the controls in (A). 0.38 $\mu$M is at the lower limit of the range of critical concentrations of villin-capped native actin filaments observed with different actin preparations (0.35–0.65 $\mu$M for native actin; 0.6–1.1 $\mu$M for Cys-374-substituted actin). The low value does not reflect incomplete capping in the presence of 8 nM villin since the capping constant of villin is lower than 1 pM. Furthermore, shearing of filaments during mixing is not a problem with an average size of 25 actin molecules per oligomer.

made a number of times with similar results using different MCP and actin preparations. Values for the concentration of sequestered actin molecules were calculated for both curves (Materials and Methods).

The resulting titration curves (Figure 6) can be fitted equally well by using the appropriate $K_d$ for binding of a single or two actin monomers to each MCP molecule. Estimation of the correct $K_d$ value, however, requires information about the ratio of actin and MCP in the complex since the concentrations of free and bound MCP are calculated on the basis of the measured concentration of complexed actin. The calculated value for free MCP, and therefore for the $K_d$, will be higher if only $^1/_2$ rather than 1 MCP is bound per complexed actin.

Additional information is provided by the fluorescence data. Mixing MCP with monomeric actin gave rise to an instantaneous fluorescence increment quite distinct from the slower

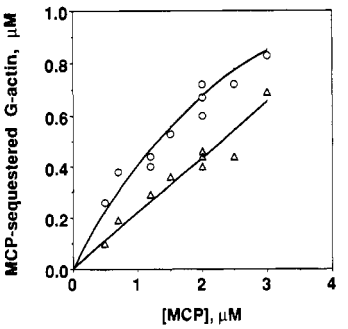


FIGURE 6: Actin monomer binding by MCP with increasing total MCP concentrations (calculated from the data shown in Figure 5). During the elongation rate measurements (upper curve), more actin was sequestered by a given concentration of total MCP than at steady state (lower curve) because, initially, the MCP-actin complexes were in equilibrium with the added 1.5 $\mu$M G-actin. At the end point of elongation, the MCP-bound actin was in equilibrium with 0.38 $\mu$M G-actin, the critical concentration for barbed end capped actin filaments. The lower curve is linear because at steady state the ratio $K_d$/free G-actin remains constant. The lines through the data points are calculated to fit a single-site binding model (MCP-A) with a $K_d$ of 1.6 $\mu$M for the upper curve and 1.35 $\mu$M for the lower curve. The lines fit a two-site random binding model (MCP + A $\leftrightarrow$ MCP–A + A $\leftrightarrow$ MCP–$A_2$) with the same $K_d$ for both sites, with $K_d$ values of 4.3 and 3.0 $\mu$M for the upper and the lower curve, respectively. The lines also fit a cooperative dimer binding model (MCP + 2A $\leftrightarrow$ MCP–$A_2$; MCP–A close to zero at all MCP/A ratios). However, in this case, the upper and lower curves cannot be fitted with similar $K_d$ values; the $K_d$ for the upper curve is 4.1 $\mu M^2$ and for the lower curve 1.2 $\mu M^2$.

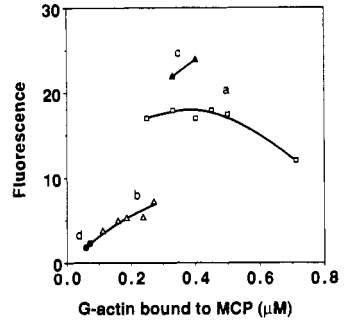


FIGURE 7: Instantaneous fluorescence increment of MCP-bound actin. The concentration of MCP-bound actin was calculated on the basis of the random binding model used to fit the data in Figure 6. The instantaneous fluorescence increments were obtained under different conditions. (a and b) [MCP] in excess of [actin]; (c and d) [actin] in excess of [MCP]; (a and b) 0.84 and 0.5 $\mu$M constant [actin] with [MCP] increasing from 0.84 to 8.4 $\mu$M and from 0.5 to 2 $\mu$M, respectively; (c and d) 0.84 and 0.3 $\mu$M constant [MCP] with [actin] increasing from 0.84 to 1.68 $\mu$M and from 0.3 to 0.5 $\mu$M, respectively.

fluorescence increase associated with oligomer and polymer formation. Furthermore, this fluorescence increment was also observed at subcritical actin concentrations where no polymerization took place (Figure 4, fluorescence trace). Presumably, the rapid fluorescence increment was due to the formation of an actin–MCP complex which was completed during the mixing time. Strikingly, however, the fluorescence increment reached a plateau with increasing [MCP] under conditions where the amount of MCP-complexed actin measured by the kinetic assays was doubled (compare Figure 4 with Figure 6). In addition, a further increase in [MCP] to very high concentrations resulted in a decrease of the fluorescence increment (Figure 4).

The fluorescence and kinetic data taken together are not compatible with the formation of an MCP–actin monomer complex, MCP + G $\leftrightarrow$ MCP–G, since the fluorescence increment is not proportional to the total bound actin (Figure 7). Both sets of data can be explained if two assumptions

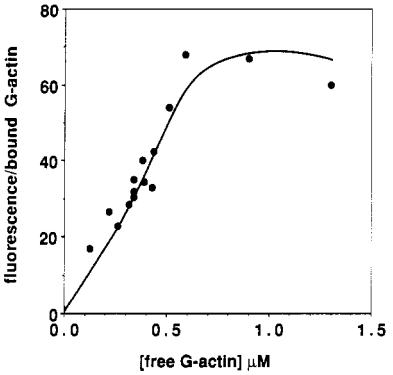


FIGURE 8: Fluorescence increment/MCP-bound actin with increasing free G-actin. The same data as in Figure 7. Free G-actin = total G-actin − MCP-G. The line through the data points was drawn by eye.

are made. The first assumption is the existence of two actin binding sites on MCP and the coexistence of two kinds of MCP–actin complexes, MCP–G and MCP–$G_2$. Binding of two rather than one actin molecule agrees with the observation that MCP nucleates actin polymerization. The second assumption is a large increase in the fluorescence increment per bound actin when two molecules rather than one actin molecule are bound to MCP. In this case, the total fluorescence increment would not increase but would remain constant if an increase in the total MCP-bound actin is associated with a shift from the strongly fluorescent MCP–actin dimer to the weakly fluorescent MCP–actin monomer complex. Eventually, the total fluorescence would decrease when most of MCP–$G_2$ had been replaced by MCP–G with increasing excess of MCP over actin (Figure 4).

MCP–actin dimer complexes would be favored over monomer complexes when the concentration of free G-actin is increased according to MCP–G + G $\leftrightarrow$ MCP–$G_2$. If the above explanation were correct and the fluorescence per MCP-bound actin molecule were greater in MCP–actin dimers than in MCP–actin monomers, then one would expect the fluorescence per bound actin to increase with increasing free G-actin concentration. This was indeed observed (Figure 8) when the data shown in Figure 7 were regraphed in this manner. It should be noted that the increasing fluorescence increment cannot be attributed to increasing concentrations of oligomers (stable nuclei and larger oligomers) displaying F-actin fluorescence. Actin in stable oligomers contributed less than 15% of the increase in the fluorescence increment between 0 and 0.4 $\mu$M free G-actin.[2]

Since the data suggest that MCP binds two actin molecules, the question arises whether the binding is random or cooperative. The data of Figure 6 were fitted quite well to a two-site random binding model with similar $K_d$ values of 4.3 and 3.0 $\mu$M for the rate and end-point measurements, respectively. Although each curve was also fitted by a strongly cooperative model according to MCP + A $\overset{K_1}{\leftrightarrow}$ MCP–A + A $\overset{K_2}{\leftrightarrow}$ MCP–$A_2$, $K_1 \gg K_2$, the two curves could not be fitted by similar values for $K_1K_2$. With values of 4.1 $\mu M^2$ for the elongation and 1.2 $\mu M^2$ for the end-point data, $K_1K_2$ for the two curves differed by a factor of nearly 3.5. The poor fit of the cooperative model was also seen by modeling the elongation data alone when $K_1K_2$ was calculated separately for each data point: $K_1K_2$ decreased systematically from 5.5 to 2.5 $\mu M^2$ on increasing [MCP] from 0.5 to 3 $\mu$M, whereas the $K_d$ values for the random binding model were scattered around a mean value. Cooperative binding is also not supported by the combined fluorescence and kinetic data which could be explained only by assuming the coexistence of MCP–actin monomer and dimer complexes in comparable concentrations. By contrast, cooperativity implies the prevalence of MCP–actin dimers over MCP–actin monomers.

[2] Estimated on the basis of the measured nuclei concentrations, an average size of 8 actin molecules per stable nucleus [calculated according to the following expression: average length = $3 + 1/(1 - \alpha)$, where $\alpha = [G]/c_\infty$, modified from Oosawa and Asakura (1975) by Martin Pring; $c_\infty = 0.5$ $\mu$M; 3 = smallest stable nucleus − 2; and fluorescence of all MCP-complexed actin molecules equal to that of F-actin].

## Discussion

We have determined the kinetic constants of a new calcium-regulated capping protein present in macrophages. Before commenting on its potential for in vivo function, we will discuss some points relating to capping and nucleation.

*Modified Capping Constant.* We defined the capping constant in terms of the various kinetic paths that result in the association and dissociation of the capping protein to and from the filament end. We limited the association reactions of actin–capper complexes to those complexes containing actin monomers bound directly to the capping protein and excluded association reactions of capped oligomers such as actin trimers, tetramers, etc. We arbitrarily restricted the dissociation reactions to the dissociation of the capper and of the capper-complexed terminal actin monomer of the filament. We excluded the dissociation reaction of the capper-complexed terminal actin dimer because its rate constants may possibly be very slow, comparable to the rate constant of filament breaking.

It can be seen from the expression for the capping constant that the apparent $K_d$ of a capping protein for the barbed filament end cannot be lower than the $K_d$ of the terminal actin molecule for the barbed filament end ($K_d = c_\infty$ at the barbed end, about 100 nM) unless the capping protein lowers the dissociation rate constant of the terminal actin molecule, i.e., strengthens its binding to the filament end. The capping protein cannot have a higher association rate constant for the barbed filament end than the actin monomer since the latter is diffusion limited [Pollard & Mooseker, 1981; Bonder et al., 1983; cf. review by Pollard and Cooper (1986)].

*Capping Constant of MCP.* The value of the capping constant in the range of 0.5–1 nM was obtained by three independent measurements. These were inhibition of the elongation and of the depolymerization rates and, third, the increase in the critical concentration to the value for the pointed end. The capping constants determined by elongation and by depolymerization measurements need not necessarily be the same since the nucleotide content of the barbed filament ends may have been different in these two assays. Free barbed ends contain ADP·$P_i$ in the presence of the high G-actin concentrations used for the elongation measurements and only ADP in the presence of the very low G-actin concentrations used for the depolymerization measurements [cf. Korn et al. (1987)]. However, it is not possible to predict the nucleotide content of the terminal actin molecules of the barbed end in the presence of a capping protein. First, the nucleotide composition should depend on the degree of saturation with capping protein. If the barbed end is capped 99% of the time, restricting the exchange with new ATP–G-actin, the capped barbed end may well contain ADP at all times. At lower MCP concentrations, capped ends with either ADP·$P_i$ or ADP alone may have been present. Furthermore, the rate constants for ATP hydrolysis and phosphate release may be altered by capping of the filament end by MCP.

The value for the capping constant of about 1 nM compared to the $K_d$ for the actin monomer of 100 nM ($c_\infty$ at the barbed

filament end) indicates that MCP strengthened the binding of the terminal actin monomer to the barbed end by a factor of 100. The data do not allow one to distinguish between two obvious possible explanations. First, actin binding may be strengthened as a result of the interaction of MCP with two actin molecules at the filament end. In this case, the difference in the actin binding constants in the presence and absence of MCP reflects the difference in the binding constants of the actin dimer and actin monomer for the filament end. Second, actin binding may have been strengthened as a result of a conformational change imposed by MCP on the terminal actin molecule.

*Rate Constants of Association and Dissociation.* The delay in capping indicates unambiguously that the association rate constant was not diffusion limited; otherwise, capping would have been completed much faster; e.g., capping by 5 nM MCP would have been 90% complete in about 1 min. On the basis of the delay in capping at different MCP concentrations, a gross estimate of the order of magnitude of the rate constant of association of MCP for barbed filament ends was made of $(0.5–1) \times 10^6$ $M^{-1}$ $s^{-1}$, about 10 times slower than expected for a diffusion-limited process. The estimate was obtained by treating MCP binding to barbed ends as a pseudo-first-order reaction with $k_{on} = ([MCP]k_+) + k_-$ and by assuming further that binding was practically complete (indicated by the beginning of the final single-exponential phase of the time course of elongation) after a lapse of four half-times (93% equilibration of MCP with barbed ends). Depending on the MCP concentration, four half-times for MCP binding took between 1 and 20 min. This approach was permissible since the concentration of MCP remained essentially constant during the initial phase of elongation. The value for the sum of the dissociation rate constants $[k_{-(MCP)} + k_{-(MCP-G)}]$ is about $(0.5–1.0) \times 10^{-3}$ $s^{-1}$ calculated on the basis of the $K_d$ of 1 nM and the estimated association rate constant of about 0.5–1 $\mu M^{-1}$ $s^{-1}$. This value is much lower than the dissociation rate constant of actin from uncapped barbed ends; the difference is 200- and 1000-fold when compared with our values and those reported by Bonder and colleagues (Bonder et al., 1983), respectively. (Whereas the equilibrium constants reported in the literature are rather comparable, there is a difference by about a factor of 5 for the rate constants reported from different laboratories.)

*Nucleation.* We would like to introduce the discussion of our data with some general comments on factors causing prolongation of the lag period of actin polymerization. The reason for the comments is that in the literature a prolongation of the lag period is always interpreted as the inability of the binding protein to promote nucleation, even when the binding protein is a known capper of the barbed filament end (Wegner & Aktories, 1988). This interpretation of the prolongation of the lag period is widely accepted, even though it is evident from the calculations by Oosawa and from subsequent treatments of nucleation that it is not necessarily correct. Modeling of spontaneous polymerization in various laboratories suggests that right after addition of salt, a rapid steady state may be set up between monomers, prenuclei, and nuclei and that at the same time nuclei present in very low concentration begin to elongate to stable oligomers and polymers [cf. Oosawa and Asakura (1975), Frieden (1983), Cooper et al. (1983), and Tobacman and Korn (1983)]. (The nucleus is conventionally defined as the oligomer that is elongated with the same $k_+$ as the filament but which has a different $k_+$ and $k_-$ for its own formation and dissociation.) With time, oligomers and polymers increase in number concentration and in size. The lag period lasts until the amount of F-actin incorporated into oligomers and polymers becomes detectable. Thus, the duration of the lag period is determined by the rate constants of formation and dissociation of prenuclei and nuclei, and by the rate constants of nuclei elongation to oligomers and polymers [cf. Oosawa and Asakura (1975)]. Capping of the barbed filament ends reduces the rate constant of nuclei elongation about 10-fold, thus prolonging the lag period unless the decrease in the elongation rate constant is compensated by a 10-fold or greater increase in the nuclei concentation. An example of this was first shown by Kurth and colleagues (Kurth et al. 1983), who observed a prolongation of the lag period with very low gelsolin concentrations and a shortening of the lag period at higher gelsolin concentrations.

Here we demonstrate directly that an increase in nucleation caused by a capping protein is associated with a prolonged lag period. This direct demonstration was possible because MCP capping of barbed filament ends can be rapidly reversed by calcium removal.

The smallest size of a stable nucleus formed by MCP was an actin pentamer, which is larger than the nucleus formed during spontaneous actin polymerization (Oosawa & Asakura, 1975; Frieden, 1983; Cooper et al., 1983; Tobacman & Korn, 1983) and the modeled size of the villin–actin nucleus (Weber et al., 1987a). However, with our assay system, only those nuclei were determined that persisted without the stabilizing influence of MCP. Therefore, it is likely that we did not measure true nuclei (according to the definition given above) but that we measured nuclei with the low $k_-$ of an actin polymer. Thus, the true MCP–actin nucleus may contain no more than four actin monomers compared to two monomers for the modeled villin–actin nucleus.

The initial phases of nucleation by MCP also appear to be different from those for either villin or gelsolin. Villin (Weber et al., 1987a) and gelsolin (Coué & Korn, 1985) bind two actin molecules in a highly cooperative fashion. MCP also binds two actin molecules but not in a highly cooperative manner. This may be related to the much weaker nucleating activity of MCP.

*Comparison between Gelsolin and Macrophage Capping Protein.* At this time, we know a great deal more about the potential functions of some of the actin binding proteins than we do about their in vivo functions during the various activities in the life of a cell. We may speculate that some of the apparently nonregulated capping proteins such as the brain protein (Wanger & Wegner, 1985) and capZ (Casella et al., 1986) may have structural functions. For instance, capZ, a capping protein of the Z band of skeletal muscle, most likely nucleates actin filaments in the Z band and functions constantly in preventing depolymerization at this end (Casella et al., 1987).

The calcium regulated capping proteins presumably are not continuously active. The macrophage has at least two calcium-regulated capping proteins, gelsolin and macrophage capping protein. Although both require calcium for activation, they differ significantly in their regulation. Only MCP is rapidly and completely inactivated by calcium removal. The functions of gelsolin, such as capping and nucleation, are greatly weakened but not abolished by calcium removal. Another factor or factors, among them phosphatidylinositol 4,5-bisphosphate ($PIP_2$), may be required for complete inactivation of gelsolin (Janmey et al., 1987). Gelsolin has a very high affinity for the barbed filament end ($K_d$ less than 1 pM; Selve & Wegner, 1986) and, as an associated property, nucleates one actin filament per gelsolin as does villin (Walsh

et al., 1984). This means it has the capacity to generate short actin filaments in direct proportion to the ratio of gelsolin and actin. In addition, gelsolin is capable of rapidly generating short filaments by cutting long filaments into short ones. The capping constant of MCP is very much weaker than that of gelsolin, and MCP does not produce very short filaments when it is copolymerized with actin. MCP also does not cut filaments (Southwick & DiNubile, 1986). Nevertheless, the capping by MCP is sufficiently tight to allow 99.9% capping at MCP concentrations of 1 $\mu$M in excess of the number concentration of filament ends present in nonmuscle cells.

While MCP appears to be well-suited for in vivo filament capping, it is not likely to function as a monomer binding protein in the cell. The $K_d$ for MCP–actin monomer and MCP–actin dimer complex formation is too high to maintain a large fraction of the cellular actin in the monomeric state as MCP–actin monomer and –dimer complexes. For instance, in the activated platelet, approximately 25% or 150 $\mu$M total actin exists as monomer [cf. Fox and Phillips (1983)]. Assuming a critical concentration of 0.5 $\mu$M (complete capping by MCP), this concentration of actin could be complexed with MCP only if the MCP concentration were about 450 $\mu$M in excess over monomeric actin! By contrast, although precise measurements under physiological salt conditions have not yet been performed, data for gelsolin suggest that it has the capacity to stoichiometrically convert actin to gelsolin actin dimers when present in a gelsolin/actin ratio of 1/2 or more.

A great deal more information about intracellular events is needed before we understand how these differences in the properties of the two capping proteins are used in the living cell.

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