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A Ca^{2+} Insensitive Actin-Crosslinking Protein From Dicytostelium discoideum

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We have isolated a 30,000-dalton protein from Dictyostelium which cosedimented with and affected the low shear viscosity of actin. At low concentrations, this protein increased the low shear viscosity to greater than that of the actin control, whereas higher concentrations decreased viscosity. The viscosity decrease correlated with the formation of actin filament bundles, as seen electron microscopically. This protein resembled a previously reported actin-binding protein from Dictyostelium [Fechheimer and Taylor, 84, J Biol Chem 259:4514] in electrophoretic mobility, Stokes radius, and ability to crosslink filaments, but was shown to be different by peptide mapping, lack of immunologic crossreactivity, and lack of sensitivity to calcium.

Key words: actin, regulatory protein, cytoskeleton

INTRODUCTION

Proteins which crosslink actin filaments have been isolated from a number of organisms [Korn, 1982; Schliwa, 1981], including Dictyostelium discoideum. Three actin-crosslinking proteins have been isolated from Dictyostelium so far, including proteins with subunit molecular weights of 120 kilodaltons [Condeelis et al, 1982], 95 kilodaltons [Condeelis and Vahey, 1982; Fechheimer et al,1982], and 30 kilodaltons [Fechheimer and Taylor, 1984] (this protein will be referred to as "p30a"). In this paper, we report the isolation of another 30-kilodalton protein which crosslinks actin (which will referred to as "p30b"). This protein differs from p30a in several ways, including sensitivity to calcium.

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MATERIALS AND METHODS

Proteins

Actin was prepared from Dictyostelium discoideum by the method of Uyemura et al [1978], and from rabbit muscle as described by Pardee and Spudich [1982].

p30a was purified by the method of Fechheimer and Taylor [1984].

p30b was purified as follows: high-speed supernatant, prepared as described in Uyemura et al [1978] from 40–50 g of cells, was diluted with 3 vol of 10 mM triethanolamine, pH 7.5, 0.4 mM dithiothreitol, 0.2 mM ATP, 0.05 mM $MgCl_2$, and loaded onto a 100 ml DEAE column preequilibrated with 10 vol of 10 mM triethanolamine, pH 7.5, 5 mM sodium pyrophosphate, 0.4 mM dithiothreitol, 0.2 mM ATP, 0.05 mM $MgCl_2$, 20 mM KCl. The flow-through from this column was applied to a 50 ml hydroxyapatite column, preequilibrated with 10 vol of deaerated 10mM potassium phosphate buffer, pH 6.5 (PO_4). The hydroxyapatite column was then washed with 10 bed vol of 0.27 M KCl in 10 mM PO_4, and eluted with a 0.27–1.5 M KCl gradient in 10 mM PO_4. Fractions eluting between 0.7 and 0.85 M KCl (measured by conductivity) were pooled, diluted 1:1 with 10 mM PO_4, and loaded onto a second hydroxyapatite column (5 ml), preequilibrated with 10 vol of 10 mM PO_4. This column was washed with 10 bed vol of 0.2 M PO_4, then eluted with a 0.2–0.7 M PO_4 gradient. p30b was seen by gel electrophoresis to elute at 0.45 M PO_4.

Anti-p30a IgG was a gift of Dr. Marcus Fechheimer, Department of Biological Science, Carnegie Mellon University.

Electrophoresis and Westerns

Proteins were run on 11% polyacrylamide SDS gels, using the buffer system of Laemmli [1970]. Gels were stained with 0.05% Coomassie Blue R, 15% acetic acid, 25% propanol, and destained in 15% acetic acid.

For Western blots [Towbin et al, 1979], proteins were transferred to nitrocellulose as follows: gels were rinsed in 20 mM Tris, 150 mM glycine, 20% methanol, pH 8.4, placed in a Hoefer Transphor, and electrophoresed for 0.5 h at 60 V in the same buffer. The nitrocellulose was rinsed briefly with distilled H_20, and dried. Parallel lanes were stained with Coomassie as above and destained with 90% methanol, 2% acetic acid or stained with antibody as follows: strips were blocked by 15 min incubation in 10% calf serum in phosphate-buffered saline (serum + PBS), rinsed 1 min in PBS, incubated 0.5 h in 10 μg/ml anti-p30a IgG in serum + PBS, rinsed with distilled water, blocked 15 min in serum + PBS, rinsed with PBS, incubated 0.5 h with goat antirabbit Ig coupled to peroxidase (Sigma) diluted 1/100 with serum + PBS, rinsed with distilled H_2O, incubated 2 min in 0.5 mg/ml 4-chloro-l-napthol (Sigma), 0.01% hydrogen peroxide in PBS, and rinsed with distilled H_2O. The above operations were performed at room temperature on a rotary shaker.

Assays for Interactions of p30a and b With Actin

The low shear viscosity of actin was measured by falling ball viscometry, essentially as described by Pollard and Cooper [1982]. Samples were prepared in triplicate and contained 0.25 or 0.3 mg/ml gel-filtered [MacLean-Fletcher and Pollard, 1980] rabbit muscle actin, 10 mM imidazole, pH 7.5, 0.2 mM dithiothreitol, 0.2 mM ATP, 0.01% azide, 50 mM KCl, 5 mM $MgCl_2$. Samples were incubated 3 h at room temperature before reading to allow the initially monomeric actin to polymerize.

Cosedimentation of proteins with actin was assayed by incubating mixtures containing the proteins in the presence or absence of polymerized actin in Airfuge tubes coated with Sigmacote (Sigma) and then centrifuging at 30 psi in a Beckman Airfuge. Supernates and pellets resuspended in an equal volume of the same buffer were electrophoresed as described above. Gel filtration of rabbit muscle actin had no effect on cosedimentation results.

Other Methods

Peptide mapping was performed on 15% SDS polyacrylamide gels by the method of Cleveland et al [1977].

Protein was determined by the method of Peterson [1977], Bradford [1976], or by optical density in the case of actin [Gordon et al, 1976].

Electron microscopy of samples stained with 1% aqueous uranyl acetate employed a Zeiss 10A operated at 60 kV.

RESULTS

Purification of p30b

p30b was purified as described in Methods, using inhibition of the low shear viscosity of actin as the assay for activity. Figure 1 is a gel of the fractions obtained at each purification step. The first step in the purification was DEAE chromatography of a high-speed supernate. The DEAE flow-through fraction inhibited the low shear viscosity of actin. The DEAE flow-through has already been reported to contain two Dictyostelium actin-binding proteins: severin [Brown et al, 1982] and p30a [Fechheimer and Taylor, 1984]. Severin should decrease, and p30a is reported to increase, the low shear viscosity of actin. When the DEAE flow-through was fractionated by hydroxyapatite chromatrography (Fig. 2), several activities were resolved. Little activity was detected in the flow-through (however, this fraction was more dilute than the other fractions; see legend to Fig. 2). A fraction eluted stepwise with 0.1 M KCl increased low shear viscosity in the presence of EGTA to a value greater than that of the actin control, and may thus contain p30a (although elution behavior of p30a from hydroxyapatite with KCl is unknown). The next fraction, eluted with 0.27 M KCl, decreased low shear viscosity below the actin control in a calcium-sensitive manner (ie, in the absence of EGTA). This activity is presumably due to severin, which elutes from hydroxyapatite at 0.17 M KCl [Brown et al, 1982]. There may be some severin in the 0.1 M pool as well, which would account for the decreased low shear viscosity in that fraction in the absence of EGTA. The third fraction, eluted with 1 M KCl, also contained low shear inhibitory activity, which, however, was calcium insensitive. Since severin and p30a are both calcium sensitive, we concluded that this third fraction probably contained a new, uncharacterized Ca^{++}-insensitive actin-binding protein.

This third fraction (obtained by gradient rather than step elution in subsequent preparations) was further purified by reapplying it to a hydroxyapatite column and then eluting with PO_4 instead of KCl [Bernardi, 1971]. A single peak of activity was seen on this column, which comigrated with a protein band with an apparent M_r of 33 kilodaltons. Since this protein migrates very similarly to p30a on SDS gels (Fig. 1), we refer to it as p30b.

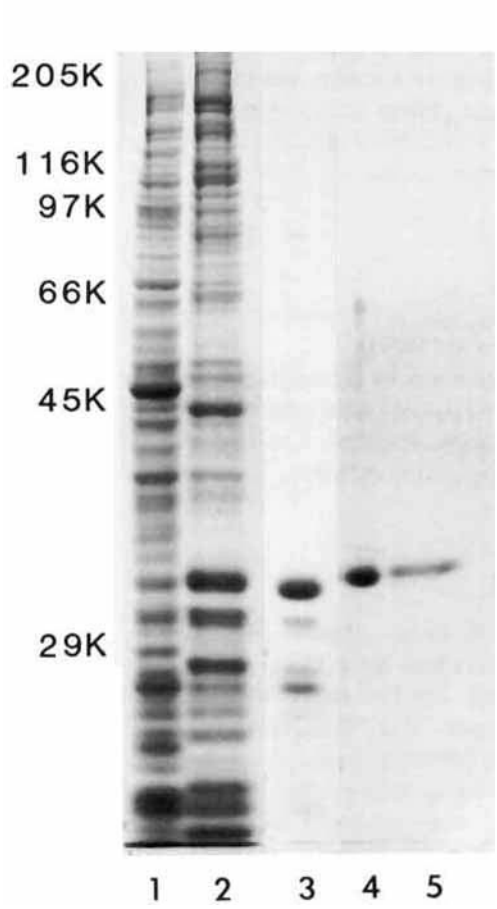

Fig. 1. Fractions obtained during purification of p30b. 1, high-speed supernatant; 2, DEAE flow-through; 3, pool from first hydroxyapatite column (KCl elution); 4, pool from second hydroxyapatite column (PO_4 elution); 5, p30a.

p30b Affects the Low Shear Viscosity of Actin and Causes the Formation of Actin Filament Bundles

As stated above, p30b was purified on the basis of inhibition of the low shear viscosity of actin. When we examined the effects of p30b on actin as a function of p30b concentration, we found that low concentrations of p30b increased low shear viscosity as compared to the control with actin alone (Fig. 3), whereas higher concentrations had the opposite effect, reducing the viscosity to less than actin alone (this is difficult to see in Figure 3, because of the scale on which the figure is drawn). If these assay mixtures were examined by electron microscopy (Fig. 4), it was observed that p30b could induce the formation of bundles of parallel actin filaments. The concentration of p30b required to induce bundles correlated closely to that causing a decrease in low shear viscosity. Figure 4 shows that 60 μg/ml p30b (molar ratio of p30b monomer to actin monomer 1:3), a concentration which causes a reduction in low shear viscosity, also causes the formation of large filament bundles.

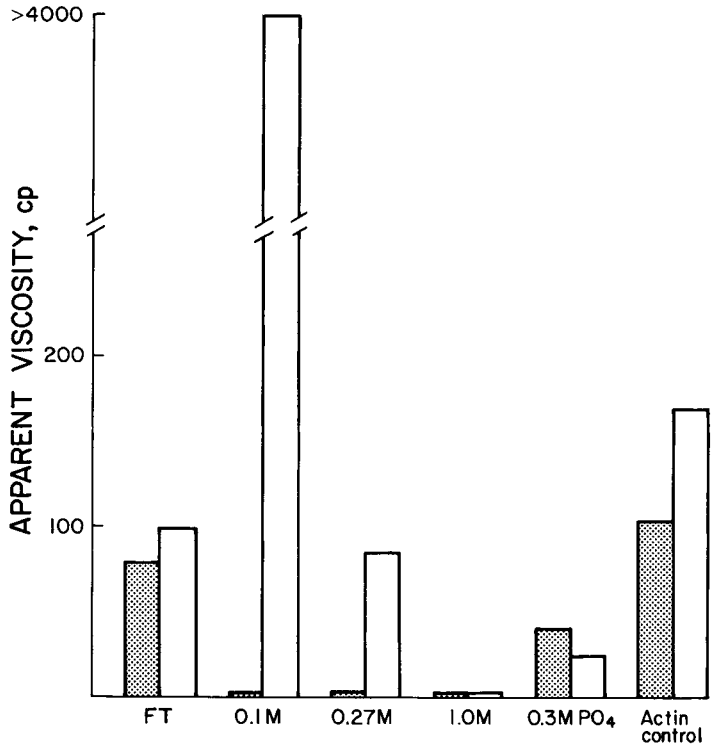

Fig. 2. Activity of fractions obtained by elution of hydroxyapatite with a KCl step gradient. A 50-ml hydroxyapatite column was loaded with a flow-through fraction from a DEAE column, and eluted successively with 5 bed vol of 0.1 M, 0.27 M, and 1 M KCl in 0.01 M PO_4, and 0.3 M PO_4. Pools of protein eluted by each step were assayed as follows: 20 μl of each pool was brought to a final volume of 340 μl containing 0.3 mg/ml rabbit muscle G-actin, 3 mM imidazole, 0.6 mM ATP and DTT, 50 mM added KCl, 5 mM $MgCl_2$, + (open bars) or − (shaded bars) 1mM EGTA, pH = 7.5. Volume of each assayed pool: FT, flow-through from hydroxyapatite, 720 ml; 0.1 M = 40 ml; 0.27 M = 40 ml, diluted 5× for assay; 1 M = 20 ml; 0.3 M PO_4 = 20 ml, diluted 5× for assay. Several dilutions of each pool were assayed; the ones illustrated in this figure were chosen on the basis of demonstrating differences in activity seen ± EGTA. This is necessary in order to see some activities (gelation activity is not seen if less of the 0.1 M pool is used, for instance), and also to assess spillover of the different activities (more 0.27 M pool shows some reduction of low shear in the presence of EGTA, and this may be due to spillover of the 1 M activity). In contrast, the Ca^{++}-insensitive nature of the activity in the 1 M pool is demonstrated by the fact that activity ± EGTA is the same at all dilutions used.

If the p30b concentration was halved to 30 μg/ml (1:6 molar ratio), a concentration which caused an increase instead of a decrease in viscosity, large bundles of filaments were not produced. Instead, much of the actin was present as tangles of individual filaments, and only a few small bundles of two to three filaments were seen (Fig. 4). At 10 μg/ml, where viscosity increase was maximum, no bundles were seen.

Effects of p30b on the low shear viscosity (Fig. 3) and electron microscopic appearance (Fig. 4) of actin appeared to be independent of the calcium concentration. The low shear experiments were complicated, however, by the fact that the viscosity of the actin alone was also calcium dependent.

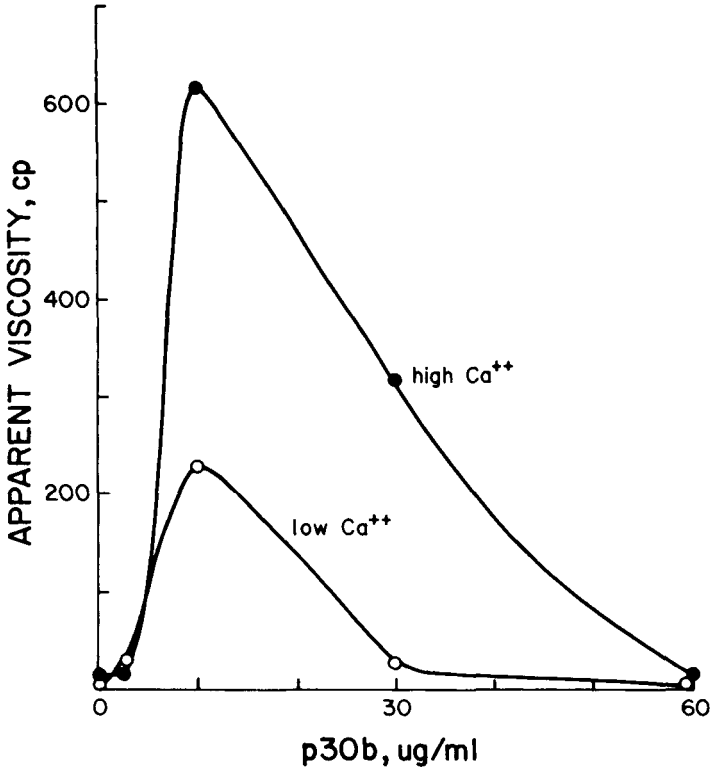

Fig. 3. Effects on the low shear viscosity of actin as a function of p30b concentration. Various concentrations of p30b were mixed with 0.25 mg/ml rabbit muscle F-actin in 10 mM Pipes, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 2 mM EGTA, and either 1.8 mM $CaCl_2$ (● ; Ca^{++}/EGTA = 1:1.1) or 0.1 mM $CaCl_2$ (○; Ca^{++}/EGTA = 1:20). Final pH = 6.8. Read after 0.5 h, room temperature.

Figure 3 demonstrates that a minimum molar ratio of p30b to actin of about 1:30 is required to obtain crosslinking under the conditions of this experiment (1:60 if p30b is dimeric; see below).

p30b Cosediments With F-Actin

To confirm the difference in calcium sensitivity between p30a and b, we examined the ability of these proteins to cosediment with polymerized actin in the presence of $CaCl_2$ versus EGTA (Figs. 5, 6). We found that p30b cosedimented quantitatively with actin, independent of the calcium concentration (Fig. 5: molar ratio of p30b to actin = 1:6). p30a, on the other hand (Fig. 6: molar ratio = 1:7), did not cosediment with actin in the presence of 0.1 mM $CaCl_2$, confirming published results [Fechheimer and Taylor, 1984]. In the presence of 5mM EGTA, some of the p30a did sediment with actin. It did not appear to bind to actin as quantitatively as p30b, even when p30b was cosedimented under exactly the same conditions or when actin concentration was increased to 2 mg/ml (data not shown). Neither of these two proteins sedimented in the absence of actin (Figs. 5, 6). Also, neither affected the amount of actin sedimented (data not shown).

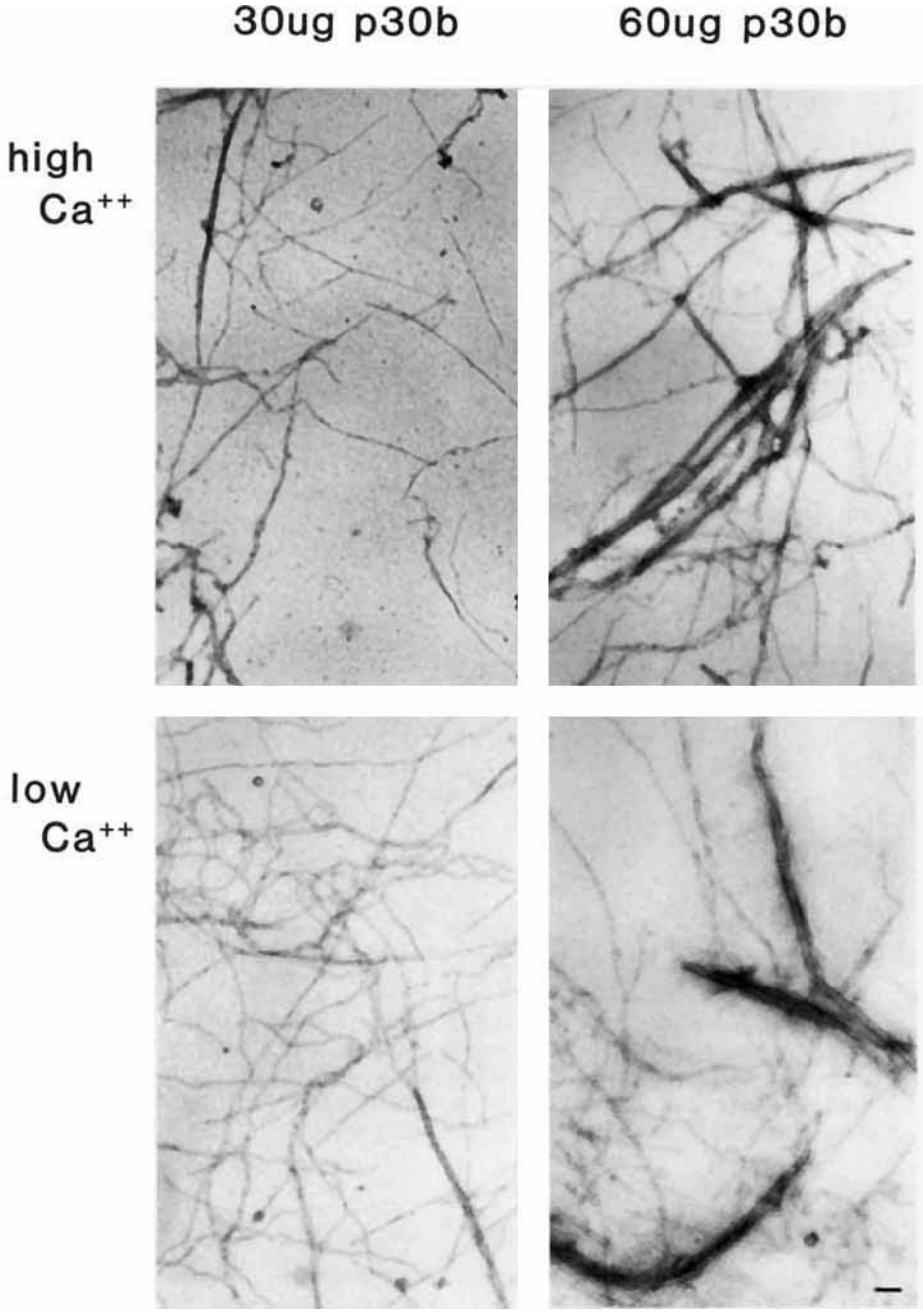

Fig. 4. Electron microscopy of p30 + actin. Aliquots of the assay mixtures shown in Figure 3 were examined in the electron microscope. Shown are the samples containing either 30 μg/ml p30b (p30/actin ratio of 1:6) or 60 μg/ml p30b (1:3) and a Ca^{++}/EGTA of 1:1.1 (high Ca^{++}) or 1:20 (low Ca^{++}). The bar in the lower right corner = 0.1 μm.

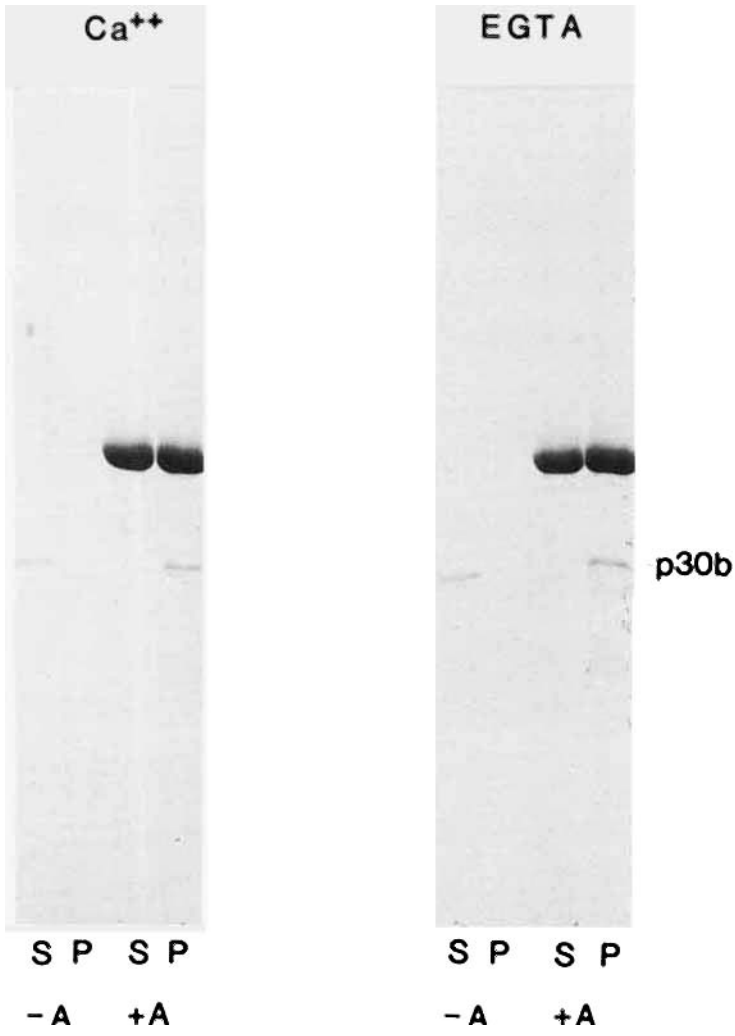

Fig. 5. Cosedimentation of p30b with F-actin; 0.03 mg/ml p30b was incubated 10 min at room temperature (RT) in the presence (+A) or absence (−A) of 0.25 mg/ml Dictyostelium F-actin in 10 mM imidazole, 50 mM KCl, 5 mM $MgCl_2$, 0.01% azide containing either 0.1 mM Ca^{++} or 1 mM EGTA, pH_{RT} = 7.4. The samples were centrifuged 20 min at 30 psi in a Beckman Airfuge, and supernatants (S) and pellets resuspended in an equal volume (P) were run on an SDS gel.

p30a and b Have Similar Stokes Radii

p30b was subjected to sizing column chromatography, to examine its native molecular weight. It migrated like a globular protein with an M_r of 68 kilodaltons (Fig. 7; Stokes radius = 35 Å). Fechheimer and Taylor [1984] have reported a Stokes radius of 30 Å for p30a. BSA is the only size standard we have in common with them, and it does not fall on the standard curve we have drawn in Figure 7B. If a standard curve is instead drawn to go through BSA and the low molecular weight standards, the Stokes radius of p30b obtained is then also 30 Å. Fechheimer and

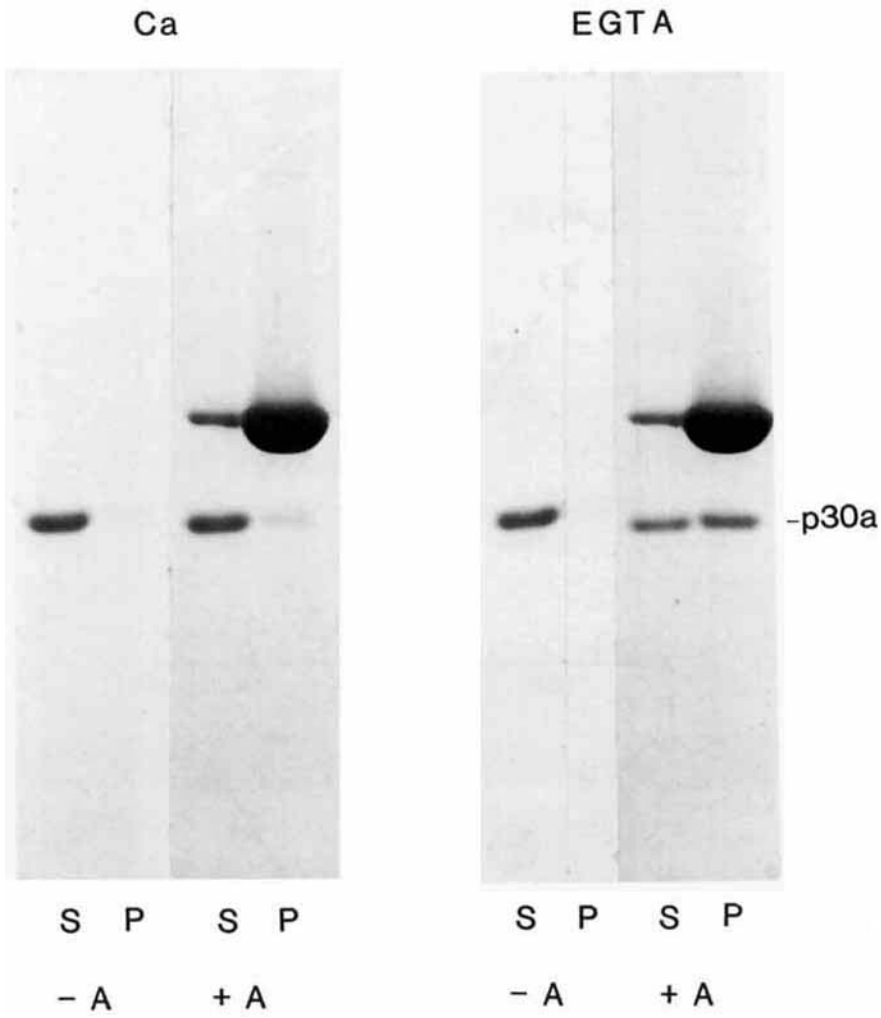

Fig. 6. Cosedimentation of p30a with F-actin; 0.05 mg/ml p30a was incubated 1 h, at RT in the presence (+A) or absence (−A) of 0.5 mg/ml rabbit muscle F-actin in 20 mM Pipes, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ATP, 0.005% azide containing either 0.1 mM Ca^{++} or 5 mM EGTA, pH_{RT} = 6.8. The samples were centrifuged, and supernates (S) and pellets (P) were run on a gel.

Taylor [1984] obtain a native M_r of 31,700 daltons for p30a by equilibrium sedimentation in an analytical ultracentrifuge, and conclude that p30a is an elongate monomer. Since p30b has not been analyzed in this way, we cannot distinguish between the possibilities that it is a dimer or also an elongate momoner.

p30b Is a Different Protein Than p30a

Because of their similar Stokes radii and electrophoretic mobility on SDS gels, we asked whether p30a and b were related proteins, by peptide mapping and immunological crossreactivity. Figure 8 shows peptide maps of these two proteins obtained

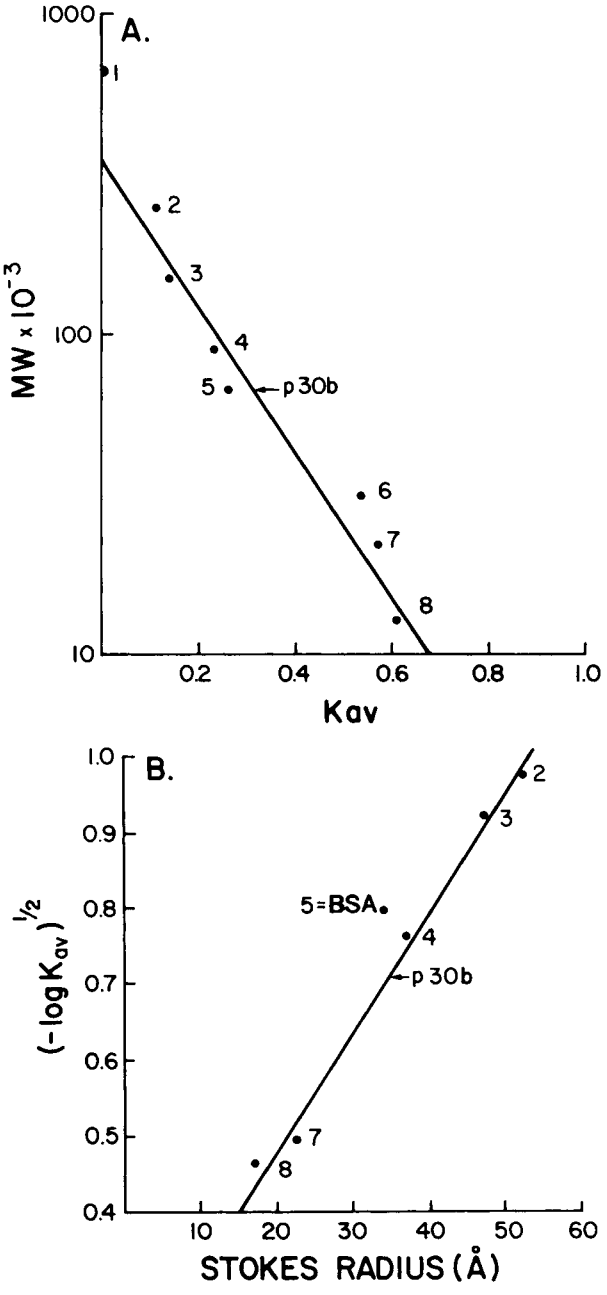

Fig. 7. Sizing chromatography of p30b. A. p30b was chromatographed on a Sephadex G 150 column in 10 mM PO_4, pH 6.5, 100 mM KCl, 0.01% azide. The position of its K_{av} is indicated by the arrow on the plot of the M_r vs K_{av} of a number of protein standards. B. The data in A is replotted as the square root of the negative log of K_{av} vs Stokes radius, for all but thyroglobulin and DNase I. The arrow indicates the position of p30b, which thus has a Stokes radius of 35 Å by this determination. Protein standards: 1, thyroglobulin; 2, catalase (52.2 Å); 3, aldolase (47.4 Å); 4, enolase (34.1 Å); 5, BSA (37.0 Å); 6, DNase I; 7, soybean trypsin inhibitor (22.5 Å); 8, cytochrome C (17.4 Å).

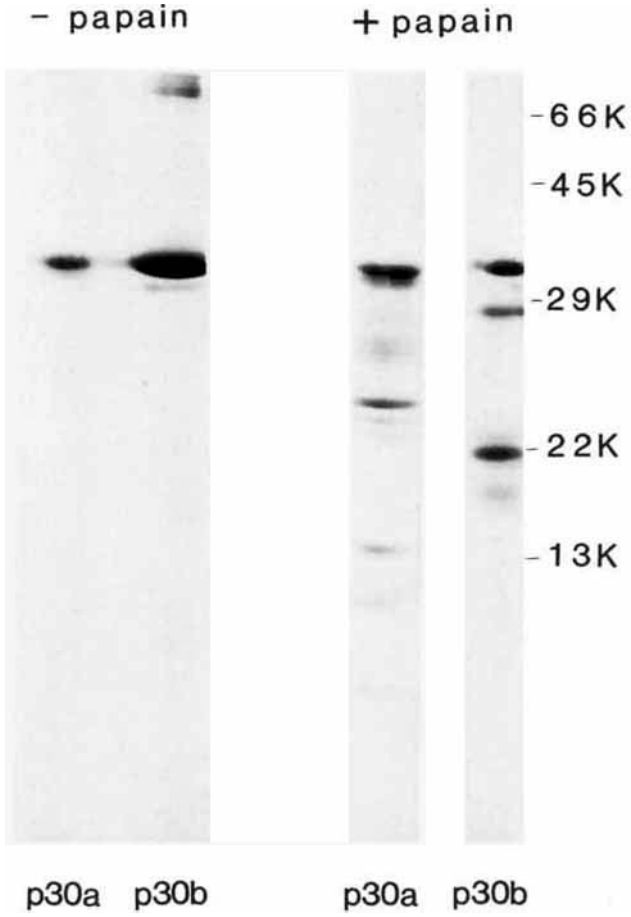

Fig. 8. Peptide maps of p30a vs p30b. Peptide mapping was carried out by the method of Cleveland et al [1977]: 5 μg p30a or b was run on SDS gels. The bands were excised and equilibrated 0.5 h in buffer. They were then applied to a second, 15% acrylamide gel and overlaid with 10 μl of 10 μg/ml papain (on the right) or buffer only (on the left; = equilibration buffer containing 10% glycerol). The proteins were run to the stacking gel/separating gel interface and incubated 0.5 h, RT, before being run on the separating gel. No bands could be seen in a lane containing 10 μl papain only.

by the method of Cleveland et al [1977]. It can be seen that a different pattern of peptides is seen for p30a versus p30b.

Western transfers of purified p30a and partially purified p30b (from the first hydroxyapatite column) were labeled with IgG directed against p30a (provided by Dr. Marcus Fechheimer, Carnegie-Mellon). Even though Coomassie staining shows that there is much more p30b than p30a transferred onto the nitrocellulose, the p30a labels intensely, whereas there is very little labeling of p30b (Fig. 9). This faint cross-reaction could be explained by approximately a 1% contamination of this crude p30b fraction with p30a. We obtained this estimate by comparing dot blots of p30a and p30b at various dilutions (data not shown). This level of contamination is possible at this purification stage, given the similarity in the purification schemes for the two proteins.

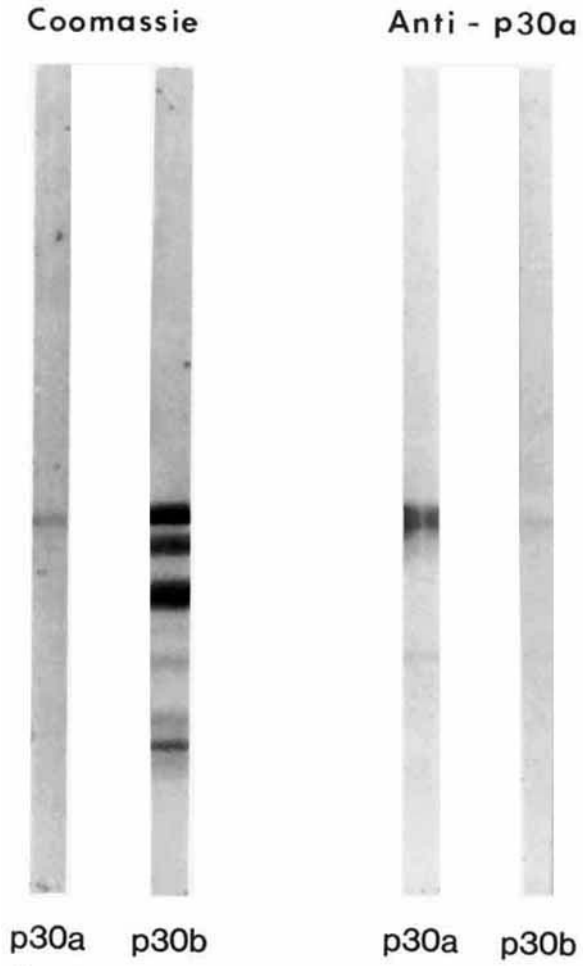

Fig. 9. Western transfers of p30a and p30b labeled with anti-p30a antibody. p30a or partially purified p30b (pool from first hydroxyapatite column) was run on a SDS gel and transferred to nitrocellulose. Strips of each were excised and either stained with Coomassie blue or antibody against p30a (see Methods).

DISCUSSION

We have purified a previously unreported actin-binding protein from Dictyostelium discoideum. This protein affects the low shear viscosity of actin, cosediments with actin, and causes the formation of actin filament bundles as seen in the electron microscope. We infer that this protein binds to the sides of actin filaments and can bind more than one actin per molecule, thereby crosslinking actin into bundles of filaments. All of these effects are insensitive to calcium.

The effects on low shear viscosity are complex; p30b increases it when present at a low ratio to actin, but decreases it at higher ratios. This could be explained as follows: a few crosslinks between filaments at a low p30b concentration could cause gel formation and elevation of low shear viscosity. More crosslinks at a higher

concentration of p30b could cause parallel alignment of filaments and consequent lowering of the low shear viscosity. The electron microscopic analysis supports this interpretation (compare Figs. 3 and 4): prominent bundles of actin filaments were seen at a p30b concentration which reduced low shear viscosity; half as much p30b both increased low shear and caused only a few small bundles. This result may not be too surprising; it has been known for some time that high concentrations of gelation factors can cause the formation of actin bundles [Schliwa, 1981], and there are a number of reports that bundling reduces actin low shear viscosity. For instance, Siegel and Branton [1985] find that band 4.9 from red cells lowers the low shear viscosity of actin by bundling filaments. (This effect is seen at a ratio of 1:30 actins, suggesting that 4.9 is a more potent bundler than p30b.)

p30b is similar in molecular weight on SDS gels (Fig. 1) to the previously reported actin-binding protein p30a, and resembles it in other ways: both bundle actin filaments, have similar Stokes' radii, and neither bind to DEAE. The two proteins differ in calcium sensitivity and in the amount of PO_4 required to elute them from hydroxyapatite (p30a is eluted with less than 0.1 M PO_4, whereas p30b requires 0.45 M PO_4 for elution). Because the similarities were so striking, we further investigated the relationship between these two proteins by peptide mapping and immunological crossreactivity. We came to the conclusion that these are not related proteins; they do not appear to have any peptides in common upon papain digestion, and p30b doesn't react appreciably with antibody against p30a.

These comparisons are useful for interpreting the difference in Ca^{++} sensitivity between the two proteins. It suggests that Ca^{++} sensitivity has not been lost or gained due to proteolysis of one of these proteins to generate the other, nor by some other modification of the same gene product. We conclude, instead, that these are truly separate proteins.

Dictyostelium has just been found [Schleicher et al, 1984] to have another actin-binding protein of very similar M_r to p30a and b. These authors have isolated a "capping protein" consisting of two subunits of 34 kilodaltons and 32 kilodaltons, which, like p30b, can reduce the low shear viscosity of actin in a Ca^{++}-independent manner. These proteins differ in their purification properties, however, and the increase in low shear viscosity seen at low p30b concentrations is not seen with the capping protein. Presumably their mechanisms of action are quite different. The capping protein blocks the end of an actin filament, and is unlikely to crosslink filaments (in analogy to the capping protein from Acanthamoeba [Cooper et al, 1984]). It is interesting, nonetheless, that several superficially similar Dictyostelium actin-binding proteins have been reported recently and it points to the need for caution when investigating actin-regulatory proteins.

Another note of caution which applies not just to p30b but to all biochemical studies of actin-associated proteins is the question of physiological relevance. This is especially true if the actin-associated protein is basic, as this might have a nonspecific electrostatic interaction with the acidic actin molecule [Griffin and Pollard, 1982]. A number of actin-associated proteins [eg, severin: Brown et al, 1982; p30a: Fechheimer and Taylor, 1984; p30b: this paper; Acanthamoeba gelactins: Maruta and Korn, 1977; profilin: Reichstein and Korn, 1979] bind to DEAE weakly or not at all and thus may be basic proteins. (Reichstein and Korn [1979], however, show that this may not always be so.) It is not easy to deal with this question of physiological relevance. Affinity of associated proteins for actin may not be a good indicator

[Pollard, 1983]. Denaturation studies can be problematic. For instance, boiling wipes out the ability of p30b to affect the low shear viscosity of actin. This does not prove that the native conformation of p30b is required, however, as boiling also causes p30b to become sedimentable under the conditions of our cosedimentation assay. Thus, the low shear result is uninterpretable, as the number of crosslinking elements has presumably been reduced by p30b precipitation. In some cases the argument can be made that the actin-associated protein is regulated by something considered to be physiologically relevant (eg, the regulation of p30a by low levels of calcium). However, Griffith and Pollard [1982] have shown effects of divalent metals on the interaction between actin and molecules presumed to be physiologically irrelevant, so even this sort of evidence must be taken with caution. Thus, it is to be hoped that other approaches, using recently developed techniques such as antibody microinjection and production of mutants by site-directed mutagenesis, will soon prove useful in addressing this question.

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