Inhibition of Actin Filament Depolymerization by the *Dictyostelium* 30,000-D Actin–bundling Protein

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Abstract. We have studied the effect of the *Dictyostelium discoideum* 30,000-D actin–bundling protein on the assembly and disassembly of pyrenyl-labeled actin in vitro. The results indicate that the protein is a potent inhibitor of the rate of actin depolymerization. The inhibition is rapid, dose dependent, and is observed at both ends of the filament. There is little effect of 30-kD protein on the initial rate of elongation from F-actin seeds or on the spontaneous nucleation of actin polymerization. We could detect little or no effect on the critical concentration. The novel feature of these results is that the filament ends are free for assembly but are significantly impaired in disassembly with little change in the critical concentration at steady state. The effects appear to be largely independent of the cross-linking of actin filaments by the 30-kD protein. Actin cross-linking proteins may not only cross-link actin filaments, but may also differentially protect filaments in cells from disassembly and promote the formation of localized filament arrays with enhanced stability.

ACTIN-binding proteins appear to function as the primary tools used by cells to mediate precise spatial and temporal control of the assembly and distribution of actin filaments during cell movements (Cooper, 1991; Devreotes and Zigmond, 1988). These actin-binding proteins have been classified as monomer binding proteins, end-binding proteins, side-binding proteins, and cross-linking proteins on the basis of detailed biochemical investigations of their interactions with actin in vitro (reviewed by Craig and Pollard, 1982; Hartwig and Kwiatkowski, 1991; Pollard and Cooper, 1986; Vandekerckhove, 1990).

Actin filament cross-linking proteins have been shown to contribute to the formation of actin gels and filament bundles (Kolega et al., 1991; Matsudaira, 1991). The formation of gels usually involves proteins such as actin-binding protein or filamin (Brotschi et al. 1978; Weihing, 1985) that form a network of the actin filaments. These proteins are found in the cortex of cells, in lamellipodia, and in stress fibers (Varlerius et al., 1981; Hartwig et al., 1980; Dharmawardhane et al., 1989). Proteins that bundle actin filaments, such as 30-kD actin-bundling protein of *Dictyostelium* (Fechheimer and Taylor, 1984), villin (Bretscher and Weber, 1980), fimbrin (Glenney et al., 1981), or fascin (Bryan and Kane, 1978), usually do not form gels but cross-link actin filaments into parallel arrays. Bundling proteins have been found in microvilli, filopodia, stereocilia of hair cells, and acrosomal processes of various sperm. The bundling of actin, in addition to organizing the F-actin, is believed to increase its mechanical strength (Sato et al., 1987; Maciver et al., 1991).

Actin filament end-binding proteins and monomer binding proteins are clear candidates for involvement in regulation of the steady state concentration and the flux of actin monomers in vivo. Filament side–binding proteins, such as tropomyosin, decrease flux by stabilizing F-actin (Broschat et al., 1989; Broschat, 1990; Weigt et al. 1990). Whether actin filament cross-linking proteins also affect the dynamic behavior of actin is unknown. Clearly, some F-actin–containing structures, such as the acrosomal bundle of *Limulus* sperm, are extremely stable. Presumably, this stability is due to lateral binding and cross-linking factors since both ends of the filaments are free to elongate (Bonder and Mooseker, 1983; Tilney et al., 1981). In addition, F-actin in lysates of polymorphonuclear leukocytes is much more stable than the same F-actin in vivo. The stability of the cytoskeletal F-actin correlates with the ability to pellet the F-actin at low centrifugal speeds suggesting that cross-linking proteins may be involved (Cano et al., 1992a).

The 30,000-D actin filament-bundling protein from *Dictyostelium discoideum* is a monomeric, calcium-regulated actin filament cross-linking protein selectively localized in filopodia of *Dictyostelium* amoeba (Fechheimer and Taylor, 1984; Fechheimer, 1987; Johns et al., 1988; Furukawa and Fechheimer, 1990; Fechheimer et al., 1991). We show here that this protein is also a potent inhibitor of F-actin depolymerization. We conclude that in addition to their role in organizing the structure of the F-actin, cross-linking proteins may also affect the stability of the F-actin, and contribute to regulation of its dynamic behavior.

Materials and Methods

Materials

Phalloidin, tetramethylrhodamine isothiocyanate–labeled phalloidin (TRITC-phalloidin), cytochalasin D, DEAE-sephacel, deoxyribonuclease I (DNase I, type II, from bovine pancreas, chromatographically purified), NP-40 and ATP, were obtained from Sigma Chemical Company (St. Louis, MO). DTT was obtained from United States Biochemical Corp. (Cleveland, OH).

Gelsolin Preparation

Rabbit plasma gelsolin was isolated from rabbit serum (Gibco Laboratories, Grand Island, NY) using methods modified from the simplified chromatographic procedure of Cooper et al. (1987). The changes from the published protocol were: (a) the first DEAE-sephacel step (in the presence of calcium) was done by a "batch" method rather than in a column; and (b) the fractions from the DEAE-sephacel column (in the presence of EGTA, eluted with 0 to 0.5 M NaCl) were analyzed for gelsolin by dot blots using a mAb to gelsolin and a peroxidase-conjugated secondary antibody (both antibodies from Sigma Chemical Co.). The activity of the gelsolin was assayed by the change in fluorescence of NBD-actin upon binding gelsolin under nonpolymerizing conditions (Bryan and Kurth, 1984; Coué and Korn, 1985). We are grateful to Dr. A. Weber (University of Pennsylvania) for this calibration. The sample was stored at −80°C or diluted 1:1 with ethyleneglycol and stored at −20°C.

Purification of the Dictyostelium 30-kD Protein

The Dictyostelium 30,000-D actin-binding protein was isolated as previously described (Fechheimer and Furukawa, 1991), dialyzed overnight in 2 mM Tris, 0.2 mM DTT, 0.02% sodium azide, pH 7.5, and stored at −70°C. Protein concentration was determined using the bicinchoninic acid method (Smith et al., 1985) with BSA as the standard.

Pyrene-labeled Actin Isolation and Labeling

Rabbit skeletal actin was isolated (Murray et al., 1981; Spudich and Watt, 1971) and labeled with pyrene and gel purified as previously described (Northrup et al., 1986). Preparations used for these studies had between 50 and 90% of the actin labeled with pyrene. Pyrene-labeled actin was stored at ∼20 μM in column buffer (0.067% [vol/vol] triethanolamine, 0.3 mM CaCl₂, 0.68 mM ATP, 0.1 mM EDTA, 0.02% sodium azide, pH 7.5). To initiate polymerization, G-actin was diluted (usually to 2 μM) into "assay buffer" (0.138 M KCl, 2 mM MgCl₂, 0.025 M Tris HCl, 1 mM EGTA, 0.2% NP-40, 1 mM ATP, pH 7.4). In some experiments, the polymerization was "seeded" by adding 50 μl of 2 μM F-actin seeds via a Hamilton syringe which sheared the F-actin into smaller filaments. In other experiments, filaments were nucleated by addition of gelsolin and 0.1 mM calcium.

Depolymerization Protocol

To initiate depolymerization, 50 μl of the 2 μM pyrene-labeled F-actin solution with or without 30-kD protein was diluted into 950 μl of "assay buffer" by pipetting slowly with a yellow micropipette tip with the tip cut off. The mixture in a 1-ml cuvette was stirred gently by four strokes with a plastic paddle before the decrease in the pyrene fluorescence intensity (370-nm excitation and 410-nm emission) was followed over time in a Perkin Elmer LS-5 fluorescence spectrophotometer.

Sedimentation Assays

Sedimentation of F-actin cross-linked by 30-kD protein with low centrifugal forces was evaluated using pyrene-labeled actin. Pyrene-labeled F-actin (2 μM) was incubated with various concentrations of 30-kD protein as described in the text. The pyrene fluorescence intensity in the sample was determined before the samples were centrifuged in a table top ultracentrifuge (Beckman Instruments, Palo Alto, CA) in a 100.2 rotor at 80,000 rpm for 1 min. During this time the centrifuge just achieved a speed of 79,000. The total g force (average r) attained was 1.5 × 10⁵ g. The pyrene fluorescence of the supernatant was used as a measure of non-pelleted F-actin. This low g force centrifugation caused less than a 5% decrease in the pyrene-labeled actin fluorescence in the supernatant of F-actin samples that did not contain 30-kD protein. The supernatant of the low g spin was subsequently centrifuged at 80,000 rpm (2.6 × 10⁵ g (r average), for 15 min to pellet the free

F-actin. The fluorescence intensity in this supernatant was used as an indication of the background fluorescence. The fluorescence intensity of control F-actin (2 μM) was decreased by >90% by the high g force spin.

Effect of 30-kD Actin–binding Protein on the Critical Concentration of Actin

2 μM pyrene-labeled actin was polymerized overnight, sonicated (to speed the rate of reaching steady state), and diluted to various concentrations. After an additional sonication, buffer or 30-kD protein (0.5 μM final concentration) was added and the samples were incubated for 1, 2, or 24 h before the pyrene fluorescence intensity was read. The fluorescence intensity reached a plateau at 2 h and did not change over the next 20 h. Data were collected for 24 h fluorescence intensities for control and 30-kD protein samples. The fluorescence from G-actin at concentrations below critical concentration was extrapolated to give the G-actin line.

Results

The 30-kD Actin–bundling Protein Inhibits F-actin Depolymerization

The presence of 30-kD protein inhibited depolymerization of F-actin. Pyrene-labeled F-actin (2 μM) was incubated for 2 h with various concentrations of 30-kD protein before the samples were diluted 20-fold (to 0.1 μM actin) and the decrease in pyrene-labeled actin fluorescence intensity was followed over time as an indication of depolymerization rate. The inhibition of the rate of depolymerization was dependent on the concentration of 30-kD protein present (Fig. 1 a).

Semilog plots of the time course of depolymerization of control could not be fit with a single exponential function. However, it was possible to fit the depolymerization time course up to 60 min, for both control actin and actin incubated with 30-kD protein, with two exponential functions, over intervals from 0 to 10 or 20 min and from 10 or 20 to 60 min. The requirement of at least two exponentials to fit depolymerization kinetics have been reported previously (Walsh et al., 1984). The slopes of both exponentials were decreased by the presence of 30-kD protein. Fig. 1 b shows semilog plots of the pyrene fluorescence intensity for both the first (between 0 and 10 min following dilution) and second (between 10 and 60 min following dilution) exponentials.

The magnitude of the effect of 30-kD protein on the apparent off-rate constant of depolymerization was estimated by comparing the slope of the semilog plot of the fraction of initial pyrene fluorescence intensity remaining from 0–10 or from 10–60 min after dilution (Fig. 2). The slope of the line is k_{off}/L_n, where L_n is a constant characterizing the exponential filament length distribution of the filament population present. By comparing the ratio of slopes of the same population of filaments with and without 30-kD protein, the length terms cancel out and the ratio gives the relative inhibition of the k_{off}. Since the time of pre-incubation with 30-kD protein did not appear to affect the depolymerization kinetics (see below), we pooled data from 28 different experiments where the incubation times with the 30-kD protein before dilution varied between 0 and 4 h. The presence of 100 nM 30-kD protein reduced the apparent off-rates to ∼30% of control values. Half maximal inhibition, evaluated by a double reciprocal plot of the second exponential, was about 4 nM 30-kD protein.

The extent of depolymerization at various times up to 24 h was also a function of the concentration of 30-kD protein
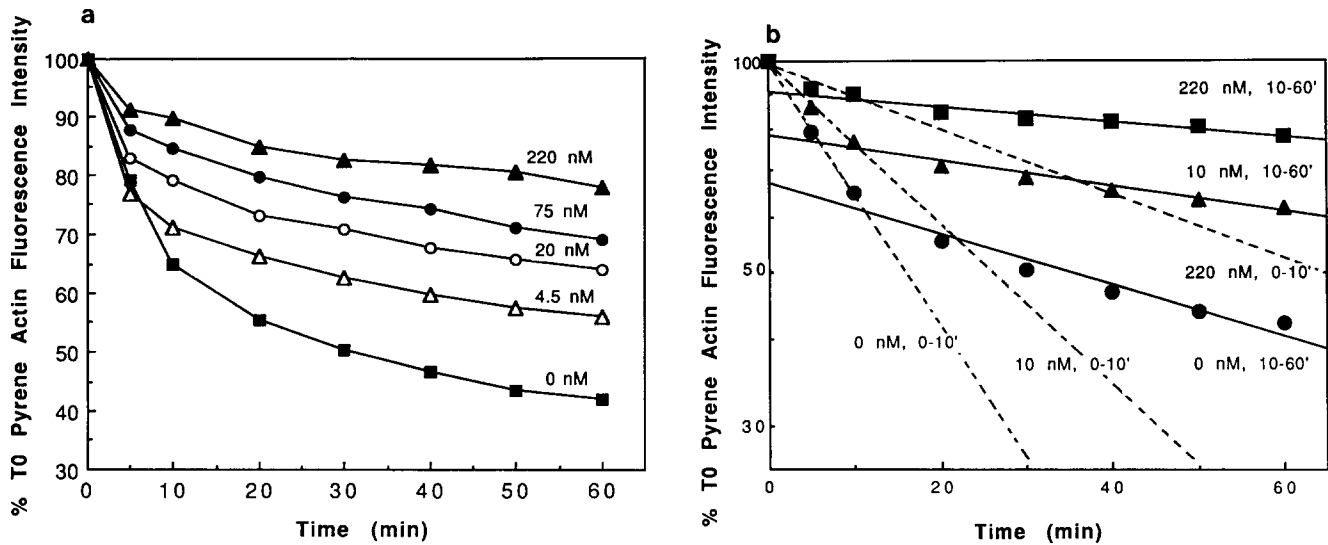
Figure 1. (*a*) Time course of depolymerization of F-actin after incubation in various concentrations of 30-kD actin binding protein. F-actin (2 μM pyrene–labeled F-actin polymerized overnight) was incubated for 2 h at room temperature with 4.4, 1.5, 0.4, 0.09, or 0 μM 30-kD protein. The samples were then diluted 20-fold (to final F-actin concentration of 0.1 μM, and 30-kD protein concentrations of 220 nM (▲), 75 nM (●), 20 nM (○), 4.5 nM (△), and 0 nM (■), and the decrease in pyrene fluorescence intensity followed. The data shown are normalized to 100% of the fluorescence intensity present in each sample at time 0. The percent of time 0 fluorescence intensity (minus background) that remained after 24 h was 58, 51, 38, 29, and 10, respectively. (*b*) Semilog plot of time course of depolymerization of data shown in *a*. Exponential curves were fit to data from 0 to 10 min (*dotted line*) and for 10 to 60 min (*solid line*) for 30-kD protein concentrations of 0, 10, and 220 nM. The *y* intercept, slope and fit (R^2) respectively, for 0 to 10 min data are: control = 99, $10^{-0.0188}$, 0.99; 10 nM = 99, $10^{-0.0115}$, 0.99; 220 nM = 99, $10^{-0.0047}$, 0.85. The comparable values for the 10 to 60 min data are: control = 67, $10^{-0.0037}$, 0.95; 10 nM = 78, $10^{-0.0019}$, 0.96; 220 nM = 90, $10^{-0.0011}$, 0.92.

(Fig. 3). A double reciprocal plot of the fluorescence intensity remaining after 24 h indicated half maximal inhibition when the concentration of 30-kD protein present after dilution was 3.6 nM (data not shown). The concentration of F-actin remaining at 24 h in the presence of 30-kD protein exceeded that predicted by the slope of the second slow exponential function indicating that a full description of kinetics of depolymerization in the presence of the 30-kD protein required more than two exponential functions. The F-actin levels re-

maining at 24 h reflected the slow depolymerization and not the steady state of F-actin in the presence of cross-linking protein, since the levels of F-actin continued to decline. Furthermore, when the F-actin remaining after depolymerization for 24 h in the presence of 5 nM 30-kD protein was sonicated, it rapidly depolymerized, indicating that this F-actin was capable of depolymerization.

The inhibition of depolymerization by 30-kD protein was rapid. Incubating 2 μM F-actin with 1 μM 30-kD protein for
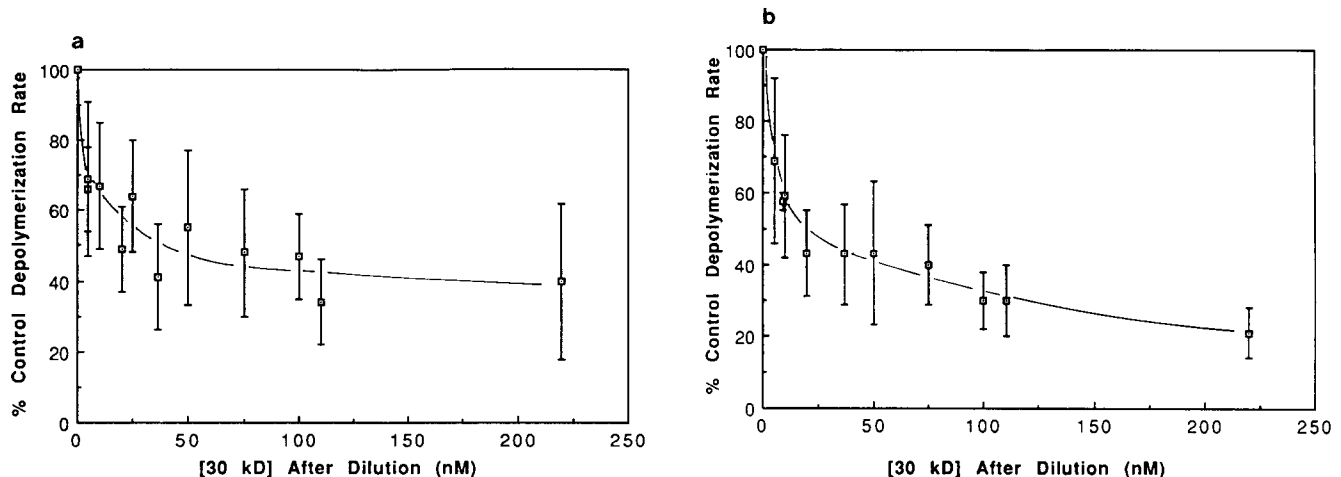

Figure 2. The fractional decrease in the apparent k_{off} as a function of 30-kD actin binding protein concentration. The percentage of control k_{off} was determined from the ratio of the slopes obtained from fit exponential functions to the depolymerization time course of 0.1 μM F-actin between 0 and 10 min (*a*) and between 10 and 60 min (*b*). The values presented are the mean values ± SD, for between 4 and 28 samples for each 30-kD protein concentration shown. One experiment, in which 30-kD protein had no apparent effect on the depolymerization was omitted from this data set.
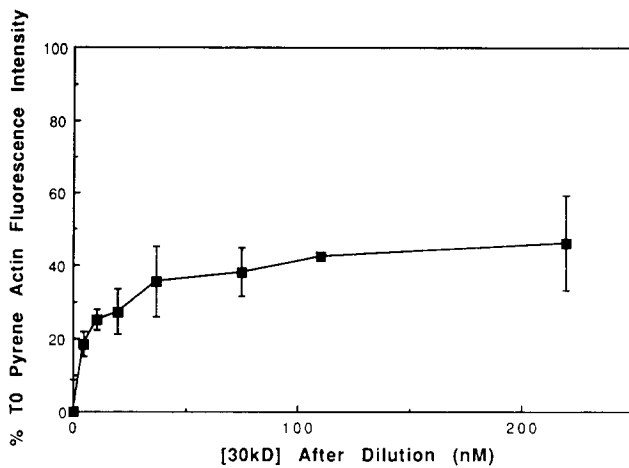
Figure 3. Percent of F-actin remaining after depolymerization for 24 h as a function of concentrations of 30-kD actin binding protein. Various concentrations of 30-kD protein were incubated for 1 or 2 h with 2 μM pyrene-labeled F-actin as in Fig. 1. The samples were diluted 20-fold and the fraction of the time 0 fluorescence intensity (minus background) that remained after 24 h at room temperature was plotted versus the concentration of 30-kD protein present during the depolymerization. The data are the mean values $\pm$ SD of three experiments.

60 min before a 20-fold dilution resulted in essentially identical inhibition of depolymerization as dilution of 2 μM F-actin directly into 50 nM 30-kD protein (Fig. 4). This suggested that the effect of 30-kD protein, at least on the early part of the time course, must involve rapid kinetics of binding. The extent of inhibition as indicated by the amount of F-actin remaining after 24 h also showed little or no dependence on the duration of incubation of F-actin with 30-kD protein before dilution.
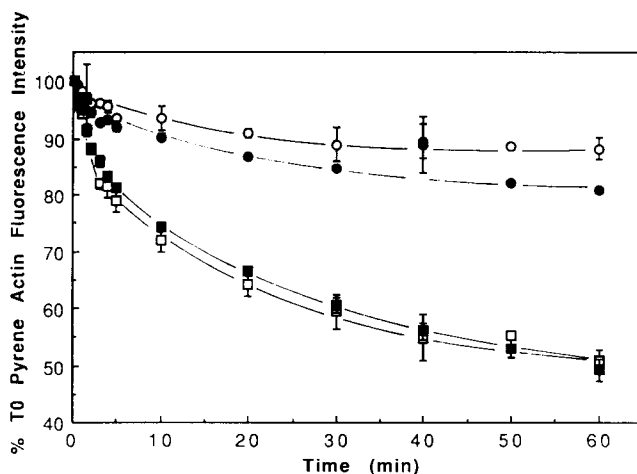
Inhibition of Depolymerization by 30-kD Actin-bundling Protein Occurs at Both the Barbed and the Pointed End of the Filament

The 30,000-D actin binding protein (50 nM after dilution) inhibited the rate of actin depolymerization by >50% (i.e., a 50% decrease in the slope of the semilog plot of F-actin remaining between 10 and 60 min, see Fig. 2 *b*). Since the barbed end off-rate is about 10 times higher than the pointed end off-rate, this extent of inhibition must involve inhibition of the barbed ends. To examine if 30-kD protein also inhibited pointed end depolymerization, actin polymerization was nucleated with 4 or 40 nM gelsolin in the presence of calcium. The number of filaments formed is approximately equal to the concentration of gelsolin and each filament is capped with gelsolin at its barbed end (Janmey and Stossel, 1986). Once capped with gelsolin in the presence of calcium, the calcium can be removed and the cap remains (since the off rate is slow) (Selve and Wegner, 1986). Using these gelsolin capped filaments, it was possible to show that 30-kD protein inhibited pointed-end depolymerization (Fig. 5). To confirm that depolymerization was occurring only at pointed ends, some samples were diluted into 0.2 μM cytochalasin D which could cap free barbed ends. While cytochalasin D slowed the depolymerization rate of control F-actin by ~90% (not shown), it slowed the rate of these gelsolin capped filaments by only 20 to 24%, indicating that most of the barbed ends were indeed capped. The inhibition of actin depolymerization by 50 nM 30-kD protein was similar (~86 to 94%) for actin filaments 50- or 500-monomers long (3 μM actin nucleated with 40 or 4 nM gelsolin; note in the presence of gelsolin the critical concentration becomes that of the pointed end, thus the F-actin concentration during incubation with 30-kD protein was 2 to 2.5 μM).
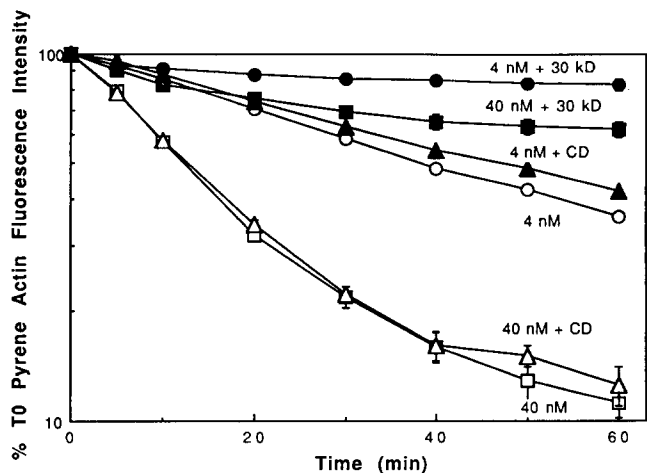

Figure 4. Effect of pre-incubation on the inhibition of depolymerization by 30-kD actin binding protein. 2 μM pyrene F-actin was incubated with 1 μM 30-kD protein for 1 h before a 20-fold dilution (●) or was diluted directly into 50 nM 30-kD protein (○). Parallel samples were diluted without 30-kD protein (□, ■). The percentage of time 0 fluorescence intensity is plotted at various times after dilution, bars represent the duplicate samples.

Figure 5. Effect on 30-kD actin–binding protein on depolymerization from the pointed end of the filament. 3 μM F-actin was polymerized overnight in 0.1 mM calcium and 4 nM (○, ●) or 40 nM gelsolin (□, ■). Samples were then incubated one to two hours with 1 μM 30 kD (●, ■) or buffer (○, □, △, ▲) before 20-fold dilution into buffer or 0.2 μM cytochalasin D (▲, △). The mean pyrene fluorescence intensity as a percentage of that present at time 0 was plotted for various times after dilution. The values of the duplicate samples are shown as bars on this semilog plot.

The Extent of Inhibition Depends on the Stoichiometry of 30 kD to Filament Number and to Actin Monomers in F-actin

In the experiments described above, the F-actin present at the beginning of depolymerization was 0.1 μM. In the presence of 20 nM 30-kD protein, the stoichiometry of 30-kD protein to F-actin monomers was 1:5. We investigated whether 20 nM 30-kD protein could also inhibit depolymerization of 2 μM F-actin where the stoichiometry of 30-kD protein to F-actin monomers was 1:100. Depolymerization of 2 μM F-actin was induced by addition of 10 μM DNase to bind the G-actin. Fig. 6 shows that the rate of depolymerization of 2 μM F-actin was inhibited ~10%, while a parallel sample diluted to 0.1 μM F-actin was inhibited ~40%. For both samples, actin was polymerized at 2 μM, and then either it was diluted 20-fold into 20 nM 30-kD protein and 10 μM DNase, or it was added with little dilution to 20 nM 30-kD protein and 10 μM DNase. If the filaments in 2 μM F-actin were sheared, by rapid pipetting just before the beginning of depolymerization, no inhibition by 30-kD protein could be detected (data not shown). Thus, the extent of inhibition depends on the concentration, the stoichiometry of 30-kD protein to F-actin monomers, and the stoichiometry of 30-kD protein to actin filaments.

Effects of 30-kD Actin–bundling Protein on Actin Assembly and Critical Concentration at Steady State

The effects of 30-kD protein on the rate and extent of actin assembly were examined to exclude the possibility that the inhibition of depolymerization was due to a decrease in filament ends that might occur if 30-kD protein either facilitated filament annealing, protected against breakage during dilution, or capped the ends of the actin filaments. Filament ends
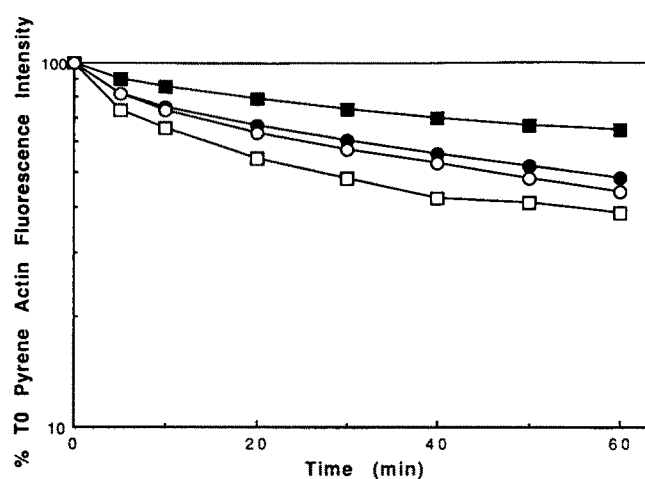
were examined from the initial rates of elongation of pyrenyl-G-actin added to filaments incubated for 1 h with or without 1 μM 30-kD protein. The elongation rate of the filaments incubated with 30-kD protein was 29% ± 14% (mean ± SD of three experiments) greater than that of F-actin incubated without the 30-kD protein. Thus, incubation with 30-kD protein did not appear to block or decrease the number of filament barbed ends. The presence of 1 μM 30-kD protein did not inhibit the rate of elongation of 2 μM pyrenyl-G-actin from F-actin seeds or from gelsolin capped F-actin seeds. Again, in both cases the net rates of elongation were slightly faster (~10%) in the presence than in the absence of 30-kD protein. Finally, several proteins that bind to the side of actin filaments have been reported to nucleate actin polymerization (see Discussion). Addition of 0.5 μM 30-kD protein to 1.5 μM G-actin did not affect the rate of actin nucleation (Fig. 7 a). However, with time, the net rate of elongation was again
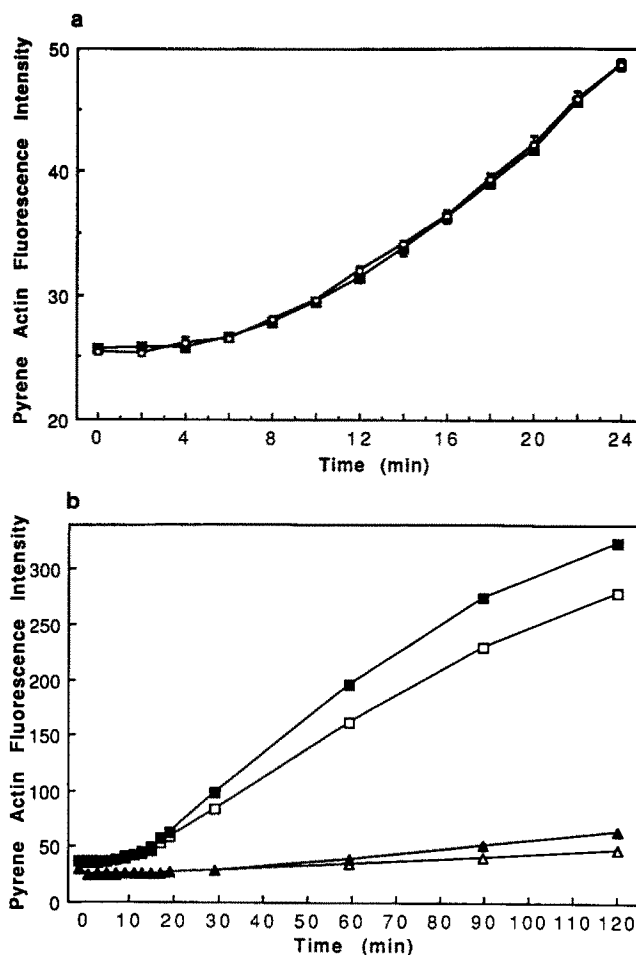
Figure 7. Effect of incubation in 30-kD actin–binding protein on nucleation of actin polymerization. (a) Effect of 30-kD actin–bundling protein on spontaneous nucleation by G-actin. 1.5 μM G-actin was incubated in buffer (□) or with 0.5 μM 30-kD protein (■) and the increase in pyrene fluorescence intensity (actin polymerization) was followed over time; error bars show duplicate samples. (b) Long time course of 30-kD actin–bundling protein on spontaneous nucleation by G-actin. 1.0 μM G-actin (△, ▲) or 2 μM G-actin (□, ■) was incubated in buffer (□, △) or with 50 nM 30-kD protein (■, ▲) and the increase in pyrene fluorescence intensity (actin polymerization) was followed over time; data presented are from a single time course which is representative of six experiments.

Figure 6. Effect of stoichiometry of 30-kD actin binding protein to F-actin. 2 μM pyrene F-actin was polymerized overnight and then (a) diluted 20-fold into 10 μM DNase with 20 nM 30-kD protein (■) or with buffer (□) or (b) diluted only 3% by addition of 10 μM DNase with 20 nM 30 kD (●) or with buffer (○). The presence of 30-kD decreased control depolymerization slope (for times between 20 and 60 min) by 39% when 0.1 μM F-actin was present and by 10% when 2 μM F-actin was present.

slightly faster in the presence than in the absence of 30-kD protein (Fig. 7 b).

One would expect that a factor that decreased the off-rate of F-actin without inhibiting the on-rate would result in a decrease in the critical concentration. However, no decrease in the critical concentration in the presence of the 30-kD protein was detected: control critical concentration = 0.12 ± 0.1 μM and with 0.5 μM 30-kD protein, critical = 0.11 ± 0.1 μM. Furthermore, incubations up to 24 h of 0.5 μM or 2 μM pyrenyl F-actin with 0.5 μM 30-kD protein produced no change in the pyrene fluorescence.

Discussion

The 30-kD protein from *Dictyostelium* has been characterized as an F-actin bundling protein (Fechheimer and Taylor, 1984; Fechheimer, 1987; Furukawa and Fechheimer, 1990) that is primarily localized in anterior pseudopods and filopodia (Fechheimer, 1987; Johns et al., 1988). This protein is also enriched both around the phagocytic cup (Furukawa et al., 1992), and in regions of cell–cell contact in developing cells (H. Ingalls, E. J. Luna, and M. Fechheimer, unpublished results). We now have shown that 30-kD protein inhibits F-actin depolymerization. The inhibition of depolymerization occurs at both ends of the filament. The extent of inhibition was dose dependent—with half-maximal inhibition of 0.1 μM F-actin at ∼4 nM 30-kD protein. These concentrations are well within the physiological range, since the concentration of 30-kD protein in a *Dictyostelium* amoeba is ∼1.2 μM (Fechheimer, 1987).

The quantitative analysis, comparing the slopes of the semilog plots as a measure of the extent of inhibition, assumes that the 30-kD protein did not affect the filament length distribution. This assumption is justified in the experiments showing that the number of filament ends as assayed by elongation with pyrene actin was similar after incubation with or without 30-kD protein. However, this analysis is limited by the fact that the filament length distribution can not be represented by a single exponential. Thus, when the data obtained between 10 and 60 min of depolymerization are compared (Fig. 2), the control samples contain less F-actin, and presumably some of the short filaments have depolymerized completely. Thus, the filament length distributions between samples may no longer be the same. This inaccuracy in the analysis would lead to an underestimate of the true extent of the inhibition by the 30-kD protein.

The extent of inhibition could depend on many factors including filament length, filament number, the concentration of 30-kD protein, the stoichiometry of 30-kD protein relative to F-actin, and the end of the filament depolymerizing. We have examined some of these factors. When 30-kD protein was present in sufficient concentrations and stoichiometry relative to the F-actin present (1:2.5), the inhibition of pointed end depolymerization was similar for filaments containing 50 or 500 actin monomers (Fig. 5). On the other hand, when the amount of 30-kD protein (20 nM) relative to total F-actin present was decreased from 1:5 to 1:100 by increasing the F-actin concentration from 0.1 to 2 μM, the extent of inhibition decreased (Fig. 6). Inhibition was lost entirely when the 2 μM F-actin was cut into many short filaments. Because we don't have a method for making a fixed number of uncapped filaments, we have been unable to directly compare the inhibition at the barbed and pointed ends.

The concentration of 30-kD protein giving half maximal inhibition of depolymerization of 0.1 μM F-actin (∼4 nM) was well below the measured dissociation constant, K_d, of 0.2 μM for the binding of F-actin by 30-kD protein. The basis for this discrepancy is not yet clear. While 30-kD protein might bind with higher affinity to a filament end, it seems surprising that it would bind selectively to both the pointed and barbed ends. Since the actin filaments present are long (∼5 μm), the inhibition may be due to a relatively low fraction of the monomers in the F-actin being bound to the 30-kD protein, but a severe inhibition of monomer off-rate when the bound monomer reaches the end of a filament. This would seem to require that 30-kD protein binding also have a slow off-rate. Given the off-rate of F-actin under our assay conditions, 2.5 s^{-1} (sum of barbed and pointed end off-rates, Cano et al., 1991 and 1992b), the off-rate of an actin monomer bound to 30-kD protein would be decreased 10- to 20-fold, if its off-rate were 0.1 s^{-1}. Then given a K_d of binding of about 0.2 μM, the on-rate would be 0.5 μM^{-1} s^{-1}. With this on rate, it would require less than one min for 50 nM 30-kD protein to reach 95% equilibrium binding with 0.1 μM F-actin. This rate is compatible with similar levels of inhibition of depolymerization with or without preincubation with the 30-kD protein. In separate experiments, adding 50 nM 30 kD during depolymerization slowed the rate to a new steady state within 5 min (data not shown).

Alternatively, the low affinity binding could align actin filaments and thus induce some highly cooperative interactions between the 30-kD protein and actin filaments that leads to the inhibition (see below). However, a significant portion of the inhibition of depolymerization by 30-kD protein did not appear to depend on the crosslinking of filaments. Negative stains of the actin filaments with and without 30-kD protein (not shown) showed F-actin either not cross-linked or in loose aggregates. The large bundles of F-actin seen earlier using much higher actin concentrations were not seen under these conditions (Fechheimer and Taylor, 1984; Fechheimer, 1987; Furukawa and Fechheimer, 1990). Furthermore, the effect of 30-kD protein upon actin depolymerization was apparent if assayed immediately after mixing, while cross-linking as assayed by actin sedimentation by 30-kD protein showed a time dependence. In addition, while cross-linking is significantly inhibited by calcium, the effects of 30-kD protein on actin depolymerization were seen in the presence of calcium (data not shown) indicating that binding by a calcium-insensitive site slowed the rate of depolymerization.

A protein that decreases the off-rate of actin monomers might be expected to affect actin polymerization. Surprisingly, 30-kD protein did not cause any major changes in the nucleation rate and only a slight increase in the net rate of elongation. No significant decrease in the critical concentration was detected at steady state. Since 30-kD protein decreases the rate of depolymerization, the critical concentration would be expected to decrease. Under our assay conditions, the critical concentration of the actin used was 0.12 μM. Thus, the maximal shift possible is 0.12 μM which would be observed if the off rate constants at both ends of all filaments became zero. Small shifts can be optimally detected using concentrations of actin near the critical concen-

tration. However, using actin concentrations near the critical concentration, we detected no decrease in the critical concentration at steady state. This is probably due to the fact that at steady state some filament ends are not bound by 30-kD protein. In the depolymerization experiments, filaments can depolymerize rapidly until a monomer bound by 30-kD protein is reached, and thus a relatively high fraction of the filament ends might be bound by 30-kD protein. However, at steady state, the monomers bound by 30-kD protein would be more evenly distributed throughout the filament. The critical concentration reflects only events at the ends of filaments. The relationship of the critical concentration to the fraction of capped filaments is an important consideration here. The critical concentration changes abruptly as the last 10% of the filament barbed ends are capped (Young et al., 1990).

Nucleation activity of actin cross-linking proteins has been studied previously. Filamin (Hartwig et al., 1980; Koteliansky et al., 1981), and *Dictyostelium* ABP 120 (Condeelis et al., 1982) have been reported to nucleate actin assembly, while alpha-actinin is reported to nucleate assembly in some studies (Blikstad et al., 1980; Koteliansky et al., 1981; Maruyama and Ebashi, 1965), but not in others (Ohtaki et al., 1985). The 30-kD protein lacked detectable nucleation activity when incubated with G-actin. However, when these assays were monitored over longer times, the net growth in samples with 30-kD protein was slightly greater. Complete inhibition of the monomer dissociation rate would be expected to increase the net polymerization rate with 2 μM G-actin from F-actin seeds by ~10% (calculated using a critical concentration of 0.2 $\times$ 10^{-6} M and an off-rate of 2.5 s^{-1} measured under our assay conditions; Cano et al., 1991). The elongation rate of the filaments diluted directly into pyrenyl-G-actin containing 50 nM 30-kD protein was 11% $\pm$ 15% (mean $\pm$ SD of three experiments) greater than that of filaments diluted into pyrenyl-G-actin elongating without 30-kD protein. Filaments pre-incubated with 30-kD protein had a slightly higher rate of elongation (29% $\pm$ 14%, mean $\pm$ SD of three experiments). This increase might be due to an inhibition of filament annealing. It also could be due to nucleation by two 30-kD protein molecules bound to adjacent monomers on the filament and thus having free actin binding sites with spacing appropriate to nucleate a new filament. This is the method of nucleation proposed for ponticulin (Schwartz and Luna, 1988).

The nature of the inhibition of depolymerization and its dependence on the concentration of 30-kD protein is complicated by the fact that the protein can presumably bind to actin via either or both of two different actin binding sites. Furthermore, because binding can affect the spatial arrangement of actin filaments, two filaments once aligned may provide sites spatially optimal for additional cooperative binding of 30-kD protein molecules. These effects may promote the action of the 30-kD protein during depolymerization, i.e., those actin filaments that are initially inhibited may become more inhibited.

We would anticipate that binding alone could inhibit the off-rate if the binding stabilized the F-actin conformation of the actin molecule or if it bound two adjacent monomers in the filament. Additional stabilization should occur if the protein cross-linked two actin filaments such that the monomer at the end of a filament was held in place by its binding to

the actin filament and its binding to an immobilized 30-kD actin–bundling protein. Other proteins that bind to filaments and inhibit depolymerization without capping include myosin (Detmers et al., 1981) and tropomyosin (Broschat et al., 1989; Broschat, 1990; Weigt et al., 1990). Interestingly, both of these proteins have binding sites on the actin filament that span more than a single adjacent actin monomer. Myosin, 30-kD protein and tropomyosin all inhibit depolymerization at both ends of the actin filament. However, while the 30-kD protein does not inhibit assembly at either end, tropomyosin delays assembly at the barbed end (Lal and Korn, 1986; Wegner and Ruhnau, 1988), and either inhibits (Weigt et al., 1990) or has no effect (Broschat et al., 1989; Broschat, 1990) on monomer addition at the pointed end of the actin filament. Effects of tropomyosin on macroscopic rates of association and dissociation from the barbed ends of filaments in bulk solution have also been ascribed to inhibition of fragmentation of filaments with bound tropomyosin (Hitchcock-DeGregori et al., 1988).

Biological Consequences of Actin Filament Stabilization by Actin Cross-linking Proteins

Factors that depress depolymerization without blocking assembly could function in cells in ways quite different from known capping proteins such as gelsolin and severin which affect assembly and disassembly equally. By slowing disassembly, cross-linking proteins could promote net accumulation of actin filaments in specific locations. The binding of cross-linking proteins could stabilize filaments sufficiently that filaments would persist for extended periods even when the free G-actin concentration is below that of the critical concentration of one or both ends. In addition to selectively stabilizing cross-linked filaments, actin cross-linking proteins could help specify the position and polarity of these cross-linked filaments. In support of this proposition, expression of villin in fibroblasts has been shown to be sufficient to induce formation of actin bundles projecting from the cell surface in cells otherwise lacking microvilli (Friederich et al., 1989).

A corollary to this idea is that noncross-linked filaments may be more susceptible to disassembly. This is interesting since regulating the activity of the cross-linking protein could promote local disassembly without requiring a change in the concentration of free G-actin. There are few uncross-linked filaments in cells. Comparisons of total F-actin in cells (pelletable at high g-forces) and cytoskeletal actin (sedimenting at low g forces) are almost identical implying that most of the filaments are cross-linked (or trapped) in cells. Yet, a significant proportion of many cross-linking proteins are found in the supernatant of detergent lysed cells. Thus, there appears to be a large reserve pool of cross-linking proteins that can be locally modified to alter rapidly F-actin stability.

Clearly, some structures such as the filaments in the hair cells of the ear, need to be very stable. In a pseudopod of a migrating cell, the actin must be polymerized and stabilized (both mechanically and from depolymerization) yet be competent for considerable and rapid refashioning of the network (Janmey et al., 1990; Sato et al., 1987). It may be that at least a portion of this flexibility comes from regulation of the cross-linking proteins. Indeed, the actin cross-linking ac-

tivities of 30-kD protein, alpha-actinin, and filamin are known to be regulated (Fechheimer and Taylor, 1984; Burridge and Feramisco, 1981; Zhuang et al., 1984). Thus, both the stability of the structures and the capability for refashioning is provided. Effects of actin cross-linking proteins on the stability of monomers in filaments as well as on the formation of filament networks may account for the recently reported abnormal behaviors of cells having depleted quantities of actin cross-linking proteins (Adams et al., 1991; Cox et al., 1992; Cunningham et al., 1992; Witke et al., 1992).

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