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.

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, 1980a), 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. (1980), as described in Stratford and Brown (1985). We used cells at a density of <5 x 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., 1980) 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 KPO4, pH 6.5, and 1 mg protein/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 \( \sim 120 \pm 30 \) 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 KCl, 5 mM imidazole, 0.2 mM ATP, 0.2 mM diithiothreitol [DTT], 100 mM KCl, 5 mM MgCl₂, 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 (1986). 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 radioactivity. 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, pH 2.7. The cells bleded 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, \(~100 \mu g\) 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 unfractonated 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 unfractonated 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).

**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).

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.

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 next used Western transfers of membrane proteins to

Table II. Extraction of Peripheral Membrane Proteins

| Extraction* | Protein       | Viscosity† |
|-------------|---------------|------------|
| 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 KCI, 1 mM phenylmethylsulfonyl fluoride (urea/KCl); 0.1 M NaOH, 1 mM DTT (NaOH/DTT); and spun for 1 h at 45 k rpm in a type 3 rotor after each extraction. 325 μg of each fraction in a volume of 0.5 ml was incubated overnight at 4°C with 1 cm² 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

Table III. Extraction of Membranes with Sodium Deoxycholate

| Fraction | Protein (mg/ml) | Viscosity (cp) |
|----------|----------------|---------------|
| Total    | 1.4            | 103 ± 1       |
| Supernatant | 0.6            | 67 ± 1        |
| Pellet   | 0.8            | 34 ± 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 cm² of nitrocellulose was incubated overnight, shaking at 4°C with 100 ml of each fraction.

| Controls | Protein (mg/ml) |
|----------|-----------------|
| Unfractionated Fab† | 17 ± 5         |
| Actin + membrane | 198 ± 70       |
| Actin alone      | 18 ± 1          |

† Mean ± 1 SD.

‡ 0.2 mg/ml.

Figure 4. Preabsorption of antibody with low molecular weight fractions of Dictyostelium membrane. 1.3 mg 35S-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 (×10⁴) 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 LC₁ (25,500), troponin I (24,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), beta-lactoglobulin (18,400), myosin LC₂ (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: cpm₁ = a given sample, cpm₂ = membranes alone, cpm₃ = 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.

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
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 extractability 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—cotedimentation. 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 studied.

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. 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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