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The 96-Kilodalton Antigen as an Integral Membrane Protein in Pathogenic *Entamoeba histolytica*: Potential Differences in Pathogenic and Nonpathogenic Isolates

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A surface antigen (EH-96) of *Entamoeba histolytica* was demonstrated to be a plasma membrane antigen by immunoprecipitation of metabolically ^{35}S-labeled antigen from live trophozoites, Triton X-114 detergent extracts, and plasma membrane-enriched fractions prepared by concanavalin A membrane stabilization and differential centrifugation. In addition, the antigen was localized to the plasma membrane by electron microscopy with colloidal gold. Antigen from *E. histolytica* strains immunoprecipitated with specific immunoglobulin M (IgM) or IgG2b monoclonal antibody was identical by one-dimensional peptide mapping with *N*-chlorosuccinimide. Additionally, antigen from different axenically cultivated amebae was demonstrated to be identical by *N*-chlorosuccinimide peptide mapping, as were peptide maps of IgG and IgM monoclonal antibody-purified antigen. The 96-kilodalton (kDa) surface antigen was identified on four axenically cultivated pathogenic isolates and on three polyxenically cultivated pathogenic isolates (zymodeme II) of *E. histolytica* but was absent or present in lesser quantity on six nonpathogenic polyxenically cultivated isolates. The 96-kDa antigen was detected in liver abscess fluid from four patients with amebic abscesses by enzyme-linked immunosorbent assay (ELISA) and immunoprecipitation. Two-dimensional gel electrophoresis profiles of the 96-kDa antigen purified from abscess material or from polyxenically cultivated trophozoites demonstrated that the antigens were related to the 96-kDa antigen found in axenically cultivated organisms.

Entamoeba histolytica is a pathogenic protozoan with worldwide distribution which causes 10^6 cases of invasive disease and at least 100,000 deaths annually (31). The mechanisms of pathogenesis of these infections are poorly understood (20). *E. histolytica* strains are capable of capping and shedding antibody (6) and of promoting contact-dependent cytolysis through initial target cell attachment via an *N*-acetylgalactosamine-inhibitable lectin (18, 21) and possess enzymes capable of degrading target cell extracellular matrices (11). At least 18 surface antigens have been identified by membrane analyses (1). However, studies of individual antigens and their biochemical and immunological properties and membrane associations are lacking, with the exception of the *N*-acetylgalactosamine-inhibitable adherence lectin (17–21), and a high-molecular-weight surface antigen with lectin-like activity (10).

Complete biochemical characterization and concrete localization of surface antigens is of paramount importance in selecting potential candidates for vaccinogens as well as in understanding the biochemistry of *E. histolytica* for chemical intervention in the pathogenesis of disease. We recently identified and partially characterized a prominent 96-kilodalton (kDa) surface antigen (by surface iodination and metabolic labeling) of *E. histolytica* by using axenic strains (28), and this antigen was shown to be serologically relevant in patients with amebic liver abscesses, ameboma, or amebic colitis (29). Accordingly, more in-depth investigation into this antigen is warranted. In this report, we have identified the antigen in amebic liver abscess fluid and have demonstrated that the antigen is highly conserved, being similar if not identical in different axenic strains and in polyxenically cultured pathogenic strains. We have further investigated the detailed biochemical nature of EH-96 by localization of the antigen as an integral plasma membrane constituent of *E. histolytica*, with a significant portion of total antigen being in the cytosol or other fractions.

MATERIALS AND METHODS

Maintenance of amebic cultures and zymodeme analysis. Axenic strains H-302:NIH (ATCC 30887), H-303:NIH (ATCC 30885), and HM1:IMSS (ATCC 30459) (28) were cultivated at 35°C in Diamond TYI-S-33 medium (9) supplemented with 15 to 20% heat-inactivated bovine serum (Bio Fluids). Logarithmic-phase organisms were harvested by chilling them in 75-cm^2 flasks, centrifuging them, and washing them twice in phosphate-buffered saline (PBS) (pH 7.0, 0.01 M) to remove loosely associated medium constituents.

Polyxenic cultures were inoculated from stool and liver abscesses into Robinson medium (22) and maintained in either Robinson's medium or TYS-GM (8) containing *Escherichia coli* 0111. Before being used in experiments, polyxenically cultivated amebae were partially purified by using a discontinuous Percoll gradient and low-speed washes to remove starch and all but tightly adherent or ingested bacteria.

Pathogenic or nonpathogenic zymodemes for clinical isolates were assigned by starch gel electrophoresis patterns of maleic enzyme, phosphoglucomutase, glucophosphoisomerase, and hexokinase from culture lysates by the method of Sargeaunt et al. (23, 24). Immunoblotting was carried out as described previously (28, 30). Protein determinations were performed by the method of Bradford (5). Monoclonal

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antibodies used (specific for *E. histolytica*; immunoglobulin G2b [IgG2b] and IgM) were described in a previous report (28). Liver tissue was obtained from recently deceased individuals on autopsy by excision of 5 to 10 g of tissue followed by freezing at −70°C until use.

Fractionation of proteins and detergent partition of membrane antigen(s). Proteins from *E. histolytica* pellets were fractionated by two methods. In the first, the method of Aley et al. (1), who used a Ca^{2+}-dependent membrane-bound ATPase and surface iodination as membrane markers, was used to obtain an enriched plasma membrane preparation (pellet IV), a soluble protein fraction (supernatant II), and two pellets purported to be nonvesiculated membranes and other debris (pellet II) and internal membranes (pellet III). In the second fractionation scheme, Triton X-114 detergent was used to partition membrane and soluble proteins by the method of Bordier (3), who demonstrated that integral membrane proteins are found in the detergent phase. Whole pelleted unlabeled or ^{35}S-labeled cells were solubilized in Triton X-114 (0.75%) and spun at 10,000 × *g* at 4°C to remove insoluble components. After partitioning, the detergent and aqueous phases were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with Coomassie blue or were transferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.) (30), stained with Coomassie blue, and analyzed by an analytical densitometer (model SLR-2D/1D; Biomed Instruments, Fullerton, Calif.). Partitioned ^{35}S-labeled protein was immunoprecipitated from the aqueous and detergent phases as described previously (28), run on an SDS-PAGE gel (13), dried, and subjected to fluorography. All procedures were carried out in the presence of proteolytic inhibitors (phenylmethylsulfonyl fluoride, leupeptin, and aprotinin) at 4°C, except for a portion of the Triton X-114 partition procedure, which was carried out at 30°C.

Immunoprecipitation from live axenically cultured organisms. Briefly, 10^7 freshly harvested live *E. histolytica* trophozoites labeled with [^{35}S]methionine were washed three times in PBS (pH 7.0) at 4°C (to prevent capping and shedding of antibody) (6), placed in a siliconized glass tube, suspended in 2 ml of PBS, and kept on ice at 4°C. To this suspension was added 1.0 ml of protein A-purified IgG2b (specific for *E. histolytica*) in PBS, pH 7.6. The mixture was kept on ice and gently mixed every 5 min. At the end of a 15-min incubation, unbound antibody was eliminated by three washes with 10 ml of cold PBS (pH 7.0, 4°C). Cells were assessed for membrane integrity by trypan blue exclusion, and >99.9% of them excluded the dye. The pellet from the final wash was treated with 2 ml of solubilization buffer (28) and centrifuged at 40,000 × *g* for 1 h at 4°C to remove insoluble constituents. The supernatant was incubated with a 10% suspension of protein A-Sepharose (Pharmacia, Inc., Piscataway, N.J.) for 2 to 3 h at 4°C. The beads were pelleted and washed four times with 6 ml of solubilization buffer and then once with PBS, pH 7.0. The sample(s) was boiled for 5 min in SDS-PAGE sample buffer (13, 28), subjected to electrophoresis on a 10% SDS-PAGE gel, treated with a fluor (En^{3}Hance; Dupont, NEN Research Products, Boston, Mass.), dried under vacuum, and subjected to fluorography for 3 to 10 days. Alternatively, immunoprecipitation was carried out as described previously (28) by using Nonidet P-40 (NP-40) to solubilize trophozoite antigen at 4°C.

Purification of EH-96. Antigen immunoprecipitated with monoclonal antibody was purified by SDS-PAGE and electroelution by using a prestained molecular mass standard (Diversified Biotech, Newton Centre, Mass.) as a marker which comigrated with the protein of interest (EH-96). A 2- to 4-mm-wide slice of gel corresponding to where EH-96 migrated was cut from each gel lane and electroeluted from the gel in an ISCO 1750 electrophoretic concentrator (ISCO Inc., Lincoln, Neb.) (in SDS-PAGE running buffer) at 4 W for 6 h at 4°C. A 200-μl electroeluate was harvested and stored at −70°C until further use as previously described (28). The integrity and purity of the 96-kDa protein were determined by SDS-PAGE followed by Coomassie blue or silver staining (29).

Iodination of purified antigen, *N*-chlorosuccinimide peptide mapping, and two-dimensional gel electrophoresis. Iodination of purified antigen was carried out by mixing 200 μl of purified EH-96 antigen with 0.3 mCi of carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.) in the presence of two Iodo-Beads (Pierce Chemical Co., Rockford, Ill.) for 12 min at 4°C. The iodinated sample was applied to a Sephadex G-25 column (Pharmacia, Inc., Piscataway, N.J.) previously blocked with 1% bovine serum albumin to remove free iodine. The labeled antigen was rerun on SDS-PAGE, excised, re-eluted to concentrate it in a 200-μl volume, and either stored at −70°C until use (no more than 3 days) or used immediately.

The purified iodinated antigen was rerun on SDS-PAGE with a reference marker (molecular mass standard). A slice of gel (1 cm by 3 mm) containing the purified iodinated antigen was then subjected to *N*-chlorosuccinimide cleavage in a urea-acetic acid system as described by Lischwe and Ochs (14).

For two-dimensional gel electrophoresis, radioiodinated antigen(s) was prepared by solubilization of 1 mg of urea for each microliter of iodinated protein sample followed by the addition of 2 volumes of nonequilibrium pH gradient electrophoresis (NEPHGE) lysis buffer (9.5 M urea, 5% β-mercaptoethanol, 2% ampholytes, pH 3 to 10 [Bio-Rad Laboratories, Richmond, Calif.], 2% NP-40). Nonequilibrium pH gradient electrophoresis was performed by using a mini-tube gel or mini-slab gel apparatus (Hoefer Scientific, San Francisco, Calif.) and the procedure described by O'Farrell et al. (16). Gels were focused at 500 V for 2 h, equilibrated in 1× SDS-PAGE sample buffer for 10 min, and then frozen in sample buffer or immediately run on the second-dimension SDS-PAGE. For the second dimension, nonequilibrium pH gradient electrophoresis gels were loaded onto 10% SDS-PAGE separating gels, and electrophoresis was performed in a discontinuous buffer system at 25 mA for 2 h. The gels were dried under vacuum and exposed to Cronex MRF-32 film (Dupont Co., Wilmington, Del.) or Kodak XRP-1 or XAR-2 film for 1 to 5 days at −70°C.

Enzyme-linked immunosorbent assay (ELISA). ELISA of *E. histolytica* fractions produced by the method of Aley et al. (1) was carried out essentially as described previously (28). Each fraction was assessed for total protein (by protein assay of a portion of the fraction and multiplication by the total volume) and equalized to yield 1 μg of protein per μl. Fifty microliters in PBS, pH 7.6, was placed in each well of an Immulon II plate (Dynatech, Richmond, Va.). The plate was incubated overnight at 4°C and then blocked with 125 μl of 5% bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) per well for 1 h at 37°C. A 50-μl portion of IgG2b or IgM monoclonal antibody (1:1,000 dilution) was added to appropriate wells and incubated for 1 h. After six washes with PBS–0.05% Tween 20 (pH 7.6), 50 μl of horseradish peroxidase-conjugated anti-mouse IgG or IgM (diluted 1:1,000 in PBS-Tween; Zymed, San Francisco, Calif.) was added. The plate(s) was washed five times with

PBS-Tween 20 and twice with PBS, pH 7.6, without Tween 20 and then was developed in a phosphate-citrate buffer (pH 5.0) with *o*-phenylenediamine and peroxide.

Electron microscopy. Preparation of samples with colloidal gold for transmission electron microscopy was performed by using a method similar to that used for scanning electron microscopy with colloidal gold (7). *E. histolytica* cultures were harvested as described above and suspended in 2 ml of ice-cold PBS (4°C). Four samples of approximately 3.5×10^4 amebae suspended in 2 ml of PBS were incubated with either IgM monoclonal antibody-bearing ascitic fluid (specific for EH-96) or irrelevant monoclonal IgM specific for *Trichomonas vaginalis* (27) on ice for 1 h with gentle agitation. A conjugate control to which no antibody was added was run in parallel.

The cells were washed gently four times in PBS (pH 7.6), suspended in 1 ml of ice-cold PBS, and incubated with 75 μl of goat anti-mouse IgM antibody conjugated to 30-nm colloidal gold particles (Janssen-Auroprobe EM, Olen, Belgium) on ice for 1 h with occasional gentle mixing. The samples were washed four times with PBS and then fixed in 5 ml of 2% glutaraldehyde (electron microscopy grade; Sigma) in PBS, pH 7.6, on ice for 30 min. After four washes in PBS, the cells were stored in 0.2% glutaraldehyde overnight on ice. The amebae were washed four times in PBS to remove excess glutaraldehyde and were prepared for dehydration by washing them twice in Tyrodes buffer (138 mM NaCl, 2.7 mM KCl, 1.8 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 120 mM $NaHCo_3$, 5.56 mM glucose). The amebae were dehydrated in 10-min steps of increasingly concentrated solutions of ethanol (30, 70, 80, and 95%) and twice for 10 min in absolute ethanol. The amebae were then washed twice for 10 min in propylene oxide (Poly Sciences, Inc., Warrington, Pa.) to prepare them for penetration by epoxy. The amebae were infiltrated for 30 min by using a 1:1 solution of propylene oxide and epoxy (Araldite/DDSA/DMP; Electron Microscopy Sciences, Ft. Washington, Pa.). After centrifugation, the pellet was reinfiltrated with epoxy for 60 min. The epoxy infiltration was repeated for another 60 min. The samples were then allowed to polymerize at 60°C for 2 days and were sectioned (90- to 110-μm sections) on a Sorvall-Porter Blum MT2 Utramicrotome (Diatome U.S.) with a 3-mm diamond knife, and the sections were fixed on 200-mesh copper grids with dilute HNO_3. The grids were then stained with uranyl acetate for 3 min and then with lead citrate (Electron Microscopy Sciences) for 4 min. The grids were dried at room temperature for 30 min, viewed on a transmission electron microscope (Phillips EM 201), and photographed by using Electron Microscopy Film (Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Identification of EH-96 from live organisms in detergent-nondetergent partition and differentially centrifuged fractions of *E. histolytica*. EH-96 was identified as a single band when immunoprecipitated from live organisms (Fig. 1A), while control antibody to *Naegleria fowleri* failed to immunoprecipitate EH-96 (data not shown). The protein migrated at the same molecular mass when electrophoresed under reducing and nonreducing conditions (Fig. 1B), although the nonreduced form migrated in a more diffuse band. The protein was isolated by immunoprecipitation from both the detergent and aqueous phases of Triton X-114 partition experiments (Fig. 2A and B) as well as from all fractions obtained by the differential centrifugation method for an enriched plasma membrane fraction (Fig. 3A and B) when the antigen was metabolically labeled. However, in addition, the intact protein could be immunoprecipitated from NP-40 extracts of *E. histolytica* after a 1-h centrifugation at 109,000 × *g* (data not shown). All membrane fractions derived from the differential centrifugation-plasma membrane enrichment procedure were reactive in an ELISA with IgM and IgG2b monoclonal antibodies specific for the 96-kDa antigen (Table 1). Total protein recovery from the fractionation was similar to that obtained by Aley et al. (1) (data not shown). When Triton X-114 partition experiments were performed, 14% of the total EH-96 protein was found in the detergent phase by analytical densitometry irrespective of the quantity of cells used (Fig. 4). Increased length of solubilization times did not significantly increase the yield of protein recovered in either phase (data not shown).

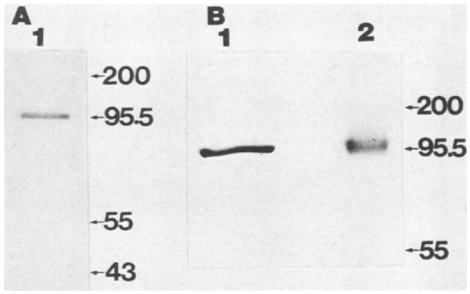

FIG. 1. (A) Immunoprecipitation of EH-96 from live ^{35}S-labeled organisms. A single band at 96 kDa was visible when IgG2b monoclonal antibody was used to immunoprecipitate antigen from live organisms. (B) Immunoprecipitation of EH-96 [^{35}S]methionine-labeled antigens with IgG2b monoclonal antibody under reducing conditions (lane 1) and nonreducing conditions (lane 2). Numbers indicate molecular mass (in kilodaltons).

Electron microscopy. Live amebae incubated with EH-96-specific IgM monoclonal antibody at 4°C to prevent

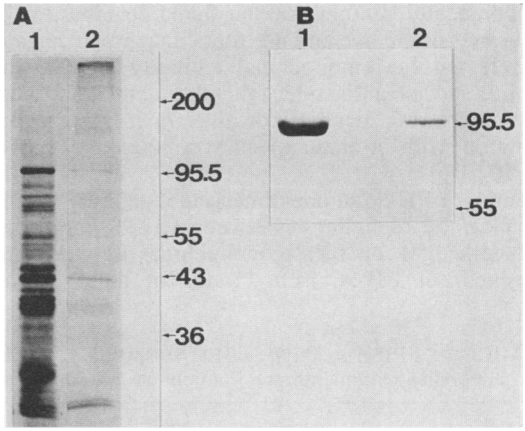

FIG. 2. (A) Fractionation of whole ^{35}S-labeled *E. histolytica* trophozoites in Triton X-114. Lanes: 1, aqueous phase of Triton X-114 fractionation after SDS-PAGE and fluorography; 2, detergent phase of Triton X-114 fractionation after SDS-PAGE and fluorography. (B) Triton X-114 detergent phase partition of ^{35}S-labeled EH-96. Lanes: 1, immunoprecipitation of ^{35}S-labeled EH-96 from the aqueous fraction of Triton X-114 detergent partition with IgG2b monoclonal antibody; 2, immunoprecipitation of ^{35}S-labeled EH-96 from the detergent fraction of Triton X-114 detergent partition with IgG2b monoclonal antibody. Numbers indicate molecular mass (in kilodaltons).

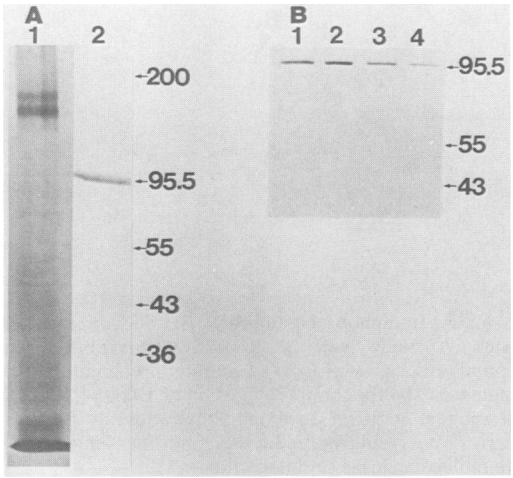

FIG. 3. (A) Immunoprecipitation of EH-96 from [^{35}S]methionine-labeled pellet IV (1). Lanes: 1, total enriched plasma membrane profile after SDS-PAGE and fluorography (50 μg of total protein loaded); 2, immunoprecipitation of EH-96 with IgG2b monoclonal antibody specific for EH-96 after SDS-PAGE and fluorography. Four pellets were used for immunoprecipitation. Numbers indicate molecular mass (in kilodaltons). (B) Detection of ^{35}S-labeled antigen EH-96 from soluble fraction(s) (supernatant II) or pellets (pellet II) of *E. histolytica* prepared by using the differential centrifugation technique of Aley et al. (1) and immunoprecipitation with anti-EH-96 monoclonal IgM or IgG2b-class antibody. Lanes: 1, EH-96 detected by IgG2b monoclonal antibody on strain 30887 (supernatant II) (1); 2, EH-96 detected by IgM monoclonal antibody on strain HM1:IMSS (supernatant II); 3, EH-96 detected by IgG2b monoclonal antibody on strain HM1:IMSS (pellet II) (1); 4, EH-96 detected by IgM monoclonal antibody on strain HM1:IMSS (pellet II). Numbers indicate molecular mass (in kilodaltons).

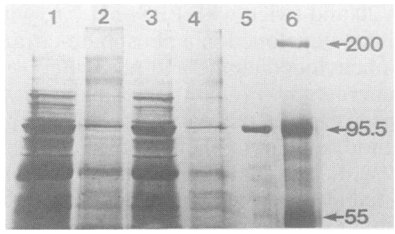

FIG. 4. Relative quantitation of EH-96 in Triton X-114 partitions with various starting cell numbers and constant detergent and aqueous-phase volumes. Lanes: 1, aqueous phase of Triton X-114 partition after Coomassie blue staining (starting with 10^6 *E. histolytica* trophozoites for the partition); 2, detergent phase from Triton X-114 partition after Coomassie blue staining (starting with 10^6 *E. histolytica* trophozoites for the partition); 3, same as lane 1 except starting with 7×10^5 *E. histolytica* trophozoites for the partition; 4, same as lane 2 except starting with 7×10^5 *E. histolytica* trophozoites for the partition; 5, purified EH-96; 6, molecular mass standards (numbers indicate kilodaltons).

capping and shedding or internalization followed by staining with colloidal gold conjugate and subsequent fixing with glutaraldehyde showed 30-nm gold particles attached to the plasma membrane surface (Fig. 5A). In contrast, colloidal gold conjugate treatment alone failed to label the plasma membranes of the live amebae (data not shown). In addition, an irrelevant IgM monoclonal antibody specific for a *T. vaginalis* surface antigen (27) failed to demonstrate colloidal gold staining after incubation with *E. histolytica* followed by incubation with μ-chain-specific colloidal gold conjugate (Fig. 5C).

Identity of EH-96 in one-dimensional peptide mapping. To determine the relatedness between EH-96 immunoprecipitated with IgM or IgG2b monoclonal antibody and the relatedness of EH-96 from strains of high and medium virulence, one-dimensional peptide mapping was performed by using *N*-chlorosuccinimide to cleave selectively at tryptophan residues. Purified iodinated antigen (Fig. 6A) was used as a template for peptide mapping. Similar cleavage patterns were seen between antigens purified with IgG2b or IgM monoclonal antibody (Fig. 6B). Similarly, EH-96 purified from strains HM1:IMSS (ATCC 30459) and NIH-302 (ATCC 30887), which differ in virulence, had cleavage patterns which were indistinguishable (data not shown).

TABLE 1. ELISA reactivity of EH-96-specific monoclonal antibodies with membrane fractions of *E. histolytica*

Fraction[a]	Optical density at 492 nm (mean ± SE; $n = 3$)[b] with:	
	IgM	IgG2b
Plasma membrane (pellet IV)	0.704 ± 0.037	0.794 ± 0.007
Nonvesiculated membranes and debris (pellet II)	0.260 ± 0.008	0.203 ± 0.011
Internal membranes (pellet III)	1.617 ± 0.008	1.445 ± 0.041

[a] Named by Aley et al. (1) and distinguished by electron microscopy and differential centrifugation (1).

[b] All samples were equalized with respect to protein concentration before ELISA adsorption (50 μg of protein per well of an Immulon II plate).

Serological reactivity and detection of EH-96 in clinical isolates and liver aspirates. Nine clinical isolates of *E. histolytica* (obtained from San Diego, Calif.) were tested for EH-96. All three polyxenically cultivated pathogenic isolates (zymodeme II, invasive disease) were found to possess EH-96 as visualized by Coomassie blue staining and immunostaining (Fig. 7A; data shown only for immunostaining) of immunoprecipitated EH-96. Bacterial controls were consistently negative. All six nonpathogenic isolates tested were also found to possess a 96-kDa protein. However, Coomassie blue staining and immunodetection of a 96-kDa protein was weakly positive only when the nonpathogenic isolates were used in immunoprecipitation reactions at 3 to 10 times the number of amebae required for pathogenic strains as evidenced in the analytical densitometric analysis of the immunoblots.

From an enriched plasma membrane preparation, a typical pattern of reactivity with acute serum from a patient with a liver abscess is shown in Fig. 8. The profile shows that EH-96 is one of few prominently stained plasma membrane antigens. In liver abscess material, EH-96 was prominently immunostained with polyclonal anti-*E. histolytica* serum after immunoprecipitation with IgG2b monoclonal antibody specific for EH-96 (Fig. 9A). EH-96 was isolated from four liver aspirates from patients with invasive *E. histolytica* liver disease (data shown for one patient), while none of eight liver samples from patients who died of nonamebic causes (some related to liver disease) was positive for EH-96.

Two-dimensional gel electrophoresis. Although antigen(s) of 96-kDa were immunoprecipitated from axenic isolates, polyxenic pathogenic and nonpathogenic isolates, and liver abscesses (Fig. 9A), we used two-dimensional gel electrophoresis to demonstrate that these comigrating species were related. EH-96 isolated from amebae grown in axenic or polyxenic culture or isolated from a liver abscess aspirate had identical migration patterns (Fig. 9B, subpanels A

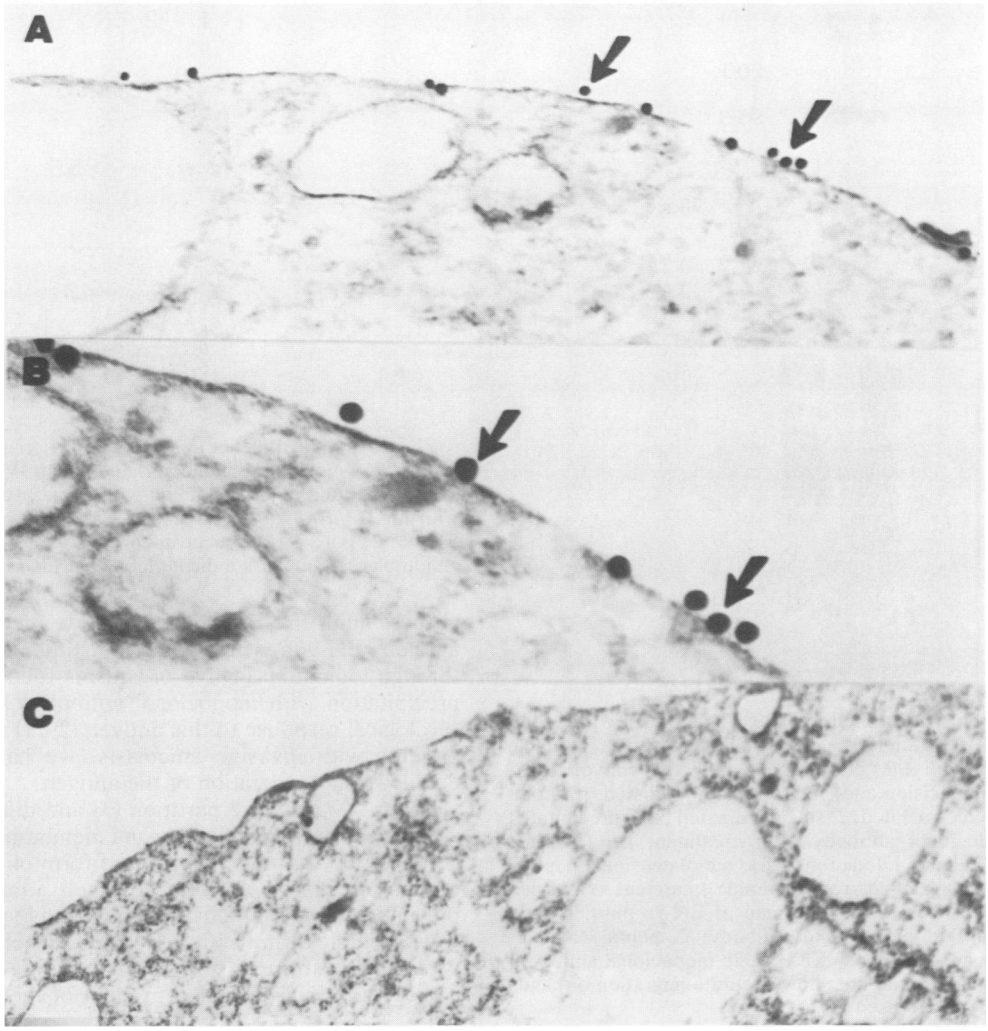

FIG. 5. Electron microscopic localization of EH-96 to the plasma membrane with colloidal gold. (A) *E. histolytica* plasma membrane reacted with IgM monoclonal antibody on live cells; the antibody was detected with goat anti-mouse μ-chain antibody conjugated to colloidal gold (magnification, ×47,000). (B) Same as panel A (magnification, ×96,000). (C) Control *E. histolytica* plasma membrane showing no staining with 30-nm colloidal gold when colloidal gold conjugate was used with prior incubation of cells with irrelevant monoclonal antibody specific for *T. vaginalis* (magnification, ×47,000).

through C). Liver abscess material contained another species in addition to EH-96 (Fig. 9B, subpanel C).

We demonstrated that the 96-kDa antigen could be detected by immunoprecipitation in all four liver abscess fluids tested. Antigens isolated from axenic culture, polyxenic culture, and from liver abscess fluid by using *E. histolytica*-specific monoclonal antibody were demonstrated to be EH-96 by their identical migration patterns on two-dimensional gel electrophoresis.

DISCUSSION

Plasma membrane constituents of protozoa play important roles in the interaction of the parasite and host. A number of parasite membrane components have been identified which mediate initial attachment and entry of parasites into cells, serve as virulence factors, or interact in an immunomodulatory capacity (up or down regulating an immunological response) with the host. In previous reports, several *E. histolytica* surface antigens were characterized as lectins or lectin-like surface antigens (10, 17). One of these surface antigens solely or in part mediates attachment to host target cells, enabling the initiation of contact-dependent cytolysis (21), which is well characterized biochemically, serologically, and functionally (17–21). Aley et al. (1) used concanavalin A as a membrane stabilizer before cell homogenization and differential centrifugation to identify a plasma membrane-rich fraction of *E. histolytica* purported to contain 18 mannose-containing glycoproteins as assessed by concanavalin A affinity chromatography. Aside from these efforts, there are no reports detailing localization and biochemical properties of other surface antigens of *E. histolytica* which may have biochemical or immunological significance.

Since whole trophozoite antigen has been demonstrated to be protective for invasive disease in several animal model systems, identification and detailed characterization of surface moieties is the logical subsequent step in selecting potential vaccinogens. Such data might be useful in selecting either antigens for use in serological diagnostic tests or DNA probes for identification of amebae in stool samples or

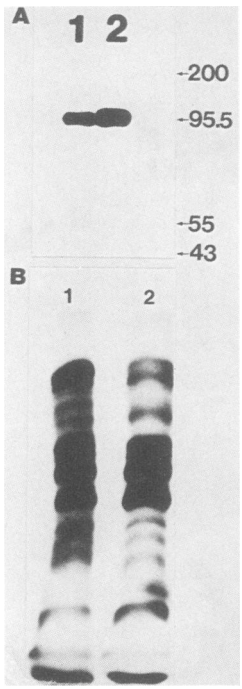

FIG. 6. (A) Profile of purified iodinated antigen used as a template for *N*-chlorosuccinimide peptide mapping. Lanes: 1, single band precipitated with IgM monoclonal antibody, run on SDS-PAGE, purified by excision and electroelution, iodinated, rerun on SDS-PAGE, and electroeluted; 2, single iodinated band precipitated with IgG2b monoclonal antibody after treatment as described above. Numbers indicate kilodaltons. (B) One-dimensional peptide map generated through *N*-chlorosuccinimide treatment of purified iodinated antigen. Lanes: 1, peptide map of EH-96 purified from strain 30887 with IgM monoclonal antibody; 2, peptide map of EH-96 purified from strain 30887 with IgG2b monoclonal antibody. (Molecular mass markers do not show accurate migration of bands because of the procedure used [14].)

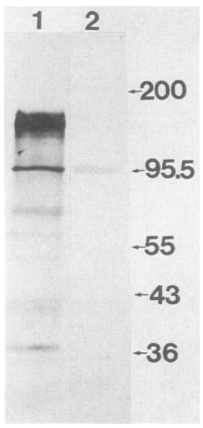

FIG. 8. Profile of pellet IV (1) after reaction with immune serum. Lanes: 1, whole plasma membrane (pellet IV) preparation after SDS-PAGE and immunostaining with human serum from a patient with an *E. histolytica* liver abscess showing prominently stained EH-96; 2, pure EH-96 electrophoresed as a marker stained with amido black. Numbers indicate molecular mass (in kilodaltons).

abscess fluid. In a previous report, we identified the 96-kDa antigen (EH-96) as a surface constituent of *E. histolytica* through iodination of live organisms followed by immunoprecipitation with monoclonal antibody. Because a strong serological response to this antigen (29) (Fig. 7) was seen in patients with invasive amebiasis, we have undertaken a detailed characterization of the antigen.

Triton X-114 phase partition (3) and differential centrifugation to isolate enriched plasma membrane components (1) demonstrate that the amphipathic form of this protein is an integral membrane antigen; however, a large proportion of the antigen is amphiphilic, as evidenced by the presence of a water-soluble form. With 86% of EH-96 being amphiphilic, it is reasonable to suppose either that the water-soluble form is a precursor or degradation product of the membrane form,

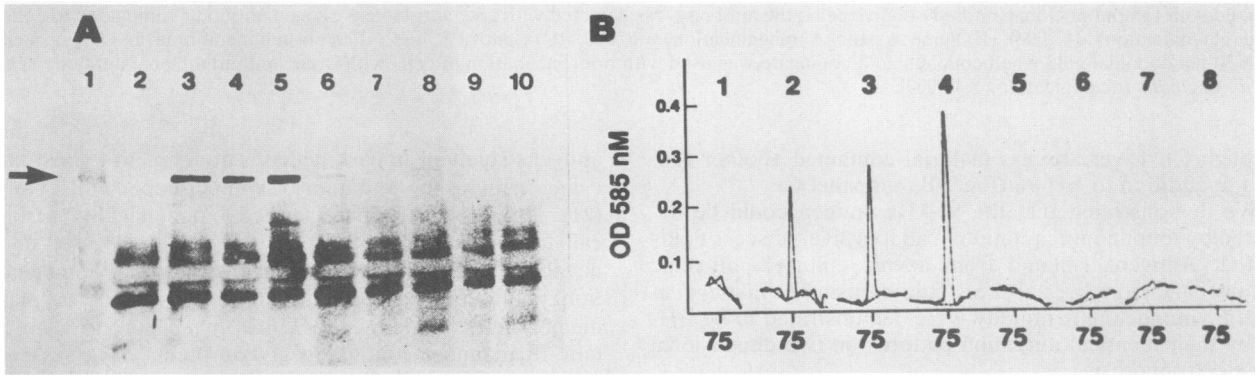

FIG. 7. (A) Immunostain of EH-96 immunoprecipitated from polyxenically cultivated amebae with EH-96-specific monoclonal antibody IgG2b, run on SDS-PAGE, transferred to nitrocellulose, and detected with rabbit polyclonal anti-*E. histolytica* serum. Lanes: 1, molecular mass standards (arrow indicates 95.5-kDa prestained marker); 2, isolate A, a nonpathogenic zymodeme II ameba with no staining of EH-96 (however, staining of murine IgG2b is evident because of cross-reactions with rabbit antiserum and peroxidase-conjugated goat anti-rabbit serum); 3 through 5, isolates B, C, and D, respectively, pathogenic zymodeme II amebae showing prominently stained EH-96; 6 through 10, isolates E, F, G, H, and J, respectively, nonpathogenic zymodeme amebae with faint staining of a 96-kDa protein. (A total of 1.5×10^6 organisms were used per immunoprecipitation. With the equivalent number of trophozoites, no band or extremely faint staining at 96 kDa was evident in the immunostains of nonpathogenic strains.) (B) Analytical densitometry of material from panel A read on a Shimadzu analytical densitometer at 580 nm in a reflectance mode. (Thermographic paper tracing was copied on a computer scanner, and traced lines were darkened by using the computer program Page-maker.) Lanes: 1, nonpathogenic zymodeme; 2 through 4, pathogenic zymodemes; 5 through 8, nonpathogenic zymodemes; (The number 75 at the bottom of each scan represents the position of the scan where EH-96 was found. Scans were started at 60 on the *y* axis and scanned to 85. The scanned lanes represent lanes 2, 3 through 5, and 6 through 9, respectively, on panel A.)

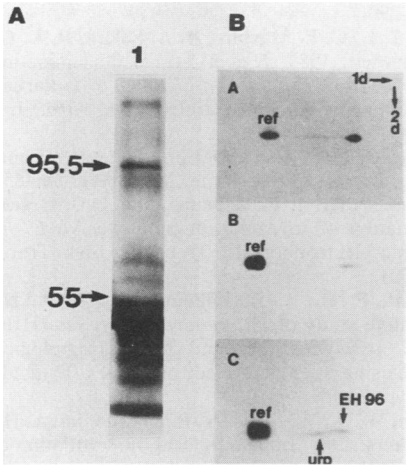

FIG. 9. Isolation of EH-96 from liver abscess and comparison with EH-96 from axenic and polyxenic *E. histolytica* with two-dimensional gel electrophoresis. (A) Immunostain of EH-96 immunoprecipitated from liver abscess aspirate with monoclonal antibody IgG2b and detected with rabbit anti-*E. histolytica* polyclonal antiserum. Multiple bands can be seen because of cross-reactivity resulting from blotting of *E. histolytica* antisera or peroxidase-labeled conjugate with liver. Numbers indicate molecular mass (in kilodaltons). (B) Two-dimensional gel electrophoresis of purified iodinated EH-96. Subpanel A, EH-96 from pathogenic axenic *E. histolytica*; subpanel B, EH-96 from polyxenic pathogenic *E. histolytica*; subpanel C, EH-96 from liver abscess aspirate; urp, Unrelated protein; ref, pure iodinated EH-96 run on the second dimension as a molecular mass marker.

possibly resulting from delipidation of the antigen or proteolysis, or that the protein has functional membrane-bound and cytosolic forms, which is not without precedent. The major membrane protein of *Leishmania* promastigotes possesses both amphipathic and amphiphilic forms (4). An alternative explanation is that the soluble form of the protein is being processed or is a peripheral membrane protein. Electron microscopy with colloidal gold localized the antigen to the plasma membrane external surface.

The EH-96 protein was detected in liver abscess fluid as well as in polyxenic and axenic cultures. Importantly, the antigen was detected on polyxenically cultivated organisms but not in spent culture medium, demonstrating the nonsecretory nature of the antigen (data not shown). We studied a small sample of *E. histolytica* strains with pathogenic and nonpathogenic zymodemes. Analytical densitometry, immunoprecipitation with EH-96-specific monoclonal antibody, and immunostaining with rabbit anti-*E. histolytica* serum demonstrated immunological differences between EH-96 in pathogenic and nonpathogenic zymodemes of *E. histolytica*. Should there be no immunological differences in pathogenic and nonpathogenic zymodemes, denser immunostaining of the small amount of 96-kDa protein from the nonpathogenic zymodemes would be expected, since rabbit polyclonal anti-*E. histolytica* serum was used in excess at a 1:100 dilution (the antiserum titer by immunoblot was in excess of 1:1,000). However, caution is indicated in the interpretation of data concerning either a complete lack of this protein or quantitative differences in concentration between strains assigned to nonpathogenic zymodemes. It is possible that the inability of anti-EH-96 monoclonal antibody to immunoprecipitate antigen may be due to either steric hindrance of the epitope by bacterial attachment or sequestering of the antigen by ingested bacteria (although the latter possibility is unlikely). Polyxenically cultivated strains were maintained with the enteric flora of the patient (which differs from person to person) or with *E. coli* 0111, and the adherent bacteria could not be completely removed. However, since the antigen was found in significant quantity in soluble form, potential bacterial surface adherence would not be expected to greatly reduce the yield from nonpathogenic organisms unless sequestration by internalized bacteria occurs. Nevertheless, the consistency of the data in the small numbers of pathogenic and nonpathogenic isolates tested so far warrants continued investigation. The observation of antigenic differences in pathogenic and nonpathogenic amebae is not without precedent. Other investigators have used monoclonal antibodies with immunofluorescence or genomic DNA probes to selectively detect or differentiate pathogenic zymodemes without identifying the antigens, if any, involved (12, 25, 26).

In summary, we have identified an integral membrane protein of *E. histolytica*, with a large proportion of it being soluble and some of it being potentially bound to internal membrane structures. Additionally, we characterized the relatedness of the protein in axenically and polyxenically cultivated *E. histolytica* and established its presence in strains of amebae associated with pathological states. This work agrees with the reports by Mahajan and Ganguly (15), who used counter immunoelectrophoresis, and Bhave et al. (2), who used ELISA, which identified trophozoite antigen in liver abscess fluid. These data demonstrate that this membrane protein of *E. histolytica* is present on clinically important strains, rendering the protein potentially useful for diagnostic purposes either in serological assays using patient sera against the protein or in antigen detection assays using the antibody to detect the antigen.

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