Purification of a High Molecular Weight Actin Filament Gelation Protein from *Acanthamoeba* That Shares Antigenic Determinants with Vertebrate Spectrins

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ABSTRACT I have purified a high molecular weight actin filament gelation protein (GP-260) from *Acanthamoeba castellanii*, and found by immunological cross-reactivity that it is related to vertebrate spectrins, but not to two other high molecular weight actin-binding proteins, filamin or the microtubule-associated protein, MAP-2. GP-260 was purified by chromatography on DEAE-cellulose, selective precipitation with actin and myosin-II, chromatography on hydroxylapatite in 0.6 M KI, and selective precipitation at low ionic strength. The yield was 1–2 μg/g cells. GP-260 had the same electrophoretic mobility in SDS as the 260,000-mol-wt alpha-chain of spectrin from pig erythrocytes and brain. Electron micrographs of GP-260 shadowed on mica showed slender rod-shaped particles 80–110 nm long. GP-260 raised the low shear apparent viscosity of solutions of *Acanthamoeba* actin filaments and, at 100 μM actin, formed a gel with a 8 μM actin. Purified antibodies to GP-260 reacted with both 260,000- and 240,000-mol-wt polypeptides in samples of whole ameba proteins separated by gel electrophoresis in SDS, but only the 260,000-mol-wt polypeptide was extracted from the cell with 0.34 M sucrose and purified in this study. These antibodies to GP-260 also reacted with purified spectrin from pig brain and erythrocytes, and antibodies to human erythrocyte spectrin bound to GP-260 and the 240,000-mol-wt polypeptide present in the whole ameba. The antibodies to GP-260 did not bind to chicken gizzard filamin or pig brain MAP-2, but they did react with high molecular weight polypeptides from man, a marsupial, a fish, a clam, a myxomycete, and two other amebas. Fluorescent antibody staining with purified antibodies to GP-260 showed that it is concentrated near the plasma membrane in the ameba.

Like many other cells, *Acanthamoeba castellanii* has a high molecular weight protein associated with actin filament gels (1) and actomyosin (2), but since this protein had not been purified, nothing was known about its properties. In particular, there was no information concerning its relation to other high molecular weight actin-binding proteins that include the macrophage ABP/smooth muscle filamin class (3–7), erythrocyte and other spectrins (8–15), and the microtubule-associated protein, MAP-2 (16–18).

Early efforts to purify the high molecular weight protein from *Acanthamoeba* actomyosin-II failed due to smearing of the protein during gel permeation chromatography (2), but several new steps now make it possible to obtain enough highly purified protein, and thus establish some of its properties. Because it forms a gel with actin filaments, it will be called GP-260 (gelation protein with a 260,000-mol-wt subunit polypeptide). This ameba also has a smaller gelation protein called GP-85 (19). Antibodies to GP-260 have been used to localize the protein near the plasma membrane in the ameba and to show that it shares some immunological determinants with vertebrate spectrins.

The existence of a spectrin-like protein in a highly motile cell with a well-characterized contractile protein system (20, 21) will expand the scope of the work possible on the spectrin class of proteins. A brief account of some of these findings was presented in March 1983 at the University of North Carolina Conference on Actin-Membrane Interactions (22).

MATERIALS AND METHODS

Materials: Dr. Susan Hagen generously made the hydroxylapatite by...
using the method of Bernardi (23). Other materials were from the following sources: Sigma Chemical Co., St. Louis, MO (ATP [grade I], imidazole [grade II], dithiothreitol, yeast hexokinase, Triton X-100 and NaN3); Fischer Scientific Co., Pittsburgh, PA (KI, D-glucose); Whatman Chemical Separation Inc., Clifton, NJ (DEAE-cellulose [DE-52]); Schleicher and Schuell, Inc., Keene, NH (Nitrocellulose paper); Miles Laboratories Inc., Elkhart, IN ("Pentex" bovine albumin solution); Schwarz/Mann, Orangeburg, NY (ultra pure ammonium sulfate).

*Acanthamoeba* actin was purified by a modification of the method of Gordon et al. (24). Pig erythrocyte spectrin, pig brain spectrin, and affinity-purified antibodies to human erythrocyte spectrin (25) were generous gifts from Vann Bennett and Jonathan Davis of The Johns Hopkins Medical School. Chicken gizzard filamin and an immunoglobulin fraction that contained antibodies to gizzard filamin (26) were kind gifts of Susan Craig of The Johns Hopkins Medical School. Pig brain microtubule protein was supplied by Stephen Rothwell of The Johns Hopkins Medical School.

**Cells** A. castellanii (Neff) was grown in aerated cultures (21) with half of the protease peptone in the medium replaced by Difco yeast extract.

**Preparation of actomyosin-II** Cells were harvested, washed, lysed by N2 cavitation, centrifuged, and the extract chromatographed on DE-52 (see Fig. 2) as described by Pollard et al. (2). Fractions that contained myosin-I and -II were identified by ATPase assay (27) and the fractions that contained myosin-II plus the leading edge of the actin peak were warmed to 25°C with 2 mM MgCl2 to polymerize the actin, followed by 1 U/ml of hexokinase and 50 mM D-glucose to precipitate actomyosin-II (2).

**Biochemical Methods** Protein concentrations were estimated by the Bradford method (28) using actin as the standard. UV absorption was measured with a Cary 219 spectrophotometer. The apparent viscosity at a low shear rate was measured with a miniature falling ball device (29). Actin polymerization was measured fluorometrically with a 5% pyrene-labeled actin (30). Gel electrophoresis was carried out in 10% polyacrylamide gels with SDS (27). Polypeptides in these gels were transferred electrophoretically to nitrocellulose paper in Tris-glycine buffer with 0.1% SDS and 20% methanol. The gels were stained with Coomassie Brilliant Blue and the nitrocellulose with 0.15% amido black in 45% methanol, 9% acetic acid.

**Immunological Methods** Two large white New Zealand rabbits were immunized by injections of 50 µg of purified GP-260 in complete Freund's adjuvant at multiple sites. After 4 wk, the rabbits were boosted by injection of 100 µg of GP-260 in incomplete Freund's adjuvant. Immune serum was collected 8–19 d later. Antibodies were purified from 1:10 dilutions of immune serum in 0.5 M NaCl, 0.1 M Tris-Cl (pH 8), (c) 3 x 5 ml of 1 M KCl, 0.1 M Tris-Cl (pH 8), and (d) 3 x 5 ml of 0.1 M NaCl, 0.1 M Tris-Cl (pH 8). Bound antibodies were eluted with 900 µl of 1 M acetic acid for 10–20 min at 4°C. The eluate was neutralized immediately with 100 µl of 2 M Tris base that contained 5 mg of bovine serum albumin, and then dialyzed against 150 mM NaCl, 20 mM Tris-Cl (pH 8). The nitrocellulose with bound GP-260 was re-used successfully several times for antibody purification. Solid phase antibody binding assay and antibody staining of polypeptides on nitrocellulose were described by Tseng et al. (33).

**Fluorescent Antibody Staining** Antibodies to GP-260 that were purified from nitrocellulose blots were used to stain Acanthamoeba exactly as described by Tseng et al. (33).

**Electron Microscopy** Purified GP-260 was dialyzed against 0.5 M ammonium formate, mixed with an equal part of glycerol, sprayed and dried in vacuo on mica, and rotary-shadowed with platinum (34). The JEOL 100 CX electron microscope was calibrated with skeletal muscle tropomyosin paracrystals.

**RESULTS**

**Purification of Acanthamoeba GP-260**

For most of this work, I used gel electrophoresis in 10% polyacrylamide with SDS. Stained with Coomassie Brilliant Blue. (Left) The mobilities of molecular weight x 10-3 standards. (Filled arrows) The interface of the stacking and separating gels. (Open arrows) The positions of the 260,000-mol-wt pig erythrocyte spectrin band and the 175,000-mol-wt Acanthamoeba myosin-II band. (A) DEAE pool. (B) Actomyosin-II precipitate. (C) Buffer G extract of the actomyosin-II. (D) Myosin-enriched actomyosin-II (the material insoluble in buffer G). (E) GP-260 pool from the hydroxylapatite column. (F) Material soluble in 10% sucrose. (G) Purified Acanthamoeba GP-260. (H) Pig erythrocyte spectrin. (I) Mixture of G and H. (J-L) Comparison of Acanthamoeba GP-260 (J) with pig brain (K) and pig erythrocyte (L) spectrin on a 5% polyacrylamide gel.

**Figure 1** Analysis of the polypeptide composition of crude and purified Acanthamoeba GP-260 by electrophoresis in 10% polyacrylamide with SDS. Stained with Coomassie Brilliant Blue. (Left) The mobilities of molecular weight x 10-3 standards. (Filled arrows) The interface of the stacking and separating gels. (Open arrows) The positions of the 260,000-mol-wt pig erythrocyte spectrin band and the 175,000-mol-wt Acanthamoeba myosin-II band. (A) DEAE pool. (B) Actomyosin-II precipitate. (C) Buffer G extract of the actomyosin-II. (D) Myosin-enriched actomyosin-II (the material insoluble in buffer G). (E) GP-260 pool from the hydroxylapatite column. (F) Material soluble in 10% sucrose. (G) Purified Acanthamoeba GP-260. (H) Pig erythrocyte spectrin. (I) Mixture of G and H. (J-L) Comparison of Acanthamoeba GP-260 (J) with pig brain (K) and pig erythrocyte (L) spectrin on a 5% polyacrylamide gel.
elutes from DEAE-cellulose in a broad zone between 175 and 275 mM KCl that overlaps the peaks of myosin-II (175 mM), GP-85 (275 mM), capping protein (200 mM), and actin (200-300 mM) (Fig. 2). GP-260 is obtained as a by-product of myosin-II purification, so the fractions eluting between 160 and 220 mM KCl that contain myosin-II, GP-260, and the leading part of the actin peak (Fig. 1, lane A) are pooled. After polymerization of the actin and removal of ATP with hexokinase and glucose, all of the myosin-II and much of the actin and GP-260 precipitate (Fig. 1, lane B) and are separated from the bulk (92%) of the protein by centrifugation. The actomyosin-II precipitate is homogenized in 50-100 ml of a

**Figure 2** Chromatography of a sucrose extract of Acanthamoeba on a 5 x 45-cm column of DEAE-cellulose. Fraction size, 20 ml. (•) A₂₅₀, (—) KCl concentrations. (O) Solid phase antibody binding assay for GP-260 using a 1:100 dilution of column fractions and a 1:100 dilution of an immune serum; units are ¹²⁵I counts per min x 10⁻³.

**Figure 3** Chromatography of a buffer G extract of actomyosin-II on a 1.5 x 7-cm column of hydroxylapatite equilibrated with 0.6 M KI, 60 mM KP, 10 mM imidazole (pH 7.5), 1 mM dithiothreitol. The column sample was brought to approximately the same concentrations of KI and KP, immediately before loading. The sample was followed with ~20 ml of equilibration buffer and then a 200-ml gradient of 60–300 mM KP, in equilibration buffer. (•) A₂₅₀, (—) KP₆ concentration. (O) Solid-phase antibody binding assay for GP-260 using a 1:12,500 dilution of the fractions and 1:100 dilution of immune serum; units are thousands of ¹²⁵I counts per min. The high molecular weight polypeptides are shown by SDS PAGE over the peak fractions. Actin runs near the dye front on these gels.
Purification of Acanthamoeba GP-260

| Step                  | Volume (ml) | Total protein (mg) |
|-----------------------|-------------|--------------------|
| Cell extract          | 1,200       | 12,000 est.        |
| DEAE peak             | 630         | 2,520              |
| Actomyosin-II precipitate | 57         | 194                |
| Buffer C extract      | 56          | 77                 |
| Hydroxylapatite peak  | 18          | 4.9                |
| 2.0–2.5 M ammonium sulfate precipitate | 2 | 2.3 |
| Sucrose precipitate 1 | 2           | 1.1                |

Protein concentrations estimated by the Bradford method (28). The starting material for this preparation was 600 g of cells estimated to contain 30 g total protein.

Table 1

Yield and Purity

The yield of GP-260 from 600 g of cells varied from 250 μg for the first preparation to 1,100 μg for the sixth; with experience there was steady improvement, attributable mainly to the selection of the original volume of buffer G to extract protein from the actomyosin. The yield can probably be improved considerably by optimizing for GP-260 at each step, because there are major losses when the DEAE fractions are pooled and during the extraction of the actomyosin. The purity was high (Fig. 1, lane G). Although not visible on gels stained with Coomassie Blue, the preparations contained a trace of myosin-II that could be identified by staining gel transfers with antibodies. No other major protein contaminants were detected by gel electrophoresis.

These preparations of GP-260 most likely contain nonprotein material. The UV absorbance spectrum had a maximum at 258 nm and the ratio of \( \lambda_{260} \) to \( \lambda_{280} \) was 0.56 to 0.72 in three preparations. The material may be a nucleic acid, but it has not yet been characterized or tested for association with GP-260. It may account for the absorbance peak on the hydroxylapatite column that elutes just ahead of the protein. It probably also accounts for why the absorbance of GP-260 is so high at 280 nm—up to a 2 A\text{280} per mg/ml of protein in one preparation.

Physical Properties

The purified Acanthamoeba polypeptide has approximately the same electrophoretic mobility as the 260,000-mol-wt alpha-chain of spectrin from pig erythrocytes (Fig. 1, lanes H, I, and L) and pig brain (Fig. 1, lane K). The electrophoretic mobility in SDS is greater than that of pig brain MAP-2 and less than that of chicken gizzard filamin.

Electron micrographs of purified GP-260 rotary-shadowed with platinum showed rod-shaped particles 70–110 nm long and ~4 nm wide (Fig. 4A and B). These particles appear to be very flexible compared with GP-85 (19) and the tail of myosin-II (2), two other rod-shaped actin-binding proteins from Acanthamoeba (Fig. 4). The size, shape, and bends of these particles are similar to isolated brain spectrin alpha- and beta-chains (35). These preparations of GP-260 also contain a small number of flexible rod-shaped particles with contour lengths between 140 and 200 nm (Fig. 4C), that could be end-to-end dimers of the major species.

At a concentration of 150 μg/ml, the purified GP-260 is insoluble in 5–10% sucrose with NaCl concentrations up to 200 mM, or with 1 mM ATP, or with 30 mM Tris-Cl (pH 8). It is soluble in 5% sucrose with 500 mM KCl, with 500 mM KI, or with 25 mM ATP. Given this insolubility at low ionic strength, it is clear why GP-260 is extracted only partially from the actomyosin-II by the actin depolymerizing buffer.

Interaction with Actin

The purified GP-260 is an actin cross-linking protein capable of forming a gel with purified actin filaments (Fig. 5). The effect on the low shear viscosity of Acanthamoeba actin filament solutions is identical in 1 mM EGTA (Fig. 5) and in 0.1 mM CaCl\text{2} (not illustrated). The traces of myosin-II in these preparations are not responsible for the cross-linking of the actin filaments, because concentrations of purified Acanthamoeba myosin-II similar to those contaminating the GP-260 have little or no effect on the apparent viscosity of actin under the conditions of this assay. At a concentration of 10 μg/ml, GP-260 had no detectable effect on the time course of polymerization of 7.5 μM Acanthamoeba actin in 1 mM MgCl\text{2}, 10 mM imidazole-Cl (pH 7.5), 0.1 mM CaCl\text{2}, and 0.1 mM ATP measured by fluorescence enhancement, so it
FIGURE 4 Electron micrographs of *Acanthamoeba* GP-260 (A–C) compared with *Acanthamoeba* myosin-II and GP-85 (D). The purified molecules in 50% glycerol were sprayed on mica, dried in vacuo, and rotary-shadowed with platinum. (A and B) Individual GP-260 particles. (C) Possible end-to-end dimers of GP-260. (D) (Left) Three *Acanthamoeba* myosin-II molecules found as minor contaminants in a preparation of GP-260; (right) several purified *Acanthamoeba* GP-85 molecules prepared in the same way. (A) Bar, 100 nm. × 116,000. (B–D) Bar, 100 nm. × 205,000.

probably has no substantial effect on the nucleation or elongation of actin filaments.

**Immunological Studies**

Both rabbits produced antibodies to the purified GP-260. When assayed by the solid-phase antibody-binding method with 0.2 μg of GP-260 in each well, these sera were positive at more than 10 times background out to a dilution of 10⁴. Purification of these antibodies by binding to and elution from electrophoretically purified GP-260 adsorbed to nitrocellulose yielded small amounts of purified antibody at a concentration 100 times lower than in the original serum.

The sera from rabbits immunized with GP-260 and purified Antibodies to chicken gizzard filamin do not bind to GP-260 antibodies to GP-260 react strongly with polypeptides with molecular weights of 260,000, 240,000, and 100,000 among all of the polypeptides of the ameba that can be separated by gel electrophoresis in SDS (Fig. 6, lane B-AM). These same antibodies react with 260,000- and 100,000-mol-wt polypeptides in the soluble extract of *Acanthamoeba* (Fig. 6, lane B-EX) and only with the 260,000-mol-wt polypeptide in samples of purified GP-260 (Fig. 6, lane B-GP). Pre-immune sera from these rabbits do not bind to any ameba peptides at the dilutions used for the immune sera (Fig. 6A). After a second boost with the purified GP-260, both rabbits also produced low levels of antibodies to *Acanthamoeba* myosin-II, the major contaminant in the samples; these sera were not used in the experiments reported here.

The immune sera and purified antibodies from both rabbits also bind to the beta-chains, but not the alpha-chains, of purified spectrin from pig erythrocytes (Fig. 6, lane B-PS) and pig brain (Fig. 7, lane E). The reaction is much weaker with pig spectrins than ameba spectrin judging from the intensity of the autoradiograms (cf. Fig. 6, lanes B-GP and B-PS) and from solid-phase antibody-binding assays. For example, a 100-fold dilution of serum from rabbit JH-20 bound 10 times over background to 500 ng of pig erythrocyte spectrin, while a 10,000-fold dilution of the same antibody bound as well to 200 ng of GP-260. The antibodies to GP-260 do not bind to either chicken gizzard filamin or pig brain MAP-2, two other high molecular weight actin-binding proteins from vertebrates, at least after gel electrophoresis and transfer of the proteins to nitrocellulose (Fig. 6D, E).

Purified antibodies to erythrocyte spectrin alpha- and beta-chains (Fig. 6, lanes C-PS and -PS') react weakly with 260,000- and 240,000-mol-wt polypeptides in samples of whole amebas (Fig. 6, lane C-AM) and purified GP-260 (Fig. 6, lane C-GP). In comparison, the reaction with the soluble extract of the ameba is much weaker (Fig. 6, lane C-EX).
or other peptides in samples of whole amebas fractionated by
gel electrophoresis and transferred to nitrocellulose paper (Fig.
6F). Antibodies to GP-85 do not react with high molecular
weight polypeptides in extracts of the ameba (Tseng, P. C.-
H., D. P. Bichell, R. C. Williams, and T. D. Pollard, manu-
script in preparation).

Judging from the reactions of the antibodies to GP-260,
most of the 260,000-mol-wt polypeptide, but little of the
240,000-mol-wt chain, is extracted into the 140,000 g super-
natant of cells homogenized in 0.34 M sucrose (Fig. 6, lanes
B-AM and -EX). Likewise, little of the material that reacts
with the antibodies to erythrocyte spectrin is extracted into the
soluble fraction (Fig. 6, lane C-AM and -EX).

The 260,000-mol-wt chain in the crude sample is most
likely GP-260. The 240,000-mol-wt chain could simply be an
insoluble fragment of the 260,000-mol-wt chain, but a more
interesting possibility is that it is another polypeptide related
to GP-260 (see Discussion). The 100,000-mol-wt polypeptide
is not myosin-II or a fragment of myosin-II, because the
purified antibodies do not bind to myosin-II and antibodies
to myosin-II do not bind to either GP-260 or the 100,000-
mol-wt polypeptide. Instead, the 100,000-mol-wt polypeptide
is probably a fragment of GP-260, because it has solubility
properties similar to GP-260 and varies in amount in different
preparations of extract (cf. Figs. 6B and 7A and M).

Purified antibodies to GP-260 also react with high molec-
ular weight polypeptides from a variety of cell types (Fig. 7).
The reaction has not been quantitated, but it is strong for
Acanthamoeba, Physarum, and Astronyxis. The reaction with
a 260,000-mol-wt polypeptide is weaker for Naegleria (an
ameboflagellate), scallop membranes, PtK cells (from a mar-
supial), fish brain, and human platelets. In the case of Dro-
sophila larvae and several chicken striated muscles (but not
gizzard), the major reactive species has the same electropho-
retic mobility as the myosin heavy chain. Since these antibod-
ies do not react with either myosin-I or myosin-II from
Acanthamoeba or myosin in samples of platelets, HeLa
cells, and PtK cells, it seems likely that they are binding to
another polypeptide about the same size as myosin rather
than myosin itself.
FIGURE 7 Reaction of purified antibodies to *Acanthamoeba* GP-260 with the polypeptides from crude cell samples and purified spectrin from various sources. The samples were prepared by gel electrophoresis and transferred to nitrocellulose paper for reaction with antibodies and 

$^{125}$I-protein A. Autoradiograms localize the sites of antibody binding. (A) *Acanthamoeba* extract. (B) Whole *Astronyxis*, another Hartmanellid ameba. (C) Whole *Physarum polycephalum*. (D) Whole *Naegleria gruberi*. (E) Purified pig brain spectrin. (F) Crude extract of *Fundulus* brain from Mr. Stephen Rothwell (Johns Hopkins Medical School). (G) Scallop gill ciliary membranes from Dr. R. E. Stephens (Marine Biological Laboratory, Woods Hole, MA). (H) Whole PtK-1 cells. (I) Whole human platelets. (J) *Drosophila* larvae from Dr. Peter Cherbas (Harvard University). (K) Chicken breast muscle. (L) Chicken gizzard. (M) Another preparation of *Acanthamoeba* extract. The exposures of the autoradiograms were varied to give strong exposures of bands in the high molecular weight region of the gel that are illustrated here. Left, Molecular weights $\times 10^{-1}$.

FIGURE 8 Indirect fluorescent antibody staining of *Acanthamoeba* with antibodies to *Acanthamoeba* GP-260. Each field is paired with a corresponding phase-contrast micrograph. (A, C, I) Purified antibody. (F) A 1:1,500 dilution of immune serum. (G) A 1:1,500 dilution of pre-immune serum. (A–D), The same field with A and B focused at the equator of the cells, and C and D focused at the base of the cells. (I and J) Six cells where the bulk of the cytoplasm was ripped away during preparation, revealing with greater clarity than in C the pattern of staining of the cortical cytoplasm that has remained attached to the cover slip. $\times 2,200$. 

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Localization of GP-260 in the Ameba

Both the immune sera (Fig. 8, E and F) and the purified antibodies (Fig. 8, A–D, I, and J) to GP-260 stain the cortical cytoplasm next to plasma membrane more strongly than other parts of Acanthamoeba. There is also less intense staining of the cytoplasm. The pattern of the fluorescence can be very complex due to convolutions of the plasma membrane, but the plasma membrane staining is obvious to an observer who can focus through the cell in the microscope. Membrane staining is clearest in single photographs focused at the equator, where there is a bright fluorescent ring around the periphery of the cell (Fig. 8, A and E).

At the base of the cell, a reticular pattern is seen in the plane of the membrane (Fig. 8 C). Some cells are torn away from their attachments to the cover slip during staining, offering a clear view, unobstructed by overlying cytoplasm, of the reticular pattern of fluorescence associated with the membrane (Fig. 8 I). A larger relative of Acanthamoeba called Astronyxis stains in the same way as Acanthamoeba (Fig. 9).

Controls without anti-GP-260 antibody or with pre-immune serum (Fig. 8, G and H) gave only very faint general staining, not the distinctive pattern of plasma membrane staining.

DISCUSSION

The GP-260 isolated here is sufficiently pure that one may be confident that it is a component of the actin filament cross-linking system in Acanthamoeba, but the mechanism of cross-linking will require a fuller analysis of its physical properties. At high ionic strength, GP-260 is soluble and appears largely as single particles 100 nm long by electron microscopy. Under conditions where the cross-linking experiments were performed, GP-260 aggregates and even precipitates if its concentration is high enough. Thus, it seems likely that multimers of GP-260 are present and participate in the cross-linking of the filaments. The electron microscopic experiments at high ionic strength suggest that end-to-end dimers similar to the ABP/filamin class of gelation proteins (36, 37, 38) might be one type of oligomer of GP-260.

An important factor to be investigated in the future is whether the 240,000-mol-wt polypeptide in the whole cell that reacts with the antibodies to GP-260 might be another component of the native molecule and form some sort of heteromer with GP-260 like the subunits of spectrins. Although not visible on stained gels of purified GP-260 (Fig. 1), there is a trace of a 240,000-mol-wt polypeptide in samples of purified GP-260, because the antibodies to erythrocyte spectrin react with a 240,000-mol-wt species (Fig. 6 C). Such heteromers might have substantially different properties than the bulk of the GP-260 studied here.

Relationship of GP-260 to Other Actin-binding Proteins of Acanthamoeba

An essential task in characterizing the actin filament system in any cell is to make a complete catalogue of the proteins that bind to actin and affect its polymerization or the organization of actin filaments in the cell. Acanthamoeba has been a rich source of actin-binding proteins: two classes of myosin called myosin-I and myosin-II (reviewed in references 1 and 2), two actin monomer-binding proteins called profilin (39) and actophorin (Cooper, J. A., J. D. Blum, and T. D. Pollard, manuscript in preparation), an actin filament capping protein (40) and six actin filament cross-linking proteins (Table II).

While this list is undoubtedly incomplete, it is already obvious that the actin system in this cell is complex and not likely to be fully understood without characterizing a rather large number of proteins.

GP-260 is the sixth actin filament cross-linking protein to be purified from Acanthamoeba (Table II). The others are an alpha-actinin–like protein called GP-85 (19) and four small proteins called gelactins (2, 41). It seems likely that additional cross-linking proteins will also be found. GP-260 is the largest by far and probably present in lower quantities than the others.

It is remarkable that proteins that differ so much in their physical properties (Table II) can have similar effects in the
assays that are commonly used to characterize proteins that bind to actin filaments. In the falling ball assay for the cross-linking of actin filaments, roughly similar molar concentrations of all of these proteins are required to form a gel (Table II). Likewise, all of these proteins form gels with actin filaments equally well in the presence or absence of Ca$^{++}$, so that the Ca$^{++}$ sensitivity of gelation of cytoplasmic extracts from Acanthamoeba (29) has not yet been explained by the intrinsic properties of the known gelation proteins. (The apparent Ca$^{++}$ sensitivity of GP-85 (19) is now known to be due to an effect of Ca$^{++}$ on the kinetics of actin polymerization, not the cross-linking by GP-85 [Bichell, D. P., D. L. Rimm, P. C.-H. Tseng, and T. D. Pollard, manuscript in preparation].) Similarly, neither GP-85 nor GP-260 has a major effect on the assembly of actin. When polymerization is measured by viscometry, GP-85 appeared to accelerate polymerization (19), but this is not observed when the polymer concentration is measured by the enhancement of the fluorescence of pyrene-actin (Bichell, D. P., D. L. Rimm, P. C.-H. Tseng, T. D. Pollard, manuscript in preparation). Thus, the effect of GP-85 is on the viscosity of the solution, not the kinetics of polymerization.

Why should a cell have so many different actin filament cross-linking proteins? The most likely possibility is that each has a unique function in the cell that may be difficult or impossible to understand from biochemical and biophysical studies on the isolated proteins. For example, although both GP-85 and GP-260 have similar effects on actin in vitro, they most likely have different physiological functions because their distributions in the cell are so different. GP-85 is spread diffusely throughout the cytoplasm and is concentrated in limited regions of the cortex (Tseng, P. C.-H., D. P. Bichell, R. C. Williams, T. D. Pollard, et al., manuscript in preparation), while GP-260 is concentrated near the plasma membrane of the amoeba, much like the spectrin in erythrocytes, neurons, and muscle (9, 13, 42, 43). Direct tests of function in the living cell will be required to fully appreciate the sort of subtlety exemplified by the existence of multiple actin filament cross-linking proteins in Acanthamoeba.

Relation of GP-260 to Other Actin-binding Proteins

Judging from the cross-reactivity of the antibodies to GP-260 with high molecular weight polypeptides from a wide range of species including protozoa, a clam, an insect, a bird, and man, there are related proteins in many cells and species. This raises intriguing questions, because it is by no means obvious how GP-260 is related to the three well-characterized classes of vertebrate high molecular weight actin-binding proteins: the spectrins, ABP/filamin, and MAP-2. The immunological analysis strongly suggests that GP-260 belongs to the spectrin family, but some of the physical properties of GP-260 suggest instead that it may be in the ABP/filamin group. A clear decision regarding classification will have to await fuller characterization of GP-260, but it is instructive to point out why the available data are ambiguous.

One can argue both for and against classifying GP-260 as a spectrin. This classification is supported by the reciprocal cross-reactivity of antibodies to GP-260 and vertebrate spectrins, the identical electrophoretic mobility of GP-260 and the alpha-chains of spectrins in SDS, the indistinguishable size and shape of GP-260 and isolated alpha- or beta-chains of spectrin, and the common localization of GP-260 and spectrins near the plasma membrane. On the other hand, there are several persuasive arguments against GP-260 belonging to the spectrin family. All known spectrins are composed of two distinct polypeptides that differ in their electrophoretic mobility, peptide maps, and antigenic sites (11, 15, 35, 42). Although each of these chains forms a slender molecule about 100 nm long (like GP-260), the alpha- and beta-chains of spectrin bind tightly enough to each other in parallel heterodimers (44) that denaturing agents are needed to separate them (35). These dimers also associate head to head to form tetramers ~200 nm long (44). Under the high salt conditions used to keep GP-260 in solution, it does not appear to form comparable molecular structures. Further, binding of spectrin to actin filaments requires both alpha- and beta-chains (35), and cross-linking actin filaments requires tetramers (44). Additional features that might identify a spectrin are binding sites for ankyrin and calmodulin (for review see reference 46), but these have not yet been tested with GP-260.

Similarly, one can argue both for and against GP-260 belonging to the ABP/filamin family. Like ABP and filamin (38), the purified GP-260 appears to consist of a single polypeptide that can form a gel with actin filaments. GP-260 also has some tendency to associate end to end like the stable

### Table II

Comparison of Actin Filament Cross-linking Proteins from Acanthamoeba

| Gelation protein | Subunit composition | Shape | Yield, µg/g (µmol/kg) | Yield, µmol/kg | µM (actin, µM) | Reference |
|-----------------|---------------------|-------|----------------------|----------------|----------------|-----------|
| Gelactin-I      | 1 × 23,000          | Globular | 11 (0.48)          | 0.30 (48)      | 42             |           |
| Gelactin-II     | 2 × 28,000          |       | 65 (1.18)          | 0.45 (48)      | 42             |           |
| Gelactin-III    | 2 × 32,000          |       | 16 (0.25)          | 0.11 (48)      | 42             |           |
| Gelactin-IV     | 2 × 38,000          |       | 8 (0.10)           | 0.04 (48)      | 42             |           |
| GP-85           | 2 × 90,000          | 3 × 50-nm rod | 100 (0.56)      | 0.08 (12)      | 19             |           |
| GP-260          | ? × 260,000         | 3 × 100-nm rod | 2 (0.004)       | 0.24 (8)       | This report    |           |

* Subunit molecular weights by SDS gel electrophoresis. Native molecular weights estimated by gel filtration except for GP-85, where the native molecular weight of 180,000 was measured by sedimentation equilibrium ultracentrifugation.

* These yield values are all underestimates of the cellular content of these proteins. For example, the content of GP-85 estimated by antibody binding assay is actually 4.3 µmol/kg of packed cells.

* The minimal concentration of gelation protein required to form a gel with the given concentration of actin. A tube inversion method was used in reference 42 and falling ball viscometry was used for the other determinations. These two assays are not strictly comparable.

* A native molecular weight of 520,000 was used for these calculations.
dimers of ABP and filamin (36). On the other hand, the individual GP-260 particles are somewhat longer than ABP and filamin subunits (38). The absence of cross-reactivity of antibodies to GP-260 and filamin is evidence against relatedness, but like most negative results, this absence is not conclusive. Likewise, ABP and filamin are typically localized in the cortical cytoplasm (47) or deeper in the cell (4) rather than being concentrated near the plasma membrane like GP-260.

There is no evidence that GP-260 is related to the third class of vertebrate high molecular weight actin-binding proteins, MAP-2 (17, 18). The antibodies to GP-260 do not react with any of the polypeptides in pig brain microtubule protein even though they react with spectrin from the same species and organ. Further, antibodies to GP-260 do not stain microtubular structures in the cell. Finally, GP-260 is considerably smaller than the MAP-2 polypeptide.

It is probably fair to conclude that the GP-260, as isolated, is not in the MAP-2 family, and that it does not fit neatly into either the spectrin or ABP/filamin families either. The immunological evidence and cellular localization persuade me to consider GP-260 to be a spectrin, at least as a working hypothesis. The main problem is the apparent absence of a second polypeptide to give GP-260 the structure characteristic of native spectrins. It could be that there are actually two different polypeptides present in the purified GP-260, but that they have the same electrophoretic mobility (like mouse brain spectrin chains [48]) and that they dissociate more readily at high ionic strength than vertebrate spectrins. Alternatively, the missing subunit might be the 240,000-mol-wt polypeptide of native spectrins. It could be that there are actually two polypeptides present in the purified GP-260, but that they have the same electrophoretic mobility (like mouse brain spectrin chains [48]) and that they dissociate more readily at high ionic strength than vertebrate spectrins. Alternatively, the missing subunit might be the 240,000-mol-wt polypeptide of native spectrins. If this speculation is true, the association of chicken gizzard filamin and heavy merofilamin.

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Since this work was completed, a report has appeared on a high molecular weight actin-binding protein from Phasusrin (49) that differs in its properties from both GP-260 and the vertebrate actin-binding proteins, indicating that other proteins may also be difficult to categorize.

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