Myosin II Is Involved in Capping and Uroid Formation in the Human Pathogen *Entamoeba histolytica*

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The redistribution and capping of surface receptors on the human pathogen *Entamoeba histolytica* was observed in the presence of concanavalin A (ConA). Capping was correlated with plasma membrane folding towards the rear of the amoeba and with uroid formation. The uroid is thought to play a role in the escape of amoebae from the host immune response. To localize myosin II during capping, amoebae were incubated in the presence of ConA and then analyzed by microscopy. Myosin II was three times more concentrated within the uroid compared with the rest of the cell, suggesting that the release of caps may depend upon mechanical contraction driven by myosin II activity. The use of drugs that disrupt cytoskeletal structure or that inhibit myosin heavy chain phosphorylation demonstrated that inhibition of capping prevents uroid formation. Biochemical analysis allowed the identification of two ConA receptors which have been previously described as major pathogenic antigens of this parasite: the 96-kDa antigen, which carries alcohol dehydrogenase 2 activity and binds extracellular matrix proteins, and the Gal-GalNAc-inhibitable surface lectin, which is involved in amoeba-cell interactions and in the degradation of complement particles attached to the parasite.

The parasitic protozoan *Entamoeba histolytica* resides in the lumen of the human large intestine and has the capacity to invade the intestinal epithelium. During invasion, amoebae phagocytose human cells, leading to the clinical symptoms of invasive amoebiasis, which is characterized by the formation of colonic ulcers. In some cases, the parasite may also spread through blood vessels and colonize organs such as the liver, where abscesses can develop (25). This invasive parasite moves through the intestinal epithelium as a polarized cell, forming a large frontal pseudopod and a posterior appendix, the latter commonly referred to as a uroid (15). During locomotion, surface-bound particles are translocated rearwards to the uroid (2), in which capped ligands accumulate. The uroid then detaches from the parasite by a yet-undefined mechanism. A correlation between motility and capping of particles has been suggested for *E. histolytica* (8) and also for other types of locomoting cells (6), including polymorphonuclear leukocytes.

During the course of the disease, *E. histolytica* encounters immune defense mechanisms. Parasite infection triggers the production of anti-amoeba antibodies and the activity of complement. A phenotype of resistance to complement-mediated lysis, however, has been observed in trophozoites freshly isolated from a liver abscess (17). Moreover, this resistance phenotype can be induced in vitro by exposure of pathogenic amoebae to sera of noninfected humans (7). The resistance of *E. histolytica* to complement-mediated cytolysis can be in part explained by the capping of proteins of the complement system. These proteins either could be degraded after endocytosis or could be translocated towards the uroid along the plasma membrane and then eliminated with the uropodial structure itself.

Capping at the surface of cells can be viewed as a two-step process: first, after exposure to ligands, receptors are recruited to particular sites on the cell surface and form patches; second,

these patches move in a rearward direction, and the local concentration of receptor-ligand complexes at the posterior part of the moving cells leads to the cap formation (3). Capping has been studied in lymphocytes (4) and in *Dictyostelium discoideum* (22). From these studies, it is now generally agreed that the cytoskeleton is involved in the movement of surface molecules forward to the cap structure.

In pathogenic *E. histolytica*, cap formation and extrusion of caps into the external medium have been observed following exposure to antibodies (7), complement (1), or glycoproteins such as concanavalin A (ConA) (31). The mechanism by which *E. histolytica* accomplishes capping has not yet been elucidated. However, the cytoskeleton of the amoeba seems to participate in this phenomenon, since the resistance to complement-mediated cytolysis is diminished when the parasite is incubated in the presence of cytochalasin B, a drug that disrupts actin filaments (17). In addition, actin and the major actin cross-linking protein, myosin II, are concentrated in the rear part of moving trophozoites (2, 24). This leads to the hypothesis that the cytoskeleton may have a role in the formation of the uroid. In this study, we analyzed the cellular localization of myosin II during capping, as well as the role of the *E. histolytica* cytoskeleton in capping and uroid formation. In addition, two receptors undergoing capping have been identified.

MATERIALS AND METHODS

Strains and culture condition. Pathogenic *E. histolytica* (strain HM1:IMSS) was kindly provided by David Mirelman, Weizman Institute of Science, Rehovot, Israel. The trophozoites were cultivated axenically in TYI-S-33 medium (10) at 35°C.

Induction of capping and isolation of caps. Caps were prepared by published methods (13). Briefly, parasites were cultivated for 48 h, washed with phosphate-buffered saline (PBS), and resuspended at 5×10^6 cells per ml in PBS. ConA (grade VI; Sigma Chemical Company) was added at 20 µg/ml, and trophozoites were incubated at 4°C for 1 h. To induce cap formation and spontaneous release, trophozoites were incubated at 37°C for 10 min and then rapidly shaken. Trophozoites and cellular debris were eliminated by two successive centrifugations at $300 \times g$ for 5 min. Caps were pelleted at $30,000 \times g$ for 30 min at 4°C. The pellet was resuspended in 50 µl of PBS containing 2 mM phenylmethylsulfonyl fluoride, 6 µM leupeptin, and 1 mM N-ethylmaleimide.

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Electrophoresis of proteins and immunoprecipitation. Protein concentrations were measured by the Bio-Rad protein assay with bovine serum albumin (BSA) as a standard. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20). Gels were stained either with silver or with Coomassie blue. For immunoprecipitation, the cap extract (50 μ l from 5×10^6 amoebae) was precleared by incubation for 1 h in 50 mM octyl- β -glucopyranoside (prepared in PBS)-2 mM phenylmethylsulfonyl fluoride-6 μ M leupeptin-1 mM *N*-ethylmaleimide in the presence of 15 μ l of protein A-Sepharose beads (Pharmacia Biotech). After centrifugation at $14,000 \times g$ for 30 s, the supernatant was collected and subjected to immunoprecipitation. Anti-ConA polyclonal antibody (Sigma) or rabbit serum was covalently bound to protein A-Sepharose CL4B beads (Pharmacia) by published methods (18). Six microliters of the anti-ConA-bead suspension (1.6 μ g of anti-ConA antibody was bound per μ l of beads) or serum-bead suspension was added to the protein preparation, and the volume was adjusted to 200 μ l with PBS containing detergent and protease inhibitors. The mixture was incubated for 2.5 h at 4°C with slow agitation. The beads were washed four times with PBS buffer containing protease inhibitors and octyl- β -glucopyranoside by centrifugation at $14,000 \times g$ for 30 s and were resuspended in 15 μ l of the same buffer. Five microliters of 4 $\times$ loading buffer was added, the sample was boiled for 3 min and centrifuged at $14,000 \times g$ for 30 s, and the entire supernatant was used for SDS-PAGE analysis. For purification of the 96-kDa protein, uroids released from 2×10^8 amoebae were used for immunoprecipitation. For identification, the protein was eluted from the polyacrylamide gel and digested by the endoprotease LysC. Peptides were fractionated by high-pressure liquid chromatography, and three of them were sequenced by sequential Edman degradation reactions.

Immunoblots. Cytoskeletal proteins from isolated caps or from ConA-immunoprecipitated complexes were resolved by SDS-7.5% PAGE as described by Laemmli (20). Proteins were transferred onto nitrocellulose membranes by the method of Towbin et al. (30). The transferred proteins were saturated with 5% nonfat milk for 2 h at room temperature or overnight at 4°C and then incubated for 12 h at 4°C, or for 1 h at room temperature with various antibodies diluted in PBS-5% nonfat milk. The following antibodies and dilutions were used: anti-ConA (Sigma), 1:500; anti-170-kDa adhesin monoclonal antibody 3F4 (kindly supplied by W. Petri, University of Virginia, Charlottesville) (14 μ g/ml), 1:100; peroxidase-conjugated goat anti-rabbit immunoglobulin (Nordic Immunology), 1:25,000; and peroxidase-conjugated sheep anti-mouse immunoglobulin (Nordic Immunology), 1:8,000. The last two sera were used as secondary antibodies to detect antigen-antibody complexes. Finally, the immunoblot was treated with the ECL Western blotting (immunoblotting) detection reagent (Amersham International) and exposed on Kodak (X-OMAT) X-ray film.

ConA overlay of capped proteins. Proteins immunoprecipitated by ConA were resolved by SDS-PAGE. The gels were equilibrated for 15 min in transfer buffer (20% methanol, 0.025 M Tris, 0.025 mM glycine, pH 9) and then electrotransferred onto a nitrocellulose membrane. Nonspecific binding was blocked by incubation overnight at 4°C in PBS containing 5% nonfat milk and 20% Tween 20, and then the blot was incubated in ConA-horseradish peroxidase (Sigma; 10 μ g/ml, prepared in PBS-Tween buffer) for 1 h at room temperature with shaking. The blot was washed three times for 10 min each with the incubation buffer, rinsed three times with PBS, and processed with the ECL Western blotting detection reagent.

Indirect immunofluorescence of *E. histolytica*. For epifluorescence labeling of trophozoites, capping was induced as described above, using fluorescein isothiocyanate (FITC)-labeled ConA (Sigma). After incubation on ice, trophozoites were transferred to a coverslip in a petri dish and incubated at 37°C for 10 min to promote amoeba attachment and cap formation. PBS was removed, and the coverslips were placed in methanol at -20°C for 3 minutes.

For direct observation of capping, coverslips were mounted on a glass slide in PBS containing 70% glycerol. Fixed trophozoites were examined under a magnification of $\times 400$ in a BHS microscope (Olympus Optical Co., Ltd.) equipped with epi-illumination.

For in situ epifluorescence labeling of myosin II or Gal-GalNAc-inhibitable surface lectin, coverslips were incubated for 30 min in PBS containing 1% nonfat milk and then washed three times with PBS. Coverslips containing fixed trophozoites were incubated with undiluted anti-myosin II serum or anti-Gal-GalNAc-inhibitable surface lectin for 1 h in PBS containing 1% nonfat milk for 30 minutes at room temperature. After two washes in PBS, samples were incubated for 30 min in a 1:100 dilution of rhodamine-labeled goat anti-rabbit immunoglobulin in PBS (Sigma) or goat anti-mouse immunoglobulin (Molecular Bioprobes) preadsorbed with trophozoites as described previously (24). The preparations were further incubated in PBS containing 1% nonfat milk for 30 minutes at room temperature, briefly washed in PBS, and mounted on a glass slide with 70% glycerol in PBS.

Confocal microscopy. Fluorescent samples were examined on a Leica DIAPLAN confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with a 63 $\times$ objective. Rhodamine-labeled samples were visualized with a RG610 narrow-band interference filter centered on 535 nm (± 8 nm) after excitation at 568 nm. For fluorescein, an interference filter centered on 535 nm, after excitation at 488 nm, was used. Observations were performed in eight planes from the bottom to the top of each cell. The distance between scanning planes was 0.5 μ m. Three-dimensional reconstruction of serial confocal sections was performed with the CLSM-Leica software. Photographs were taken on

Kodak T-max 400 film with a 35-mm camera mounted on a Polaroid Freeze-Frame video recorder monitor.

Transmission electron microscopy (TEM). After induction of capping, 10^7 amoebae were harvested by centrifugation at $700 \times g$ for 5 min, washed in PBS, and then resuspended in 200 μ l of PBS. Trophozoites (or uroid pellets) were fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.3) at room temperature. Sedimented amoebae were briefly washed with phosphate buffer and then postfixed for 15 min at 4°C with 0.2% OsO₄ in the same buffer. The pellet was rinsed with distilled water, dehydrated in a graded ethanol series, and embedded in Spurr's resin according to the recommendations of the supplier (Ladd Research Industry, Burlington, Vt.). Thin sections were prepared on an LKB Nova ultramicrotome (Leica, Vienna, Austria) fitted with a diamond knife and were observed in a JEOL 1010 electron microscope operating at 80 kV.

Staining of ConA binding sites. After induction of capping, trophozoites were washed with PBS and incubated for 15 min in 50 μ g of peroxidase per ml prepared in PBS. The cells were then carefully washed with PBS four times and fixed with glutaraldehyde as described above. The trophozoites were gently pelleted by centrifugation, washed with PBS, and then incubated in 0.5 mg of diaminobenzidine per ml-0.03% hydrogen peroxide in 50 mM Tris-HCl buffer (pH 7.5) for 15 min at room temperature with mixing by inversion every 2 min. The enzymatic reaction was stopped by dilution with PBS. Amoebae were washed with PBS, postfixed with 0.2% OsO₄, embedded, and processed as described above.

Immunogold electron microscopy. After induction of capping, amoebae were fixed for 1 h at 4°C in 0.1 M phosphate buffer (pH 6.3) containing 1% acrolein. The trophozoites were then pelleted by centrifugation at $800 \times g$ for 10 min, washed twice with phosphate buffer, and treated with 50 mM NH₄Cl in PBS (pH 7.4) for 15 min at 4°C. Samples were then embedded with 2% agarose in Microvette CB 300 capillary tubes (Sarstedt, Nümbrecht, Germany). Small blocks (1 by 0.2 mm) were dehydrated and embedded in Lowicryl HM20 with a progressive lowering of the temperature to -50°C (9). Thin sections were incubated for 10 min at room temperature in PBS containing 5% BSA and 5% normal goat serum (NGS); briefly washed with PBS containing 1% BSA, 1% NGS, and 0.1% Tween 20; and incubated with the appropriate dilution of purified antibodies in PBS for 1 h at room temperature in a humidified chamber. Before use, the anti-myosin II antibody was purified on Sepharose-protein A beads by published methods (18). Sections were washed five times in PBS containing 0.1% BSA for 3 min and incubated for 30 min at room temperature with 10-nm-diameter colloidal gold-conjugated antirabbit antibodies (British Biocell Laboratories, Cardiff, United Kingdom) which were diluted in PBS containing 0.01% gelatin (Sigma catalog no. G7765). Grids were rinsed sequentially with PBS-gelatin and distilled water and were fixed for 5 min with 1% glutaraldehyde in phosphate buffer. The grids were rinsed with water, dried, and counterstained either with 1% aqueous uranyl acetate for 5 min at room temperature or with a saturated aqueous solution of uranyl acetate for 35 min followed by Millonig's stain, second formulation (21), for 1 min. Sections were observed with a Philips CM12 electron microscope operating at 60 kV.

Confocal analysis of ConA-FITC fluorescence of *E. histolytica* after treatment with cytochalasin D and kinase inhibitors. Growing trophozoites were harvested, washed in PBS, allowed to attach to coverslips in the presence of PBS at 37°C, and then incubated for 15 min at 37°C in 100 μ M cytochalasin D (Sigma) (dissolved in dimethyl sulfoxide), 300 μ M protein kinase C (PKC) inhibitor H7 (Biomolecular Research Lab, Inc.), or 100 μ M casein kinase II inhibitor A3 (Biomolecular Research Lab, Inc.). Subsequently, 20 μ g of ConA-FITC (Sigma) per ml was added. Trophozoites were incubated for 15 min at 37°C and fixed with -20°C methanol for 3 min.

For direct observation of capping, coverslips were mounted on a glass slide with 70% glycerol in PBS. Slides were examined on a Leica DIAPLAN confocal laser scanning microscope as described above.

RESULTS

Ultrastructural analysis of the uroid in association with the protozoan and after release. The interaction of *E. histolytica* with ligands such as immunoglobulins from patients with amoebiasis, anti-amoeba antibodies, or certain glycoproteins results in the capping of receptors to these ligands. Cytoskeletal activities have been proposed to account for capping in *E. histolytica* (14, 15). To analyze the cellular mechanisms involved in capping, growing trophozoites were treated with ConA, a lectin that binds to receptors containing D-mannose and D-glucose (16), and analyzed by microscopy. To stain ConA binding sites, cells were incubated in the presence of peroxidase and subsequently treated for TEM.

Capping parasites were identified as cells with a unique uroid, in which ConA-receptor complexes were concentrated (Fig. 1). ConA attachment sites were visualized by the peroxidase activity, which specifically labeled the membrane invagi-

FIG. 1. TEM of capping induced with ConA on *E. histolytica* trophozoites. (A) General view of *E. histolytica*, showing redistribution of ConA receptors to the uroid region of the parasite as observed by the labeling of peroxidase. Magnification, $\times 6,000$. (B) Detailed view of an induced uroid. The membrane region showing a high concentration of ConA receptors, which are stained in black by oxidized diaminobenzidine, is delimited by the arrows. Magnification, $\times 20,000$. (C to E) After the capping, the released material was concentrated by centrifugation and fixed, and then thin sections were prepared from the upper part of the pellet (C), the middle region of the pellet (D), and the bottom of the pellet (E). Mainly membrane vesicles heterogeneous in size were found in all the sections. Magnification, $\times 40,000$. Examples of ConA receptors stained in black by oxidized diaminobenzidine are indicated by the arrowheads.

nations on the uroid. Numerous vacuoles that were heterogeneous in size were found in the cytoplasm. These vacuoles were absent from the uroid region, indicating polarization of the cell. The uroid was enriched in membrane invaginations of heterogeneous forms and sizes, as well as vesicles (0.1 to 0.7 μm in diameter).

To study the relationship between amoebic surface receptors and the cytoskeleton, the identification of ConA receptors undergoing capping was undertaken. We took advantage of the fact that the material accumulated in the uroid would be released by incubation of capping trophozoites at 37°C and by simple shaking of the preparation. The released material was separated from the amoebae and concentrated by differential centrifugation. The pellet obtained in these preparations was fixed, and thin sections were examined by TEM. The results presented in Fig. 1C to E show that the preparation was composed essentially of membrane vesicles with a very heterogeneous morphology. The vesicles (0.1 to 0.7 μm in diameter) were found in all observed sections. Although the possibility of contamination by cellular debris cannot be excluded, the presence of specific material released from the uroid was confirmed by the ConA peroxidase labeling found in these vesicles. Unfortunately, the quantitation of the uroid-specific material within that preparation could not be done, since the revelation of the intensity of the labeling depends on the plane from which the TEM section was obtained. However, amoeba membrane fractions obtained from noncapping cells were treated by the same procedure and were not found to be labeled by peroxidase (data not shown). From these results and further biochemical analysis, we conclude that uroid-specific material from capping amoebae was obtained in the purification.

Analysis of ConA-receptor complexes from the uroid-released protein fraction. To investigate specifically the nature of the ConA-receptor complexes, the proteins bound to ConA were immunoprecipitated by a polyclonal serum raised against ConA. To estimate the degree of contamination by cellular debris, amoebae were treated with the same methods in the absence of ConA and the glycoprotein was added to the purified protein fraction before immunoprecipitation. Either nonionic detergents, such as Triton X-100 or octyl- β -glucopyranoside, or zwitterionic detergents, such as CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}, were tested to solubilize the proteins. Consistent results were obtained when octyl- β -glucopyranoside was used at 50 mM. Protein concentrations were measured by the Bio-Rad protein assay. The fraction obtained from ConA-capping amoebae contained 4.7 μg of protein per 10^6 amoebae (average of four purifications; standard deviation [SD], 1.3), and the fraction obtained from noncapping amoebae contained 0.4 μg of protein per 10^6 amoebae (average of three experiments; SD, 0.1). These results indicate that during capping of ConA by *E. histolytica*, membrane shedding increased 12-fold. Antigen-antibody com-

FIG. 2. Electrophoretic analysis of proteins from the ConA-immunoprecipitated complex. Uroids prepared from 10^7 amoebae were subjected to immunoprecipitation with the anti-ConA antibody. Recovered immunoprecipitated proteins were resolved by SDS-PAGE and silver stained. Lanes: A, proteins immunoprecipitated with an anti-ConA antibody from the uroid-released material of amoebae undergoing capping of ConA; B, proteins immunoprecipitated with rabbit immunoglobulin-beads from the uroid-released material of amoebae undergoing capping of ConA; C, proteins immunoprecipitated with an anti-ConA antibody from the released material from noncapping amoebae incubated in the presence of ConA.

plexes formed during capping were analyzed by SDS-PAGE. The analysis allowed the visualization of peptides with masses of 170, 150, and 96 kDa (Fig. 2, lane A). Comparison of the ConA-immunoprecipitated protein profile with that of the proteins obtained with beads coupled to rabbit immunoglobulins (Fig. 2, lane B) showed that the proteins of 96 and 150 kDa also bound immunoglobulins. In contrast, the 170-kDa protein specifically appeared within the ConA-immunoprecipitated complex. Antigen-antibody complexes were also immunoprecipitated from the fraction obtained with noncapping amoebae (Fig. 2, lane C): a protein of 170 kDa was immunoprecipitated, as well as several species at 100 kDa and the immunoglobulin G at 55 kDa.

Identification of ConA-interacting proteins from uroid-released material. Immunoprecipitation with ConA had revealed three proteins of 96, 150, and 170 kDa directly or indirectly associated with this lectin. To investigate which of these peptides contained ConA binding motifs, an overlay test was performed. Proteins immunoprecipitated by ConA were resolved by electrophoresis, transferred to a nitrocellulose membrane, and then incubated in the presence of ConA coupled to peroxidase. The interaction of ConA with the immunoprecipitated protein was revealed by the peroxidase activity. Under these experimental conditions, the 170-kDa protein, the 96-kDa protein, and two proteins at 80 and 58 kDa (not visible by silver staining) were detected (Fig. 3, lane A). These results indicate the presence of multiple ConA receptors undergoing capping. ConA, as revealed by immunoblotting, was found in the monomeric conformation at 29 kDa (Fig. 3, lane B).

Identification of the nature of the ConA receptors in uroid-released material. To identify the nature of the two ConA receptors revealed by the ConA overlay test, a molecular analysis of ConA-immunoprecipitated proteins was performed. Using the immunoblotting technique, we found that the cap-associated protein of 170 kDa cross-reacted with a monoclonal antibody prepared against the purified 170-kDa subunit of the Gal-GalNAc-inhibitable surface lectin of *E. histolytica* described by several groups (Fig. 4) (23, 26, 28). Taking advan-

FIG. 3. ConA overlay on ConA-immunoprecipitated complexes. Proteins from the uroid-released material were immunoprecipitated with an anti-ConA antibody. After resolution by SDS-PAGE, immunoprecipitated proteins were transferred to a nitrocellulose membrane and incubated in the presence of ConA-peroxidase. The binding of ConA was revealed by the peroxidase activity (lane A). In the same preparation immunoprecipitated ConA was revealed by an anti-ConA antibody (lane B).

tage of the monoclonal antiadhesin antibody reactivity, we analyzed the cellular localization of this protein during capping. Amoebae were incubated in the presence of ConA-FITC, and cells were fixed after induction of capping. After immunofluorescence staining for the adhesin, trophozoites were analyzed with a laser confocal microscope (Fig. 5). Staining of amoebae with an antiadhesin antibody revealed that this protein was highly concentrated in the uroids of capping amoebae. Interestingly, this lectin enriched within the caps is a protein necessary to inhibit the late complement attack complex C5b-9 (5).

To identify the nature of the 96-kDa protein, ConA immunoprecipitation was performed with uroids from 2×10^8 amoebae. This protein was eluted from the gel, and the N-terminal sequences of three internal peptides were obtained: (i) KTRN PIVFSFHPSA, (ii) KFMFGVNAYSADVN, and (iii) KAA YPIA. Sequence comparison of these peptides with those in the National Center for Biotechnology Information protein data bank revealed significant similarities to the alcohol dehydrogenase 2 from *E. histolytica* (33). In this parasite the alcohol

FIG. 4. Immunoblotting analysis of protein fractions obtained with an anti-ConA antibody from the uroid-released material. After protein electrophoresis, an immunoblot was performed with the antiadhesin antibody. Lanes: A, proteins immunoprecipitated with an anti-ConA antibody from the uroid-released material of amoebae undergoing capping of ConA; B, proteins immunoprecipitated with rabbit immunoglobulin-beads from the uroid-released material of amoebae undergoing capping of ConA; C, proteins immunoprecipitated with an anti-ConA antibody from the released material from noncapping amoebae incubated in the presence of ConA.

FIG. 5. Gal-GalNAc-inhibitable surface lectin distribution in moving *E. histolytica* cells. Cells were incubated with ConA-FITC to induce capping and then were fixed and stained with an anti-Gal-GalNAc-inhibitable surface lectin antibody. The distribution of ConA is shown on the left, and the distribution of Gal-GalNAc-inhibitable surface lectin is shown on the right. Optical sections were 0.5 μm , and plane 5 is shown.

dehydrogenase 2 enzyme has the alcohol dehydrogenase iron-dependent activity and also has the capacity to bind proteins from the extracellular matrix (33). The 96-kDa protein has been described as a membrane-associated antigen, synthesized particularly in pathogenic strains; it induces a strong immune response in patients developing amoebiasis (29), leading to the conclusion that this protein corresponds to one of the major pathogenic markers of *E. histolytica*.

Observation of ConA-receptor complexes and myosin II after capping. In a previous study, we had shown that myosin II was concentrated at the rear part of moving amoebae, some of which formed a uroid (24). To examine whether myosin II colocalizes with the caps, capping was massively induced in growing trophozoites by incubation with ConA-FITC. The relative distributions of myosin II and ConA were analyzed by using immunolabeling and fluorescence. Cells were treated with ConA-FITC, allowed to cap during 15 min, and then fixed. These cells were immunofluorescently stained for myosin II. Under these conditions, most trophozoites showed formation of caps containing ConA-receptor complexes. The proportion of cells with a uroid among the total population was 78%. Analysis of capping trophozoites by laser confocal microscopy (Fig. 6) showed polarized amoebae that had one pseudopod and one uroid of different sizes. Staining of amoebae with an anti-myosin II antibody and observation of the optical plane corresponding to the middle of the cell revealed a low density of myosin II along the cell membranes and on the frontal parts of amoebae. Myosin II reached a high concentration in the uroid (Fig. 6A). Visualization of ConA by FITC fluorescence showed that it was concentrated in the uroids of the amoebae (Fig. 6B). As shown in Fig. 6C, myosin II and ConA colocalized in the uroid region. Computer analysis of the section corresponding to myosin II and ConA labeling (Fig. 6D) confirmed that both were in the uroid.

FIG. 6. Distributions of myosin II and ConA in moving *E. histolytica* cells. Cells were incubated with ConA-FITC to induce capping and then were fixed and stained with an anti-myosin II antibody. (A) Distribution of myosin II; (B) distribution of ConA. Optical sections were 0.5 μm , and plane 5 is shown. (C) Position of labeling of both myosin II and ConA; (D) computer analysis of section of colocalization. Red corresponds to myosin II labeling, and green corresponds to ConA fluorescence. The computer analysis of the localization of pixels leads to the position of overlap, which is represented as yellow. In areas of high fluorescence intensity, the overlap of the labelings appears white.

FIG. 7. Ultrastructural localization of myosin II. Cells were induced to cap ConA and then were fixed and incubated with an anti-myosin II primary antibody and a gold-conjugated secondary antibody. Colloidal gold is mainly present over the uroid. The cytoplasm of the amoebae shows a marker density that is 3.7 times less than that in the uroid. The inset shows the area that is presented at a higher magnification in the main part. Counterstaining was with 1% uranyl acetate. Magnification, $\times 36,000$ (inset magnification, $\times 11,000$).

FIG. 8. ConA distribution in *E. histolytica* after inhibition of the cytoskeleton activity or myosin II phosphorylation. Cells were incubated with ConA-FITC to induce capping under regular growth conditions (A) or after treatment with a PKC inhibitor (B), a casein kinase II inhibitor (C), or cytochalasin D (D). Fluorescent ConA was observed in a confocal microscope. Optical sections were 0.5 μm , and plane 5 from each observation is shown. In the presence of inhibitors, ConA-receptor complexes appeared in fluorescent patches.

To quantitate the amount of myosin II concentrated within the caps, growing trophozoites were incubated with ConA, labeled with an anti-myosin II antibody, incubated with secondary antibodies conjugated to gold particles, and then examined by TEM. The anti-myosin II antibody revealed that this protein was localized adjacent to membrane invaginations in the posterior portions of the amoebae, as well as in cytoplasmic regions. The quantification of gold granules showed that the myosin II distribution was 3.12 times more intense in the uroid region (SD, 0.7) than in other areas of the cell (Fig. 7). Quantification of the number of gold granules in 10 amoebae showed 462 granules per mm^2 (SD, 123) in the uroid region, whereas there were 152 granules per mm^2 (SD, 43) in the cytoplasm region. Because the cytoskeleton in this parasite was difficult to fix during the TEM preparation, the myosin II concentration was probably underestimated.

Cytoskeleton activity during capping: localization of myosin II and Gal-GalNAc lectin. Cellular analysis indicated that myosin II preferentially accumulated intracellularly underneath receptor-capped structures. To determine whether a cytoskeleton contractile process is responsible for the capping of ConA receptors in *E. histolytica*, the capping efficiency was quantified in the presence of drugs that affect cytoskeletal activities. Amoebae were incubated in the presence of cytochalasin D, a drug that disrupts actin polymerization. In untreated samples or samples treated with dimethyl sulfoxide, 50% of trophozoites were found to cap fluorescein-labeled ConA. In contrast, in the presence of cytochalasin D, only 2% of the cells showed

capping of ConA, supporting the hypothesis that actin polymerization is necessary for capping.

In the tail region of the myosin heavy chain A of *E. histolytica*, two putative sites of phosphorylation by PKC and casein kinase II were found (27); their inhibition dramatically reduced the capping activity of the parasite. In fact, incubation of amoebae in the presence of H7, a PKC inhibitor, and in the presence of A3, a casein kinase II inhibitor, reduced the capping activity in the population of treated amoebae to 20 and 2%, respectively.

To further characterize the role of these drugs during uroid formation, an examination of cap formation with ConA-FITC in the presence of inhibitors of the cytoskeletal activities was carried out by confocal microscopy. In untreated samples, fluorescein-treated ConA bound the surface of the plasma membrane and was polarly accumulated in the uroid region (Fig. 8A). Trophozoites that were initially exposed to the drugs prior to incubation with ConA bound ConA, since their surfaces were fluorescent. In addition, we observed in all cases that the majority of treated cells showed areas on their surfaces that had dense fluorescence (Fig. 8B, C, and D), indicating that ConA-receptor complexes were clustered in patches. Some of these patches were internalized, because fluorescent ConA was also found inside the cells. Moreover, their translocation towards the uroid was inhibited. These experiments revealed significant differences in the degree of capping, as well as dramatic changes of morphology in the amoebae. In the presence of cytochalasin D or kinase inhibitors, trophozoites

FIG. 9. Distributions of myosin II and Gal-GalNAc adhesin in *E. histolytica* after inhibition of the cytoskeleton activity or myosin II phosphorylation. Cells were incubated with ConA-FITC under the same conditions as described for Fig. 8. The ConA distribution was determined by fluorescence analysis. The myosin II and lectin distributions were determined by using specific antibodies and indirect immunofluorescence. Labeling was observed in a confocal microscope. Optical sections were 0.5 μ m, and the plane located at the middle of the cell for each observation is shown. CD, cytochalasin D; H7, PKC inhibitor; A3, casein kinase II inhibitor.

rounded up but remained attached to the substratum. The absence of caps was correlated with the absence of the uroid.

Immunofluorescence was used to localize myosin II and the Gal-GalNAc lectin in amoebae treated with the drugs. In all cases, myosin II labeling was found to be diffuse below the plasma membrane; an enrichment of myosin II colocalizing with the ConA-receptor patches was not observed (Fig. 9). In contrast, the Gal-GalNAc lectin was found in some of the ConA-receptor patches, indicating that clustering of the lectin occurred in the presence of inhibitors of the cytoskeletal activities, whereas cap formation was inhibited (Fig. 9). Fluorescent ConA-receptor patches, not labeled by the antilectin antibody, may correspond to the clustering of other ConA receptors.

DISCUSSION

During capping, *E. histolytica* undergoes striking morphological changes in the rear part of the cell, including highly active membrane folding and localization of surface receptors. Accumulation of folded membrane and exfoliation occurred during capping, indicating that the turnover of membrane components is particularly active in *E. histolytica*. Uroid formation is triggered by the capping activity. This fact appears to be an important feature in pathogenesis, since the uroid seems

to contribute to the escape of amoebae from the immune response. Capping is followed by a process of vesicle budding that is particular to this amoeba. The mechanism by which the folded membranes fuse to create the vesicles may, as in other eukaryotic cells, be mediated by specialized membrane proteins that remain to be described.

Studies with *D. discoideum* and lymphocytes have led to two hypothetical mechanisms for capping (3, 12). In the first one, patches are formed via clustering of receptors. Patch translocation to a pole of the cells occurs by continual membrane flow. In the second proposed mechanism, clustered receptors interact with the contractile system of actomyosin filaments which mediate the receptor movement to a pole of the cells. Receptors could be recycled by endo- and exocytoses. Capping lymphocytes have also been reported to have a dramatic change in cell shape, generally referred to as uropod formation (3). The nature of these lymphoid structures has not yet been studied. In contrast, morphological studies of lymphocytes show cap formation in the absence of ligands but after incubation of cells in hypertonic medium. In the latter case, caps show a direct association with microvilli; these are finger-like projections that contain bundles of actin filaments supported by a terminal web rich in myosin II (32).

In the case of *E. histolytica*, capping seems also to be achieved by activity of actomyosin filaments, as suggested by the enrichment of myosin II observed beneath the caps. This conclusion is reinforced by experiments in which cytochalasin D was used to prevent actin polymerization and by experiments in which inhibitors of protein phosphorylation were used. These drugs inhibited capping and uroid formation. These results are consistent with the standing hypothesis that capping activates a myosin II-dependent cytoskeletal function. Genetic evidence obtained for *D. discoideum* supports this hypothesis. For these nonpathogenic amoebae, it was demonstrated that capping is associated with cellular stiffening and that both capping and stiffening fail to occur in cells lacking myosin II (19). With cells treated with H7 or A3 kinase inhibitors, we observed that the phosphorylation of the myosin heavy chain may be necessary for receptors to accumulate into a cap structure but not to form patches. This is not the case for nonpathogenic amoebae such as *D. discoideum*, in which inhibition of myosin heavy chain phosphorylation does not inhibit cap formation (11). One explanation for such differences might be that the nature of the phosphorylation sites on the myosin II heavy chain from *D. discoideum* has diverged from that of these sites in *E. histolytica*. Besides the role of myosin II in the rearward movement of surface-attached ligands, the presence of myosin II in the uroid would indicate that a contraction produced by mechanical forces is necessary to detach the caps from the cells and that the membrane invagination taking place in the uroid could be driven by myosin activity. Thus, both the surface redistribution of receptors and the subsequent release of the caps through the contraction of a constriction ring might be regulated by cytoskeletal components in *E. histolytica*. When pathogenic *E. histolytica* organisms are incubated in the presence of ConA, a cascade of events occurs: (i) binding of ligands induces the formation of surface receptor patches, a function occurring independently from the actomyosin filament activities; (ii) patches are translocated rearward to form a cap by the activity of actin and myosin II; (iii) the plasma membrane is folded in the same spatial direction as the cap formation and results in the establishment of the uroid, and at this time amoebae glide as morphologically polarized cells; and (iv) membrane vesicles formed on the uroid are extruded from the amoeba by a contraction mechanism that may require myosin II activity. Ligands complexed to surface receptors

accumulate on the uroid and are, in part, eliminated by vesicle exfoliation. When these ligands are proteins of the complement system or anti-amoeba antibodies, cap release is most likely to contribute to survival of the parasite. Surface receptors that undergo capping must maintain a connection with the cytoskeleton, as suggested by the colocalization of myosin II and the Gal-GalNAc adhesin in the uroid. It remains to be demonstrated whether the protein complexes are formed by direct interaction of receptors with the cytoskeleton or through the activity of other proteins acting as intermediates between the receptors and the cytoskeleton.

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fluorescence
confocal
63.0 / 1.40 OIL
xy: 48x48 um

E. histolytica
HM1: IMSS

anti-myoII
conA-FITC

A

B

C

D

5 um