ISOLATION OF CONCANAVALIN A CAPS DURING VARIOUS STAGES OF FORMATION AND THEIR ASSOCIATION WITH ACTIN AND MYOSIN

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ABSTRACT

Regions of plasma membrane of *Dictyostelium discoideum* amoebae that contain concanavalin A (Con A)-receptor complexes are more resistant to disruption by Triton X-100. This resistance makes possible the isolation of Con A-associated membrane fragments in sufficient quantity and homogeneity to permit the direct biochemical and ultrastructural study of receptor-cytoskeletal interactions across the cell membrane. After specific binding of Con A to the cell surface, a large amount of the cell's actin and myosin copurifies with the plasma membrane fragments. Myosin is more loosely bound to the isolated membranes than actin and is efficiently removed by treating membranes with ATP and low ionic strength. If cells are not lysed immediately after lectin binding, all of the Con A that is bound to the cell surface is swept into a cap in a process requiring metabolic energy. When cells are lysed at different stages of cap formation, the amount of actin and myosin that copurifies with the isolated membranes remains the same. Thick and thin filaments that are attached to the protoplasmic surface of the isolated membranes underlie lectin-receptor complexes during all stages of cap formation. Once the cap is complete, the amount of actin and myosin that copurifies with the isolated caps decreases. Therefore, cortical actomyosin that is tightly bound to the plasma membrane is concentrated into the cap along with the Con A-receptor complexes. These results suggest that the ATP-dependent sliding of membrane-associated actin and myosin filaments is responsible for the accumulation of Con A-receptor complexes into a cap on the cell surface.

KEY WORDS *Dictyostelium discoideum* · amoeboid movement · cell surface receptor mobility · transmembrane linkages

A model system that has been extensively used to examine the possible mechanism by which ligand binding affects cell behavior is the induction of patching and capping of cell surface molecules by antibodies and lectins. There is considerable agreement that both the actin- and tubulin-containing cytoskeletons are involved in the changes of cell surface receptor mobility that are induced by ligand binding, (1, 2, 3, 7, 8, 9, 10, 16, 18, 19, 21).

To determine the mechanism by which the actin cytoskeleton influences the formation, subsequent topography, and endocytosis of the ligand-surface-receptor complex, I have chosen to study the interaction between the amoeboid stage of *Dictyostelium discoideum* and various lectins. This cell is useful for such a study because it does not contain an extensive microtubule cytoskeleton at this stage of development, and the properties of the actin cytoskeleton have been characterized (5).

In this paper, I describe initial progress made on the first direct characterization of the interaction between actin and myosin and concanavalin A (Con A)-associated fragments of plasma membrane that have been isolated before, during, and

after cap formation.

MATERIALS AND METHODS

Dictyostelium discoideum, strain AX-3, was grown in axenic culture (13). Amoebae were harvested at a density of 10^7/ml, washed in 20 mM Na-K phosphate, pH 6.2, (Buffer 1) at 22°C, and resuspended to the same density in Buffer 1. Either Con A or fluorescein-labeled Con A (F Con A) was added to a final concentration between 20 and 75 μg/ml, and the cells were swirled on an orbital shaker in Buffer 1 at 22°C. Cells were periodically withdrawn, plated on clean glass slides to observe cell motility or fixed in isotonic 1% paraformaldehyde, and monitored for cap formation by visualizing the cell surface topography of F con A.

To isolate plasma membrane ghosts and caps that are free of contamination by cytoplasmic organelles, the cytoplasmic gel in which the organelles are suspended must be solated. We have previously observed that actin-containing gels which form in warmed extracts of *Dictyostelium* amoebae are solated at low ionic strength in the absence of Ca^{2+} at pH >7.0 (5). To apply this observation here, cells were lysed in 1 mM EGTA, 5 mM Tris, pH 7.6 (Buffer 2). This treatment results in solation of the cytoplasmic gel which then permits the organelles to be squeezed out of the perforated membrane ghosts and caps by centrifugation. If the cells are lysed in buffers of lower pH or higher ionic strength, the material isolated after detergent lysis and centrifugation contains the cytoplasmic gel and its suspended organelles surrounded by plasma membrane (12, 17).

Cells that were in the process of forming caps after a 7-, 12-, 30-, or 45-min incubation with Con A were washed and resuspended to a density of 5×10^7/ml in ice-cold Buffer 2. The cell suspension was then made 0.2% in Triton X-100, swirled for 1 min, and centrifuged at 480 *g* for 20 s at 4°C. The pellet, which contained whole cells, nuclei, and cytoplasmic particles, was discarded, and the supernate was centrifuged at 2,000 *g* for 4 min at 4°C. This second pellet (P2) contained two layers. The loose white flocculent top layer contained the plasma membrane caps while the tightly packed bottom layer contained residual cytoplasmic debris. The two layers were separated by tipping the tube, allowing the top layer to slide aside, and removing the bottom layer by vacuum aspiration. The top layer was then washed at least once in either Buffer 1 or 2 at 2,000 *g* for 4 min, and the procedure outlined above for separating the two layers in the pellet was repeated until the final pellet was uniformly white and contained only plasma membrane caps (final uniform pellet) as judged by light and electron microscopy.

To prepare plasma membrane ghosts, cells were collected at 480 *g* for 2 min after a 2-min incubation with Con A, resuspended in ice-cold Buffer 2, and immediately lysed with 0.2% Triton. The ghosts were isolated by using a protocol essentially the same as the one used above for caps. However, because the ghosts were less dense than caps, subsequent washes of the top layer of P2 were carried out at 7,700 *g* for 4 min to insure efficient sedimentation of all ghosts. The final uniform pellet contained only plasma membrane ghosts as judged by light and electron microscopy.

Plasma membrane ghosts and caps were further purified by centrifugation (SW27 rotor [Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.], 50,000 *g* for 40 min at 4°C) on 20–55% sucrose gradients containing 20 mM sodium phosphate, pH 6.8, in either the presence or absence of 1 mM MgATP. Because the isolated ghosts and caps had morphologies that were easily recognized, the yield of each was calculated by counting the number of ghosts or caps in the final preparation on a hemocytometer and comparing these counts to the starting number of intact cells or intact cells with caps, respectively (Table I).

Membranes were also prepared from *Dictyostelium* amoebae in the absence of Con A as previously described by Spudich (20).

The amount of actin, myosin, and Con A that was associated with the various membrane preparations was estimated by densitometry of Coomassie Blue-stained SDS-polyacrylamide gels in a range of protein concentration that was directly proportional to the absorbance.

Three-times crystallized Con A was purchased from Sigma Chemical Co., St. Louis, Mo. Tetrameric Con A was prepared by dissolving the lyophilized powder in 1 M NaCl, 0.25 mM $CaCl_2$, 0.25 mM $MnCl_2$, and 40 mM potassium phosphate, pH 7.0, overnight at 4°C. F Con A was prepared by a direct mixing method modified by

TABLE I

Con A and Time Dependence of Membrane Association

Time of incubation before lysis	Form of membrane	Percent of component in whole cell recovered with membrane		
		Myosin	Actin	Con A
0 (no Con A added)	Vesicles	2	8	0
7	Ghosts and ¼ capped	62	22	33
12	½ capped	55	22	33
30	Complete cap	33	18	33

Membranes were prepared from cells that were lysed after incubation with Con A for the time indicated. All membrane samples were centrifuged on sucrose gradients in the absence of ATP. Preparations of completed caps used here were isolated from cells that were >¾ capped but before extensive endocytosis occurred. The percent of each component (myosin, actin, and Con A) available in the whole cell that was recovered with the membrane (% yield of component ÷ yield of membrane) was calculated by using percent yields of 10.8% for caps, 18% for plasma membrane ghosts, and 12% for membranes isolated in the absence of Con A (20).

the procedure of Mallucci (15). Samples were prepared for electron microscopy by fixation at 4°C for 20 min in 2% glutaraldehyde in Buffer 1 at pH 7.0 (intact cells were fixed at 22° for 30 min). Samples were postfixed in 1% OsO_4 in Buffer 1 at pH 6.0 for 10 min and stained for 30 min at 4°C in 1% aqueous uranyl acetate. Samples were washed twice in water, embedded in gelatin, dehydrated in alcohol, and embedded in Epon 812. To visualize Con A in the electron microscope, both intact cells and plasma membrane preparations were incubated with *Busycon* hemocyanin for 10 min in Buffer 1 and then washed in Buffer 1 to remove unbound hemocyanin. Actin filaments were identified in the electron microscope by labeling with heavy meromyosin (HMM) in the absence of ATP. HMM was prepared from rabbit skeletal muscle myosin (14).

RESULTS

Upon initial binding of tetrameric Con A, cytoplasmic streaming in the amoebae is depressed. Con A remains uniformly distributed on the cell surface for the first 2–5 min after binding. If the cells are lysed within this 5-min interval, ghosts are isolated by the above procedure. These ghosts are approximately the size of the original cell, have a uniform distribution of Con A on the extracellular surface (ES), and are free of contamination by cytoplasmic organelles (Fig. 1). If Con A that is bound to the plasma membrane is labeled with hemocyanin either before or after cell lysis, the same sidedness of labeling is ob-

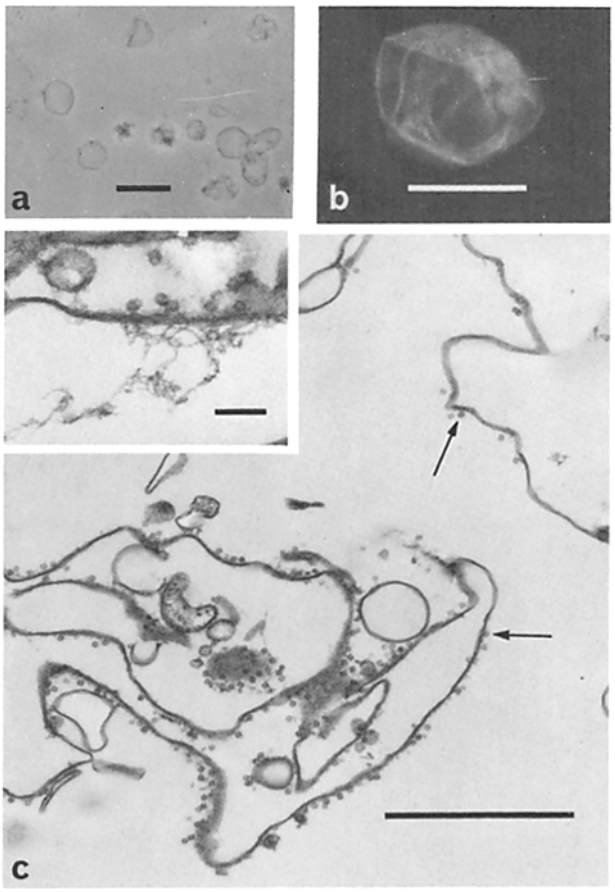

FIGURE 1 Micrographs of ghosts prepared from cells after a 2-min incubation with Con A. (*a*) Phase micrograph of ghosts in the top white layer of pellet P2. Bar, 20 μm. (*b*) Fluorescence micrograph of a ghost that has been stabilized with F Con A, from the final uniform pellet. Bar, 10 μm. (*c*) Electron micrograph of a thin section of the final uniform pellet of ghosts. Con A bound to the plasma membrane has been labeled with hemocyanin (arrows). The *inset* shows the association of 6-nm filaments with the PS of the plasma membrane opposite several Con A-hemocyanin complexes on the ES. Bar, 1 μm. *Inset* bar, 0.1 μm.

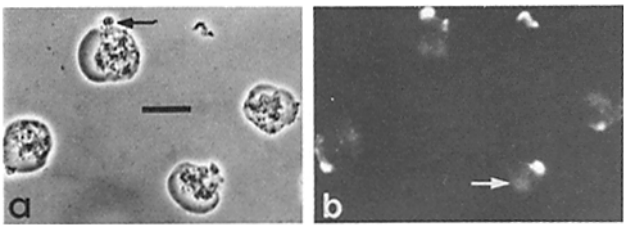

FIGURE 2*a* and *b* (*a*) Phase, and (*b*) fluorescence light micrographs of cells capped with Con A. Dense protrusions (Fig. 2*a*, arrow) correspond to the position of the F Con A caps (*b*). Endocytosis of F Con A can be visualized as fluorescence inside the cell (Fig. 2*b*, arrow). Bar, 20 μm.

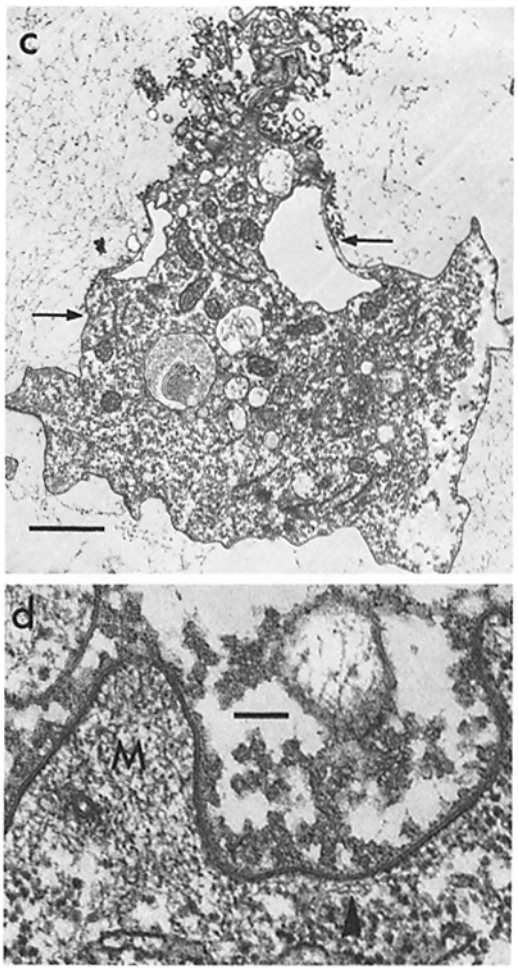

FIGURE 2*c* and *d* (*c*) Electron micrograph of an intact cell capped with Con A. Con A was labeled with hemocyanin. The cap occupies the portion of the cell surface above the arrows. Bar, 1 μm. (*d*) Higher magnification of an intact cell in the region of the cap. Thin filaments (arrowhead) and filament meshworks (*M*) are present in the cortex under the Con A-hemocyanin complexes. Bar, 0.1 μm.

served, thus indicating that the asymmetric sidedness of the Con A-receptor complex is maintained during plasma membrane isolation. A few 6-nm filaments are usually associated with the protoplasmic surface (PS) of the plasma membrane (Fig. 1, *inset*).

If the cells are incubated with Con A at 22°C in Buffer 1 for >5 min, the bound Con A moves into a cap on >80% of the cells in 30 min (Fig. 2). Cap formation but not patch formation is inhibited by 15 mM NaN_3 and by cytochalasin B (50% inhibition of the extent of capping by 2 μm cytochalasin B). All of the Con A bound to the cell surface is concentrated into the cap (Fig. 2*b*). Once the cap has formed, vigorous cytoplasmic streaming and pseudopod activity resume. Pseudopods formed at this stage most often extend from the side of the cell opposite the cap.

The binding of Con A to *Dictyostelium* amoebae is specific because it is competitively inhibited by alpha-methyl mannoside (6). Treatment of cells with 0.2% Triton X-100 in the absence of tetrameric Con A or the presence of tetrameric Con A plus alpha-methyl mannoside results in vesiculation and dissolution of the plasma membrane. However, in the presence of surface-bound tetrameric Con A, the plasma membrane is perforated by the detergent but does not vesiculate and dissolve. Furthermore, during cap formation, the agglutination of cells by surface-bound Con A decreases and titration of capped cells with additional tetrameric Con A does not result in further agglutination, indicating that the Con A receptors have been swept into the cap and are no longer available for the agglutination reaction. Because the specific binding of tetrameric Con A to the cell surface is essential to stabilize the plasma membrane against Triton X-100, it follows that lysis of capped cells with detergent results in the disruption of the plasma membrane, except for that

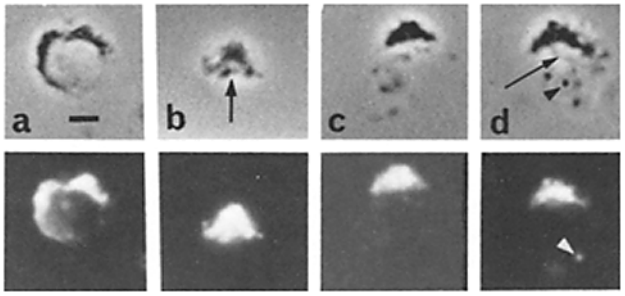

FIGURE 3 Phase (top) and fluorescence (bottom) light micrographs of isolated caps. Pair *a*, cells lysed when 1/4 capped; pair *b*, lysed when fully capped; pairs *c* and *d*, caps from cells that have capped and begun endocytosis of Con A. The arrow in Fig. 3*b* points to the concave side of the isolated cap corresponding to the PS before lysis. The arrow in Fig. 3*d* points to a fibril on the concave side of the cap, and the arrowheads point to vesicles that appear to be associated with branches of the fibril. Note fluorescence of vesicle (Fig. 3*d*, bottom panel). Bar, 5 μm.

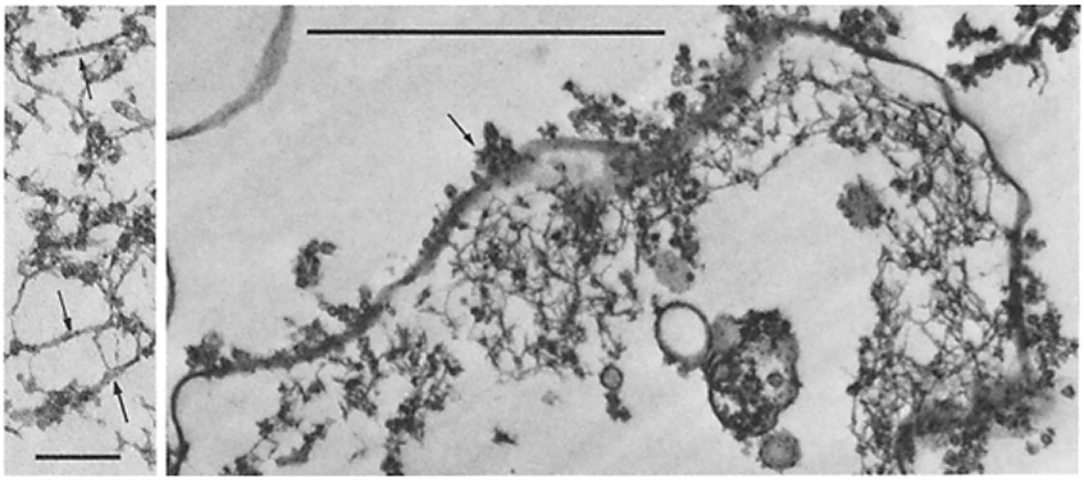

FIGURE 4 Electron micrograph of an isolated cap. The arrow in the main figure, right, denotes the ES with attached Con A-hemocyanin complexes. Thin filaments, which are found exclusively on the PS, appear to make numerous attachments with the plasma membrane. The *inset* (left) shows thick filaments (arrows) at higher magnification. Bar, 1 μm. *Inset* bar, 0.2 μm.

which is in immediate association with the cap itself (Fig. 3).

Because the movement of Con A on the ES of the suspended amoebae was relatively synchronous, it was possible to isolate the cap at different stages of formation by incubating cells with Con A for various durations before lysis. Caps isolated from cells that were 1/4 capped (cells lysed after a 7-min incubation with Con A), fully capped (30 min), and fully capped and endocytic (45 min) are shown in Fig. 3.

The isolated caps in the final uniform pellet are identical in appearance to the phase-dense caps that are present on intact capped cells (compare Figs. 2 and 3). The organization of the cap is maintained in the isolated state with a convex side corresponding to the ES and a concave side corresponding to the PS before lysis (Fig. 3*b*). Caps prepared after endocytosis has begun have cytoplasmic fibrils extending down from the concave side (Fig. 3*d*). Vesicles appear to be attached to these fibrils, and they undergo rapid Brownian motion in association with the fibrils. These vesicles are fluorescent, indicating that they contain F Con A and hence might be endocytic in origin. Electron microscopy of the isolated caps reveals extensive Con A-hemocyanin complexes and thick and thin filaments attached to opposite sides of the same section of unit membrane (Fig. 4). The thin filaments range

in diameter from 3.6 to 7.3 nm with a mean diameter of 5.9 ± 1.4 nm. The thin filaments label with HMM (not shown), thus indicating that they are actin-containing filaments. The thick filaments have average dimensions 0.2 μm long × 13 nm wide (arrows, Fig. 4, left *inset*.) and do not label with HMM. Numerous contacts are made between the actin-containing filaments and the plasma membrane.

The result of centrifuging final uniform pellets of ghosts and caps on sucrose gradients is shown in Fig. 5. The ghosts and caps band sharply at densities of ~1.16 and 1.20 g per ml, respectively. Actin and myosin are not removed from the membrane in the gradients, while actin and myosin isolated from *Dictyostelium* amoebae (4, 5) do not sediment on control gradients with the same density as the membranes. Roughly 60% of the Con A that was initially bound to the membrane is lost from the membrane during centrifugation on sucrose gradients.

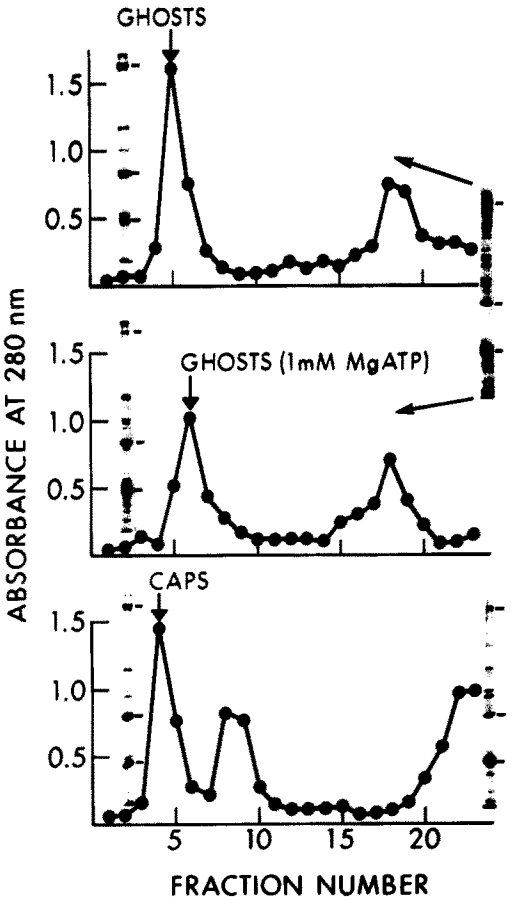

FIGURE 5 Sucrose gradient profiles of ghosts and caps. SDS-gels show the material applied to the gradients (right) and the peak fractions (left) that contain ghosts or caps. Purified actin and myosin did not enter the gradient. Bars indicate the major components of the membrane preparations which are (top to bottom) myosin, actin, and Con A.

Centrifugation of ghosts on gradients containing 1 mM MgATP released 77% of the myosin and 5% of the actin from the ghosts. Subsequent incubation of the myosin-depleted ghosts in 1 mM EDTA at pH 7.5 rapidly removed 82% of the myosin and 62% of the actin that remained associated with the membrane after ATP treatment. These results indicate that the bulk of the myosin and actin is associated with the membrane as peripheral components.

Concomitant with the removal of actin and myosin, the ghosts became unstable and underwent rapid vesiculation (50% vesiculation in 20 min at 22°C). Control ghosts maintained at 22°C in 1 mM potassium phosphate at pH 7.0 in the absence of ATP were not depleted of actin and myosin and did not vesiculate.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of isolated plasma membrane and Con A ghosts and caps is shown in Fig. 6. The most remarkable feature of these gel patterns is the large amount of Con A, actin, and myosin that remains bound to the Con A membrane complex after repeated washing and gradient centrifugation. The identity of the actin and myosin was established as follows: (*a*) The actin and myosinlike components comigrated with purified *Dictyostelium* actin and myosin on SDS-PAGE. (*b*) Actinlike filaments that were associated with the isolated membranes labeled with HMM. (*c*) The myosinlike component that was released from the membrane by MgATP had a MgATPase activity that was activated by 4- to 6-fold with purified *Dictyostelium* actin.

Three additional major components were consistently present on SDS-PAGE of the isolated membranes. These were the 75,000–58,000-, and 52,000-dalton components (Fig. 6). The identity and functional significance of these components are not currently known.

The amount of actin and myosin that is associated with the isolated membranes from *Dictyostelium* depends on the method of isolation and the length of time that the cells have been associated with Con A (Fig. 6 and Table I). Membranes that have been isolated in the absence of Con A by the method of Spudich (20) contain small amounts of associated actin and

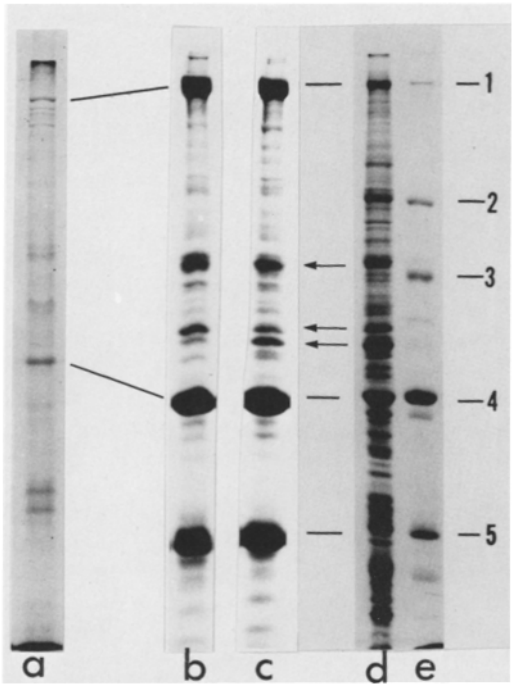

FIGURE 6 SDS-PAGE of membrane preparations centrifuged on sucrose gradients in the absence of ATP. (*a*) Membrane prepared in the absence of Con A by the method of Spudich (20); (*b*) plasma membrane ghosts prepared from cells that were lysed with Triton after a 5-min incubation with Con A (15 μg protein); (*c*) caps prepared from cells that were lysed with Triton after a 15-min incubation with Con A (18 μg). The arrows point to three unidentified bands that measure (top to bottom) 75,000, 58,000, and 52,000 daltons. (*d*) Whole cells with bound Con A (20 μg); (*e*) standards: *1*, *Dictyostelium* myosin, 225,000 daltons; *2*, Phosphorylase A, 94,000 daltons; *3*, Bovine serum albumin, 68,000 daltons; *4*, *Dictyostelium* actin, 43,000 daltons; *5*, Con A, 27,000 daltons.

myosin. However, membranes that are isolated by the Triton procedure described above from cells that have been incubated with Con A contain much greater amounts of actin and myosin. When the membranes are isolated from cells at different stages of cap formation, the amount of membrane-associated actin and myosin remains roughly the same. However, when caps are isolated by the Triton procedure from fully capped cells, the amount of membrane-associated actin and myosin is smaller. This decrease is believed to reflect the actual dissociation of actin and myosin from the cytoplasmic surface of the membrane during the final stages of cap formation. It is not caused by differences in the recovery of the Con A membrane complex from partially capped vs. fully capped cells because the same percent of the total Con A that was originally available on the intact cell is recovered in both (Table I).

DISCUSSION

The electrophoresis and electron microscope results presented above indicate that binding Con A to the external surface of *Dictyostelium* amoebae results in an increase in either the amount of actin and myosin that is associated with the cytoplasmic surface of the plasma membrane or the stability of this association. The amount of actin and myosin that remains bound to the isolated Con A-membrane complex remains roughly the same when membranes are prepared from cells during different stages of cap formation. However, when Con A-membrane complexes are isolated from cells that have finished capping, the amount of actin and myosin that remains bound to the membrane is less. Furthermore, after a tight phase-dense cap has formed, the cell resumes cytoplasmic streaming and pseudopod activity. This suggests that myosin, as it leaves the cap, may be recruited to fulfill these added motile functions.

The mechanism by which actin and myosin remain bound to the membrane is unknown. That these components are not removed from the membrane by repeated washing and centrifugation on sucrose gradients indicates that they are tightly bound. However, substantial amounts of myosin are removed by MgATP, thus indicating that this myosin fraction may be bound to the membrane through its association with actin (20). Subsequent low ionic strength treatment is required to remove the remaining myosin, indicating either that this fraction is associated with the membrane by a mechanism different from actin binding or that the cortical actomyosin forms such a dense tangle that low ionic strength depolymerization of actin is required before more myosin can be removed.

Unlike myosin, large amounts of actin remain bound to the plasma membrane after extraction with both MgATP and low ionic strength. This suggests either that actin is more tightly bound than myosin, perhaps forming the primary link for the contractile proteins to the membrane, and/or that actin remains in a polymerized form that is protected from complete depolymerization and MgATP dissociation by being cross-linked to other peripheral components besides

myosin (5).

What is the role of actin and myosin associated with regions of membrane that contain bound ligand? The results presented in this paper indicate that the contractile proteins are directly linked to regions of membrane that contain Con A receptors and that actin and myosin remain in tight association with the membrane during cap formation. It is hypothesized that the ATP-dependent sliding of actin and myosin filaments that are bound to the ligand-receptor complexes would reduce the volume of the cortical actomyosin complex (i.e., contraction) and hence would reduce the percent of the protoplasmic surface of the plasma membrane that is covered by the contractile proteins. As a result, the linked receptors would be pulled into a cap on the external surface above the contracted actomyosin. The actin and myosin initially recruited into the cortex in response to ligand binding would, therefore, be sufficient to move the occupied receptors into caps, and the release of this myosin and actin would not occur until the cap was completed.

Alternate explanations that ascribe an indirect role for actomyosin in capping are based on the observation that recruitment of actomyosin into cortical protrusions can precede capping and does not inhibit subsequent capping upon addition of Con A (1). However, the results presented here argue that substantial amounts of actin and myosin remain available in the cytoplasm after cap formation to account for additional motile phenomena, including secondary cap formation.

The precise nature of the interaction between the actin cytoskeleton and the Con A-receptor complexes described here remains unknown. Our electron microscopy results demonstrate that the lipid bilayer appearance of the plasma membrane survives detergent extraction and that such regions are associated with cytoplasmic actin and myosin. Hence, the conclusion that the association between cell surface receptors and the actin cytoskeleton is solely dependent on a protein-protein interaction because it occurs in the presence of detergent must be viewed with caution, especially when the association is documented by cosedimentation experiments alone (11). Further studies employing nearest-neighbor analysis with cleavable cross-linking agents and the isolation of the components of the purified plasma membrane ghosts and caps are in progress to resolve this question.

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