Using Antibodies against *Dictyostelium* Membranes To Identify an Actin-binding Membrane Protein

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Abstract. Polyclonal antibodies made against *Dictyostelium discoideum* membranes were used to block the interaction of those membranes with actin. As expected, actin interacted mostly with the internal surface of the membrane, demonstrated by the fact that whole cells could only absorb out a minor fraction of the blocking antibody. The antibody was used to show that the membrane component(s) which interacted with actin were probably integral; they could be extracted with detergent but not with solutions designed to extract peripheral membrane proteins. To identify the responsible protein(s), Western transfers of membranes were cut into fractions which were tested for their ability to absorb out the blocking activity of the antibody. We observed a single peak at a molecular weight of ~20,000, and thus conclude that a 20,000-mol-wt protein is a major integral membrane actin-binding protein in *Dictyostelium*. This approach to the identification of proteins involved in actin–membrane interaction has allowed us to make the first identification of an actin-binding membrane protein which is based on its activity in native membranes.

ALTHOUGH it is clear that actin is associated with membranes in all cells which have been studied (for reviews, see Geiger, 1983; Jacobson, 1983), this interaction is well understood only in the case of the red blood cell. The red cell is not likely to be a good paradigm for all actin–membrane interactions, however, and progress in studying other systems has been slow (Brown, 1985). A promising start was made by Luna et al. (1981), who used low shear viscosity as an assay to examine actin–membrane interaction in *Dictyostelium*. Their studies suggest that actin can interact directly with integral membrane proteins. The next step is to fractionate membrane proteins and determine which ones interact with actin. Luna et al. (1984), Schleicher et al. (1984), and Stratford and Brown (1985) have carried out such studies with detergent-solubilized *Dictyostelium* membrane proteins. A possible problem with these studies is that detergent solubilization of the membrane removes proteins from their native lipid environment, and might give rise to artifactual actin binding. Therefore, we have been interested in finding a way to identify membrane actin-binding proteins that avoids the perturbations of detergents.

In this paper, we report our success in developing such an approach. Taking our lead from the method used to identify the neural cell adhesion molecule (Thiery et al., 1977), we have made antibodies against whole *Dictyostelium* membranes that block their interaction with actin. We have then gone on to fractionate the antibody using Western transfers, and conclude that an integral membrane protein with a molecular weight of ~20,000 is responsible for much of the actin–membrane interaction in *Dictyostelium*.

Materials and Methods

Preparation of Proteins

Rabbit muscle actin was prepared from acetone powder (Spudich and Watt, 1971) and further purified by gel filtration (MacLean-Fletcher and Pollard, 1980*a*), essentially as described by Pardee and Spudich (1982).

Crude *Dictyostelium* membranes were prepared by a modification of the methods of Spudich (1974) and Luna et al. (1981), as described in Stratford and Brown (1985). We used cells at a density of $<5 \times 10^6$ per ml, and usually obtained 2–4 mg of membrane protein per gram wet weight of cells. Most of the actin which is endogeneous to the membrane is removed during the sucrose gradient and subsequent washes described by Stratford and Brown (1985). Dialysis vs. 1 mM imidazole, 1 mM EDTA, or more often a urea/KCl treatment (as described in Table II and Luna et al., 1981) was used to remove residual actin and peripheral proteins, as indicated.

Antibody was prepared as follows. To remove highly antigenic sugar moieties, 80 mg/ml (wet weight) *Dictyostelium* were incubated 100 min at room temperature in 1 mg/ml almond meal (a crude source of a variety of glycosidases; Sigma Chemical Co., St. Louis, MO) and 15 μg/ml neuraminidase (Sigma Chemical Co.) in 50 mM sodium acetate, pH 5.5. Cells were washed well with 10 mM triethanolamine, pH 7.5, and membranes made as described above. These membranes were extracted with urea and KCl, resuspended in 10 mM KPO_4, pH 6.5, and 1 mg membrane protein was injected as a 1:1 mixture with Freund's complete adjuvant (Gibco, Grand Island, NY). 1 mo later, the rabbit was re-injected, using three parts Freund's incomplete adjuvant (Gibco) to one part membranes. The rabbit was bled approximately every 2 wk. Crude serum was passed over a Protein A–Sepharose CL-4B column (Sigma Chemical Co.) to obtain IgG.

Fab was prepared from IgG as described by Mage (1980). A Protein A column was used to separate Fab from Fc after papain digestion and iodoacetamide treatment. This Fab was >95% pure as assessed by SDS gels.

Low Shear Viscosity Assay for Actin–Membrane Interaction

Membranes in PBS (urea/KCl extracted, unless otherwise indicated) were

sonicated four times for 30 s before use in the assay. This gave a vesicle size of ~120 ± 30 nm, and increased the effect of the membranes in the assay at least 10-fold relative to unsonicated membranes (presumably by increasing the number of pieces of membrane available to cross-link actin; Luna et al., 1984). 10 μl sonicated membrane (0.25 mg/ml stock in PBS) was mixed with Fab and incubated for 10 min at room temperature. Controls included replacing membrane and/or Fab with a corresponding volume of the appropriate buffer. Filamentous rabbit muscle actin (0.5 mg/ml stock in 10 mM imidazole, 0.2 mM ATP, 0.2 mM dithiothreitol [DTT], 100 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA) was then added to a final concentration of 100–150 μg/ml in an assay volume of 100 μl. Low shear viscosity was assayed essentially as described by MacLean-Fletcher and Pollard (1980*b*). The mixture was vortexed and immediately loaded into three 100-μl capillary tubes. The capillary tubes were then incubated for 4 h at room temperature. Viscosity was read by measuring the time required for a steel ball to roll 2 cm through the solution, with the capillary at an angle of 20° (only a single measurement was made per capillary tube). The readings for the three aliquots of each sample were averaged. Seconds/cm was then converted to centipoise using a standard curve generated with glycerol. The rare readings in which the steel ball did not roll at a constant rate were not used.

Variability was seen in the low shear viscosity of actin alone in different experiments. This could be due to such factors as extremely low levels of contaminants (Griffith and Pollard, 1982) or the age of the actin preparation. The variability did not present a problem in our experiments, however, as the fold enhancement by membranes and the amount of blocking of that enhancement by antibody was consistent from experiment to experiment.

Cosedimentation Assay for Actin–Membrane Interaction

Samples were prepared as for the low shear assay, with the following modifications. Membranes were metabolically labeled with [^{35}S]methionine (Stratford and Brown, 1985) and sonicated four times as long, to reduce sedimentation of membranes alone. The actin concentration was reduced to 20 μg/ml, and 2 mg/ml BSA was added to reduce nonspecific losses of radioactive membranes. Samples were incubated for 30 min at room temperature, then centrifuged for 1 min at 20 psi in an Airfuge (Beckman Instruments, Inc., Palo Alto, CA). 150 μl supernatant was counted in 1 ml TS-1 plus 9 ml Cocktail Neutralizer (Research Products International Corp., Mt. Prospect, IL) to determine how much of the membrane had cosedimented with actin.

Preabsorption and Acid Elution of Antibody from Whole Cells

Antibody was mixed 1:1 with 50 mg/ml cells (wet weight) in PBS and the supernatant saved. Cells were then washed twice in PBS, and bound Fab was eluted with 0.2 M glycine, 1 mM EGTA, pH 2.7. The cells blebbed but did not lyse during elution (>90% intact by trypan blue exclusion). The supernatant was neutralized with NaOH, and 0.1 mg/ml BSA was added as carrier protein.

Preabsorption of Antibody with Membrane Fractions on Nitrocellulose

1 cm^2 nitrocellulose (Schleicher & Schuell, Inc., Keene, NH; capacity, ~100 μg protein) was incubated on a shaker with membrane fractions or buffer alone as a control. Protein determination of the sample before and after exposure to nitrocellulose gave an estimate of the amount of protein bound. Alternately, protein was transferred to the nitrocellulose from an SDS gel. Next the nitrocellulose was washed three times with PBS and blocked for 1 h with 5% BSA in PBS. After another three washes in PBS, the nitrocellulose was incubated, shaking, with 50 μl Fab for 3 h. A volume of supernatant was assayed which was equivalent to the volume of unfractionated antibody which blocked about 90% of the membrane-induced increase in viscosity (determined for each batch of Fab with a dose–response curve). It should be noted that preabsorption of blocking antibody by a fraction bound to nitrocellulose results in a decrease in the amount of blocking activity in the supernatant, and therefore the viscosity of actin + membrane + that supernatant in the assay would be higher than a sample with the equivalent amount of unfractionated antibody.

Other Techniques

SDS gel electrophoresis was performed with 10% (or 15% in Figs. 3 and 4) separating, 5% stacking gels using the buffer system of Laemmli (1970). Western transfers were performed as described by Towbin et al. (1979), using horseradish peroxidase–coupled secondary antibody (Hawkes et al., 1982).

Protein concentration was determined by the method of Bradford (1976), unless there was detergent present, in which case the method of Peterson (1977) was used.

^{35}S-Labeled membranes were counted in a Beckman LS 9000 in 10 ml Aqueous counting scintillant (background = 40 cpm) (Amersham Corp., Arlington Heights, IL). If the membranes were adsorbed to nitrocellulose, the nitrocellulose was first dissolved in 1 ml ethoxyethanol for 10 min. If they were in gel slices, the gel was first dissolved in 0.2 ml 60% perchloric acid and 0.4 ml 30% hydrogen peroxide overnight at 60°C . Known amounts of membrane radioactivity were also counted in these solutions to control for quenching.

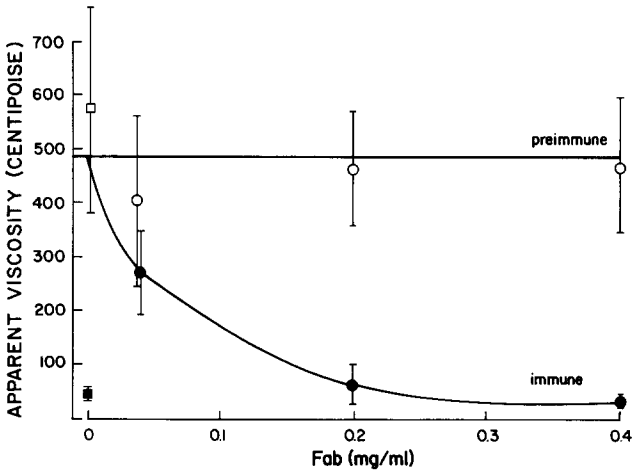

Figure 1. The inhibition of the actin–membrane interaction by antibody, assayed by low shear viscometry. Various amounts of immune (*solid circles*) or preimmune (*open circles*) Fab were incubated with 150 μg/ml rabbit muscle actin and 25 μg/ml EDTA-treated *Dictyostelium* membrane, and assayed by low shear viscometry. The viscosity of the actin alone (*solid square*) and actin + membrane (*open square*) are shown on the ordinate. Symbols indicate averages; bars indicate ± 1 SD.

Results

Antimembrane Antibody Blocks the Interaction of Actin with Membranes

Luna et al. (1981) have reported that *Dictyostelium* membranes increase the low shear viscosity of actin, apparently by cross-linking actin filaments. We have made an antibody against *Dictyostelium* membranes and have assayed for its ability to block this actin–membrane interaction. To maximize the sensitivity of the assay to antibody, we have used a minimum amount of actin (100–150 μg/ml) and sonicated membrane (25 μg/ml), and have picked a ratio of membranes to actin which increases the low shear viscosity about an order of magnitude over that of actin alone. These conditions put our assay in a range where the relation between viscosity and membrane concentration is pseudolinear (but not truly linear; Luna et al., 1981).

Fig. 1 shows that the effect of membranes on the low shear viscosity of actin can be completely blocked by adding immune Fab to the assay mixture; the viscosity is reduced to that of actin alone at sufficiently high antibody concentrations. Preimmune Fab, on the other hand, has little or no effect on the viscosity of actin plus membranes. We have used Fab fragments in all experiments rather than intact IgG to

avoid the possibility of cross-linking membrane vesicles (which could have given an artifactual reduction in membrane effects). The Fab has no effect on the viscosity of actin in the absence of membranes.

This blocking suggests that our Fab preparation includes antibody which binds to membrane proteins and blocks their ability to bind actin in the assay mixture. Since Fab fragments are generated by papain, we wanted to confirm that their blocking of membrane effects was not simply an effect of residual protease. This possibility seemed unlikely, as preimmune Fab did not block membranes. To further rule out this possibility, we showed that the length of time (0–3 h) that Fab was preincubated with membranes before addition of actin did not increase the amount of blocking seen. (This experiment was performed at a Fab concentration which only blocked about a third of the membrane activity, so that any increases would be readily seen.) Furthermore, no evidence of proteolysis was seen when membranes ± Fab were compared on SDS gels.

The validity of using low shear viscometry to assay antibody blocking of actin–membrane interactions was confirmed by using a cosedimentation assay to obtain the same results. Fig. 2 shows that immune but not preimmune Fab can block the ability of radioactive membranes to cosediment with actin. The half maximal concentration for inhibition by immune Fab (60 μg/ml) is the same as in the low shear assay.

Blocking by Antibody Occurs at the Internal Surface of Membranes

It would be logical to assume that all actin-binding sites, and thus all ability to absorb out blocking antibody, would reside on the internal surface of the membrane. To test this assumption, we asked whether the external surface (of intact cells) could remove any of the blocking antibody. Controls were done to show that little of the total Fab was absorbed out by cells (<10%), and that the supernatant from cells incubated without Fab had little or no effect on the assay (Table I). As predicted, cells were not nearly as effective as membranes in removing blocking antibody. We found (Table I) that intact cells were capable of removing a maximum of about one-third of the blocking activity of the antibody. Increasing the concentration of cells or the time of incubation of Fab with cells (from 5 to 90 min) did not increase the amount removed. This was in contrast to the almost complete removal obtained with isolated membranes in comparable amounts (based on average yields of 3 mg membrane protein/g wet weight cells; data not shown).

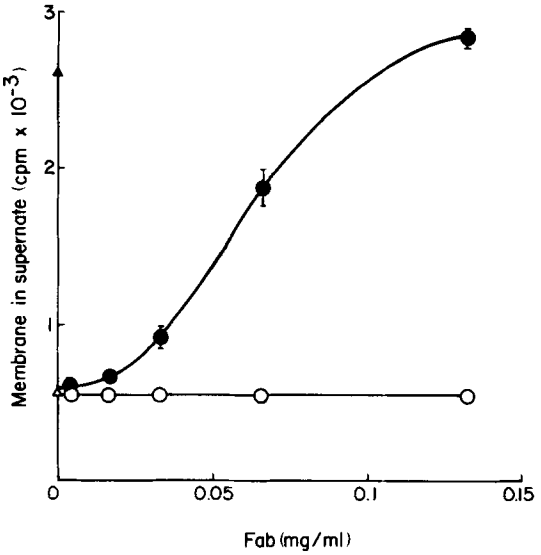

Figure 2. The inhibition of the actin–membrane interaction by antibody, assayed by cosedimentation. Various amounts of immune (*solid circles*) or preimmune (*open circles*) Fab were incubated with 20 μg/ml rabbit muscle actin and 25 μg/ml *Dictyostelium* urea/KCl-extracted membrane, and the samples assayed by cosedimentation. Circles indicate averages, and bars the range. The range is not shown if it is less than the diameter of the circle. The open triangle on the ordinate indicates the supernatant cpm for actin + membranes in the absence of antibody; the solid triangle indicates the cpm for membranes in the absence of actin.

Table I. Comparison of Antibodies That Do and Do Not Bind to Cells

Fraction*	Viscosity‡
	cp
Unbound	
Fab	67 ± 6
PBS	250 ± 8
Bound	
Fab	154 ± 18
PBS	222 ± 35
Controls	
Unfractionated Fab§	30 ± 4
Actin + membranes	270 ± 19
Actin alone	11 ± 1

* 50 mg/ml cells were mixed with 1.2 mg/ml Fab or PBS alone, incubated 5 min, and centrifuged (20 min, 30 psi, Beckman Airfuge). The supernatant equals the unbound fraction. The bound fraction was obtained by acid elution (Materials and Methods). These fractions were assayed for blocking antibody by low shear viscometry, as described in Materials and Methods.
‡ Mean ± 1 SD.
§ An equivalent amount (0.2 mg/ml).

We concluded that the removal of blocking activity by cells was not simply due to lysis of cells to expose the internal surface of the membrane, for the following reasons. (*a*) Since roughly comparable amounts of cells and membranes were used, many of the cells would have had to lyse, and we could demonstrate by cell counts and trypan blue exclusion that there was little or no cell lysis during the incubation with antibody. (*b*) Increasing the concentration of cells did not increase the amount of antibody removed. (*c*) Antibodies that did not bind to whole cells vs. those that bound gave different labeling patterns on Western transfers of *Dictyostelium* membranes. The fraction of antibody which was eluted from whole cells did not label any distinct bands, but instead labeled a faint smear over most of the lane. In contrast, equivalent amounts or considerably less of the fraction of antibody which did not bind to whole cells labeled many distinct polypeptide bands (Fig. 3). Fractionated preimmune antibody gave no labeling on the Westerns at any of the dilutions used.

Thus a likely conclusion is that a minor fraction of the blocking antibody is specific for the external cell surface. This in turn suggests that some of the membrane-mediated increase in the low shear viscosity of actin is due to the interaction of actin with the external cell surface. We found that actin did indeed bind to whole cells (measured as described

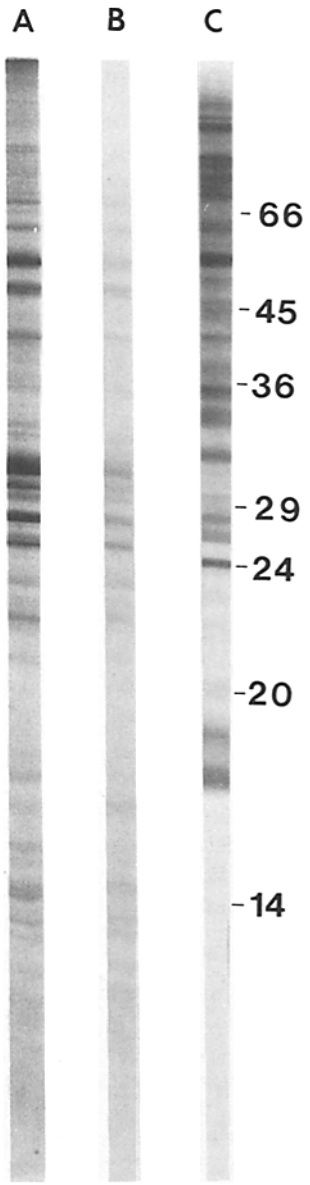

Figure 3. Western transfer of *Dictyostelium* membranes. Urea/KCl-treated *Dictyostelium* membrane was subject to electrophoresis on a 15% SDS polyacrylamide gel. A portion of the gel was either stained with Coomassie Blue (*A*) or transferred to nitrocellulose and stained with Amido Black (*B*) or labeled with antibody which had been preabsorbed with whole cells (*C*). The region of lane *C* that corresponds to the peak of activity in Fig. 4 contains both a heavy band and a lighter-staining band.

by Stratford and Brown, 1985). However, Scatchard analysis (not shown) revealed that isolated membranes had an affinity for actin at least an order of magnitude higher than that of whole cells.

Membrane Extractions Suggest That Blocking Antibody Is against an Integral Membrane Protein(s)

Luna et al. (1981) have shown that the actin cross-linking activity of membranes remains associated with the membrane through a series of extractions designed to remove peripheral membrane proteins. We have repeated their extractions, applied the fractions to nitrocellulose, and assayed for the ability of these fractions to preabsorb blocking antibody (Table II). The controls, in which nitrocellulose was treated with buffer instead of sample, did not absorb any blocking antibody (Table II). We found that ATP + EDTA, which extracted residual actin and a 200,000-mol-wt protein which we assume is myosin, released material capable of absorbing out some of the blocking activity, but much of this ability remained associated with the membrane (Table II). The two subsequent extractions, with urea/KCl and NaOH/DTT (Table II), did not extract blocking activity, even though NaOH was quite effective at extracting protein. Since NaOH treatment resulted in a smeary appearance on SDS gels, with a few apparent shifts in band position and a significant amount of the protein running at the stacking/separating gel interface, we used urea/KCl-extracted membranes in subsequent experiments. (A possible cause might have been reaction of proteins with O-linked oligosaccharides released by NaOH. We tried to offset this possibility by adding 0.1 M sodium borohydride, which did not improve the pattern.)

Our results with membrane extractions are thus consistent with those of Luna et al. (1981); i.e., extractions which should remove peripheral membrane proteins did not extract components which react with blocking antibody. Thus, we conclude as they do that integral membrane protein(s) may interact with actin. If this is the case, the putative actin-binding membrane proteins should be extractable with detergents. To test this prediction, we treated urea/KCl extracted membranes with 0.5% sodium deoxycholate and applied fractions to nitrocellulose (Table III). The supernatant absorbed out about two-thirds of the blocking activity relative to whole membranes, whereas the pellet absorbed out about one-third. Thus, we conclude that much of the putative actin-binding protein(s) is easily extracted with detergent. Western transfers showed that most of the membrane protein which reacted with antibody was extracted by 0.5% sodium deoxycholate (data not shown).

Fractionation of Antibody Using Western Transfers Indicates That There Is an Actin-binding Protein with a Molecular Weight of ~20,000

We next used Western transfers of membrane proteins to

Table II. Extraction of Peripheral Membrane Proteins

Extraction*	Protein	Viscosity‡
	mg	*cp*
ATP/EDTA		
Supernatant	10	55 ± 17
Pellet	23	83 ± 9
Urea/KCl		
Supernatant	4	26 ± 2
Pellet	13	114 ± 5
NaOH/DTT		
Supernatant	27	15 ± 0.2
Pellet	2	102 ± 35
Controls		
Unfractionated Fab§		11 ± 0.5
Fab preabsorbed with blank nitrocellulose		10 ± 3
Actin + membranes		98 ± 48
Actin alone		12 ± 2

* *Dictyostelium* membranes were sequentially extracted by sonication in 3 mM imidazole, 1 mM ATP, 0.1 mM EDTA, pH 7.5 (ATP/EDTA); 3.5 M urea, 1 M KCl, 1 mM phenylmethylsulfonyl fluoride (urea/KCl); 0.1 M NaOH, 1 mM DTT (NaOH/DTT); and spun for 1 h at 45 krpm in a type 50 rotor after each extraction. 325 μg of each fraction in a volume of 0.5 ml was incubated overnight at 4°C with 1 cm^2 of nitrocellulose.

‡ Viscosity is expressed as mean ± 1 SD.

§ An equivalent amount treated antibody (0.2 mg/ml) was assayed. This experiment was repeated with antibody which had been pretreated with whole cells, and essentially the same results were obtained.

fractionate antibody (adapting an approach developed by Olmsted [1981] for affinity purification of antibody). A Western transfer of urea/KCl-extracted membranes was cut into fractions and used to absorb antibody. In our initial experiments, we found that a low molecular weight region of the Western transfer was the most active in removing blocking antibody. Therefore, we increased the percent acrylamide to increase resolution in the low molecular weight range; this revealed a peak of activity at ~20,000 (Fig. 4).

Discussion

Our studies with antibodies indicate that there is a major 20,000-mol-wt actin-binding protein which is probably integral to *Dictyostelium* membranes. The most important contribution of our approach is the identification of an actin-binding membrane protein on the basis of its behavior in its native state. We have avoided perturbations of detergent solubilization of membranes, which exposes regions of proteins ordinarily buried in the lipid bilayer, and which may cause conformational and other changes (Grasberger et al., 1986). Another advantage of the assay is that it allows us to assess the relative contribution of a given membrane actin-binding protein to the total membrane activity. Thus it is clear that the 20,000-mol-wt protein is responsible for a sizeable fraction of the total membrane activity, as it is the only peak of activity we have discerned. Technical limitations (amount of protein we can load on a gel and transfer onto nitrocellulose) do not at present allow us to determine the maximum fraction of the total activity which can be removed by the 20,000-mol-wt protein.

Preabsorption of antibody with cells has shown that most of the actin–membrane interaction we are studying is taking place at the internal cell surface. However, our finding that whole cells can absorb out a minor fraction of the blocking antibody suggests that the assay is detecting an interaction of actin with the external cell surface as well. We found using radioactive actin that actin could indeed bind to the external surface of whole cells, but with a lower affinity than to membranes. Thus it is important to control for such an artifact when studies of this kind are done. We do not know what is responsible for this binding. Our experiments suggest that it is unlikely that this is due to cell breakage to expose the internal cell surface, but we cannot completely rule out several other somewhat unlikely explanations: (*a*) cross-reactivity between some epitopes at the external and internal cell surfaces; or (*b*) cytoplasmic actin-binding proteins from broken cells sticking to the external cell surface.

We have used a low shear viscosity assay to detect an actin–membrane interaction and its blocking by antibody. Luna et al. (1981) have already provided several pieces of evidence that the low shear assay is detecting the interaction of actin

Table III. Extraction of Membranes with Sodium Deoxycholate

Fraction*	Protein	Viscosity‡
	mg/ml	*cp*
Total	1.4	103 ± 1
Supernatant	0.6	67 ± 1
Pellet	0.8	34 ± 10
Controls		
Unfractionated Fab§		17 ± 5
Actin + membrane		198 ± 70
Actin alone		18 ± 1

* *Dictyostelium* membranes which had been urea/KCl-extracted (Table II) were sonicated in 0.5% sodium deoxycholate in 10 mM Tris-Cl, pH 8, 1 mM EDTA, 0.2 mM DTT. An aliquot was saved (Total) and the rest spun for 10 min, 30 psi (Beckman Airfuge). The pellet was resuspended by sonication into an equal volume of the same buffer. 1 cm^2 of nitrocellulose was incubated overnight, shaking at 4°C with 100 μl of each fraction.
‡ Mean ± 1 SD.
§ 0.2 mg/ml.

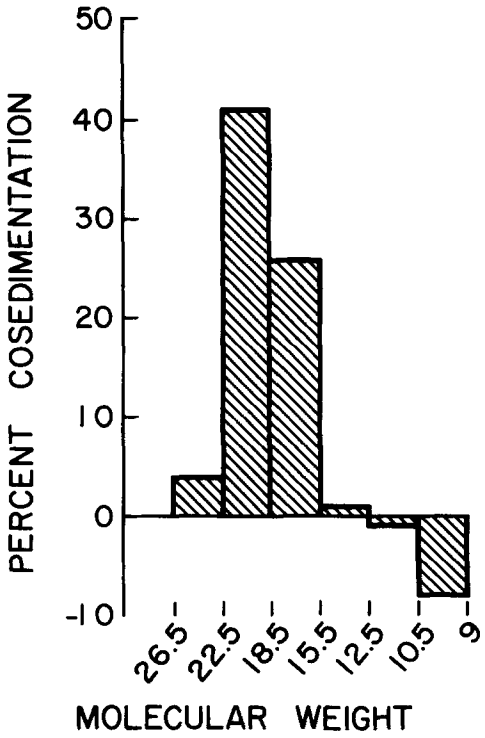

Figure 4. Preabsorption of antibody with low molecular weight fractions of *Dictyostelium* membrane. 1.3 mg ^{35}S-labeled *Dictyostelium* membrane protein from urea/KCl-extracted membranes was loaded into a 6.5-cm slot, separated on a 15% SDS polyacrylamide gel, and transferred to nitrocellulose. The nitrocellulose was cut into 1-cm horizontal strips, each of which contained 22–32 μg membrane protein (determined by counting strips). The range of molecular weights ($\times 10^{-3}$) on each strip is listed on the abscissa, and was determined by alignment with the following standard proteins which were also transferred to the nitrocellulose: ovalbumin (45,000), actin (42,000), troponin T (37,000), tropomyosin (35,000), carbonic anhydrase (29,000), myosin LC_1 (25,500), troponin I (24,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), beta-lactoglobulin (18,400), myosin LC_2 (17,500), and lysozyme (14,300). The nitrocellulose strips were each incubated with 26.4 μg immune Fab in 200 μl. This pretreated Fab was then assayed for its ability to block the cosedimentation of membranes with actin. Counts per minute in the pellet were calculated by subtracting supernatant cpm from total cpm, and used to calculate a "percent cosedimentation" using the following formula: $cpm_1 - cpm_2/cpm_3 - cpm_2 \times 100$, where cpm_1 = a given sample, cpm_2 = membrane + actin in the presence of Fab which had been preabsorbed with blank nitrocellulose, and cpm_3 = actin + membranes alone, in the absence of Fab. The peak of activity appears to be close to the lower edge of the 18,500–22,500 fraction, as some of the activity spills over into the next lower fraction. This same peak of activity was seen when antibody which had been preabsorbed with whole cells was used, demonstrating that the 20,000-mol-wt protein is interacting with actin at the internal cell surface.

with protein(s) in *Dictyostelium* membranes. They have shown (*a*) that membrane activity is destroyed by proteases and boiling and (*b*) that vesicles prepared from lipids extracted from membranes are not active. (*c*) They have also shown that the activity is not extracted by chaotropes, suggesting that the active protein(s) is integral. We have extended some of these findings to our studies using antibodies. If we repeat the membrane extractions of Luna et al. (1981), we find that component(s) which bind blocking antibody behave as though they were integral membrane protein(s). The extractibility of these components with detergent also supports this conclusion.

To confirm the validity of using the low shear assay, we have shown that we can obtain the same results with a different type of assay—cosedimentation. In both assays, complete inhibition of an actin–membrane interaction can be obtained with immune Fab, whereas preimmune Fab has no effect. Both assays indicate that there is a peak of activity in the 20,000-mol-wt range of membrane protein.

A 24,000-mol-wt putative integral membrane protein which we have studied earlier (Stratford and Brown, 1985) is close in molecular weight to the 20,000-mol-wt activity which we report here. However, this protein clearly does not co-migrate with the peak of activity, but instead migrates in the middle of the next higher piece of nitrocellulose in Fig. 4. Another possible candidate on the basis of molecular weight is a 17,000-mol-wt membrane protein isolated from *Dictyostelium* by Schleicher et al. (1984). However, this protein is not integral, as these authors obtain it by high salt extraction. Thus, it should have been removed from our urea/KCl-extracted membranes. We have recently learned, after completion of this work, that Wuestehube and Luna (1986) have independently found evidence for a 17,000-mol-wt actin-binding integral membrane protein, using different techniques. This may well be the same protein as the one we have identified.

Finally, we should point out that we cannot be sure that the actin-binding activity we are studying resides in the cytoplasmic membrane. Our membrane preparation is crude and certainly contains other organelles. Once we have monospecific antibody against the 20,000-mol-wt protein, we will localize it in the cell by immunofluorescence.

The next step is to obtain such a monospecific antibody. We will produce monoclonals against membrane proteins in the very narrow molecular weight range we have defined.[1] Additionally, we will produce polyclonals against spots of the appropriate molecular weight on two-dimensional gels.

1. We decided not to use monoclonal antibodies for the initial studies for several reasons. First, we did not know how many membrane-associated actin-binding proteins there might be. If there were a number, each responsible for a small fraction of the total membrane activity, no monoclonal would have given detectable blocking. Second, monoclonals are in general less likely to block a function than polyclonals.

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References

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Brown, S. S. 1985. The linkage of actin to non-erythroid membranes. *BioEssays.* 3:65–67.

Geiger, B. 1983. Membrane–cytoskeletal interaction. *Biochim. Biophys. Acta.* 737:305–341.

Grasberger, B., A. P. Minton, C. DeLisi, and H. Metzger. 1986. Interaction between proteins localized in membranes. *Proc. Natl. Acad. Sci. USA.* 83: 6258–6262.

Griffith, L. M., and T. D. Pollard. 1982. Cross-linking of actin filament networks by self-association and actin-binding macromolecules. *J. Biol. Chem.* 257:9135–9142.

Hawkes, R., E. Niday, and J. Gordon. 1982. A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* 119:142–147.

Jacobson, B. S. 1983. Interaction of the plasma membrane with the cytoskeleton: an overview. *Tissue & Cell.* 15:829–852.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Luna, E. J., V. M. Fowler, J. Swanson, D. Branton, and D. L. Taylor. 1981. A membrane cytoskeleton from *Dictyostelium discoideum.* I. Identification and partial characterization of an actin-binding activity. *J. Cell Biol.* 88:396–409.

Luna, E. J., C. M. Goodloe-Holland, and H. M. Ingalls. 1984. A membrane cytoskeleton from *Dictyostelium discoideum.* II. Integral proteins mediate the binding of plasma membranes to F-actin affinity beads. *J. Cell Biol.* 98:58–70.

MacLean-Fletcher, S., and T. D. Pollard. 1980*a*. Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. *Biochem. Biophys. Res. Commun.* 96:18–27.

MacLean-Fletcher, S., and T. D. Pollard. 1980*b*. Viscometric analysis of the gelation of *Acanthamoeba* extracts and purification of two gelation factors. *J. Cell Biol.* 85:414–428.

Mage, M. G. 1980. Preparation of Fab fragments from IgGs of different animal species. *Methods Enzymol.* 70:142–150.

Olmsted, J. B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955–11957.

Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *In* Methods in Cell Biology. Vol. 24. 271–289.

Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* 83:346–356.

Schleicher, M., G. Gerisch, and G. Isenberg. 1984. New actin-binding proteins from *Dictyostelium discoideum. EMBO (Eur. Mol. Biol. Organ.) J.* 3:2095–2100.

Spudich, J. A. 1974. Biochemical and structural studies of actomyosin-like proteins from non-muscle cells. *J. Biol. Chem.* 249:6013–6020.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin–troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Stratford, C. A., and S. S. Brown. 1985. Isolation of an actin-binding protein from membranes of *Dictyostelium discoideum. J. Cell Biol.* 100:727–735.

Thiery, J.-P., R. Brackenbury, U. Rutishauser, and G. M. Edelman. 1977. Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J. Biol. Chem.* 252: 6841–6845.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Wuestehube, L. J., and E. J. Luna. 1986. A 17 kd integral membrane protein in *D. discoideum* plasma membrane binds F-actin. *J. Cell Biol.* 103(5, Pt. 2):111*a*. (Abstr.)