Effect of Actin-binding Protein on the Sedimentation Properties of Actin

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ABSTRACT Actin and actin-binding protein (ABP) have recently been purified from human platelet cytoskeletons (S. Rosenberg, A. Stracher, and R. C. Lucas, 1981, J. Cell Biol. 91:201-211). Here, the effect of ABP on the sedimentation of actin was studied. When ABP was added to preformed F-actin filaments, it bound until a maximum ratio of 1:9 (ABP:actin, mol:mol) was reached. However, when actin was polymerized in the presence of ABP, two and a half times more ABP was able to bind to the actin—that is, every 3.4 actin monomers were now bound by an ABP dimer. ABP was not able to induce the sedimentation of actin under nonpolymerizing conditions but was able to reduce the time and concentration of actin required for sedimentation under slow polymerizing conditions. ABP, therefore, exerts its effect on G-actin by either nucleating polymerization or by cross-linking newly formed oligomers into a more sedimentable form.

In muscle cells, a stable actin lattice is required for the generation of contractile force, whereas in nonmuscle cells, the more transitory contractile processes make a flexible actin cytoskeleton more desirable. Changes in the state of actin aggregation have been reported during cell division (30), mitosis (29), viral transformation (26), active phagocytosis (15), and adhesion to a substratum (8). These changes can either be a result of the reorganization of actin from filament bundles to single filaments simply by a cross-linker going off and on again or by a monomer to polymer mechanism.

In platelets, several investigators have found the majority of the cells' actin to be in a nonfilamentous form (6, 9, 10, 23, 25), whereas others found a significant amount of the actin to be in a filamentous form (2, 28, 35, 38). There are reports of a rapid polymerization of the cells' actin upon platelet activation (6, 9, 23, 25) which might be responsible for subsequent filopodial formation though there is also the alternative that the preexistent actin filaments of the cytoskeleton are rearranged to form these filopodia (28). In either case, platelets, like all nonmuscle cells, must have mechanisms by which the state of assembly of their actin lattice is regulated. Various accessory proteins may be involved in this regulation.

Actin-binding protein (ABP), a 260,000-dalton protein originally isolated from macrophages by Hartwig and Stossel (12, 32), is present in high concentrations in platelets (19, 28). ABP has the ability to cross-link and cause the low-speed sedimentation of F-actin (5, 28, 32). Profilin, a 16,000-dalton protein which binds to G-actin in a 1:1 molar ratio rendering it polymerization-resistant has also been found in platelets (7, 11, 20). A 90,000- to 95,000-dalton protein in platelet extracts which binds to G-actin in the presence of Ca$^{2+}$ has also been identified (21, 33). This protein may or may not be similar to gelsolin described by Yin and Stossel (36). Gelsolin has the ability to break actin filaments in the presence of Ca$^{2+}$, thereby increasing the amount of ABP required for incipient gelation of the actin (37). Platelets have also been found to contain a whole array of other proteins known to interact with actin including myosin, $\alpha$-actinin, troponin, and tropomyosin (1).

In 1976, Lucas et al. (18) introduced a procedure for the direct isolation of the platelets' cytoskeleton, using a solution containing 1% Triton X-100 and 10 mM EGTA. This structure, seen by negative staining in the electron microscope as branched bundles of actin filaments, consists mostly of actin, ABP, and $\alpha$-actinin (27, 28). The individual components of the cytoskeleton have been purified and their in vitro interactions studied. We have previously reported that both ABP and $\alpha$-actinin exhibit Ca$^{2+}$-sensitive interactions with the actin cytoskeleton in vivo (28), though only $\alpha$-actinin remains Ca$^{2+}$-sensitive in its binding to actin in vitro (27). The binding of ABP to actin in vitro is insensitive to the presence of calcium ions. In this report we describe the effect of ABP on the sedimentation properties of actin.
MATERIALS AND METHODS

Preparation of Platelets

Human platelet concentrates, fresh from the Greater New York Blood Center, were centrifuged at 350 g for 20 min to pellet any contaminating erythrocytes and leukocytes. Platelets were sedimented from the platelet-rich-plasma at 1,000 g (20 min) and were washed twice by resuspension in (126 mM NaCl, 5 mM KCl, 0.3 mM EDTA, 10 mM NaPO₄, pH 7.4 (platelet wash) and reconstituted (1,000 g, 20 min). All utensils used in the platelet preparation were plastic or siliconized glass and all operations were carried out at 4°C.

Preparation of Proteins

Cytoskeletons were prepared as previously described (28). Essentially, 1 vol of washed platelets (resuspended 1:1, vol:vol, in platelet wash) was added to 10 vol of Triton X-100 (Calbiochem-Behring Corp., La Jolla, CA), 40 mM KCl, 10 mM imidazole-HCl, 10 mM EGTA, 2 mM NaN₃, pH 7.0. After 12 min on ice, the cytoskeletons were sedimented at 3,000 g (2 min) and washed once in the Triton buffer and twice in 100 mM Pipes (Calbiochem-Behring Corp.), 2 mM NaN₃, pH 6.8 (buffer A). Platelet actin and platelet actin-binding protein were purified from the cytoskeleton as previously described (28). Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (31).

Protein concentrations were determined by the method of Lowry et al. (17) using bovine serum albumin as a standard.

SDS PAGE

Samples to be electrophoresed were precipitated with cold 10% trichloroacetic acid, neutralized with NH₄OH, resolubilized with 1% SDS, 1% 2-mercaptoethanol, pH 8.0 (depolymerization buffer), and clarified. No structure was visible in the tube and none could be sedimented even when the mixture was centrifuged at speeds sufficient to pellet F-actin (148,000 g) (Fig. 1). However, when the G-actin was added to a solution containing ABP and 2 mM MgCl₂, the G-actin presumably began to polymerize and was then able to form a complex with the ABP. This structure is easily seen with the naked eye (Fig. 2) and can be sedimented at 3,000 g.

It was then of interest to compare the nature of the interaction between ABP and polymerizing actin to that of ABP with polymerized actin. When a constant amount of filamentous actin (either human platelet or rabbit skeletal muscle) was added to increasing amounts of ABP, and any ABP-actin complex that formed was sedimented out at 10,000 g, it is seen that the amount of ABP sedimenting increased until a saturating level was reached (Fig. 3). At this point, the excess ABP began appearing in the supernatant. Since neither ABP nor F-actin alone sedimented under these experimental conditions, it can be assumed that the ABP was bound to the F-actin and caused its precipitation at this relatively low centrifugal force. Some free F-actin was still present in the supernatants of the four lowest ABP concentrations, but after this point greater than 85% of the actin pelletted at this speed. The ABP-binding sites on platelet F-actin became saturated at a weight ratio of 1.36:1 (ABP:actin) or when 1 mol of ABP was bound to 9 mol of actin (using a dimer molecular weight of 520,000 for ABP).

Lucas et al. (18), consist mostly of actin, ABP and α-actinin (27, 28). We purified both ABP and actin from the platelet cytoskeleton and found that they interacted in vitro to form a structure which looked very similar to the platelets’ native cytoskeleton (28). Formation of this low-speed (3,000 g) sedimentable complex of ABP and actin from purified, high-speed (25,000 g) clarified solutions of these two proteins occurred only when the actin (either human platelet or rabbit skeletal muscle) was in the filamentous state. No ABP-actin complex formed when the two solutions had previously been dialyzed into depolymerization buffer (2 mM Tris-HCl, 0.2 mM CaATP, 0.5 mM 2-mercaptoethanol, pH 8.0) and clarified. No structure was visible in the tube and none could be sedimented even when the mixture was centrifuged at speeds sufficient to pellet F-actin (148,000 g) (Fig. 1). However, when the G-actin was added to a solution containing ABP and 2 mM MgCl₂, the G-actin presumably began to polymerize and was then able to form a complex with the ABP. This structure is easily seen with the naked eye (Fig. 2) and can be sedimented at 3,000 g.

RESULTS

Human platelet cytoskeletons, isolated by the procedure of

FIGURE 1 5.5% SDS polyacrylamide gels of the recombinant of ABP and actin in depolymerization buffer. Platelet ABP and rabbit skeletal muscle actin were dialyzed overnight against 2 mM Tris-HCl, 0.2 mM CaATP, 0.5 mM 2-mercaptoethanol, pH 8.0 (depolymerization buffer), and clarified for 25 min at 148,000 g in a Beckman airfuge before their use in this experiment. Immediately upon clarification of these protein samples, 81 μg of the G-actin supernatant were added to either 25 μg of the ABP supernatant (2) or to an equivalent volume of depolymerization buffer (1), to a final volume of 0.15 ml. After 15 min at room temperature, the samples were centrifuged at 148,000 g for 18 min in a Beckman airfuge and the resulting pellets (P) and supernatants (S) were separated and processed for SDS polyacrylamide gel electrophoresis as described in Materials and Methods.

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binding to every 3.4–3.5 mol of actin. Thus, two and a half times more ABP was able to bind to polymerizing actin than to polymerized actin allowing much more highly cross-linked structures to be formed. Again, except for the first three points where there was not enough ABP to sediment all of the F-actin, >85% of the actin pelleted under these experimental conditions.

It seemed therefore, that although ABP could not induce G-actin to polymerize under nonpolymerizing conditions (Fig. 1), it might have some effect on G-actin undergoing salt-induced polymerization (compare Figs. 3 and 4). We chose slow polymerizing conditions to investigate this possibility (26 mM KCl at room temperature) so that any effect of ABP could be more easily discerned. It is seen in Fig. 5 that when G-actin, in depolymerization buffer at a final concentration of 0.7 mg/ml, was added to a solution containing a final concentration of 26 mM KCl, a lag period of 15 min occurred before F-actin filaments could be sedimented from the solution. When ABP was present, however, no lag period was seen and the amount of actin sedimenting within 20 min had required 35 min in the absence of ABP.

We then looked at the effect of ABP on the critical actin

Fig. 2 Recombination of purified platelet ABP and actin. 1.0 ml of 0.8 mg/ml platelet G-actin was added to 1.0 ml of 3.0 mg/ml ABP in buffer A + 4 mM MgCl₂. The cuvette was photographed after 8 min of intermittent agitation at room temperature. Note the fibrous nature of the structure formed. These fibers were shown, by negative stain electron microscopy, to be made up of bundles of actin filaments (28).

Fig. 3 Maximum binding of ABP to platelet F-actin. 44 µg of platelet F-actin were added to increasing amounts of ABP in buffer A to a constant final volume (0.2 ml) of 40 mM KCl, 8 mM PIPES, 0.8 mM EGTA, 2 mM MgCl₂, pH 6.8. After 15 min at room temperature, the samples were centrifuged at 10,000 g for 10 min. The supernatants were removed, the pellets washed once and the wash combined with the supernatants. The pellets were solubilized directly in the gel sample buffer, whereas the supernatants were first precipitated with cold 10% trichloroacetic acid. Equivalent loads of the pellets and supernatants were electrophoresed on 5.5% SDS polyacrylamide gels which were subsequently scanned and the areas under the protein peaks determined. The empty and filled circles represent the data points for two different experiments using different preparations of ABP.

and a monomer molecular weight of 42,500 for actin). This is in close agreement with Wang and Singer (34), who reported that 1 mol of chicken gizzard filamin bound stoichiometrically to 8–12 mol of F-actin.

When the binding studies were performed with G-actin which was allowed to polymerize in the presence of ABP (G-actin was exposed to ABP and 2 mM MgCl₂ at the same time), a different picture emerged (Fig. 4). The ABP binding sites on the actin were not saturated until a ratio of 3.5–3.6:1, ABP:actin (wt:wt), was reached. This is equivalent to 1 mol of ABP

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monomer concentration required for sedimentation under these low salt conditions (Fig. 6). A 45-min incubation period was chosen for this experiment since it appeared from Fig. 5 that the maximum amount of sedimentation would have occurred by this time. In the absence of ABP, an actin monomer concentration of 0.25 mg/ml was required before actin filaments could be detected by sedimentation. The presence of ABP lowered the critical concentration for sedimentation of actin in 26 mM KCl to ~0.16 mg/ml.

DISCUSSION

The polymerization of actin occurs in two distinct steps: (a) the formation of nuclei consisting of three to four G-actin monomers (slow) and (b) the elongation to filaments by the addition of monomers to the nuclei (fast) (14). Polymerization can be elicited in vitro by the addition of 100 mM KCl or 2 mM MgC12 to a G-actin solution though the in vivo regulation of the state of actin polymerization is unknown. Gordon et al. (10) found, after studying various highly purified nonmuscle actins, that there were no qualitative differences in their polymerization properties as compared to muscle actin. Therefore, changes in the state of actin aggregation (as described at this paper's beginning) are not due to intrinsic differences in nonmuscle actins but may be under the control of various accessory proteins which either function to keep the actin monomeric or which stabilize the polymer.

One candidate for an actin filament stabilizer is ABP. It has been shown here that although ABP cannot induce the polymerization of G-actin under nonpolymerizing conditions (Fig. 1), it appears to have an effect on actin undergoing salt-induced polymerization (Figs. 5 and 6).

When ABP was added to preformed F-actin filaments, steric hindrance seems to have prevented the addition of more than one mole of ABP to 9 mol of actin (Fig. 3). When ABP was added to G-actin in the presence of 2 mM MgCl2, 2.5 times more ABP was able to bind to the actin. Now, every 3.4 actin monomers were bound by an ABP dimer (Fig. 4). The low, constant amount of actin found in the supernatants of these binding experiments provide evidence that the different binding ratios observed are not due to different degrees of polymerization of the actin. Since the polymerization of actin seems to have blocked some of its ABP binding sites, it is possible that even more ABP would bind to actin under slow polymerizing conditions (26 mM KCl as opposed to 2 mM MgCl2) perhaps approaching ABP:actin ratios of 1:2 or 1:1.

The data presented in Figs. 5 and 6 provide evidence that ABP increases the sedimentability of actin by reducing both the time and concentration of actin required to see the onset of this sedimentation. These data can be interpreted in one of two ways: either ABP is accelerating the salt-induced polymerization of actin by acting as a nucleating site upon which actin filaments can grow or ABP cross-links newly formed actin oligomers into larger aggregates which are more easily sedimented. Whichever event is actually occurring, one would predict the result of such copolymerization to be highly cross-linked short actin filaments. Although we have not performed the electron microscopy, Hartwig et al. (13) have shown in a similar type of copolymerization experiment that this is indeed the case. Thus the presence of ABP during the polymerization of actin can have a profound effect on the framework of the final structure—the higher ABP:actin ratios leading to more cross-linked, highly branched structures of shorter actin filaments. The data in Fig. 4 showing more ABP bound to actin polymerized in its presence than to preformed actin filaments is consistent with this model.

High molecular weight complexes from platelets and red blood cell membranes (16), isolated by their ability to bind to cytochalasin B, have the property of causing the rapid polymerization of actin under slow polymerizing conditions. Brenner and Korn (3) also reported that the spectrin-actin complex they isolated on a Sepharose 4B column (probably short oligomers of actin crosslinked by spectrin tetramer) induced actin polymerization under slow polymerizing conditions. Since the actin achieved the same steady state viscosity with or without the spectrin-actin complex, the major effect of the complex was to accelerate the rate of actin polymerization as a source of stable actin nuclei. (The same effect was seen using sonicated F-actin as nuclei [4]). Spectrin dimer and spectrin tetramer, however, were each unable to induce the polymerization of G-actin though spectrin tetramer (like ABP) can cross-link F-actin (3). Therefore ABP is functionally different from spectrin in that ABP alone (without actin oligomers) can increase the sedimentability of added G-actin under slow polymerizing conditions.

In conclusion, we suggest that platelet ABP belongs to a class of proteins which promote the formation of actin filaments or large actin aggregates. Two other proteins also found in the platelet, myosin (24) and a-actinin (22), have been shown to increase the rate of actin polymerization. For this reason, care must be taken in the interpretation of data on the state of actin in whole cell lysates when lysate preparation conditions may either promote or prevent the association of actin with its various modifying proteins.

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