A Membrane Cytoskeleton from *Dictyostelium discoideum*.

III. Plasma Membrane Fragments Bind Predominantly to the Sides of Actin Filaments

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ABSTRACT The binding between sonicated *Dictyostelium discoideum* plasma membrane fragments and F-actin on Sephacryl S-1000 beads was found to be competitively inhibited by myosin subfragment-1. This inhibition is MgATP-sensitive, exhibits a $K_i$ of $\approx 5 \times 10^{-8}$ M, and is reciprocal, since membranes inhibit the binding of $^{125}$I-heavy meromyosin to F-actin on beads. These experiments demonstrate that membrane binding and S-1 binding to F-actin on beads are mutually exclusive and, therefore, that the membrane fragments bind predominantly to the sides, rather than to the ends, of the actin filaments. This conclusion is supported by electron micrographs that show many lateral associations between membrane fragments and bead-associated actin filaments. Such lateral associations could play an important role in the organization and lateral movement of membrane proteins by the cytomusculature.

Two types of interactions between membranes and actin filaments have been proposed. The first type is the binding of filament ends to cytoplasmic membrane surfaces, an interaction that has been documented by the decoration of membrane-associated actin filaments with myosin fragments (1, 22, 24). The "arrowheads" of decorated actin filaments associated with plasma membranes from many different cell types point away from the cytoplasmic face of the membrane and toward the center of the cell (1, 10, 22, 24, 35, 39). Therefore, it is often assumed that the physiologically preferred mode of attachment involves interactions between membranes and the “barbed” ends of actin filaments. However, a second type of F-actin–membrane interaction, the binding between membranes and the sides of actin filaments, has recently attracted attention. This mode of attachment is supported by electron micrographic observations of membranes associated with undecorated actin filaments (20, 34, 35, 41), by the magnitude of the viscosity increases induced by membrane vesicles in solutions that contain F-actin (15, 16, 28), and by the observation of lateral associations between F-actin and membrane-associated actin-binding proteins in solution (6, 7, 14, 21, 23).

The present study was initiated to explore the mode of attachment of F-actin to the plasma membranes of the cellular slime mold, *Dictyostelium discoideum*. In the preceding paper (29), we demonstrated that acetamido fluorescein-F-actin on antifluorescein Sephacyr S-1000 beads (F-actin beads) specifically binds $^{125}$I-labeled, sonicated membrane fragments from *D. discoideum*. Since myosin fragments also interact specifically with F-actin beads (31), we examined the effect of myosin fragments on the interactions between F-actin beads and sonicated *D. discoideum* plasma membranes. We found that myosin subfragment-1 (S-1)$^1$ and sonicated membranes compete for mutually exclusive binding sites along the sides of actin filaments. This result suggests a mechanism for lateral movement of membrane proteins. Furthermore, we suggest that caution should be exercised in the interpretation of electron microscopic images of decorated actin filaments associated with membranes.

This work was presented in preliminary form at the Annual Meeting of the American Society for Cell Biology in November, 1982 (30).

MATERIALS AND METHODS

Chemicals: Dithiothreitol (DTT), BSA, imidazole (Grade I), and Tris, were supplied by Sigma Chemical Co. (St. Louis, MO). Ovalbumin was secured from Worthington Biochemical Corp. (Freehold, NJ). Ultrapure ammonium sulfate was obtained from Schwarz/Mann, Div. of Becton Dickinson (Orangeburg, NY) and fluorescein-5-isothiocyanate was obtained from Molecular Probes (Junction City, OR). Both Sephacyr S-1000 and Sephadex G-25 were

$^1$Abbreviations used in this paper: Con A, concanavalin A; DTT, dithiothreitol; HMM, heavy meromyosin; S-1, myosin subfragment-1.
purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Other reagents used were procured from the sources listed in the accompanying paper (29).

**Myosin and Myosin Fragments:** Heavy meromyosin (HMM) and S-1 were produced by chymotryptic digestion of rabbit skeletal muscle myosin as reported previously (31). 1 mM ATP was routinely added to the HMM in storage buffer to improve its shelf life. Both 1 mM ATP and 1 mM DTT were added to the S-1 before storage. Occasionally, S-1 and HMM were stored as precipitates in 50% saturated ammonium sulfate, 5 mM EDTA, 10 mM KCl, 0.5 mM DTT, 0.01% sodium azide, 5 mM imidazole-HCl, pH 7.0. Before use, DTT, ATP, and ammonium sulfate were removed from the myosin fragments by dialysis against a buffer appropriate to assay requirements. HMM and S-1 were used within 2 wk of preparation to ensure maximum binding activity.

**Sedimentation Binding Assays:** Myosin fragments, F-actin beads (28), and membrane vesicles from Dictyostelium discoideum membranes were sonicated, centrifuged, and stored as a 25% suspension in 0.5 M Tris-base, pH 7.0 (Buffer A). These beads, as well as F-actin and control beads (anti-fluorescein IgG-Sepharose) were produced by the method of Laemmli (25). Details were previously reported (28).

**RESULTS AND DISCUSSION**

**S-1 Inhibits Binding of D. discoideum Plasma Membrane Fragments to F-Actin Beads**

In sedimentation assays containing F-actin beads and 125I-labeled, sonicated plasma membrane fragments, the inclusion of S-1 substantially inhibits the binding of the membranes to bead-bound F-actin (Fig. 1). This inhibition is caused by a specific interaction between S-1 and F-actin since it is reversed by MgATP, an agent that dissociates actin-S-1 complexes (17, 40). MgATP, in the absence of S-1, does not affect the binding of sonicated membranes to F-actin beads (Fig. 1). Inhibition is not due to the presence of residual chymotrypsin in our highly purified S-1 since chymotryptic digestion of the membranes or bead-bound F-actin would not be MgATP sensitive.

The reduction in binding of 125I-labeled, sonicated membrane fragments to F-actin beads observed in the presence of...
S-1 is not caused by an S-1-mediated release of actin from the beads. SDS PAGE of pellets and supernatants from sedimentation assays containing S-1 and F-actin beads shows that the amount of actin in the supernatant does not increase as the beads become saturated with S-1 (Fig. 2). Similar analyses of sedimentation assays containing sonicated membrane fragments and F-actin beads show that the membranes also are incapable of releasing actin from the beads (not shown).

**FIGURE 2** SDS PAGE of pellets (P) and supernatants (S) from sedimentation assays containing 5.3 μg of bead-bound F-actin. F-actin beads alone (lane 1) or F-actin beads plus 2.5 μg (lane 2), 11 μg (lane 3), or 22 μg of S-1 (lane 4). SDS PAGE of the pellet and supernatant from an assay containing control beads and 22 μg of S-1 (lane 5). Actin, BSA, S-1, and IgG heavy chain are labeled. A component of the S-1 preparation co-migrates with BSA.

**Myosin Fragments and Membrane Vesicles Compete for Common Binding Sites on F-Actin Beads**

Both unlabeled HMM (not shown) and unlabeled S-1 (Fig. 3a) compete the binding between 125I-labeled, sonicated membrane fragments and F-actin beads to levels just above those observed with control beads. When averaged data from the low ends of these competition curves are plotted according to the method of Dixon (Fig. 3b; reference 9), apparent inhibition constants (K_is) are obtained that can be compared with reported equilibrium constants for the S-1 association with F-actin. For example, the apparent K_i in Fig. 3b is ~5 ± 3 μg/ml or ~4 ± 2 × 10^{-8} M, assuming a molecular weight of 120,000 for S-1. In independent determinations, we have obtained the following approximate K_is: 2.5 × 10^{-8}, 4 × 10^{-8}, and 8 × 10^{-8} M. These values are consistent with the dissociation constant (K_d) for the interaction between S-1 and F-actin. Using various techniques and assay conditions, other researchers have measured the following K_is for S-1 binding to actin: 2 × 10^{-8} M, 5 × 10^{-8} M, 20 × 10^{-8} M, 30 × 10^{-8} M, and 50 × 10^{-8} M. The differences among these reported K_is are probably due to the different temperatures, ionic strengths, and divalent cation concentrations used by the different experimenters.

The excellent agreement between published equilibrium constants and the apparent K_is obtained from competition experiments (Fig. 3) suggests that the binding of S-1 to F-actin beads is directly responsible for the diminution of membrane binding to these beads. However, experimental error and the low 1/B_max for the membrane interaction with F-actin beads preclude the use of Dixon plots to distinguish between the various types of binding inhibition. To ascertain whether S-1 is a true competitive inhibitor of membrane binding to F-actin beads, we measured the binding of 125I-labeled, sonicated membrane fragments to F-actin beads in the presence of...
of different, fixed concentrations of S-1 (Fig. 4a). In the presence of low concentrations of S-1, inhibition is observed with low amounts, but not with relatively large amounts, of added 125I-labeled, sonicated membrane fragments. As the S-1 concentration is increased, ever larger amounts of membranes must be added before the S-1-induced inhibition is overcome (Fig. 4a).

When averaged data from binding curves such as those in Fig. 4a are plotted according to the method of Lineweaver and Burk (26), curves are obtained that intersect on the y-axis at 1/Bmax (Fig. 4b). This result indicates that S-1 and membranes do compete for the same, or overlapping, sites on F-actin (38). In addition, the curvature changes observed with increasing S-1 concentration suggest that at least one other phenomenon may occur in this system. One possibility is that S-1 reduces the apparent Hill coefficient (29) of the F-actin-membrane interaction from ~1.1–1.2 to <1.0. A more likely possibility is that interactions between F-actin and different membrane fragments, or proteins within a membrane fragment, are unequally sensitive to inhibition by S-1 (38). Specifically, the inhibition of binding induced by S-1 (Figs. 1, 3, and 4) may have both competitive and noncompetitive components.

As expected when two ligands compete for the same or overlapping sites, we observe competition for binding sites regardless of which ligand is radiolabeled. Although 125I-labeled S-1 binds poorly to F-actin either in solution or on beads (not shown), 125I-labeled HMM with only one label per molecule binds to F-actin beads with saturation kinetics (31). The binding of 125I-labeled HMM to F-actin beads is substantially reduced by the inclusion of unlabeled, sonicated membrane fragments in the assay (Fig. 5). Since about half of the 125I-labeled HMM binding is competed by sonicated membrane fragments and about half the binding is not (Fig. 5), this inhibition of binding also appears to have both competitive and noncompetitive components (see reference 38). However, the noncompetitive component in this situation is probably an artifact caused by the differential penetration of 125I-labeled HMM and sonicated membrane fragments into the bead interiors (see below). In general, when using gel filtration beads as affinity matrices in competition studies, it is obvious that small molecules will compete more effectively with large structures than will large structures with small molecules.

Electron Microscopy

To visualize the interactions between F-actin, S-1, and plasma membrane fragments, we present transmission elec-
FIGURE 6  Transmission electron micrographs of the surfaces of antifluorescein IgG-Sepharocryl S-1000 beads taken from the same experiment. (a) Control bead, (b) F-actin bead, (c) F-actin bead with bound S-1, and (d) F-actin bead with bound plasma membrane vesicles. The external surfaces of the beads are at the tops of the micrographs. Bar, 0.5 μm. × ~40,000.

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electron micrographs of antifluorescein IgG-Sephacryl S-1000 beads bound to one or more of these moieties (Figs. 6 and 7). Thin sections of control beads show scattered regions of electron-dense, amorphous material surrounded by extensive electron-lucent pores (Fig. 6a). In sections of F-actin beads, filaments are observed weaving around the electron-dense regions and extending into the bead pores (Fig. 6b). Only an occasional filament extends beyond the bead periphery. When S-1 is added to F-actin beads, the filaments in the pores assume the "arrowhead" appearance characteristic of S-1-decorated actin filaments (Fig. 6c). After the addition of sonicated plasma membrane fragments to undecorated F-actin beads, vesicular and linear membrane profiles are observed at or near the bead surface, apparently in close association with the actin filaments (Fig. 6d). Control beads bind relatively few membrane fragments (not shown).

Although many associations between sonicated membrane fragments and the sides of actin filaments are observed in micrographs of beads containing undecorated F-actin (Fig. 6d), most of the F-actin-membrane interactions are ambiguous with respect to F-actin orientation, probably because the actin filaments are weaving in and out of the plane of section. The orientation of membranes on F-actin is more readily identified when F-actin beads are incubated with both membranes and S-1 at concentrations such that membranes and S-1 both bind to the F-actin on the beads (Fig. 7). Near the surface of such a bead (Fig. 7a), many plasma membrane fragments are observed bound to the decorated actin filaments. While some membrane fragments may be bound to filament ends, many more membrane fragments appear to bind along the sides of the filaments (Fig. 7, a and inset). In the core of the bead (Fig. 7b), few membrane fragments are observed although the actin filaments are completely decorated with S-1. This observation supports the hypothesis, proposed above, that myosin fragments and membrane fragments penetrate the bead to different extents.

F-Actin Associations with Intact D. discoideum Plasma Membranes

Using sonicated plasma membrane fragments and phal-loidin-stabilized, rabbit F-actin on antifluorescein IgG Sephacryl S-1000, we have shown that most of the binding between these membranes and the F-actin can be inhibited by S-1. To show that this observation is applicable to native associations between plasma membranes and actin, we present electron micrographs of freshly prepared D. discoideum plasma membranes before (Fig. 8a) and after (Fig. 8b) decoration with S-1. These plasma membrane domains, obtained by Con A stabilization and detergent extraction, are associated with endogenous actin and myosin (8, 29, 37). Electron micro- graphs of undecorated plasma membrane domains show filamentous material in close association with the cytoplasmic surfaces of the membranes (Fig. 8a). In contrast, the external membrane surfaces, coated with Con A and visualized as described in the accompanying paper (29), are not associated with the filamentous material (Fig. 8a and reference 8). After decoration with S-1, little or no endogenous actin is released from the membranes in sedimentation assays, as quantified by SDS PAGE (not shown), but far fewer contacts are ob-

![Figure 7](https://example.com/fig7.png)
served between the decorated filaments and the cytoplasmic
membrane surfaces (Fig. 8b). Although we have not at-
ttempted to quantify the number or type of actin-membrane
associations before and after S-1 decoration, Bennett and
Condeelis (2), using stereo microscopy, report that several
types of attachment exist for both lateral and end-on binding
of actin filaments to D. discoideum membranes and that S-1
decoration diminishes the number of filament-membrane
interactions. This result is in gratifying agreement with the
predictions made on the basis of our competition binding
assays (Figs. 3 and 4).

CONCLUSION

Our results show that S-1 competitively inhibits most of the
binding between sonicated plasma membrane fragments and
F-actin beads (Figs. 1, 3, and 4). This inhibition is abolished
in the presence of MgATP (Fig. 1) and exhibits an inhibition
constant that is essentially identical to the reported dissocia-
tion constant of the interaction between S-1 and F-actin (Fig.
3). In addition, sonicated membrane fragments compete with
125I-HMM for binding to F-actin beads (Fig. 5). From these
experiments, we conclude that most of the binding of the D.
discoideum membrane fragments is along the sides of the
actin filaments at or near the well-characterized S-1 binding
sites (36). This conclusion is supported by electron micro-
graphs of sonicated membrane fragments bound to F-actin
beads. These micrographs show many lateral interactions
between the membrane fragments and bead-associated actin
filaments (Figs. 6 and 7). Lateral interactions between endog-
ous actin and plasma membranes are consistent with the
observation that S-1 disrupts many of the native interactions
between Con A-stabilized plasma membranes and actin fila-
ments (Fig. 8). These results agree with the suggestion, based
on low shear viscosity data, that associations between plasma
membranes and the sides of actin filaments are extensive in
D. discoideum (28).

The demonstration of binding to the sides of actin filaments
does not exclude binding to filament ends. Indeed, end-on
associations may be responsible for that component of the
binding between 125I-labeled, sonicated membranes and F-
actin beads that is not competitively inhibited by S-1 (Figs. 3
and 4). End-on associations may also explain the binding of
endogenous actin to Con A-stabilized plasma membranes that
persists in the presence of relatively high concentrations of
S-1 (Fig. 8).

It is noteworthy that the membrane fragments used in this
study are derived from plasma membrane domains involved
in Con A-induced patching and capping. In conjunction with
the observation that integral, possibly transmembrane, pro-
teins mediate much of the binding between F-actin and D.
discoideum plasma membrane fragments (29), our data sug-
gest that D. discoideum plasma membranes contain one or
more integral membrane proteins that bind to the sides of
actin filaments. The laterally bound actin filaments may then
interact with cytoplasmic myosin filaments during the cap-
ping process, as previously proposed (4, 5, 8, 11, 13). In fact,
a lateral association between membranes and actin filaments
is intuitively the most efficient orientation for the lateral
movement of membrane proteins by the cytomusculature.

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