Brevin and Vitamin D Binding Protein: Comparison of the Effects of Two Serum Proteins on Actin Assembly and Disassembly†

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ABSTRACT: Actin depolymerizing activity in serum can be attributed to the two proteins brevin and vitamin D binding protein (DBP). To investigate their mechanisms of action, we used a number of techniques, including procedures involving the fluorescent pyrene-labeled actin probe, to compare the interaction of the two proteins with G- and F-actin in vitro. With a fluorescence enhancement assay, we determined that brevin forms a 1:2 complex and DBP forms a 1:1 complex with pyrene–G-actin. We also found that both proteins reduce the viscosity of F-actin measured with high-shear and low-shear viscometers, with brevin effective at much lower concentrations than DBP. In polymerization experiments, brevin inhibits filament elongation at substoichiometric levels by inhibiting monomer addition at the barbed end but can also accelerate polymerization by nucleating assembly of filaments which grow from the pointed end. DBP does not nucleate filament assembly and inhibits filament elongation at either end only at near-stoichiometric levels. Brevin, but not DBP, accelerates disassembly of filaments diluted into a depolymerizing medium. This is consistent with the capability of brevin to sever preformed filaments associated with erythrocyte membranes and to increase the number of filament ends as estimated by a cytochalasin binding assay. In steady-state experiments involving the use of pyrene–actin, brevin produces only a small increase in the apparent monomer concentration when the critical concentrations at the two ends of the filaments are the same (i.e., in 0.1 M KCl). However, when the critical concentration at the pointed end is higher than that at the barbed end (i.e., in 2 mM $MgCl_2$), low molar ratios of brevin sharply increase the monomer concentration to the critical concentration of the pointed end. This allows substoichiometric amounts of brevin to completely depolymerize filaments when the total actin concentration is at or below that of the pointed end. In contrast to brevin, DBP increases the amount of nonfilamentous actin in a stoichiometric and dose-dependent manner regardless of the nature of the salt in the medium. We conclude from this study that brevin is similar in its mechanism of action to other proteins known to bind to the barbed end of filaments and that DBP is related in its action to proteins that complex monomers and prevent them from participating in the polymerization process.

Actin has an important role in many motile and cytoskeletal processes in eukaryotic cells. Besides undergoing reversible polymerization, actin in the monomeric (G-actin)[1] and filamentous (F-actin) forms interacts with a wide variety of proteins (Craig & Pollard, 1982; Korn, 1982; Weeds, 1982). Current research is increasingly concerned with the molecular mechanisms by which these proteins control the structure and function of actin filaments. One class of such proteins seems to act in a manner similar to the cytochalasins, fungal metabolites which bind to the "barbed" end (as defined by heavy meromyosin decoration) of actin filaments [e.g., see Flanagan & Lin (1980)]. Depending on experimental conditions, these filament-capping proteins inhibit monomer addition, reduce the high- and low-shear viscosity of F-actin, nucleate assembly of new filaments, and may also sever filaments. Another class of actin-modulating proteins, e.g., profilin and DNase, apparently bind only to actin monomers.

Several groups have demonstrated that serum has an actin depolymerizing activity (Chaponnier et al., 1979; Norberg et al., 1979; Harris et al., 1980). The nature of this activity is of interest since true actin depolymerizing factors have not commonly been observed among intracellular proteins (Korn, 1982). Weeds and co-workers identified a 92 000-dalton protein from porcine serum, which they named "actin depolymerizing factor" (ADF) because of its ability to depolymerize F-actin in a rapid, stoichiometric manner (Harris et al., 1980, 1982; Harris & Gooch, 1981). Subsequently, another laboratory (Harris & Schwartz, 1981) isolated a 90 000-dalton protein from rabbit serum and showed that its principal action is to shorten, rather than to depolymerize, actin filaments. Accordingly, this protein was named "brevin". More recently, Thorstensson et al. (1982) also demonstrated that a 93 000-dalton protein from human serum shortens rather than depolymerizes filaments.

Serum also contains other proteins that can apparently depolymerize F-actin in a manner different from those proteins described above. It has been shown that a 58 000-dalton human serum protein, vitamin D binding protein (DBP), interacts with G-actin and depolymerizes F-actin to form a 1:1 DBP–actin complex (Haddad, 1981; Van Baelen et al., 1980). Vandekerckhove & Sandoval (1982) isolated a pair of polypeptides of 56 000 and 60 000 daltons from rabbit serum that bind G-actin, inhibit polymerization, and slowly depolymerize filaments. They named the preparation "serum actin inhibitory protein" (SAIP).

To better understand the potential role of serum proteins in the assembly and disassembly of actin filaments, we un-

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[1] Abbreviations: G-actin, monomeric actin; F-actin, filamentous actin; DBP, vitamin D binding protein; ADF, actin depolymerizing factor; SAIP, serum actin inhibitory protein; CB, cytochalasin B; [^{3}H]actin, [^{3}H]-*N*-ethylmaleimide-labeled actin; K_d, dissociation constant; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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dertook a comparative study of the interaction of brevin and DBP with G- and F-actin in vitro. We found that while both of these proteins can act as depolymerizing factors, brevin can be classified as a filament-capping protein, whereas DBP is a G-actin binding protein.

Experimental Procedures

Actin was purified from acetone powder of rabbit skeletal muscle by the method of Spudich & Watt (1971) and stored in buffer A (5 mM Tris, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, and 0.01% NaN_3). Some of the actin was further purified on a Sephacryl S-200 column in buffer A and used for the indicated experiments. A crude preparation of erythrocyte spectrin–band 4.1–actin complex was prepared from fresh human red blood cells (generously donated by the Baltimore Red Cross Blood Center) as described by Lin & Lin (1979). Inside-out erythrocyte membrane vesicles reconstituted with the spectrin–band 4.1–actin complex were prepared according to Cribbs et al. (1982).

Human DBP was prepared from serum as described by Haddad & Walgate (1976). For some experiments, the protein was obtained by chromatography on actin-affinity and hydroxylapatite columns, a procedure which has been shown to yield a protein indistinguishable in its properties from material isolated by the earlier method (J. G. Haddad, unpublished results).

Brevin was isolated from human plasma by an improved method adapted from the procedure of Harris & Gooch (1981). Falling-ball viscometry (MacLean-Fletcher & Pollard, 1980) was used to follow the F-actin-modulating activity of the protein. Additional $CaCl_2$ was added when the buffers contained EGTA. The active material from the DEAE column as described by Harris & Gooch (1981) was dialyzed against 50 mM Tris, pH 8.0, 1 mM EGTA, and 0.01% NaN_3 and further purified on a 1 × 8 cm column packed with Reactive Blue cross-linked to agarose (Sigma Chemical Co.). The column was washed with buffer containing 0.08 M NaCl and then eluted with a 0.08–1 M NaCl gradient (100 mL). Brevin emerged as a single peak at about 0.1 M NaCl. The protein was concentrated by ultrafiltration on a YM10 membrane (Amicon) and dialyzed into buffer A. Brevin prepared by this method had a molecular weight of 90 000, similar to that reported by Harris & Schwartz (1981) for brevin from rabbit serum, but slightly lower than the molecular weight 92 000–93 000 reported by others for ADF from human and porcine serum (Thorstensson et al., 1982; Harris & Gooch, 1981). When 1 unit of human plasma was used, a typical preparation gave 1–2 mg of brevin of 95–99% purity as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. These gels also showed that the brevin and DBP preparations were essentially free of the other species.

Radiolabeled actin was prepared by reaction of the protein with [^{3}H]-*N*-ethylmaleimide as described by Cohen et al. (1978). Pyrene-labeled actin (referred to as pyrene–actin) was prepared by labeling the actin with *N*-(1-pyrenyl)iodoacetamide (Molecular Probes) essentially as described by Cooper et al. (1983). Typically, preparations have 0.75–0.95 mol of pyrene/mol of actin, as determined on the basis of an extinction coefficient of 2.2×10^4 M^{-1} cm^{-1} at 344 nm (Kouyama & Mihashi, 1981).

Relative fluorescence was determined either with a Perkin-Elmer MPF or with a Perkin-Elmer 650-10S spectrofluorometer in the ratio mode, with buffer A as the blank. Except where indicated, the excitation wavelength was 368 nm and the emission wavelength 407 nm, and the slits were set at less than 10 nm. Acrylic cuvettes (Sarstedt) were used.

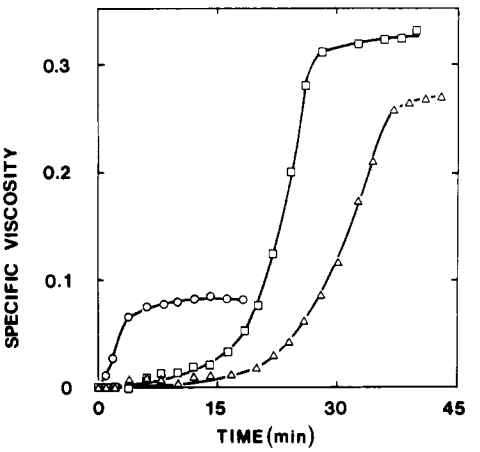

FIGURE 1: Effect of brevin and DBP on salt-induced actin polymerization as determined by Ostwald viscometry. Polymerization of column-purified actin (8.1 μM) was followed with an Ostwald viscometer at 28 °C after addition of KCl (to 0.1 M) at time zero. Samples contained actin in the absence (□) and presence (○) of 0.06 μM brevin or 0.8 μM DBP (△).

The temperature in the spectrofluorometer was maintained at 25 °C with a circulating water bath.

The concentration of unlabeled actin was determined from the extinction coefficient, $E^{1\%}_{290nm} = 6.37$ (Rich & Estes, 1976), while the concentrations of labeled actin preparations were determined by using the Bio-Rad protein assay (Bradford, 1976) with native actin as the standard. All other protein concentrations were measured with the same dye-binding assay, but with bovine serum albumin as the standard.

All reagents were of analytical grade or better. [^{3}H]Cytochalasin B and [^{3}H]-*N*-ethylmaleimide were from New England Nuclear. Cytochalasin D was from Aldrich. Acrylamide, sodium dodecyl sulfate, and protein standards for electrophoresis were obtained from Bio-Rad.

Results

Viscometric Studies on Actin Assembly. Using Ostwald viscometry to follow actin polymerization, we found that both brevin and DBP affected the rate and extent of the viscosity increase. Under conditions where nucleation occurred slowly, i.e., when 0.1 M KCl was used to initiate polymerization, brevin at 0.07 μM enhanced the rate of viscosity increase but decreased the final viscosity level (Figure 1). The apparent nucleating activity of brevin was less evident in 2 mM $MgCl_2$ because spontaneous nucleation is so rapid. However, the final viscosity level in the presence of brevin was similar in both 0.1 M KCl and 2 mM $MgCl_2$. DBP was also able to decrease the final viscosity, but in contrast to brevin, under both slow and rapid nucleation conditions, there was an inhibition of the rate of increase of the viscosity (Figure 1). These dose-dependent effects of both brevin and DBP are reminiscent of the actions of proteins which cap filaments or bind G-actin, respectively (Korn, 1982).

The effects of brevin and DBP on the apparent low-shear viscosity of F-actin measured with the falling-ball method (MacLean-Fletcher & Pollard, 1980) were also examined. Both proteins reduced the apparent viscosity of F-actin, but brevin was effective at considerably lower concentrations than DBP. A reduction in apparent viscosity was seen at brevin:actin ratios as low as 1:4000; at 13 nM (1:894 actins), the protein reduced the apparent viscosity by 50%. In contrast, 0.83 μM DBP (1:14 actins) was required to produce that level of effect. This difference in relative potency for the two proteins presumably reflects the ability of brevin to shorten filaments (Harris & Schwartz, 1981) in the manner described

for villin and gelsolin (Glenney et al., 1981; Yin et al., 1981b). DBP could be reducing the apparent viscosity of F-actin by any of a variety of mechanisms, such as reducing the amount of F-actin or affecting interfilament interactions.

Fluorescence Studies on Protein Binding to G-Actin. In order to study the interaction of G-actin with brevin and DBP, we used actin labeled with the fluorescent probe *N*-(1-pyrenyl)iodoacetamide. Lee et al. (1982) have reported that profilin affects the fluorescence spectra of this species. Figure 2 shows the excitation and emission spectra of pyrene–G-actin in buffer A in the presence and absence of a molar excess of brevin or DBP. While the excitation spectrum of pyrene–actin is known to exhibit a new peak at 368 nm when the labeled actin polymerizes (Kouyama & Mihashi, 1981), binding of pyrene–G-actin to either brevin or DBP did not result in the formation of this peak. As seen in Figure 2A, the excitation spectra still generally resembled that of the pyrene–G-actin alone, although the fluorescence at most of the wavelengths measured increased. The emission spectrum was changed considerably upon binding of the pyrene–actin to the serum proteins. In the presence of a molar excess of DBP, the peak at 385 nm was enhanced, and a new peak appeared at about 405 nm. With a molar excess of brevin, the emission spectrum showed a similar enhancement at 390 nm, but only a shoulder appeared at 400 nm.

The following control experiments demonstrate that this type of fluorescence enhancement was due to a specific interaction of the serum proteins with the pyrene–actin: (1) Neither brevin nor DBP by themselves had any intrinsic fluorescence signal. (2) No fluorescence enhancement was seen when the DBP or brevin was first incubated with excess unlabeled actin before the addition of the pyrene–actin. (3) Brevin boiled for 5 min did not produce the increased fluorescence intensity; DBP that was boiled for 5 min did produce a minor increase in fluorescence, but this protein is known to be very heat stable (Bouillon et al., 1976). (4) A 3-fold molar excess of bovine serum albumin did not affect the fluorescence of the pyrene–actin. (5) The results of Figure 2B were similar over a 10-fold range of actin concentrations (0.16–1.5 μM), indicating that the fluorescence enhancement was not due to a minor component in the buffer.

With the excitation wavelength set at 348 nm and the emission recorded at 390 nm, the addition of DBP or brevin to pyrene–G-actin caused the fluorescence to increase 2.3 and 2.5 times, respectively, over that of the pyrene–actin alone. This increase is significant but much smaller than the 25–30-fold increase seen when the pyrene–actin polymerizes. At the excitation and emission wavelengths used to follow the polymerization of the actin (368/407 nm), there was no fluorescence enhancement when the DBP was added to the labeled G-actin. The emission and excitation spectra indicate that this wavelength pair is an isosbestic point for DBP binding to the pyrene–actin (data not shown, but a similar result obtained with the 368/390-nm wavelength pair is seen in Figure 2A). In contrast, addition of brevin to pyrene–G-actin produced an approximately 2.5-fold increase in fluorescence intensity at this pair of wavelengths.

We used the fluorescence enhancement observed with excitation at 348 nm and the emission recorded at 390 nm to estimate the stoichiometry of the binding of G-actin to brevin and DBP. Increasing amounts of pyrene–G-actin were added to a solution of 0.1 μM brevin or DBP in buffer A (Figure 3A). With brevin, we found that the fluorescence enhancement reached a plateau at a brevin:actin ratio of 1:(2.0–2.6). For DBP, a plateau was reached at a DBP:actin ratio close

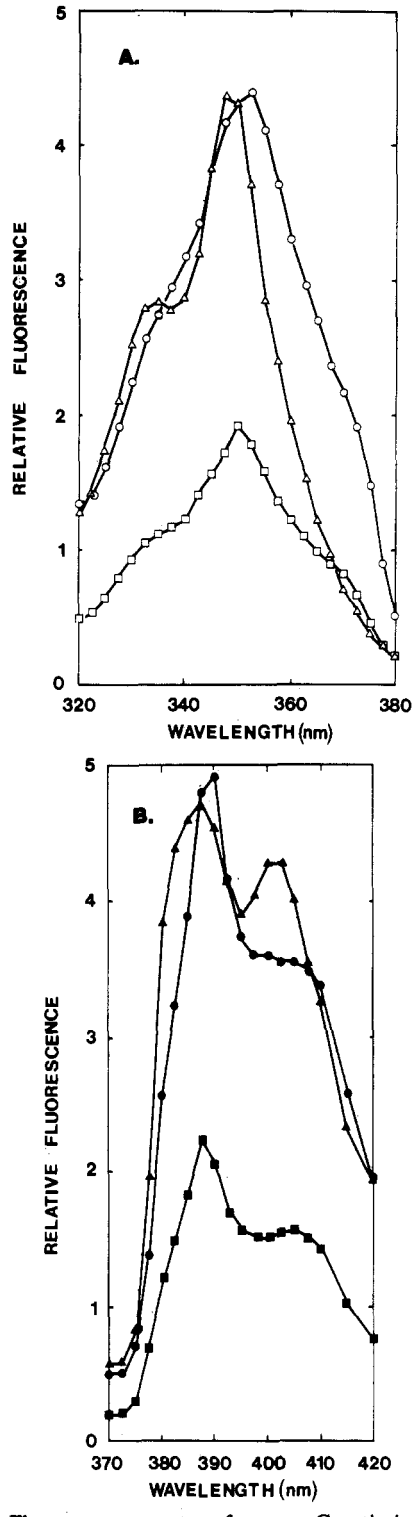

FIGURE 2: Fluorescence spectra of pyrene–G-actin in the presence and absence of brevin and DBP. The samples contained 0.275 μM pyrene–G-actin alone (□, ■) or with 0.66 μM brevin (○, ●), or with 0.43 μM DBP (△, ▲) in buffer A. The excitation spectra (A) were recorded at 390 nm, and the emission spectra (B) were obtained with excitation at 348 nm. The slits were both set at 3 nm. The small contribution of the buffer has been subtracted from the spectra. Spectra were recorded in the ratio mode to compensate for lamp fluctuations but are uncorrected for variations in detector sensitivity with wavelength.

to 1:1. The reverse experiment, adding increasing amounts of brevin or DBP to a fixed amount of pyrene–G-actin (0.194 μM), was also performed (Figure 3B). At saturation, we again found that DBP bound approximately one actin and that brevin bound approximately two actins.

Using an electrophoretic method, Cooke et al. (1979) de-

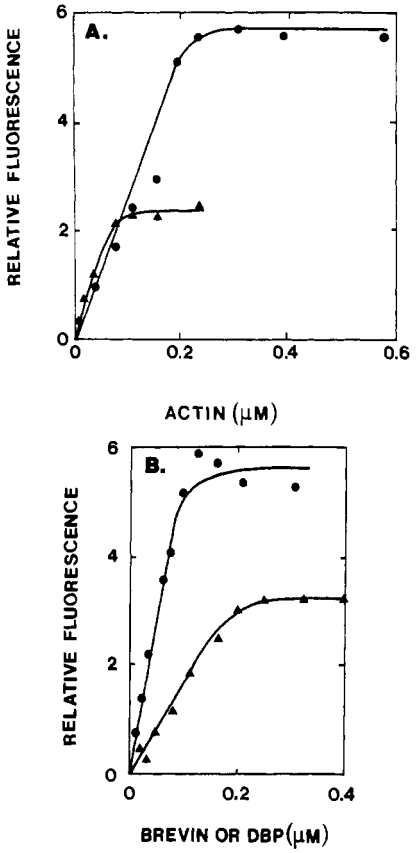

FIGURE 3: Fluorescence enhancement accompanying the binding of brevin and DBP to pyrene–actin. Fluorescence was determined after 2–4 h of incubation at 25 °C. In this experiment, relative fluorescence is the increase in fluorescence intensity over a control sample containing only the labeled actin. (A) The samples contained 0.1 μM brevin (●) or DBP (▲) and the indicated amounts of pyrene–actin. (B) The samples contained the indicated concentrations of brevin (●) or DBP (▲) and 0.194 μM pyrene–actin. For DBP, the solid lines indicate the theoretical binding curve for a K_d of 10 nM.

termined the K_d of actin binding to DBP to be less than 50 nM. Our data points were better fitted by using a K_d of 10 nM. The theoretical curve for this K_d is indicated by the solid lines in Figure 3. The analysis of actin binding to brevin is complicated considerably by the presence of two binding sites, since each actin need not contribute equally to the fluorescence enhancement. Indeed, Bryan et al. (1983) reported that for the binding of platelet gelsolin to 4-nitro-2,1,3-benzoxadiazole-labeled actin, there is fluorescence enhancement only upon binding of the second actin. Because the first actin bound virtually irreversibly, they were able to estimate that the dissociation constant for the second actin was in the nanomolar range. Our results are consistent with the K_d of the brevin–actin complex being in this range. In related experiments, we found that the shift in the peak position on a calibrated Sephadex G-200 column for brevin with and without preincubation with G-actin suggested that approximately two to three actins are bound to brevin (data not shown).

Fluorescence Studies on Actin Assembly. To extend our viscometric studies described above, we used the sensitive fluorescent probe pyrene–actin (Kouyama & Mihashi, 1981; Tellam & Frieden, 1982; Tobacman & Korn, 1982; Cooper et al., 1983). Using an excitation wavelength of 368 nm and an emission wavelength of 407 nm to follow actin filament assembly, we typically find a 25–30-fold increase in the fluorescence intensity upon polymerization of the pyrene–actin.

In the presence of $MgCl_2$ and ATP, actin filaments have different critical concentrations at each end (Pollard & Mooseker, 1981). We took advantage of this difference to specifically assay for barbed-end elongation by employing conditions such that the total actin concentration was below the critical concentration at the pointed end but above that at the barbed end. At 0.93 μM actin, there was no increase in fluorescence when 2 mM $MgCl_2$ was added, indicating that spontaneous filament assembly was minimal (Figure 4A). With the addition of the erythrocyte spectrin–band 4.1–actin complex as a source of exogenous nuclei (Lin & Lin, 1979), there was a rapid increase in fluorescence, indicating polymerization of the actin (Figure 4A). Since 0.2 μM cytochalasin D completely blocked the fluorescence increase (Figure 4A), the nucleated growth is presumably at the barbed end.

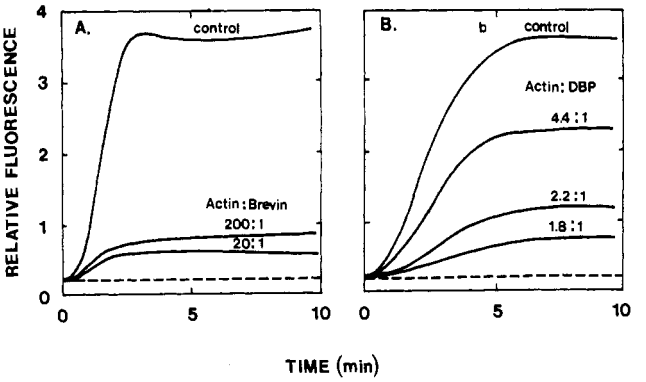

FIGURE 4: Effect of brevin and DBP on nucleated barbed-end assembly. Samples of pyrene–actin (0.93 μM) and the indicated molar ratios of brevin (A) or DBP (B) were incubated at 25 °C for 1 min with exogenous nuclei in the form of the erythrocyte spectrin–band 4.1–actin complex (50 μg of protein/mL). At time zero, polymerization was initiated by the addition of $MgCl_2$ to 2 mM. There was no polymerization in the absence of added nuclei (dashed lines).

Under the assay conditions used, both brevin and DBP were capable of inhibiting barbed-end growth in a dose-dependent manner. However, the stoichiometry of the inhibition was very different. Brevin inhibited most of the growth at a brevin:actin ratio of 1:200 (Figure 4A); inhibition was detectable at ratios below 1:500 (data not shown). In contrast to brevin, DBP inhibited barbed-end growth only at high DBP:actin ratios. As shown in Figure 4B, near-stoichiometric amounts of DBP to actin were required to slow nucleated actin filament assembly in 2 mM $MgCl_2$. A DBP:actin ratio of 1:10 caused only a slight decrease in the elongation rate. At a DBP:actin ratio of 1.3:1, the fluorescence curve over a 30-min time course was indistinguishable from that of the control without added nuclei (not shown). As shown in Figure 4B, there was a progressive decrease in the rate of filament assembly as the concentration of DBP was increased. The fluorescence polymerization curves were similar regardless of the order in which actin, nuclei, and brevin or DBP were added.

Many of the known actin binding proteins have been shown to nucleate filament assembly (Korn, 1982). The ability of brevin and DBP to nucleate assembly was assayed in 50 mM KCl with 11.6 μM column-purified actin containing 5.6% pyrene–actin. Spontaneous nucleation is slow under these conditions [e.g., see Cribbs et al. (1982)], and filament growth in the absence of a nucleating agent is therefore negligible. As shown in Figure 5, substoichiometric levels of brevin induced rapid polymerization of the actin in a dose-dependent fashion. With 0.046 μM brevin (1:253 actins), 50% of the maximum fluorescence was reached in about 5 min (Figure 5), and with 0.018 μM brevin, this level was reached in about 13 min (not shown). In contrast to the barbed-end filament elongation nucleated by the erythrocyte spectrin–band 4.1–actin complex, the brevin-induced polymerization was not

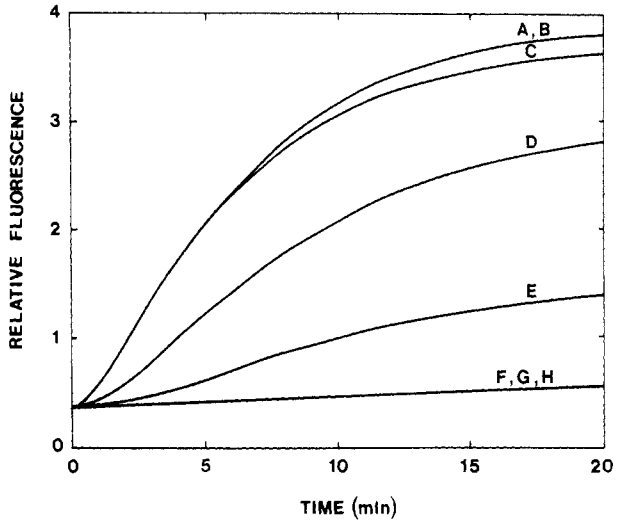

FIGURE 5: Effect of brevin and DBP on nucleation and pointed-end elongation of filaments. The indicated proteins were added to a solution of column-purified actin containing 5.6% pyrene–actin (11.6 μM final concentration) in buffer A and incubated for 1 min at 25 °C. At time zero, KCl was added to 50 mM to initiate polymerization. In the nucleation experiments, the samples contained actin alone (H) or actin plus 0.046 μM brevin (A), 0.046 μM brevin + 0.2 μM CD (B), 0.033 μM DBP (F), or 0.5 μM DBP (G). For the pointed-end elongation experiments, samples contained actin and 0.046 μM brevin plus the following amounts of DBP: 0.5 μM (C); 2 μM (D); 5 μM (E).

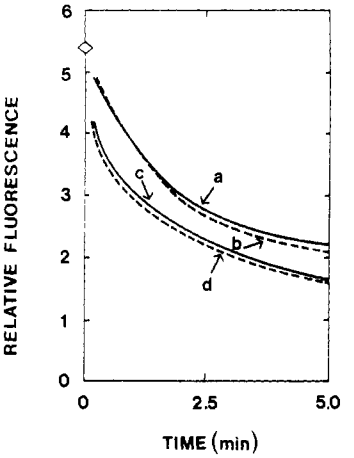

FIGURE 6: Effect of brevin and DBP on filament disassembly in a depolymerizing medium. A 90 μM actin solution containing 10% pyrene–actin was induced to polymerize with 1 mM $MgCl_2$ in buffer A for 2 h at room temperature. A small volume of this F-actin solution (40 μL) was pipetted onto the bottom of a cuvette thermostated at 25 °C. After 1 min, 1.5 mL of buffer A with or without brevin or DBP was added and mixed with three quick inversions, and the fluorescence was determined. The final actin concentration was 2.3 μM. Samples contained actin alone (a) or actin plus 0.57 μM DBP (b), 0.074 μM brevin (c), or 0.074 μM brevin plus 0.57 μM DBP (d). The fluorescence intensity in the absence of depolymerization (◇) was calculated by dividing the fluorescence of the stock F-actin solution by the dilution factor.

blocked by the addition of 0.2 μM CD (Figure 5). On the other hand, the addition of a 70 μg/mL sample of a skeletal muscle preparation that has been shown to block monomer addition at the pointed end (Lin et al., 1982) inhibited brevin-nucleated assembly. Under the conditions of Figure 5, after 30 min, this sample had less than 25% of the fluorescence intensity of the brevin control. These results indicate that brevin nucleates the assembly of actin filaments which grow from the pointed end [cf. Lin et al. (1982)].

Unlike brevin, DBP did not show any nucleating activity in the range of concentrations tested. Neither low (1:351) nor high (1:23) DBP:actin ratios caused an increase in the polymerization rate over a period of 1 h. It should be noted that these ratios are lower than the 1 DBP:10 actins used in the Ostwald viscometry experiments (Figure 1), where a significant reduction in the rate of polymerization was observed.

The ability of DBP to affect pointed-end growth was examined by assaying its effect on filament assembly nucleated by brevin as described in the previous section. This type of pointed-end elongation assay is similar to the one described by Lin et al. (1982), in which a platelet protein preparation with cytochalasin-like activity was used to nucelate assembly of filaments that elongate at the pointed end. A range of 0.033–0.5 μM DBP was assayed with a fixed amount of brevin (0.046 μM). The polymerization curves did not deviate significantly from the brevin control curve over the first 5–10 min (about 40–50% of the maximum fluorescence). In this concentration range, DBP caused a slight dose-dependent inhibition late in the polymerization process. At 20 min, the curve of the sample with 0.5 μM DBP was at 95% of the control curve (Figure 5). At lower DBP concentrations, there was even less inhibition. This inhibition presumably occurs because as the polymerization process proceeds and free monomer is depleted, the percentage of the DBP–actin complex to free monomer increases.

Just as for barbed-end filament growth, near-equimolar ratios of DBP to actin were needed to cause significant inhibition of the brevin-nucleated polymerization. At 5 min, when the brevin control had reached half of its maximum fluorescence, the samples with brevin plus 2 and 5 μM DBP had reached only 23% and 12%, respectively, of the brevin control maximum (Figure 5). The order of addition of the various components was not found to affect the fluorescence polymerization curves.

The stoichiometry of brevin and DBP inhibition of actin assembly suggests that the former acts by blocking the growing end of nuclei and filaments while the latter binds to monomers to form a complex, sequestering them so that they cannot add to nuclei and filaments.

Studies on Actin Disassembly. We studied the rate of diassembly of actin filaments by following the decrease in fluorescence when pyrene–F-actin was diluted into a depolymerizing medium (i.e., buffer A). Immediately after dilution, the G-actin concentration is very low (below 0.01 μM), making the forward addition reaction negligible. Initially, the amount of fluorescence is essentially proportional to the amount of F-actin, since the monomeric form of actin has less than $^1/_{20}$th the fluorescence of the polymeric form. Therefore, the initial rate of decrease in fluorescence is an approximate measure of the rate of loss of monomeric actin from the filaments.

We found that brevin accelerated the rate of depolymerization of actin filaments (Figure 6). The increase in the depolymerization rate, as measured by the initial slope of the fluorescence curve, was dependent on the amount of brevin added. In the presence of 42 nM brevin, the depolymerization rate was twice that of the control; 84 nM brevin caused the rate to be three times that of the control (Table I). In contrast, the depolymerization curve in the presence of 0.57 μM DBP (one DBP per four actins) was very similar to that of the control (Figure 6), indicating that this protein does not affect the kinetics of filament disassembly. Furthermore, as seen in Figure 6, simultaneously adding both brevin and DBP gave a depolymerization curve similar to that of brevin alone.

We found that if brevin preincubated with G-actin was used in the depolymerization experiment, there was a slight inhibition rather than enhancement of the depolymerization rate

Table I: Effects of Preincubation of Brevin with G-Actin on the Rate of Depolymerization of F-Actin[a]

	(A) without preincubation	(B) with preincubation
control (no brevin)	1.0 ± 0.1 (8)	0.9 ± 0.1 (3)
42 nM brevin	2.1 ± 0.4 (3)	0.9 ± 0.4 (3)
84 nM brevin	2.7 ± 0.3 (3)	0.6 ± 0.2 (3)

[a] A stock solution of 70 μM actin (10% pyrene–actin) was polymerized with 1 mM $MgCl_2$ for 2 h; 20 μL was pipetted into one corner of a plastic cuvette. Buffer A alone or brevin in buffer A, each with (B) or without (A) enough G-actin to make with final concentration 1.8 μM, was pipetted into the opposite corner. After 1.5 min at 25 °C, buffer A was added to bring the total volume to 0.6 mL and the fluorescence recording begun as described in the legend of Figure 6. The rates are determined from the initial slopes of curves similar to Figure 6 and are normalized to the control rate. Data are mean ± standard deviation (number of determinations).

(Table I). In these experiments, an equivalent amount of G-actin was included in the dilution buffer for the control samples. There was essentially no change in the depolymerization rate (compare controls of Table I), but the addition of 84 nM brevin–actin complex reduced the depolymerization rate by about one-third (Table I).

Brevin could be increasing the depolymerization rate by affecting the intrinsic rate constants, by increasing the number of filament ends, or by a combination of these mechanisms. To quantitatively assay for the ability to sever preformed filaments under low-shear conditions, we used the reconstituted erythrocyte membrane method described previously (Cribbs et al., 1982). Radiolabeled actin was allowed to polymerize onto inside-out erythrocyte membrane vesicles reconstituted with spectrin–band 4.1–actin complexes. The membrane-associated filaments were separated from unattached filaments by centrifugation through a 20% sucrose solution. Figure 7 shows that brevin caused a decrease in the amount of membrane-associated F-actin. After a 5-min incubation, a ratio of 1 brevin per 300 actins was sufficient to remove more than half of the membrane-associated actin. With increasing brevin concentrations, the amount of membrane-associated actin was reduced to the background level of the cytochalasin B control (Figure 7). When brevin was first preincubated with a 3-fold molar excess of G-actin to form the brevin–actin complex, no decrease in the amount of membrane-associated actin was observed (Figure 7). These experiments suggest that free brevin, but not the brevin–G-actin complex, has the ability to sever actin filaments associated with the membranes.

DBP also decreased the amount of membrane-associated actin in the above assay system, but did so much more slowly than brevin and only at high molar ratios to actin. When the incubation time was short (5 min), a sample with more than one DBP per two actins still contained more than 80% of the control level of membrane-associated actin. After a 2-h incubation, there was a linear decrease in the amount of actin in the membrane pellets (Figure 7), corresponding to somewhat less than an equimolar reduction with added DBP. This experiment suggests that DBP does not rapidly sever filaments. It also supports the notion that DBP does not enhance the rate of depolymerization since at short time periods the amount of actin removed from the membrane was essentially independent of the quantity of DBP added. Thus, the dissociation of monomers from the filament is apparently the rate-limiting step in the depolymerization of the filament by DBP.

Cytochalasin Binding Studies. Tritiated cytochalasin B ([^{3}H]CB) binding to F-actin can be used to estimate the number of filament ends (Flanagan & Lin, 1980). We found

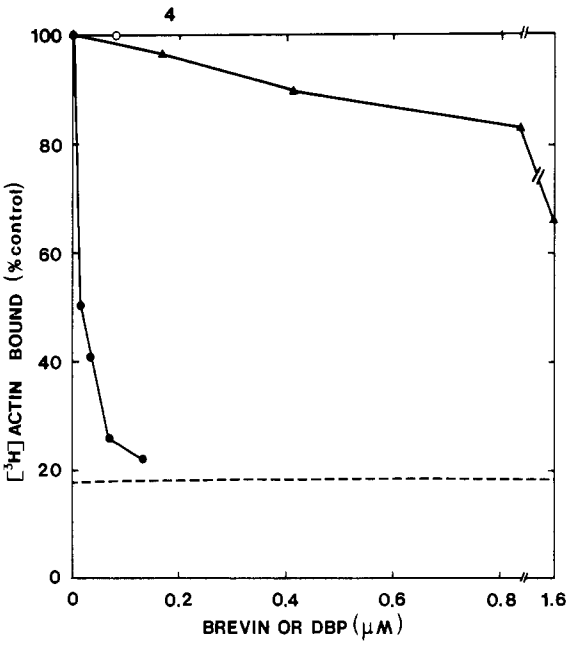

FIGURE 7: Effect of brevin and DBP on actin filaments attached to membranes. Tritium-labeled G-actin at 3.1 μM (●) or 3.4 μM (▲) was allowed to polymerize for 1 h at 25 °C onto spectrin–band 4.1–actin complexes which had been reconstituted on inside-out erythrocyte membrane vesicles (Cribbs et al., 1982). Brevin (●) or DBP (▲) was added in the indicated amounts and incubated for 5 min (brevin) or 2 h (DBP), and the amount of membrane-associated actin was determined as described by Cribbs et al. (1982). The dashed line indicates the amount of [^{3}H]actin bound to membranes when 2 μM CB was included to block actin polymerization and serves as an indicator of nonspecific binding. In order to test the activity of the preformed brevin–G-actin complex, 0.08 μM brevin was preincubated with 0.24 μM unlabeled actin for 10 min before addition to membranes (○). Each point is an averaged value from duplicate determinations. Results are expressed as a percent of the appropriate control.

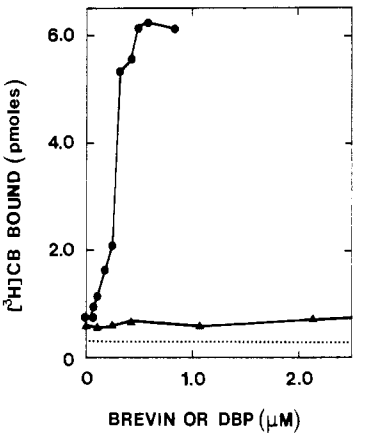

FIGURE 8: Effect of brevin and DBP on the binding of [^{3}H]CB to F-actin. Actin was induced to polymerize in buffer A with 0.1 M KCl and 2 mM $MgCl_2$ for 1 h. The amount of cytochalasin binding to F-actin (11.63 μM) was measured with 40 nM [^{3}H]CB and the indicated concentrations of brevin (●) or DBP (▲) in buffer A by equilibrium dialysis (Lin & Lin, 1978) for 24 h at 4 °C. The brevin samples also contained 1 mM $CaCl_2$. Points are the average of duplicate determinations. The dotted line represents nonspecific binding defined as bound [^{3}H]CB not displaceable by an excess of unlabeled CB.

that at a brevin:actin ratio of 1:20, cytochalasin binding increased 8-fold (Figure 8), indicating that at steady state brevin increases the number of filament ends. Furthermore, since brevin is acting at the barbed end of the filament, the same end of the filament as the cytochalasins, the result indicates

that these two factors can apparently bind to F-actin at the same time. We could not tell from this experiment whether the K_d of cytochalasin binding was affected by brevin. DBP did not increase CB binding to actin filaments over the concentration range of 0.1–2.0 μM (Figure 8). This corresponds to a range of DBP:actin ratios of 1:116 to 1:6. This result is consistent with the protein's lack of effect on the F-actin depolymerization rate and its inability to sever or nucleate filament assembly.

Fluorescence Studies on Critical Concentration. The experiments described thus far examined some of the effects of brevin and DBP on the kinetic aspects of actin assembly and disassembly. We also determined the effects of these two proteins on the steady-state concentration of G-actin. The critical concentration of the actin is determined from the intercept of the G- and F-actin standard curves. In 2 mM $MgCl_2$, the intercepts as a function of DBP concentration are plotted in Figure 9A. It indicates that DBP caused a near-stoichiometric increase in the amount of nonfilamentous actin (Figure 9A). This observation is consistent with a mechanism of DBP shifting the actin equilibrium away from the polymeric state through the sequestering of monomers.

The increase in the apparent critical concentration in the presence of DBP can be used to estimate the dissociation constant (K_d) of the DBP–actin complex in the manner described for the profilin–actin complex (Tseng & Pollard, 1982). The data points of Figure 9A indicate a K_d of the DBP–actin complex in the range of about 20–100 nM, similar to the value of about 50 nM estimated by Cooke et al. (1979).

Since brevin affects the fluorescence of the pyrene–G-actin, it was added to all samples used for preparing the G- and F-actin standard curves. It is worth noting that all of the F-actin curves were linear with a nearly identical slope over the concentration range used (1.16–9.3 μM actin). A plot of the intercepts of the G- and F-actin standard curves is shown in Figure 9B. In 2 mM $MgCl_2$, the addition of up to 0.01 μM brevin caused the apparent critical concentration to increase from about 0.35 μM to about 1.4 μM; with the presence of additional brevin, there was only a small additional increase in unpolymerized actin. Thus, it appears that in 2 mM $MgCl_2$ (where the two ends have different critical concentrations), there is a relatively large increase in the amount of unpolymerized actin at nanomolar concentrations of brevin, followed by a tapering off to a more gradual increase in the critical concentration (Figure 9B). The initial increase is to about 1.4 μM, which is near the critical concentration of the pointed end in the presence of $MgCl_2$. In contrast, in 0.1 M KCl (where the two ends have similar critical concentrations), the results were quite different (Figure 9B). There was no sharp increase in the amount of unpolymerized actin. Instead, there was only a gradual increase in the critical concentration, similar to that seen at high brevin concentrations in 2 mM $MgCl_2$. In both 0.1 M KCl and 2 mM $MgCl_2$, the slope of the gradual increase in G-actin indicates approximately two G-actins per mole of brevin.

Discussion

The experiments reported here permit a detailed comparison of the properties of the two extracellular proteins brevin and DBP with each other and with the cytoplasmic actin binding proteins. The evidence presented in this paper suggests that DBP interacts directly with G-actin. The observed inhibition of actin assembly and the increase in the critical concentration in the presence of DBP can be explained by its tight binding to G-actin, leading to a shift in the equilibrium of actin away from the polymeric state. By reducing the concentration of free G-actin, DBP causes the inhibition of both the nucleation and the elongation steps of the polymerization. Its effects on the former are more pronounced because of the dependence of nucleation on an exponential power of the actin concentration (Kasai et al., 1962). A similar sequestration mechanism has been proposed for the effect of profilin on the polymerization and depolymerization of actin (Tobacman & Korn, 1982; Tseng & Pollard, 1982). A previous report (Norberg et al., 1979) may have missed the depolymerizing activity of DBP in the serum because, compared with brevin, the former acts relatively slowly and depolymerizes only a stoichiometric amount of actin.

Several reports have described the ability of preformed actin nuclei to promote the polymerization of the G-actin in the profilin–actin complex and the inability of profilin to increase the critical concentration (Blikstad et al., 1980; Grumet & Lin, 1980; Markey et al., 1982a). We observed no such effects with DBP. These differences between DBP and profilin can be explained on the basis of the much higher affinity of the former for actin. The K_d's of muscle and *Acanthamoeba* actin for *Acanthamoeba* profilin are about 50 and 5 μM, respectively (Tobacman & Korn, 1982; Tseng & Pollard, 1982), several orders of magnitude higher than the 50 nM range estimated for the complex of muscle actin and DBP (Cooke et al., 1979). These differences in affinity may be indicative of the different roles of DBP and profilin. The former has been proposed as an extracellular scavenger of actin (Van Baelen et al., 1980) while the latter has been postulated to be important in regulating the amount of free actin available for polymerization (Carlsson et al., 1977).

In some of its binding characteristics, DBP resembles DNase, another G-actin binding protein. The K_d of the DNase–actin complex is about 5 nM (Mannherz et al., 1980), similar to that of the DBP–actin complex. We have observed that the tight binding of DBP to actin, like that of DNase, is not dependent on ionic conditions. However, it has been reported that DNase can increase the rate of filament disassembly (Hitchcock et al., 1976) and may bind with high affinity to the barbed end of the filament (Pinder & Gratzer, 1982). We found no indication that DBP does either of these things. Supporting the notion that DBP does not bind to the filaments with high affinity, Van Baelen et al. (1980) found that tritium-labeled DBP did not bind to erythrocyte ghosts (which should contain short actin filaments), although it did bind to proteins from an acetone-dried powder of the ghosts (which should contain G-actin).

The SAIP isolated by Vandekerckhove & Sandoval (1982) shows many similarities to DBP, although they did not test whether their protein binds vitamin D. In addition to having similar molecular weights, these proteins were isolated by similar procedures (actin affinity chromatography), have similar isoelectric points and similar amino acid compositions, and bind G-actin. Using radiolabeled SAIP binding to actin attached to Sepharose beads, they estimated a K_d of about 0.12 μM, a slightly weaker association than has been found between DBP and actin (Cooke et al., 1979). SAIP, like DBP, inhibited actin polymerization only at near-equimolar ratios. As these researchers observed no other serum actin binding proteins besides the one with a molecular weight near that of brevin, and since rabbit serum is known to contain a vitamin D binding protein (Haddad et al., 1981), it is quite likely that DBP and SAIP are the same protein existing in different species.

The data presented in this paper strongly indicate that brevin binds with high affinity to the barbed end of the filament. In addition, brevin is apparently able to break intrafilament

actin–actin bonds, as demonstrated in the erythrocyte membrane–actin assay (Figure 7). This type of severing action would initially result in the creation of numerous short filaments without reducing the mass of F-actin and would account for the short filaments seen by electron microscopy in the presence of brevin (Harris & Schwartz, 1981; Thorstensson et al., 1982). The shortening of filaments would also account for brevin's effectiveness at reducing the viscosity of F-actin measured with high- and low-shear viscometers. Consistent with this action, [^{3}H]CB binding to F-actin is increased by brevin, indicating that there are more filament ends. The increased number of ends is presumably responsible for the enhanced rate of F-actin depolymerization with added brevin.

If brevin is bound tightly to the barbed end, it would be expected to reduce the monomer off rate at that end. We found that the brevin–G-actin complex, which does not appear to sever filaments, indeed inhibits rather than enhances the bulk depolymerization rate (Table I). Thus, it appears that it is the severing action of free brevin which causes a net increase of ends which leads to an acceleration in the depolymerization rate. These observations suggest that with increasing concentration of a filament-capping protein such as brevin, there would be an initial inhibition of the rate, as the barbed ends of the filaments are capped, followed by an increase in the rate, as severing increases the number of pointed ends. This pattern of inhibition and enhancement of the depolymerization rate has been described for villin (Wang et al., 1982).

How can a capping protein act like a depolymerizing factor? We have already described how filament-capping proteins which can sever filaments can accelerate filament disassembly under depolymerization conditions. The experiments of Figure 9 suggest that filament-capping proteins can also induce filament depolymerization under particular circumstances. When conditions are present such that the two ends have equal critical concentrations, e.g., 0.1 M KCl or in the absence of ATP, capping the barbed end of the filament should not appreciably increase the amt. of G-actin. Our results indicate that the monomer concentration was essentially unchanged when brevin was added to F-actin in 0.1 M KCl (Figure 9B). However, under other more physiological conditions, a filament-capping protein like brevin could certainly act like a depolymerizing factor. In buffer with ATP and 2 mM $MgCl_2$, the barbed end has a lower critical concentration than the pointed end. Therefore, capping the barbed end will cause the filament to depolymerize until the monomer concentration reaches the critical concentration at the pointed end [see Brenner & Korn (1979) and Hill & Kirschner (1982) for further discussion]. Since the filament number concentration is low when compared with the total actin concentration, a protein that binds to the barbed end with high affinity can produce this depolymerizing effect at very low molar ratios. The model predicts that a filament can be entirely depolymerized by a barbed-end capping protein whenever the total actin concentration is below the critical concentration at the pointed end. As shown in Figure 9B, very low concentrations of brevin were found to depolymerize filaments when the actin concentration was 1.16 μM, which is near the critical concentration of the pointed end. Conditions that raise the critical concentration at the pointed end (e.g., low concentrations of Mg^{2+}) should increase the total actin concentration at which full depolymerization could occur. Conversely, at a high total actin concentration, the percent of the filament depolymerized by capping the barbed end would be very small, although the absolute number of subunits lost from the filaments should be the same as at low actin concentrations. Barbed-end capping proteins that can also sever filaments to produce more ends will speed the rate at which the new steady state is reached.

A true depolymerizing factor has been defined as one which would shift the G- and F-actin equilibrium toward the monomeric form by increasing the monomer off-rate constant (Korn, 1982). According to this definition, the factor would increase both the rate of depolymerization and the critical concentration, which is the ratio of the on- and off-rate constants. As indicated above, although it reduces the monomer off rate at the barbed end, brevin would appear to be exhibiting both these effects and so could operationally be defined as a depolymerizing factor. However, it would do so only under particular conditions and through a different mechanism than by affecting rate constants.

The data in Figure 9B suggest that besides any increase in G-actin caused by capping the barbed end, brevin also causes an apparent additional gradual increase in the amount of unpolymerized actin. This increase is approximately two actins per brevin. While this paper was in preparation, Harris & Weeds (1983) reported a similar phenomenon for porcine ADF. In addition, Tellam & Frieden (1982) have observed this gradual increase in the critical concentration when cytochalasins were used as filament-capping factors. As in the experiments described here, both of these groups used the pyrene–actin probe to determine the extent of actin polymerization. There are a number of explanations for this apparent increase in unpolymerized actin. One possibility is that the kinetics of the monomer on and off reactions may be different for short oligomers than for long filaments. Since brevin creates short filaments, this could result in the release of G-actin from such filaments and an increase in the critical concentration. While the concentration of free brevin–G-actin complexes would be expected to be very low, since the complexes can nucleate assembly of new filaments or add to the barbed end of existing filaments, theoretical considerations suggest that a filament-capping protein could increase the complex concentration by affecting the filament length distribution. An F-actin solution normally contains filaments with an exponential length distribution. When brevin randomly cuts these filaments, the exponential distribution would remain the same but the mean filament length becomes shorter. Therefore, the proportion of the smallest unit, the brevin–G-actin complex, would increase. The net result is that a capping protein would appear to be sequestering monomers and effectively increasing the critical concentration. A similar argument can be made for a protein or complex which only nucleates and caps but does not sever filaments.

The apparent increase in G-actin could also be an artifact of the fluorescence assay. It is not known, for example, if the actin molecules on the filament ends have the same fluorescence properties as those in the middle of the filaments. Since brevin creates new ends, in the presence of high concentrations of this protein, any "end" effects would become magnified. Furthermore, brevin bound to the filament end could conceivably quench the fluorescence signal of the end pyrene–actins. This would cause a decrease in the fluorescence level with added brevin without an actual increase in monomer concentration. In support of this notion, it has been observed that heavy meromyosin (HMM) quantitatively reduces the fluorescence signal of pyrene–F-actin to that of G-actin with no disassembly of the filaments (Kouyama & Mihashi, 1981).

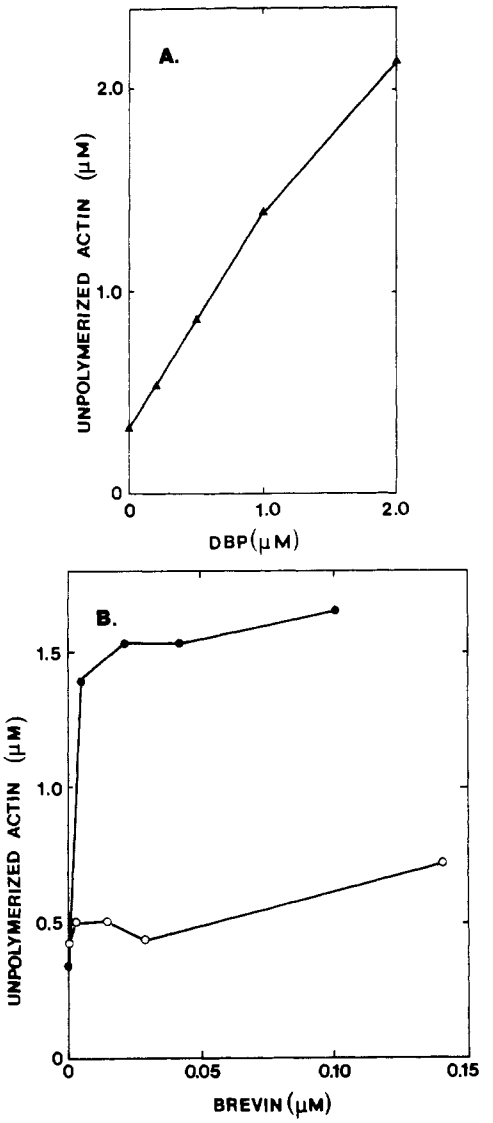

FIGURE 9: Effect of brevin and DBP on the critical concentration as determined by fluorescence. (A) Critical concentrations of F-actin in 2 mM $MgCl_2$ in the presence of the indicated amounts of DBP (▲) were determined from the intercepts of standard curves of G- and F-actin in the presence of increasing amounts of DBP. Eight different actin concentrations (5% pyrene–actin) between 0.58 and 8.14 μM were used at each DBP concentration. Samples were incubated 18 h. (B) Critical concentrations of F-actin in 2 mM $MgCl_2$ (●) or 0.1 M KCl (○) in the presence of brevin. Brevin was included in both the G- and F-actin standard curves. Five different actin concentrations (10% pyrene–actin) between 1.16 and 9.3 μM were used at each brevin concentration. Samples were analyzed after an incubation period of 12 h (KCl) or 6 h ($MgCl_2$).

A serum protein of 93 000 daltons has recently been shown to be immunologically related to the intracellular protein gelsolin, a calcium-sensitive protein of molecular weight 91 000 (Yin et al., 1981a; Thorstensson et al., 1982). Limited proteolytic digestion analysis of a platelet protein of about 90 000 daltons and brevin from human or chicken serum showed that these proteins have considerable structural homology (Markey et al., 1982b). In a preliminary study, we have observed that brevin resembles gelsolin in its dependence on calcium ion, ability to reduce the low-shear viscosity of F-actin, inhibition of barbed-end elongation of filaments, and release of F-actin from erythrocyte membranes (unpublished results). Thorstensson et al. (1982) have also shown that their 93 000-dalton serum protein shortens actin filaments only when micromolar calcium is present.[2] The physiological significance of the requirement of brevin for this ion is unclear since serum contains millimolar amounts of calcium.

We conclude that the high serum concentration of brevin and DBP (approximately 10 μM each) (Haddad et al., 1976; Harris & Schwartz, 1981; Thorstensson et al., 1982) can account for the depolymerizing activity that has been reported to be present in plasma. For the experiments where whole serum was used or where the proteins were only partially purified (Harris et al., 1980, 1982), the activity would be due to a combination of the action of brevin and DBP: the brevin would increase the depolymerization rate by severing filaments, and the DBP would maintain the actin in the G form. In the published experiments with purified material, near-stoichiometric amounts of the 92 000-dalton protein were used (Harris & Gooch, 1981). Since brevin can rapidly sever and cap filaments as well as bind G-actin, it is not surprising that the protein was found to rapidly depolymerize F-actin.

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Registry No. CB, 14930-96-2.

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[2] While this paper was in preparation, Harris & Weeds (1983) reported that their ADF showed both calcium-dependent and calcium-independent interactions with actin. Unlike brevin (Harris & Schwartz, 1981) and the 93 000-dalton serum protein (Thorstensson et al., 1982), Harris & Weeds (1983) found the effect of ADF on the length of actin filaments was not reversed by removing the calcium. Nevertheless, although there may be some differences among them, these proteins are all closely related if not identical. It may therefore be appropriate to call these proteins serum gelsolin, as has been suggested by others (Yin et al., 1981a).

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