Decoration with Myosin Subfragment-1 Disrupts Contacts between Microfilaments and the Cell Membrane in Isolated *Dictyostelium* Cortices

HOLLY BENNETT and JOHN CONDEELIS
Department of Anatomy, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT We used isolated cortices from ameboid cells of *Dictyostelium discoideum* to examine the structural nature of attachments between microfilaments and the cell membrane and to determine the effect of myosin subfragment-1 (S-1) on such contacts. By varying several parameters in our previously described isolation procedure (Condeelis, J., 1979. *J. Cell Biol.*, 80:751–758), we have improved this procedure and have been able to isolate stable cortices. In this paper we identify two types of contact sites between microfilaments and the cell membrane similar to those seen in the brush border of intestinal epithelial cells: (*a*) an end-on attachment between the barbed end of actin filaments and the cell membrane; and (*b*) a lateral attachment mediated by rod-shaped bridges measuring ~6 × 15 nm. The spacing between bridges averages 36 nm, which suggests that the helical twist of the actin filament influences bridge location. Together these contacts account for an average of ~25,000 attachments per cell. Incubation of cortices with concentrations of S-1 sufficient to saturate binding sites on the microfilaments caused disruption of the contacts. This observation was confirmed by quantitative morphometry to show a threefold loss in the number of contact sites following S-1 decoration. These results indicate that S-1 decoration should be used with caution when information about the precise location of microfilaments and their attachment to the membrane is required.

Amebae such as vegetative cells of *Dictyostelium discoideum* exhibit motility that is associated with the cell cortex such as growth of microfilament filled projections, capping, and endocytosis. These events are associated with reorganization of actin, myosin, and several actin-binding proteins in the cell cortex (4).

To investigate the molecular mechanisms that are responsible for ligand-induced capping and changes in cell shape, we isolated actin-containing cortices from *Dictyostelium* amebae. We have chosen to use isolated cortices instead of whole cells in these studies for several reasons. First, isolated cortices have a simpler protein composition and are enriched for actin and myosin as compared with the whole cell (5). They are permeable to cytochemical probes like subfragment-1 (S-1)[1] and colloidal gold (18). Finally, the observation of cortical microfilaments in thin section by transmission electron microscopy is greatly facilitated in isolated cortices, compared with whole *Dictyostelium* amebae, in which they are obscured by the dense surrounding cytoplasm. Hence, isolated cortices are particularly suitable for studies of the fine structure of actin-containing filaments in situ.

Decoration of microfilaments with S-1 could be a powerful tool for investigating in situ the mechanisms of assembly and force production within actin containing structures. For example, decoration with S-1 has been used to identify actin filaments in situ (11), define filament polarity (10), determine the preferred direction of filament growth (25), and demonstrate the relative direction of filament sliding during muscle contraction (10).

In this paper we describe the use of these cortices isolated from *Dictyostelium* amebae to identify two types of interactions between microfilaments and the cell membrane and investigate the effect of S-1 decoration on the stability of these interactions.

This study was reported in preliminary form at the 23rd Annual Meeting of the American Society for Cell Biology (1).

MATERIALS AND METHODS

Isolation of Cell Cortices: Isolation of cell cortices was by the following procedure (which is a modification of that used by Condeelis [5]

[1] Abbreviations used in this paper: S-1, subfragment 1.

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for the isolation of ghosts and caps formed in response to concanavalin A [Con A]). *D. discoideum* amebae strain AX-3 were grown axenically in HL-5 medium to a density of 1×10^7 cells/ml. Cells were pelleted (700 *g* for 2 min) washed, and resuspended in PDF buffer (20 mM KCl, 30 mM $NaH_2PO_4 \cdot H_2O$, 10 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 2 mM $MgSO_4$, pH 6.2) and challenged with 70 μg/ml Con A (Sigma Chemical Co., St. Louis, MO) for 4 min at room temperature while swirling in an Erlenmeyer flask. Con A was prepared as described previously (5). All further steps were performed at 0–4°C unless noted. Cells were washed in PDF and swirled by hand for 5 min with 0.5 mg/ml hemocyanin to label the Con A. Hemocyanin was prepared by the method of Karnovsky (12). Cells were washed in lysis buffer I (1 mM EGTA, 5 mM Tris, pH 7.6) (Sigma Chemical Co.), resuspended to a density of 5×10^7 cells/ml in lysis buffer I containing 0.4 ml/ml Trasylol (Mobay Chemical Co., New York) and 0.01 mg/ml chymostatin (Sigma Chemical Co.), and lysed on a vortex mixer for 5 s with 0.2% Triton X-100 (Sigma Chemical Co.). Cortices were obtained by pelleting the lysed cells at 2,000 *g* for 4 min and discarding the supernatant and underlying hard brown pellet. Cortices in the fluffy white pellet were washed and centrifuged at 2,000 *g* for 4 min and resuspended in either 40 mM potassium phosphate, pH 7 or H3 buffer (1 mM EGTA, PIPES, 5 mM $MgSO_4$, 20 mM KCl, pH 7) containing 0.01 mg/ml chymostatin.

Variable conditions in the experimental protocol that correspond to procedures *A* through *D* in Table I are as follows. In procedure *A*, cortices were prepared as described above, washed in 40 mM potassium phosphate, pH 7.0, and labeled with S-1 at a final concentration of 3 mg/ml for 30 min. In procedure *B*, cortices were prepared as described above, resuspended in H3 buffer containing 45 μM phalloidin for 3 min, and then mixed with an equal volume of buffer II (10 mM PIPES, 1 mM EDTA, 1 mM dithiothreitol, pH 7.0) containing 20 mg/ml S-1. In *C*, cortices were lysed in buffer I containing 20 mM potassium phosphate, pH 7.6, resuspended in H3 buffer with 45 μM phalloidin for 3 min, and mixed with an equal volume of buffer II with 20 mg/ml S-1 as in *B*. In *D*, cortices were prepared and labeled with S1 as in procedure *C* except that EGTA was absent from the lysis buffer. For each procedure, samples were also processed in an identical manner with S-1 omitted. Rabbit skeletal muscle myosin was prepared by the method of Perry (19). S-1 was prepared by papain digestion according to Murray (17) (procedures *B* through *D*) or Margossian and Lowey (14) (procedure A).

Preparation of Thin Sections for Transmission Electron Microscopy: After incubation with S-1 or buffer, cortices were washed with the buffer used in the S-1 incubation and fixed in suspension with 2% glutaraldehyde (Polysciences, Inc., Warrington, PA) in 60 mM potassium phosphate, pH 7, containing 0.2% tannic acid for 20 min at room temperature. They were pelleted, washed in 40 mM potassium phosphate, pH 6, at room temperature, resuspended in 5 vol ice-cold 40 mM potassium phosphate, pH 6, and fixed with 0.5% OsO_4 (Polysciences, Inc.) in 40 mM potassium phosphate, pH 6, for only 3 min on ice. They were washed twice with ice cold H_2O, stained en bloc for 15 min in 1% uranyl acetate, resuspended in H_2O at room temperature and pelleted through 0.5% agarose at 36°C in a microfuge (Beckman Instruments, Inc., Palo Alto, CA). After gelling on ice, the pellet was cut axially and carried through a series of dehydration steps in ethanol and propylene oxide. The cortices were embedded in a 50:50 EPON-araldite mixture.

Analysis of Thin-sectioned Cortices: Silver-gray sections were cut from EPON-araldite blocks with a diamond knife on an ultramicrotome (Reichert, Austria), picked up on grids coated with formvar and carbon, and stained with uranyl acetate and lead citrate. The sections were examined on a JEOL 100 CX electron microscope operating at 80 kV. Only cortices that appeared intact and contained the cell membrane (identified by its association with hemocyanin) were examined. A low magnification photograph was taken of each cortex. Photographs taken of regions in each cortex were analyzed at a final magnification of × 60,000. Stereo microscopy was used to confirm that microfilaments made physical contact with the cell membrane. We found that if a filament touched the membrane when the membrane was viewed in exact profile (i.e., as unit membrane), stereo tilt would confirm the contact. For this reason, only microfilaments touching the membrane that was seen in profile or could be tilted to profile were counted as contacts. The amount of membrane seen in profile was measured with an electron graphics calculator (Numonics Corp., Landsdale, PA). The number of links per micrometer of membrane was averaged for each cortex. The undecorated and decorated cortices for each experiment were compared by the Student's *t* test assuming the null hypothesis.

RESULTS

Effect of Isolation Procedure on Cortical Structure

To assess the effect of S-1 on the organization and membrane attachment of cortical microfilaments, it was necessary to prepare cortices in which interactions of microfilaments with themselves and the cell membrane survived during lysis, isolation, and subsequent incubation. We have described previously a method for isolating regions of the cell surface containing patches and caps of Con A (5). This is procedure *A* in the current study. Starting with this method, we varied several parameters to determine conditions that minimized disruption of the cortices as viewed in the electron microscope. The results are summarized in Figs. 1 and 2 and Table I.

Cortices were most disrupted when either a low ionic strength lysis buffer was used followed by incubation in the absence of phalloidin (procedure *A*) or EGTA was deleted from the lysis buffer (procedure *D*). In procedure *A*, few microfilaments were observed and the membranes were more vesiculated as shown previously (5, 6). This was confirmed in the present study, in which we also observed few interactions between the cell membrane and microfilaments (Table I). In procedure *D*, microfilaments appeared to be much shorter than usual and displaced from the cell membrane with fewer membrane contacts (Table I and Fig. 2).

More intact cortices were obtained by adding phalloidin to incubation buffers used subsequent to cell lysis to prevent microfilament depolymerization (13) and by inclusion of EGTA in both the lysis and wash buffers (procedure *B*).

The most intact cortices were obtained with procedure *C* (Table I), in which the ionic strength of the lysis buffer was increased and phalloidin was added following lysis. Under these conditions, a large network of microfilaments remained associated with the cell membrane, the cell membrane underwent only a small degree of vesiculation, and many contacts between microfilaments and the cell membrane persisted (Table I and Fig. 1).

Stereo Microscopy Reveals Both Lateral and End-on Contacts between Microfilaments and the Cell Membrane

Two types of contacts between microfilaments and membranes were commonly seen, end-on and lateral. The term end-on describes the contact of either end of a microfilament with unit membrane. By lateral contacts we mean interactions that are mediated by short bridges that link the lateral aspects of a microfilament to the membrane.

End-on contacts occurred with the highest density in cortical meshworks as shown in Figs. 3 and 4. Few of these meshworks could be found in S-1 decorated preparations (see below). In those which remained the filaments contacted the membrane at their barbed ends (Fig. 5).

The most frequent type of contact between microfilaments and membranes was by lateral bridges measuring 15 ± 4.9 nm in length and 6.4 ± 3.5 nm in width ($n = 32$) (Figs. 6, 7, and 8). Lateral bridges were usually found on microfilaments which remained parallel to the membrane for some distance and in such cases many bridges could be found on a single filament. These bridges exhibited a periodicity along the microfilament of 36 ± 18 nm ($n = 23$).

Effect of S-1 on Interactions of Microfilaments with Themselves and the Cell Membrane

The largest number of contacts between microfilaments and membranes was found in cortices isolated by procedure *C* in which an average of 4.7 contacts were observed per

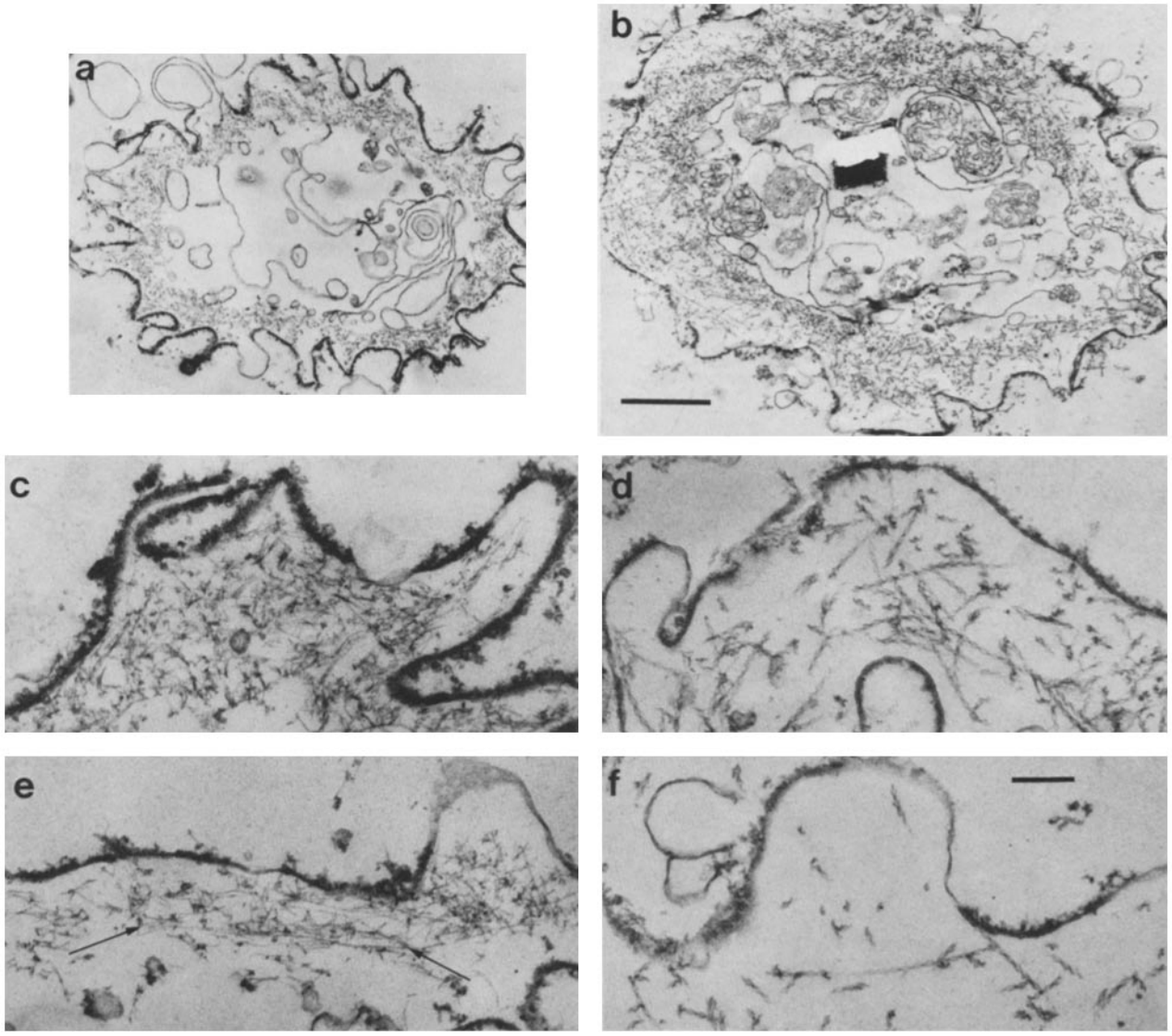


FIGURE 1 Electron micrographs of cortices prepared by procedure C before (*a*, *c*, and e) and after (*b*, *d*, and *f*) labeling with S-1. (*a* and *b*) At low magnification the isolated cortices are found to consist of a dense network of actin containing microfilaments underlying the residual cell membrane. Hemocyanin, used to label Con A on the cell surface, is seen in all of these micrographs. (*c* and *e*) In the absence of S-1, microfilaments are present as dense meshworks (c) or parallel arrays (*e*, arrows) closely associated with the cell membrane. (*d* and *f*) After labeling with S-1 the microfilaments are dispersed from each other and displaced from the cell membrane. Bars, 1.0 μm (*a* and *b*); 0.2 μm (*c–f*). × 13,000 (*a* and *b*); × 46,000 (*c–f*).

TABLE I

Effect of Isolation Procedure and S-1 on Linkage of Microfilaments to Cell Membrane in Cortices

	Procedure						
	S_1	Ph*	PO_4‡	EGTA	Links/μm Membrane	P Value	−S-1 Mean/ +S-1 Mean
					mean ± SD (n)		
A	−	−	−	+	0.79 ± 0.50 (10)	<0.28	1.39
	+	−	−	+	0.57 ± 0.49 (17)		
B	−	+	−	+	1.86 ± 0.57 (9)	<0.003	2.45
	+	+	−	+	0.76 ± 0.72 (9)		
C	−	+	+	+	4.69 ± 1.27 (17)	$<7 \times 10^{-7}$	3.19
	+	+	+	+	1.47 ± 0.93 (19)		
D	−	+	+	−	1.60 ± 0.41 (6)	<0.69	0.91
	+	+	+	−	1.76 ± 1.12 (6)		

N, number of cortices counted; *P* value is the statistical probability. The level of confidence is 0.01.
* Phalloidin.
‡ Potassium phosphate in lysis buffer.

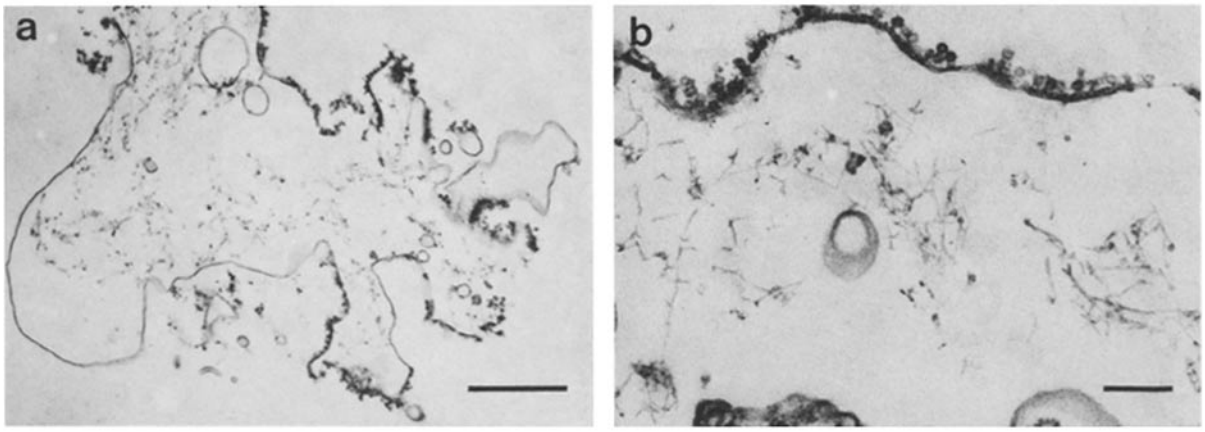


FIGURE 2 Electron micrographs of cortices prepared by procedure *D*. In the absence of EGTA, microfilaments in the isolated cortices appear shorter and more disorganized than those found in cortices isolated by procedure *C*. Few contacts between microfilaments and the cell membrane were observed in these preparations. Bars, 1.0 μm (*a*); 0.2 μm (*b*). × 13,000 (*a*); × 46,000 (*b*).

micrometer of unit membrane profile (Table I).

Addition of S-1 to such preparations at concentrations sufficient to uniformly decorate all the cortical actin filaments resulted in marked loss of contacts between microfilaments and membranes (Table I and Fig. 1). This effect was most dramatic in cortical preparations that contained large numbers of contacts before addition of S-1 as in procedure *C* (compare Fig. 1, *c* and *d* with *e* and *f*), less marked in preparations with fewer contacts (procedure *B*), and statistically insignificant in preparations with either few contacts before addition of S-1 (procedure *A*) or microfilaments that were disrupted by the isolation procedure (procedure *D*, Table I). In S-1 decorated cortices the microfilaments were displaced from the cell membrane (Fig. 1 *d*). Most of the contacts between microfilaments and membranes that persisted after S-1 decoration were of the end-on type shown in Figs. 3–5. Lateral bridges (Figs. 6–8) were almost completely absent in decorated preparations.

S-1 decoration also had an effect on the appearance of the microfilament network occurring within isolated cortices. Usually two changes were evident: first, decoration resulted in a decrease in density of the filament network (Fig. 1, *c–f*), and second, the small filament bundles containing between three and six filaments running parallel to the membrane that were common in undecorated cortices (Fig. 1 *e*), were absent after decoration.

DISCUSSION

Of the procedures tried, procedure *C* resulted in isolated cortices which were intact, with numerous contacts between microfilaments and the cell membrane. Crucial changes that resulted in this improvement were the presence of EGTA and 20 mM potassium phosphate in the lysis buffer and phalloidin in the incubation buffer.

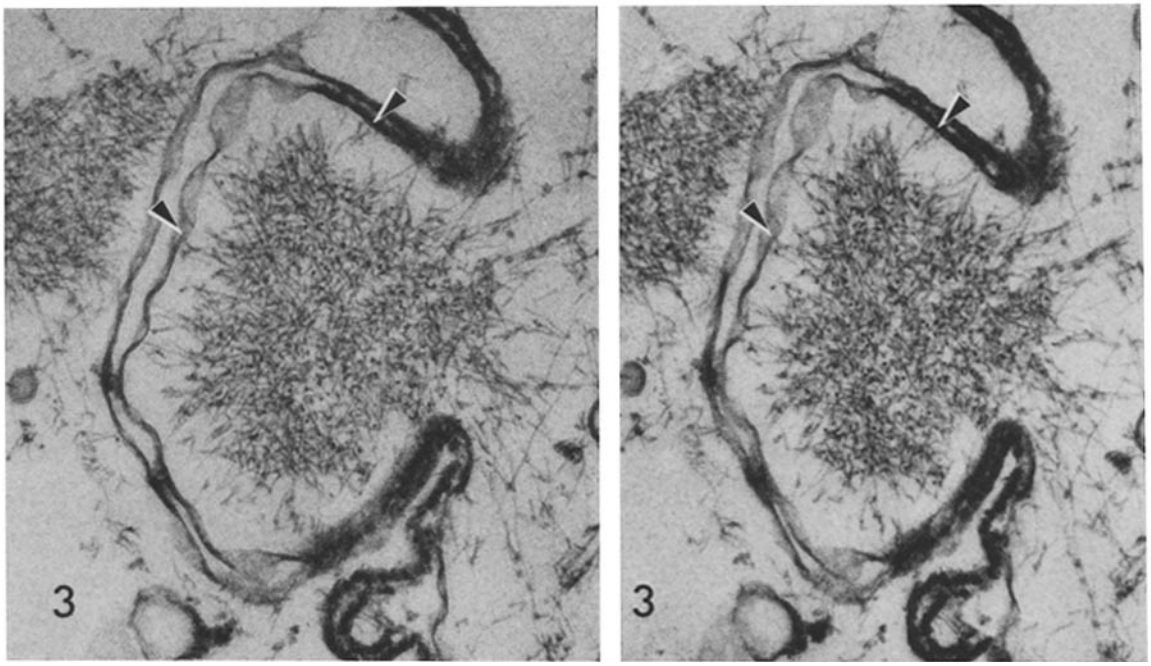
EGTA was necessary for preservation of microfilaments. In the absence of EGTA cortical microfilaments were fragmented. This observation is consistent with the results of Giffard et al. (7), who found that actin was lost from Triton X-100 cytoskeletons of *Dictyostelium* amebae in the presence of calcium but not EGTA. In both cases, fragmentation of microfilaments may have occurred due to the action of severin, a Ca^{2+} dependent microfilament severing protein (3, 26), which was activated in the presence of calcium released from the cells during Triton X-100 lysis.

Addition of potassium phosphate to the lysis buffer was important in preventing membrane vesiculation. Decreased vesiculation may have occurred for several reasons: first, the increased ionic strength could help preserve membrane associated microfilaments during lysis, which may prevent membrane vesiculation (5); second, the higher ionic strength may shield charge interactions on the cell membrane that drive vesiculation.

Inclusion of phalloidin in incubation buffers stabilized microfilaments against depolymerization (13). Presumably this accounts for the greater frequency of contacts between microfilaments and membranes seen in preparations containing phalloidin. Since phalloidin was added after cell lysis and isolation of cortices, it could not have induced polymerization of microfilaments from the pool of endogenous, free, unpolymerized actin which is removed during these steps.

Contacts between Microfilaments and Membranes

Using stabilized cortices isolated by procedure *C* we have identified two distinct types of contacts between microfilaments and the cell membrane, end-on and lateral. An important question is whether contacts observed in isolated *Dictyostelium* cortices are true mechanical links between microfilaments and the membrane involving specific molecular interactions or whether contacts are random, nonspecific associations induced by rearrangement of microfilaments during cell lysis. Several pieces of evidence argue that these are specific interactions. First, end-on contacts with the membrane tend to survive S-1 labelling better than lateral contacts suggesting that these two types of contact result from distinct mechanisms. Further, these contacts are almost always at the barbed end of the filament. It is unlikely that this would be true if these contacts were caused by filament rearrangement during lysis, which would result in filaments with random polarity relative to the membrane. Finally, filaments that make lateral contact with the membrane are not simply lying on the membrane surface. Lateral contacts are mediated by bridges with distinctive size, morphology, and periodicity, which argues that these represent specific molecular interactions. The periodicity of the bridges along the microfilament is similar to the periodicity of the microfilament. Although



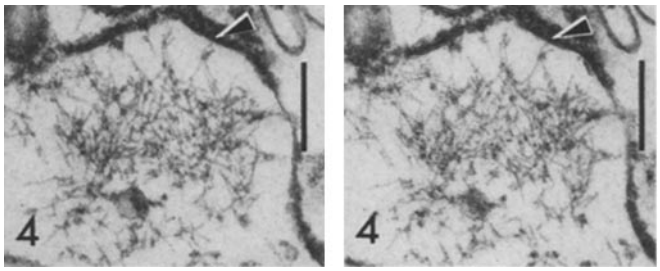


FIGURE 3 and 4 Stereo views of dense microfilament meshworks in the cell cortex prepared by procedure C. End-on contacts between microfilaments and the cell membrane occurred with highest frequency in these cortical meshworks (arrowheads). Bar, 0.2 μm. × 60,000.

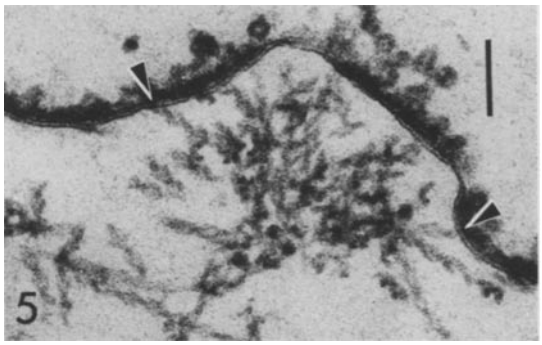


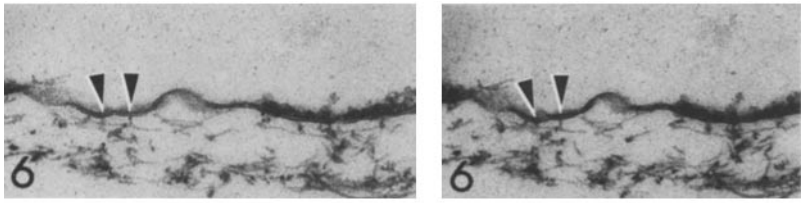
FIGURE 5 A microfilament meshwork after S-1 decoration of a cortex prepared by procedure C. There are fewer microfilament meshworks remaining in S-1 decorated preparations. However, in those which survive, microfilaments make end-on contacts at their barbed ends with the cell membrane (arrowheads). Bar, 1.0 μm. × 102,000.

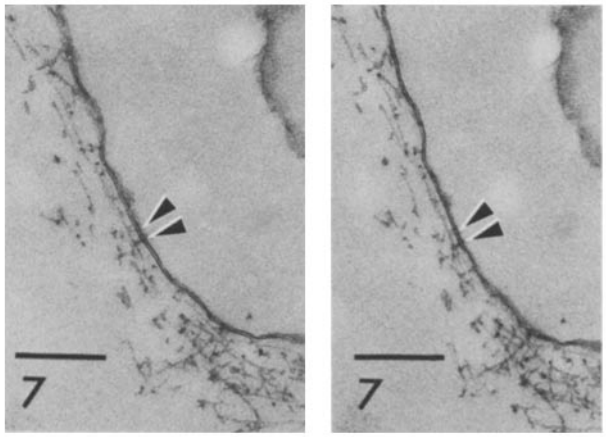
the filament may not be saturated with lateral bridges, the periodicity of the bridges suggests that the location of a bridge on the filament may be determined by the helical conformation of the microfilament.

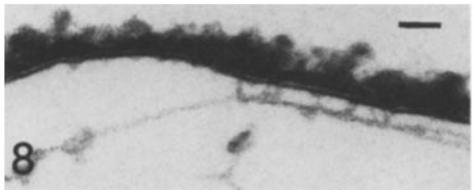
The two types of contacts observed are reminiscent of those found between microfilaments and the membrane in brush borders of intestinal epithelial cells (8, 15, 16). In brush borders, end-on contacts occur between the barbed ends of filaments in the microvillar core bundle and the inner tip of the microvillar membrane. Lateral bridges measuring 7 × 20 nm and exhibiting a periodicity of 33 nm (15, 16) link these same filaments to the parallel surface of the microvillar membrane. ATP treatment of the demembranated brush border extracts the lateral bridges from the core microfilaments of the microvilli (15). The lateral bridges extracted by ATP are composed of a 110-kdalton protein and calmodulin (9). Experiments are in progress to determine if the lateral bridges found in *Dictyostelium* are sensitive to ATP since they show great similarity in length, width, and periodicity to the lateral bridges of the brush border.

Microfilaments have been observed contacting the membrane by the end-on mode in other cell types such as *Acanthamoeba castellanii* (20) and peritoneal macrophages from mice (22). However, lateral bridges have not been widely reported. It is possible that with adoption of the techniques we employed for isolation and preservation of the *Dictyostelium* cortex, lateral bridges will be preserved and found to be common in other cell types.

Under optimal conditions, approximately 4.7 microfilament contacts were observed per micrometer of cell membrane in 60-nm thin sections or 78.3 contacts per square micrometer. Assuming that the cell is a sphere with a diameter of 10 micrometers, the total number of contacts between microfilaments and the cell membrane per cell would be







FIGURES 6 and 7 Stereo views of contacts between microfilaments and the cell membrane mediated by lateral bridges found in a cortex prepared by procedure C. Lateral bridges, ~15-nm long (arrowheads) were usually found on microfilaments running parallel with the cell membrane. In these cases more than one bridge could be found on a single filament. Bar, 0.2 μm.

FIGURE 8 Several lateral bridges linking a microfilament to the cell membrane viewed at higher magnification. The lateral bridges exhibit a periodicity of 36 ± 18 nm ($n = 23$) suggesting that their distribution is influenced by the helical twist of the microfilament. Bar, 36 nm. × 150,000.

24,600. By comparison, human erythrocytes that contain a dense, stable membrane bound cytoskeleton have a maximum of 200,000 contacts between actin oligomers and the membrane per cell (2).

Effect of S-1

S-1 labeling caused a threefold loss in the number of contacts between microfilaments and the cell membrane S-1 also caused redistribution of cortical microfilaments suggesting that linkage to the membrane is an important influence determining the location of cortical microfilaments and/or that cross-links between microfilaments are susceptible to S-1 disruption. This latter possibility is consistent with the findings that heavy meromyosin causes solation of actin gels and inhibits binding of high molecular weight actin-binding proteins to actin filaments (24).

This is the first quantitative demonstration of S-1 induced loss of contacts between microfilaments and cell membranes. Although our analysis was done with cortices isolated from *Dictyostelium* amebae, we believe that our results are general. For example, Hirokawa et al. (8) reported that S-1 treatment of brush borders isolated from intestinal epithelial cells under conditions that saturate the actin filaments with S-1 appears to detach microfilaments in the microvillar core bundles from the membrane and disrupts crossbridges between microfilaments in the terminal web.

Therefore, we believe our data demonstrate that caution is necessary when interpreting results of S-1 decoration experiments if amounts of S-1 sufficient to saturate the endogenous F-actin present are used to obtain information about the precise location and membrane attachment of microfilaments.

Does Disruption of Contacts Between Microfilaments and Membranes by S-1 Have Biological Significance?

Certain types of motility that are associated with the cell surface, such as capping (4, 21) and phagocytosis (23), are correlated with the concentration of cellular myosin in a small region of the cell cortex. The concentration of myosin in such regions may be sufficient to saturate local actin filaments resulting in their detachment from the cell membrane. During a sliding filament type of interaction between myosin and actin filaments, myosin might act to detach microfilaments from the membrane, thereby facilitating sliding, while more distal regions of the same microfilaments retain membrane contact. Also, myosin may be recycled from bipolar filaments that were assembled for contraction to the soluble form that could act to detach microfilaments more efficiently from the membrane. The ability of S-1 to compete endogenous membrane links from a microfilament as reported here gives evidence to substantiate this hypothesis. However, a definitive demonstration will require experiments to measure competition for binding to actin filaments between myosin and linker proteins isolated from the membrane.

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Note Added in Proof: While this paper was in press, an article appeared by Goodloe-Holland and Luna (1984, *J. Cell Biol.*, 99:71–78) demonstrating that S-1 inhibits the binding of F-actin coated beads to vesicles derived from the plasma membrane of *D. discoideum*.

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