ABSTRACT

The cytoskeletal components, macrophage actin-binding protein and filamin, were dried from glycerol and examined by low-angle rotary shadowing electron microscopy. Both are elongate, flexible molecules whose general morphology is similar to that of erythrocyte spectrin. Neither actin-binding protein nor filamin binds to spectrin-depleted erythrocyte membranes.

Proteins that bind and cross-link actin filaments have been identified. Several of these proteins, including filamin (21), macrophage actin-binding protein (10), and spectrin (5, 12, 13), share certain properties. The three molecules are composed of high molecular weight subunits that can self-assemble to form dimers; they have relatively low sedimentation coefficients, large Stokes’s radii, and high frictional ratios. These properties have suggested that the three molecules may exist in solution as prolate ellipsoids or as flexible rods. Electron microscopy of filamin (7) and actin-binding protein (15) with the use of negative stain techniques has tended to support the assignment of a prolate ellipsoid shape to these molecules. However, examination of spectrin after low-angle rotary shadowing indicates that this molecule forms an extended, flexible rod in solution (14). Because of the striking biophysical similarities between the three proteins, and in the light of our experience with spectrin which binds with high affinity to membranes, we have reexamined the structure and membrane-binding properties of filamin and actin-binding protein.

MATERIALS AND METHODS

Purification of Human Spectrin

Spectrin was prepared by well-established methods (1, 2, 3, 18). Low ionic strength extraction of ghosts in 1 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) 0.1 mM EDTA, pH 7.6 was followed by column chromatography on Sepharose 4B in a buffer consisting of 5 mM NaPO₄, 1 mM EDTA, 20 mM KCl, 0.2 mM dithiothreitol, pH 7.6. For low-angle rotary shadowing, purified spectrin was sprayed onto mica in a buffer containing 1 mM NaPO₄, 0.2 mM EDTA, 20 mM KCl, 0.2 mM dithiothreitol, pH 7.6, and 60% glycerol.

Purification of Filamin

Filamin from chicken gizzards was prepared by the method of Wang (20) with some modifications. Disopropyl fluorophosphate (iPr₂FP) (0.015% vol/vol) was used in place of phenylmethylsulfonyl fluoride as an inhibitor of serine proteases. Chromatography was carried out on Sepharose 4B (2.5 × 90 cm column) rather than on Bio-Gel A-15m (Bio-Rod Laboratories, Richmond, Calif.). DEAE cellulose (DE52, Whatman) chromatography of peak fractions from Sepharose 4B yielded essentially pure filamin, free from contamination by low molecular weight polypeptides. Purified filamin was sprayed for rotary shadowing in a buffer containing 5 mM Tris acetate, 50 mM KCl, pH 7.6, and 60% glycerol.

Purification of Actin-binding Protein

Actin-binding protein prepared from rabbit macrophages by the method of Hartwig and Stossel (10) was a gift from T. Stossel and J. Hartwig (Harvard Medical School). For rotary shadowing, the protein was sprayed in a buffer containing 2 mM Tris acetate, 30 mM KCl, 0.1 mM EGTA, 0.1 mM dithiothreitol, pH 7.6, and 60% glycerol.

Low-angle Rotary Shadowing of Proteins

The purified proteins in glycerol (10–50 μg/ml) were sprayed onto freshly cleaved mica as previously described (17). In some experiments, before the addition of glycerol, the purified protein was incubated for at least 22 h at 4°C in a buffer containing 0.1% glutaraldehyde (16). The mica, bearing droplets of protein in glycerol buffer, was dried under vacuum, and platinum/carbon (0.7–0.8 nm) was deposited on the rapidly rotating samples from
an electron bombardment gun at a shadow angle of 45°. Replicas were coated with a 10-nm-thick supporting film of carbon and floated onto distilled water. They were then picked up on bare 400-mesh copper grids and examined with a Philips 301 electron microscope at 60 kV with a 50-µm objective aperture.

Analysis of Protein Binding to Membranes

Binding of 125I-labeled spectrin and filamin to erythrocyte membranes was assayed by a modification of the method of Bennett and Branton (3). Erythrocyte ghosts were stripped of spectrin and actin by incubation for 30 min at 37°C in 50 vol of 1 mM TES, 0.1 mM EDTA, pH 7.6. Pelleted vesicles were washed once in this buffer at 2°C and stored for a maximum of 24 h in a buffer consisting of 5 mM NaPO4, 1 mM EDTA, 20 mM KCl, and 0.5 mM dithiothreitol, at pH 7.6. Just before use, the vesicles were diluted with appropriate buffers to achieve a protein concentration of 1.0 mg/ml. Spectrin and filamin were labeled with 125I-Bolton-Hunter reagent (4) (Amersham Corp., Arlington Heights, Ill.) as previously described (2,18). Varying amounts of either 125I-spectrin or 125I-filamin were added to a constant amount of spectrin-depleted inside-out vesicles (20 µg) and incubated for 90 min on ice. Spectrin-binding assays were carried out in a buffer consisting of 5 mM NaPO4, 1 mM EDTA, 130 mM KCl, 10 mM NaCl, 0.5 mM dithiothreitol, pH 7.6. Filamin binding was measured in this buffer and in similar buffers that contained less salt (10 mM KCl) or more salt (300 mM KCl). After incubation, membrane-bound and free protein were separated as described (3).

RESULTS AND DISCUSSION

Filamin Purification

Fig. 1 shows the elution profile, sodium dodecyl sulfate polyacrylamide electrophoretic gels, and low-angle platinum/carbon rotary shadowed replicas of material from the Sepharose 4B filamin purification column. As expected, the void volume fraction, denoted by A, contains high molecular weight aggregates as well as monomeric myosin. The ascending side of the filamin peak fraction contains, in addition to filamin dimers, higher-order aggregates of filamin. Fraction C, the filamin peak, appears by electron microscopy as two flexible strands that are joined at one end. These two strands presumably correspond to the two monomers that comprise the filamin homodimer. Wang has shown that the filamin homodimer elutes from an agarose gel filtration column at a position corresponding to fraction C on Sepharose 4B (20).

The descending side of the filamin peak fraction, denoted by D, consists of a polypeptide whose molecular weight is indistinguishable from that of intact filamin in our gel electrophoresis system. In addition, a number of bands that exhibit higher electrophoretic mobilities are present. These presumably correspond to the short linear fragments observed in rotary shadowed replicas of this material. We rarely see dimeric structures in such preparations and, on the basis of these observations, it appears likely that proteolytic cleavage has occurred near the terminus of the molecule responsible for dimer formation. Davies et al. (7,8) have digested purified filamin with calcium-activated protease to produce two fragments, heavy merofilamin (M, 240,000) and light merofilamin (M, 10,000). Heavy merofilamin exists in solution as a monomer, whereas intact filamin exists predominantly in the dimer form, the result of a monomer-dimer equilibrium which strongly favors dimerization under the conditions used in their study. They note that although both intact filamin and heavy merofilamin are capable of binding to actin in solution, only intact filamin is also capable of producing gelation. On the basis of these studies and our results with low-angle rotary shadowing, a working hypothesis of filamin morphology has evolved: intact filamin monomers have a single binding site for actin and another discrete association site for other filament monomers. The latter site is located within 10,000 daltons of one terminus. At normal equilibrium under in vitro conditions, two monomers are joined to produce a divalent molecule that is capable of cross-linking actin filaments. The formation of filamin tetramers and higher-order oligomers is reported to be essentially irreversible (7), a finding that indicates that such structures may not be physiologically relevant.

The final fraction assayed by platinum/carbon shadowing contains primarily G-actin and other low molecular weight polypeptides. Rotary shadowed preparations of this material (denoted by E) reveal small globular structures ranging in size from 8.0 to 11.0 nm in diameter.

Comparison of Molecular Morphologies

Human erythrocyte spectrin has been the subject of numerous detailed biochemical and structural studies. Shotton et al. (14) and Tyler et al. (18) have examined the molecule by rotary shadowing of dilute solutions dried from glycerol. A representative electron micrograph is presented in Fig. 2a. The molecule consists of the α, β-heterodimer of two polypeptide chains (M, 240,000 and 220,000), loosely intertwined and joined at the ends to form a structure ~100 nm long. The α2β2 tetramer is in thermodynamic equilibrium with the dimer (19) and appears to consist of two heterodimers joined end-to-end with a total length...
FIGURE 1  Purification of filamin from Sepharose 4B. The upper panel shows platinum/carbon low-angle rotary shadowed replicas of material in fractions A, B, C, D, and E eluted from a Sepharose 4B column. Bar, 100 nm. All, × 60,000. Lower panel: elution profile and sodium dodecyl sulfate polyacrylamide gels of designated fractions run essentially by the method of Fairbanks et al. (9).
of ~200 nm (Fig. 2a). The ends of the dimer always appear closed, as do the ends of the tetramer.

The filamin dimer, comprised of two identical polypeptide chains of mol wt 250,000, has a morphology distinct from that of spectrin in rotary shadowed preparations (Fig. 2b). In contrast to spectrin, filamin dimers appear to be joined at only one end, with the other ends unassociated in the majority of cases. Each monomer has a length of ~80 nm and the overall length of a dimer is therefore 160 nm. The single strands appear to have a greater shadowed diameter than the individual sister strands of spectrin. This does not appear to be a function of differing salt concentrations in preparations of the two proteins but is consistent with a more compact tertiary structure for the polypeptide chains of filamin than for spectrin. If each strand observed in rotary-shadowed preparations is a monomer, filamin averages ~26 amino acids/nm (assuming a mean mol wt of 120/amino acid), whereas the average for spectrin is ~19 amino acids/nm. We discern no clearly differentiated domains along the length of the filamin strands at the present level of resolution, although the strands frequently assume a "beaded" appearance when shadowed very lightly with platinum.

Actin-binding protein from rabbit macrophages exists in solution predominantly as a homodimer comprised of two 250,000-dalton polypeptide chains. Rotary shadowed preparations of this protein (Fig. 2c) are indistinguishable from preparations of filamin, although the two proteins have been shown to be chemically distinct (20).

Effects of Fixation

Spectrin dimers or filamin dimers cross-linked in 0.1% glutaraldehyde were also examined after low-angle rotary shadowing. Glutaraldehyde should fix the molecules to preserve their native structures in solution. The majority of fixed molecules were of the same general morphology as unfixed molecules (Fig. 3) and sodium dodecyl sulfate polyacrylamide electrophoretic gels clearly showed that the glutaraldehyde was capable of cross-linking the polypeptides. But, because relatively few high molecular weight aggregates were seen in electron microscopy, we postulate that some of the cross-linking observed in gels occurred during heating and solubilization of the samples in sodium dodecyl sulfate. Alternatively, the larger
FIGURE 3 Structural comparison of native and glutaraldehyde-fixed molecules. Electron micrographs and sodium dodecyl sulfate electrophoretic gels of (A) native and (B) fixed spectrin. C shows native and D shows glutaraldehyde-fixed filamin. Bar, 50 nm. All, × 125,000.

aggregates may have been swept from the field of view by the drying glycerol in rotary shadowed preparations (17).

**Binding of Spectrin, Filamin, and Actin-binding Protein to Erythrocyte Membranes**

The similarity of filamin and actin-binding protein to erythrocyte spectrin has led to the suggestion that these molecules share common functional roles (11, 20). Both filamin and actin-binding protein are capable of binding and cross-linking actin in solution (6), and recent work indicates that spectrin has similar properties (5). Spectrin is clearly capable of sustaining a number of other protein-protein interactions, including specific binding to bands 2.1 and 4.1 of the erythrocyte membrane (18). To explore other functional similarities which might exist among these structurally related proteins, we compared the membrane-binding properties of spectrin, filamin, and actin-binding protein.

Purified spectrin bound to spectrin-depleted inside-out vesicles derived from human erythrocytes with high affinity ($K_D = 5 \times 10^{-8}$ M), saturation of membrane-binding sites occurring at concentrations of 120–150 µg of spectrin/mg of membrane protein (Fig. 4). These values are consistent with those reported previously (2). Parallel experiments, in which purified filamin was incubated with spectrin-depleted vesicles under a variety of salt conditions, showed no measurable binding. As a further test, nonradiolabeled filamin was incubated in vast molar excess with $^{125}$I-spectrin in the presence of inside-out vesicles in an effort to competitively displace spectrin. No competition for membrane-binding sites could be detected, even at molar ratios in excess of 100:1 (filamin:spectrin).

We conclude that filamin is not capable of binding to membrane attachment sites for spectrin. Similar experiments performed with actin-binding protein also failed to detect binding to spectrin-depleted erythrocyte membranes. This finding does not prove that filamin- and actin-binding protein have no sites analogous to the 2.1 and 4.1 binding regions of spectrin, but it does suggest that, even though these proteins have many properties in common, their binding affinities diverge extensively.

**CONCLUSION**

Our results show that, like spectrin, filamin and macrophage actin-binding protein appear as elon-
gate, flexible molecules that assume a number of configurations when dried on mica. The appearance of these molecules in our preparations is consistent with their hydrodynamic properties (13, 14). This consistency, as well as our observation that molecules cross-linked in solution have a morphology similar to unfixed molecules, suggests that filamin and actin-binding protein are also flexible molecules when in solution. As with spectrin (14), filamin and actin-binding protein are probably loose, flexible, convoluted structures whose time-averaged diameter in solution is significantly less than the length of the extended molecules seen in shadowed preparations.

Electron microscopy has provided a clear image of actin filaments, microtubules, and intermediate filaments in eukaryotic cells. The stereo-specific associations that maintain such polymers inevitably dictate relatively rigid structures. Much less is known about the structure of those components that may link these cytoskeletal units to each other or to other cellular components. Typically, cells and their membranes are remarkably flexible. It is unlikely that this flexibility could be a function of rotation or pivoting about the stereo-specific, non-covalent bonds that associate subunits into filaments. Such deformations can be tolerated in extended, covalently bonded molecules of which spectrin, filamin, and actin-binding protein appear to be clear examples. The structure and organization of these flexible molecules in cells remains to be explored.

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