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Role of the N- and C-Terminal Actin-Binding Domains of Gelsolin in Barbed Filament End Capping†

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ABSTRACT: Gelsolin is a bivalent Ca^{2+}-modulated actin-binding protein that severs, nucleates, and caps filaments. In order to gain a better understanding of the capping mechanism we have studied N- and C-terminal gelsolin fragments, 14NT and 41CT, each of which contains a single functional actin-binding site. The very tight binding measured between gelsolin and the barbed filament end requires gelsolin to greatly decrease the dissociation rate constant of the terminal actin from this end. A mechanism that could account for the observed decrease in dissociation is one in which gelsolin links two actin monomers so that they dissociate more slowly as a dimer. This cannot be the only mechanism, however, since, as shown here, 14NT and 41CT, fragments with single actin-binding sites, decrease the dissociation rate of the capped terminal actin molecule. The observations suggest that these fragments induce a conformational change in the actin monomer that either increases the affinity or alters the kinetics of the terminal actin–actin bond. The available data argue for strengthening of the terminal actin–actin bond.

Gelsolin is a Ca^{2+}-dependent actin-binding protein found in vertebrate cytoplasm (Yin & Stossel, 1979; Wang & Bryan, 1981) and blood plasma (Harris & Gooch, 1981; Nodes et al., 1987; Kwiatkowski et al., 1988). Three functional activities have been defined for this molecule. First, gelsolin forms a very tight, cooperative complex with two actin monomers in the absence of salt under conditions that will depolymerize actin filaments (Bryan & Kurth, 1984; Doi & Frieden, 1984; Coue & Korn, 1985). Second, in the presence of salt, gelsolin caps the barbed ends of actin filaments very tightly, inhibiting the addition and dissociation of monomers (Yin et al., 1981; Bryan & Coluccio, 1985). Third, free gelsolin binds laterally to filaments, weakening actin–actin interactions so that filaments break, producing capped polymers with a shorter average length (Yin et al., 1980; Wang & Bryan, 1981; Bryan & Coluccio, 1985). All of these activities require calcium binding at a regulatory site on the extreme C-terminal end of gelsolin (Yin & Stossel, 1980; Bryan & Kurth, 1984; Kwiatkowski et al., 1989).

There are three distinct actin-binding sites to carry out these activities (Bryan, 1988; Yin et al., 1988; Kwiatkowski et al., 1989), one on the C-terminal half, domains 4–6, according to the terminology of Way et al. (1989), and two on the N-terminal half of the molecule, domains 1–3. Proteolytic cleavage of the amino-terminal half separates a high-affinity G-actin-binding site, 14NT, domain 1, from an F-actin-binding site, situated on domains 2–3, whose activity is modulated by phosphoinositides (Yin et al., 1988).

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Capping of the barbed filament end is associated with inhibition of elongation at this filament end. Capping proteins can be divided into two groups according to their ability to inhibit depolymerization from the barbed filament end. Profilin, for instance, does not reduce the rate of depolymerization and has an apparent K_d, or K_{cap}, for the barbed end above 50 μM (Pollard & Cooper, 1984; Kaiser et al., 1986). Gelsolin, an example of the other group, strongly inhibits depolymerization and has a K_{cap} for the filament end of about 1 pM (Selve & Wegner, 1986). A low K_{cap} can be achieved with a high on-rate and a low off-rate constant. However, the on-rate constant of a capping protein cannot exceed that of actin for the barbed end since actin is already almost diffusion limited [Bonder & Mooseker, 1983; cf. Pollard and Cooper (1986)]. A further consideration is that dissociation of a capping protein from a filament end can occur by two routes, either alone or complexed to actin, and the apparent K_{cap} will be determined by the faster of the two off-rate constants. Actin has a K_d for the barbed end of about 0.1 μM—the critical concentration for the barbed end. Therefore, a K_{cap} of less that 0.1 μM implies that the capping protein itself has a low off-rate constant and also decreases the off-rate constant of the terminal actin molecule to which it is bound; i.e., it inhibits depolymerization. Two mechanisms for the decreased dissociation rate constants suggest themselves: cross-linking of two actin molecules by the capping protein or an induced conformational change in the capped actin molecule. Gelsolin can bind two actin molecules (Kwiatkowski et al., 1985; Bryan & Ho, 1986), and there is evidence that domains 1–3 and domain 6 (Kwiatkowski et al., 1989; Way et al., 1989) cooperate, since deletion of either domain greatly diminishes capping by the altered gelsolin. Evidence for an induced conformational change is less clear. We have investigated this further by studying the capping activities of 14NT, domain 1, and 41CT, domains 4–6, two gelsolin fragments with single functional actin-binding sites obtained by cloning or by proteolytic cleavage. Both fragments cap barbed ends and inhibit depolymerization, suggesting that gelsolin can induce a conformational change.

Barbed end capping has generally been associated with nucleation of polymerization, and all of the barbed end capping proteins capable of binding actin dimers nucleate filament assembly, although the activity of some, like the macrophage capping protein, is rather weak (Young et al., 1990). Gelsolin binds an actin dimer in calcium and retains only one tightly bound actin molecule after inactivation of domains 4–6 by calcium removal (Bryan & Kurth, 1984; Coue & Korn, 1985). This gelsolin–actin complex retains some nucleating activity, even though it has lost the ability to bind more than one actin monomer. Nucleotide-exchange studies (Bryan, 1988) indicate that the tightly bound actin is associated with 14NT, domain 1. Therefore, we have remeasured the nucleating activity of 14NT with a more sensitive test than that previously used (Kwiatkowski et al., 1985; Way et al., 1989).

Materials and Methods

Protein Preparation. Actin was prepared from a muscle acetone powder as previously described (Murray et al., 1981) with some modifications in the chromatography step (Young et al., 1990). Pyrenyl-actin was prepared according to Kouyama and Mihashi (1981) with the modifications described previously (Northrop et al., 1986). NBD–actin was prepared according to Detmers et al. (1981). Actin was stored in liquid nitrogen and defrosted as previously described (Young et al., 1990). The critical concentration of various actin preparations varied between 0.07 and 0.1 μM for uncapped filaments and between 0.4 and 0.6 μM for filaments capped at their barbed ends.

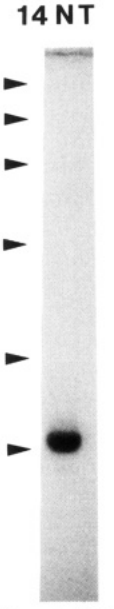

FIGURE 1: SDS–polyacrylamide gel of 14NT fragment purified by HPLC. Approximately 5 μg of 14NT was subjected to electrophoresis in a 15% polyacrylamide gel as described previously (Bryan & Hwo, 1986) and then stained with Coomassie Blue. The estimated molecular mass is 14–16 kDa. The arrows give the molecular masses of a set of marker proteins (top to bottom): phosphorylase *b* at 94 000; albumin at 67 000; ovalbumin at 43 000; carbonic anhydrase at 30 000; trypsin inhibitor at 20 100; α-lactalbumin at 14 400.

Acrosomes, a generous gift from Dr. Louis G. Tilney, were isolated from *Limulus* sperm according to Tilney (1975) with slight modifications as previously described (Young et al., 1990).

Villin, a generous gift from Dr. Mark Mooseker, was prepared according to Coleman and Mooseker (1985) and stored for weeks at 4 °C. Human plasma gelsolin was purified as described by Bryan (1988). Gelsolin was cleaved by use of chymotrypsin as described by Bryan and Hwo (1986). 14NT and 41CT were purified by HPLC as described previously (Bryan & Hwo, 1986). A cloned C-terminal fragment of gelsolin starting with amino acid residue Gly_{310} was purified from bacterial extracts by use of actin–agarose chromatography. Extracts were loaded in the presence of calcium, 0.2 mM, and the column was washed with 100 mM NaCl/0.2 mM $CaCl_2$/50 mM Tris-HCl, pH 8, and then eluted with the same buffer containing 5 mM EGTA in place of the calcium chloride. Further purification was done with HPLC (Bryan & Hwo, 1986). All of these fragments are homogeneous when analyzed by rechromatography on HPLC columns or with use of overloaded SDS–polyacrylamide gels as described previously (Bryan & Hwo, 1986; Bryan, 1988). A gel indicating the purity of the 14NT fragment that has not been published previously is shown in Figure 1.

Protein concentrations were calculated for actin with E_{290} = 24.9 mM^{-1} cm^{-1} and for gelsolin, 14NT, and 41CT with E_{280} = 117.23, 21.08, and 67.39 mM^{-1} cm^{-1}, respectively (Bryan, 1988).

Fluorescence and Kinetic Measurements. Changes in G- and F-actin were calculated from fluorescence measurements as previously described (Weber et al., 1987) with a Perkin-Elmer MPF-3L spectrofluorometer. All experiments were carried out with Mg–actin, the physiological form of actin (Weber et al., 1969; Kitasawa et al., 1982); in a KCl–Mg medium elongation starts at the maximal rate with Mg–actin, but not with Ca–actin. The conversion to Mg–actin was

carried out as previously described (Young et al., 1990).

Measurements of the elongation rates at either filament end, of depolymerization, and of cutting were carried out as previously described (Walsh et al., 1984; Northrop et al., 1986; Young et al., 1990) in a medium containing 10 mM imidazole buffer, pH 7.4, 0.1 M KCl, 2 mM $MgCl_2$, 1 mM azide, 1 mM dithiothreitol, 0.5 mM ATP, and either 0.2 mM Ca^{2+} or 5 mM EGTA. The data were fitted to one exponential with use of a nonlinear fitting routine.

Known number concentrations of pointed filament ends in the presence and absence of Ca^{2+} and of barbed filament ends in the absence of Ca^{2+} were obtained as previously described (Northrop et al., 1986; Walsh et al., 1984). Actin stock solutions containing a known number concentration of filaments were prepared by copolymerizing actin with a strong nucleating protein, either villin or gelsolin, which results in the formation of one filament per nucleating protein molecule (Walsh et al., 1984, Coleman et al., 1985).

Results

Determination of Capping Constants: 41CT Capping. The extent of capping by 41CT and 14NT was estimated by the inhibition of elongation at the barbed ends of actin filaments in *Limulus* acrosomes. Acrosomes consist of highly cross-linked actin filaments that do not depolymerize in the absence of monomeric actin. At actin concentrations above the critical concentration of the barbed end, but below that of the pointed end, the net rate of elongtion reflects growth at barbed ends with no treadmilling, i.e., without simultaneous depolymerization at the pointed filament end. The observed rates of elongation are linear functions of the number of free barbed ends in the acrosomes and of the concentration of polymerizable G-actin ($=C_0 - C_c$, where C_0 is the total G-actin concentration and C_c is the critical concentration).

Since we wanted to determine whether 41CT, like gelsolin, was capable of decreasing the dissociation rate constant of the terminal actin molecule, we had to ensure that 41CT was not binding to an acrosomal actin molecule, i.e., one tightly cross-linked to the filament end. Therefore, we preelongated acrosomes with muscle actin before adding 41CT and additional G-actin. The time course of elongation can be described by a single exponential in either the absence or the presence of increasing concentrations of 41CT or the 14NT–actin complex indicating that the extent of barbed end capping does not change during the period of measurement.

In the experiment shown in Figure 2, 4 nM 41CT reduced the initial rate of elongation by about 50%. Inhibition reflects the extent of capping according to the equation

$$\text{capped filaments/total filaments} = 1 - \text{rate}_{\text{capped}}/\text{rate}_{\text{uncapped}}$$

A double-reciprocal plot (data not shown) of the fraction of capped filaments versus total 41CT concentration gave a value for K_{cap} of about 5 nM. A definition of K_{cap} is given in the Discussion. The total concentration of 41CT was taken as equal to the concentration of free 41CT + 41CT–G-actin since the fraction of 41CT bound to filament ends was negligible. This value of K_{cap} is in the same range as values obtained without preelongation of the ends. This could indicate that G-actin, which is present in large excess over 41CT, binds to acrosomes faster than 41CT. Alternatively, the extent of capping may be limited by the dissociation rate constant of 41CT, rather than that of the 41CT–G-actin complex. We consider this further in the Discussion.

The capping constant, K_{cap}, is a mixed constant reflecting both the binding of 41CT to terminal actin molecules and the effect of 41CT on actin–actin bonds at the filament end as outlined in the introduction. An understanding of the interrelationship between these two components requires knowledge of the binding constant between 41CT and actin monomers.

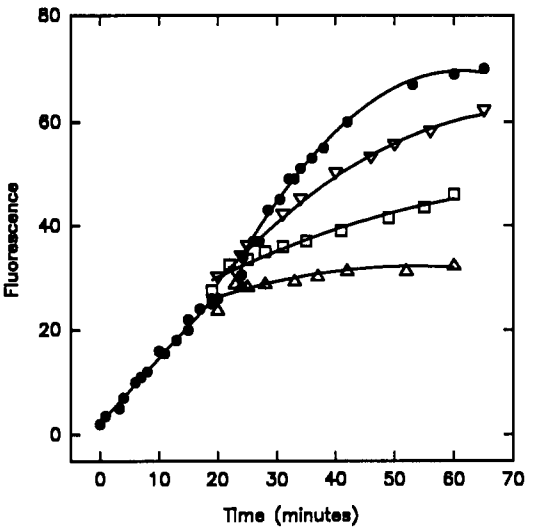

FIGURE 2: Estimation of the 41CT capping constant from the inhibition of filament elongation at the barbed end. Acrosomes were preelongated by incubation with 0.5 μM Mg–actin, 10% pyrene labeled, which is equal to or below the critical concentration for the pointed end. After 18–22 min, when approximately 0.3 μM actin had been incorporated into the acrosomal ends, more G-actin (0.3 μM) was added with increasing amounts of 41CT. (●) Control values in the absence of 41CT; (▽) 4 nM 41CT; (□) 20 nM 41CT; (△) 50 nM 41CT.

Determination of the Monomer Binding Constant of 41CT. Neither NBD– nor pyrenyl-actin shows a fluorescence change upon binding of 41CT, the C-terminal fragment of gelsolin. Therefore, we evaluated monomer binding to 41CT by determining the extent to which this fragment inhibits monomer incorporation onto the pointed end of actin filaments. 41CT does not bind to the pointed end, as shown by the cross-linking studies of Sutoh and Yin (1989) and a consideration of the structure of actin monomers and their organization into filaments (Kabsch & Holmes, 1990; Holmes, 1990). Therefore, inhibition of the initial rate of elongation at the pointed ends of villin-capped actin filaments reflects monomer sequestration. As shown in Figure 3, 41CT decreased the initial elongation rate (Figure 3A) and decreased the total amount of actin incorporated after 24 h (Figure 3B). In Figure 3B, the intersection of the polymer and G-actin fluorescence curves gives the steady-state G-actin concentration. There is an increase from a control value of 0.6 μM to a value of about 1.0 μM in the presence of 0.5–0.6 μM 41CT, consistent with a K_d for the 41CT–actin interaction of 0.3 μM. The elongation rate measurements in Figure 3A give a similar K_d value of 0.2 μM. The difference between the K_d of 0.2 μM obtained from the elongation rate measurements and 0.3 μM from the end point fluorescence values is small and may be accounted for by 41CT denaturation over the 24-h room temperature incubation used to obtain the end points. In a number of experiments, with both cloned and proteolytic 41CT, the estimated K_d for actin monomer binding ranged from 0.15 to 0.5 μM.

Capping by the 14NT–G-actin Complex. The effect of 14NT on the terminal actin–actin bond was studied directly by measuring capping with use of the 14NT–G-actin complex. In the presence of calcium, 14NT forms a stoichiometric, very high-affinity complex with actin monomers with a K_d of about 5 pM at depolymerizing (Bryan, 1988) and polymerizing salt concentrations (Figure 4A). Removal of calcium raises the K_d significantly: NBD–actin complexed to 14NT could not

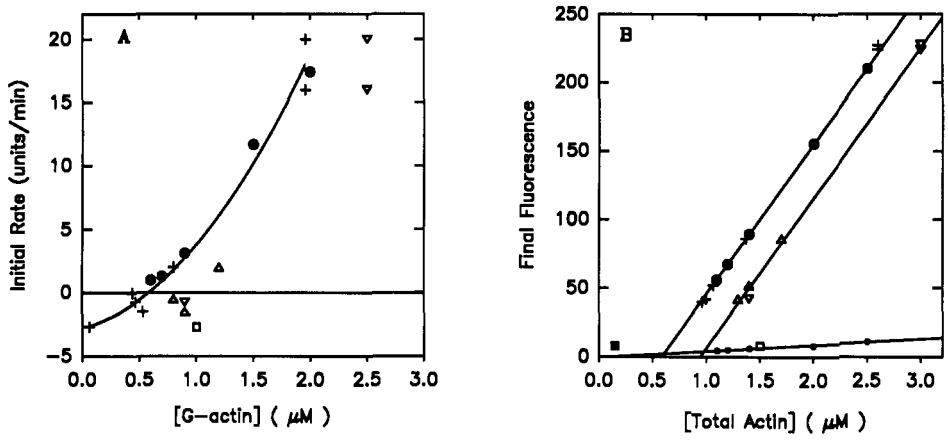

FIGURE 3: Estimation of the monomer binding constant of 41CT. Estimations were made with use of two approaches. (A) inhibition of pointed end elongation by 41CT was used. Elongation was started by the addition of villin-capped actin filaments (actin/villin = 50; F-actin final concentration = 0.5 μM; 10% pyrene labeled) and polymerizing salts, containing 100 μM calcium, to Mg–G-actin either in the absence of 41CT [control (●)] or in the presence of different concentrations of 41CT [0.5 μM (△), 0.6 μM (▽), 4 μM (●)]. Rates in the presence of 41CT are plotted against the total added G-actin concentration and against the calculated free G-actin concentration with a K_d of 0.2 μM (+). The plusses fall reasonably well on the control curve, indicating that the K_d for monomer sequestration by 41CT was about 0.2 μM. (B) The second method of estimation utilized the decrease in the end point of polymerization in the presence of 41CT. The data are the overnight fluorescence end points of the assays in panel A. The end points are plotted against the total added actin (added G- + added F-actin), with the same symbols used as in panel A, and against the calculated values of the total actin available for polymerization in the presence of 41CT (+ and □), i.e., total actin − 41CT–actin. The concentration of 41CT–actin was calculated with a K_d of 0.3 μM for monomer binding. The smaller filled circles are the G-actin fluorescence values.

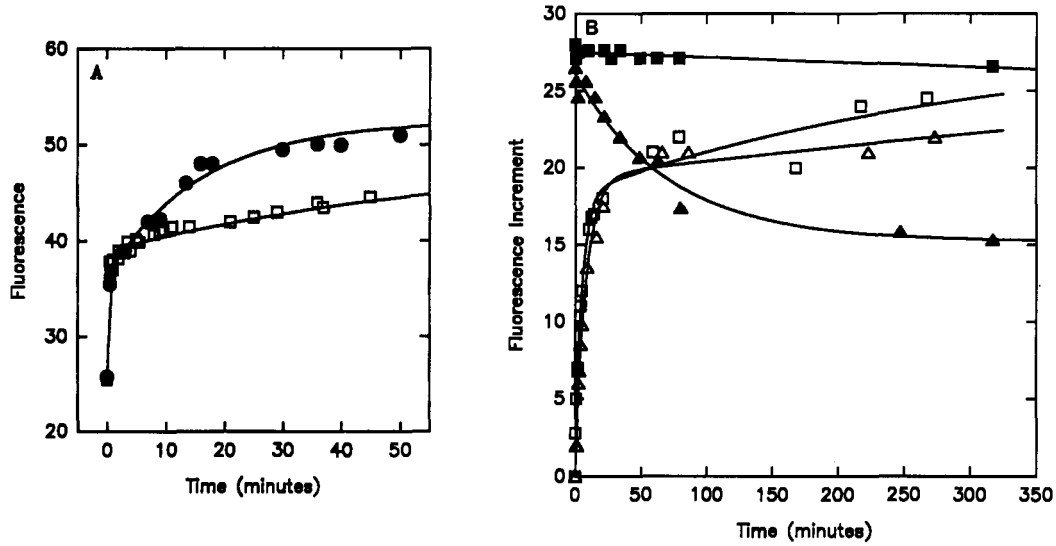

FIGURE 4: (A) Formation and stability of the 14NT–actin complex in the presence and absence of calcium. The time course of the fluorescence increase during complex formation between NBD–G-actin and 14NT (□) and gelsolin (●). NBD–G-actin (0.1 μM)/Mg–actin was mixed with either 2.0 μM 14NT or 2.0 μM gelsolin in polymerizing medium (0.1 M KCl and 2.0 mM $MgCl_2$ containing 100 μM $CaCl_2$). The biphasic time course suggests a slow conformational change subsequent to complex formation. The data can be fit with a value of 5×10^4 M^{-1} s^{-1} for the second-order rate constant and a value of 0.0003–0.0006 s^{-1} for the first-order rate constant of the conformational change. (B) Exchange of NBD-actin bound to 14NT with added unlabeled actin in the presence and absence of calcium. 0.1 μM NBD–G-actin was incubated with 0.2 μM 14NT in either the presence of 100 μM Ca^{2+} [(□) end point = 28 units) or 5 mM EGTA [(△) end point = 26 units). After 12 h, 0.6 μM unlabeled actin was added and the decrease in fluorescence, resulting from the release of bound NBD–actin, was followed in the presence of calcium (■) or 5 mM EGTA (▲). The amount of unlabeled actin was limited in order to minimize polymerization of actin. It was impossible to avoid polymerization altogether because the critical concentration is low when the extent of barbed end capping (by 14NT–G-actin) is only about 67% (Walsh et al., 1984; Selve & Wegner, 1986) as would be expected if the K_{cap} is 20–40 nM in EGTA. The measured exchange occurred mainly during the lag period.

be exchanged with unlabeled NBD–actin in the presence of calcium, but it did exchange at a measurable rate in its absence (Figure 4B). From the on-rate constants in the absence of calcium, estimated from the biphasic binding curve shown in Figure 4A, and the off-rate constant calculated from the exchange data, an effective K_d of 10 nM was calculated according to the reaction model

$$14NT + G\text{-actin} \underset{0.0009\ s^{-1}}{\overset{2.5 \times 10^4\ M^{-1}\ s^{-1}}{\rightleftharpoons}} 14NT\text{–}G\text{-actin} \underset{0.0003\ s^{-1}}{\overset{0.0008\ s^{-1}}{\rightleftharpoons}} 14NT\text{–}G\text{-actin}^*$$

This model accounts for the biphasic time course of 14NT binding to NBD–actin by a slow conformational change of 14NT–G-actin to 14NT–G-actin* subsequent to complex formation.

With this low K_d more than 90% of 14NT was bound to actin during the capping constant measurements that were carried out in a similar manner as described for 41CT. In addition, the exchange of 14NT between the complex and cross-linked actin at the acrosomal ends is expected to be minimal because the exchange rate of 14NT-bound actin

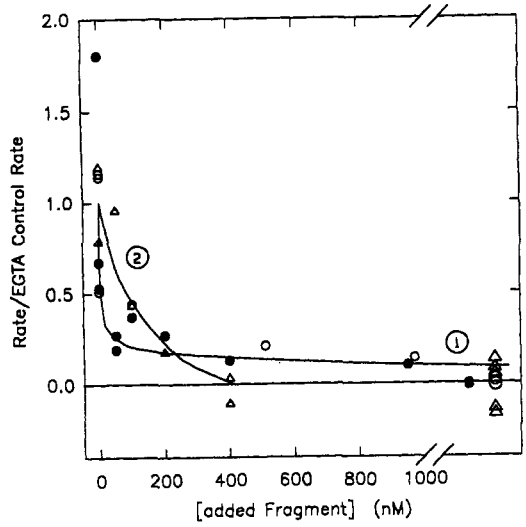

FIGURE 5: 41CT and the 14NT–actin complex inhibit actin depolymerization. Curve 1 (○ and ●): Actin (3 μM) 35% pyrene labeled, was polymerized the day before the experiment in either the absence or presence of 41CT at an actin to 41CT ratio of 5. Depolymerization was initiated by diluting F-actin to 50 nM into a polymerizing medium containing 0.1 mM Ca^{2+} and increasing concentrations of 41CT. All rates are given as a fraction of the maximal rate after subtraction of the pointed end rate. The maximal rate was obtained by use of 5 mM EGTA in the absence of any binding protein. The depolymerization rate at the pointed end was obtained after the addition of 10 nM gelsolin–actin dimers. The points after the break in the abscissa, which cluster around zero, indicate the scatter in the rates determined for the pointed end. The scatter of the maximal rates is indicated by the values at [41CT] = 0. (●) F-actin diluted into 41CT; (○) F-actin copolymerized with 41CT and diluted into 41CT. Curve 2 (△) is the data obtained with 14NT–G-actin. Villin-capped actin filaments (actin/villin = 50) were diluted to a total actin concentration of 50 nM into a salt solution containing 5 mM EGTA and 14NT–G-actin at the concentrations given on the abscissa. In EGTA, villin dissociates from the barbed filament ends and is replaced by 14NT–G-actin. All depolymerization measurements, including the determination of the control rates, were carried out as described for curve 1. The 14NT–G-actin complex and villin-capped actin filaments were prepared the day before as described in Figure 3.

monomers is unmeasurably low in the presence of calcium (Bryan, 1988) and quite slow, with a $t_{1/2}$ of about 1 h in its absence (Figure 4B). Since all the 14NT was bound to actin monomers that were not cross-linked to acrosomal filaments, it was not necessary to preelongate the acrosomes. With a K_{cap} of 10 nM in the presence of calcium, 14NT must have decreased the dissociation rate constant of the terminal actin 8–10-fold. This capping by 14NT was not calcium dependent, but it was slightly calcium modulated; in the absence of calcium K_{cap} was 20–40 nM. This small calcium effect, a 2–4-fold difference in the capping constant of 14NT in the presence and absence of calcium, contrasts with the very large calcium effect on complex formation between 14NT and actin monomers, a 1000-fold difference in the binding constant in the presence and absence of calcium. The calcium sensitivity of domain 1 has been studied in detail by Way et al. (1990).

Effects of 41CT and 14NT on Depolymerization Rates. 41CT- and 14NT–actin both inhibit depolymerization at the barbed filament end. This was determined from measurements of the initial rate of depolymerization of actin polymers diluted to subcritical actin concentrations. In the presence of saturating concentrations of 41CT or 14NT, the initial rates of depolymerization were lower than that of control filaments of the same average length and were similar to that of gelsolin-capped actin filaments (Figure 5).

Nucleation by 41CT and 14NT. Typically, nucleation has been assessed on the basis of a shortening of the lag period of polymerization, even though simultaneous filament end capping and monomer sequestration can give misleading results with this method (Young et al., 1990; Kurth et al., 1983).

We evaluated the ability of 41CT and 14NT to promote nucleation by testing their ability to reduce the steady-state average filament length. Increased nucleation results in a decrease in average filament length and an increase in filament number (Oosawa & Asakura, 1975). Strong nucleating proteins, like villin and gelsolin, shorten the average filament length in direct proportion to their ratio with actin (Coleman & Mooseker 1985; Northrop et al., 1986).

Steady-state conditions were established by overnight polymerization in the absence or presence of 14NT and 41CT at high fragment-to-actin ratios. Decreases in average filament length, compared to the actin controls, were evaluated from a comparison of the depolymerization rates measured after dilution to a constant subcritical concentration of actin. Barbed end capping was equalized in the presence and absence of fragments in two different ways: When 41CT was used, 41CT was added to the controls during depolymerization. When 14NT was used, a saturating concentration of gelsolin–actin complexes was added to the depolymerization assays for both controls and for actin copolymerized with 14NT. At fixed concentrations of total actin, the number concentration of filaments, and therefore the rate of depolymerization, is inversely proportional to average length. Within the scatter of the data we saw no significant effect of either 41CT (Figure 5, curve 1, compare open with closed circles marking the controls) or 14NT (Figure 6A), after copolymerization at ratios of 41CT or 14NT to actin as high as 1:5. The resolution of the measurement with 41CT is limited by shearing of the long control actin filaments during dilution, making it impossible to obtain a true control length by any measurement that involves handling of actin after polymerization. For comparison, copolymerization with gelsolin–actin complexes in EGTA at the same ratio (Figure 6B) increased the depolymerization rate about 50-fold over control values, corresponding to an average length of 20 monomers per filament as determined by calibration with villin-copolymerized actin filaments (Walsh et al., 1984).

Discussion

Mechanism of Capping by Gelsolin. Capping constants have been determined in several ways, including measuring the inhibition of elongation (Young et al., 1990; Caldwell et al., 1989), determining the depolymerization rate (Caldwell et al., 1989), or measuring increases in critical concentration (Walsh et al., 1984; Selve & Wegner, 1986). The last method allows an estimation of the capping constant when its value is below 0.1 nM. However, it has the disadvantage that the critical concentration does not increase proportionately with the saturation of barbed ends with capping protein. The critical concentration will only increase significantly when 90–100% of the ends are capped (Walsh et al., 1984; Selve & Wegner 1986; Young et al., 1990). Furthermore, if there is significant monomer sequestration by the capping protein the increase in free G-actin cannot be accurately assessed in the concentration range needed for 90–100% capping. This latter problem is probably the reason why Kwiatkowski et al. (1985) failed to observe capping by either of the two gelsolin domains studied here.

The inhibition of elongation rates, on the other hand, is a linear function of saturation with capping protein. Measurements can be carried out at relatively high actin concentrations so that the fraction of sequestered monomer becomes insignificant. Furthermore, elongation measurements have an

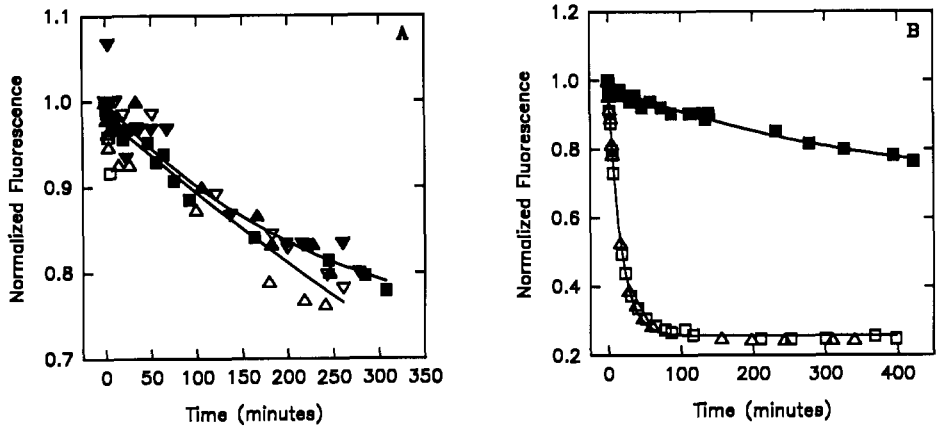

FIGURE 6: Comparison of nucleation by 14NT and the gelsolin–actin monomer complex in EGTA. Nucleation activity was evaluated from the reduction of the average steady-state filament length. Steady state was obtained by overnight polymerization of 3 and 6 μM actin in the absence and presence of either 1.2 μM 14NT or 1.2 μM gelsolin–actin complexes in EGTA, respectively. To evaluate differences in average length from differences in depolymerization rates, the polymerized actin was diluted to a concentration of 150 nM into polymerizing medium containing a saturating (50 nM) concentration of gelsolin–actin complex to obtain complete barbed end capping. Depolymerization resulting from dilution below the pointed end critical concentration is indicated by the decrease of fluorescence with time (20% pyrenyl labeling). Closed symbols: controls in triplicate in panel A, single in panel B. Open symbols: (A) 14NT copolymerized actin in triplicate, (B) actin copolymerized with gelsolin–actin complexes in EGTA in duplicate.

advantage over depolymerization measurements in that they avoid some technical difficulties, for example filament shearing during dilution to subcritical actin concentrations.

Capping constants estimated by any of these methods are usually described as the K_d of the capping protein for the filament end. This is not strictly correct since the measured extent of capping depends not only on the binding of the capping protein but also on the kinetics of binding of the capping protein–actin complex to the filament end. This is described by the expression

$$b/b_{cap} = \{k_{-(capper)} + k_{-(capper-actin)}\}/\{k_{+(capper)}[capper] + k_{+(capper-actin)}[capper-actin]\}$$

and

$$b/b_{cap} = K_{cap}/[\text{total capper}]$$

where k_+ and k_- are the association and dissociation rate constants for the indicated species and b and b_{cap} are the concentrations of free and capped barbed filament ends, respectively. Once steady-state capping has been achieved, these expressions show that a high-affinity capping protein with high rate constants may associate with the barbed end as the free protein and dissociate as the actin complex, provided the actin complex has both high rate constants and low affinity. Tight binding of the capping protein, tighter than the binding of the terminal actin molecule to the barbed end, is possible only if the off-rate constant of the terminal actin complexed to the capping protein is lower than the off-rate constant of the uncomplexed terminal actin molecule.

The decrease in the off-rate constant of the complexed actin could be accomplished in two ways: One mechanism is for a capping molecule to bind two actin molecules at the filament end, allowing only dissociation of actin dimers. Since dimer dissociation from either single- or double-stranded helices will involve the breaking of more bonds than monomer dissociation, this should decrease the rate of dissociation. This "dimerization" mechanism is restricted to capping proteins with two or more actin-binding sites, such as gelsolin, villin [cf. Mooseker (1985)], macrophage capping protein (Young et al., 1990), and capZ (Caldwell et al., 1989), and cannot be invoked for molecules with a single actin-binding site. ADP-ribosylated actin has been shown to cap barbed filament ends (Wegner & Aktoris, 1988). This is a special case because actin itself has several actin-binding sites and ribosylation of an actin monomer strengthens its binding to the barbed filament end but prevents association with other ribosylated actin molecules (Wegner & Aktoris, 1988). Single binding site capping proteins, by contrast, must alter the kinetics or the thermodynamics of actin–actin binding by a second mechanism, by imposing a conformational change on the actin molecule.

In the presence of calcium, gelsolin probably achieves very strong capping, a capping constant <10 pM (Selve & Wegner, 1986), by a combination of both mechanisms. Intact gelsolin is generally acknowledged to bind two actin monomers in the presence of Ca^{2+}. Presumably the C-terminal half, domains 4–6, and the N-terminal half, domains 1–3, each bind one monomer (Bryan & Kurth 1984; Kwiatkowski et al., 1989; Way et al., 1989). Contributions from conformational changes are suggested by our observations that fragments with single actin-binding sites (Kwiatkowski et al., 1985, 1989; Bryan, 1988; Way et al., 1989), 41CT, domains 4–6, and 14NT, domain 1, both inhibit depolymerization and cap with K_{cap} values below the critical concentration for the barbed end. The existence of second, weak actin-binding sites on these fragments, as has been demonstrated for the macrophage capping protein (Young et al., 1990), is unlikely since neither domain is capable of nucleating actin polymerization as we confirm by using a very sensitive test.

The conformational change could reduce the rate constant of actin dissociation either by a purely kinetic effect or by a strengthening of the terminal actin–actin bond. If we make the assumption that the free energy change contributed by ATP hydrolysis is equivalent when either G-actin or 41CT–G-actin binds to the the barbed end, the K_d of 41CT–actin for the barbed end can be calculated and compared to that of G-actin. The basis for this calculation is given by the top energy square in Figure 7. The reactants in the upper left are converted to the products in the lower right corner. The standard free energy change, determined by the product of the equilibrium constants, is independent of the pathway; i.e., the products of the K_d values in both pathways must be equal. The K_d values for the first step in each path have been determined by direct measurements. The K_d for monomer binding by 41CT is higher than the K_d for actin binding to the filament end; therefore, the K_d value for binding of the 41CT–actin complex to an end must be lower than that of

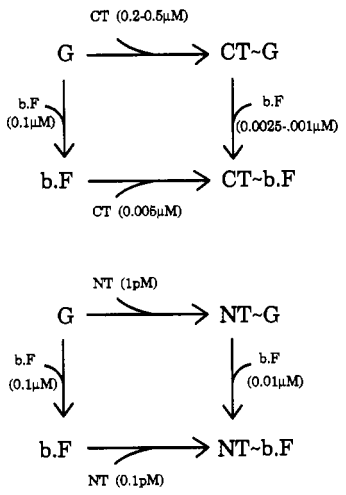

FIGURE 7: Energy squares for capping with 41CT and 14NT. G-actin monomer is in the upper left corner in both panels with reactants added to form the appropriate capped barbed end in the lower right corner. The reactants are identified as follows: G-actin (G); 41CT (CT); 14NT (NT); barbed filament ends (b.F). The numbers in brackets are the appropriate K_d values. Vertical arrows indicate binding of one actin molecule to the barbed end, either as actin alone (left arrows) or actin complexed to 41CT (top panel, right arrow) or to 14NT (bottom panel, right arrow). The horizontal arrows indicate binding of the fragment to either G-actin (upper arrow) or the barbed end (lower arrow).

41CT alone. In short, the complex appears to bind more tightly to the barbed end than 41CT alone, and therefore, capping is limited by the binding of 41CT alone. We have therefore assigned the measured K_{cap} value to the interaction between 41CT and the barbed end.

The opposite is true of capping by 14NT, which is limited by the relatively low affinity of the 14NT–actin complex for the barbed end as can be seen from the lower energy square. All K_d values were measured except that for 14NT binding to the filament end, which is calculated to have the lowest value. This means that in the presence of calcium 14NT binds extremely tightly to the barbed filament end, even more strongly than to actin monomers.

Nucleation by 14NT and Its Implication for the Mechanism of Nucleation of the Gelsolin–Actin Monomer Complex in EGTA. A comparison of the 41CT and the 14NT binding constants for actin monomers and for actin at the barbed filament end shows that both fragments prefer the filament end conformation of actin. Therefore, one might expect that fragment binding to monomers would force them into this conformation and increase nucleation. However, this is not the case since using a very sensitive assay we confirm that neither 41CT nor 14NT promotes nucleation. The difference in nucleating capacity between the fragments and the gelsolin actin monomer complex isolated in EGTA could be used to argue that gelsolin prefers filament ends even more strongly than the fragments. Alternatively, it seems more plausible that nucleation requires a protein that can bind two actin molecules and that nucleation by the gelsolin–actin monomer complex in EGTA (Kurth et al., 1983) involves both N-terminal actin-binding domains. Gelsolin fragments reported to promote nucleation, the amino-terminal half of gelsolin (Bryan & Hwo, 1986; Chaponnier et al., 1986; Kwiatkowski et al., 1985), and a mutated gelsolin molecule missing domain 1 (Way et al., 1989) are known to have two actin-binding domains.

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Characterization of Maize Microtubule-Associated Proteins, One of Which Is Immunologically Related to Tau†

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ABSTRACT: Microtubule-associated proteins (MAPs) are identified as proteins that copurify with tubulin, promote tubulin assembly, and bind to microtubules in vitro. Higher plant MAPs remain mostly unknown. One example of non-tubulin carrot proteins, which bind to neural microtubules and induce bundling, has been reported so far [Cyr, R. J., & Palewitz, B. A. (1989) *Planta 177*, 245-260]. Using taxol, we developed an assay where higher plant microtubules were induced to self-assemble in cytosolic extracts of maize cultured cells and were used as the native matrix to isolate putative plant MAPs. Several polypeptides with an apparent molecular masses between 170 and 32 kDa copolymerized with maize microtubules. These putative maize MAPs also coassembled with pig brain tubulin through two cycles of temperature-dependent assembly-disassembly. They were able to initiate and promote MAP-free tubulin assembly under conditions of nonefficient self-assembly and induced bundling of both plant and neural microtubules. One of these proteins, of about 83 kDa, cross-reacted with affinity-purified antibodies against rat brain tau proteins, suggesting the presence of common epitope(s) between neural tau and maize proteins. This homology might concern the tubulin-binding domain, as plant and neural tubulins are highly conserved and the plant polypeptides coassembled with brain tubulin.

Most essential functions of eukaryotic cells, such as chromosome segregation during mitosis, intracellular organelle motility, determination of cell shape, and cell cleavage, are mediated by microtubules. These dynamic polymers are assembled from two major soluble and highly conserved proteins, the α- and β-tubulins. Additional polypeptides, collectively known as microtubule-associated proteins (MAPs), copolymerize with tubulins and bind to the outside surface of the microtubule (Olmsted, 1986). Originally isolated from mammalian brain tissue, MAPs were named MAP 1, MAP 2 (>200 kDa), and tau (35-65 kDa) according to their molecular mass. The presence of MAPs in animal nonneural systems is now well documented (Vallee & Collins, 1986; Aizawa et al., 1989) and suggests that they are universal components of the cytoskeleton. Recently, a wealth of data, primarily based on molecular genetics, has uncovered new properties of MAPs, particularly concerning tau and MAP 2, which highlight their potential role in microtubule function (Mandelkow & Mandelkow, 1990; Cleveland, 1990). It has been demonstrated that multiple tubulin isoforms of higher plant (Hussey et al., 1987) and animal (Lewis et al., 1987) cells or tubulins from various origins (Guens et al., 1989; Vantard et al., 1990) coassemble in functionally distinct microtubules, suggesting that different tubulins might be interchangeable. As a result, much attention is now focused on microtubule-associated proteins as factors that can modulate the assembly of tubulin and microtubule activity in vivo.

In the present debate, higher plant MAPs remain mostly unknown. Only one example of non-tubulin proteins that bind to neural microtubules in vitro and are named microtubule-binding proteins (Cyr & Palewitz, 1989) has been described so far. This lack of information represents a serious handicap in understanding the molecular mechanisms that regulate intracellular plant tubulin assembly and the differential stability and bundling properties of plant microtubules. Higher plant cells represent a peculiar model in that they lack distinct centrosomes, which are known to control microtubule nucleation and spatial distribution in animal cells including neurons. Therefore, the regulation of plant microtubule assembly and dynamics remains poorly documented. Higher plant cells possess peculiar microtubule-organizing domains, such as the nuclear surface (Vantard et al., 1990; Baskin & Cande, 1990; Lambert et al., 1991), whose function seems to involve "MAP"-mediated interactions with cell membranes.

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