The Cell Surface Proteome of *Entamoeba histolytica**

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Surface molecules are of major importance for host-parasite interactions. During *Entamoeba histolytica* infections, these interactions are predicted to be of prime importance for tissue invasion, induction of colitis and liver abscess formation. To date, however, little is known about the molecules involved in these processes, with only about 20 proteins or protein families found exposed on the *E. histolytica* surface. We have therefore analyzed the complete surface proteome of *E. histolytica*. Using cell surface biotinylation and mass spectrometry, 693 putative surface-associated proteins were identified. *In silico* analysis predicted that ~26% of these proteins are membrane-associated, as they contain transmembrane domains and/or signal sequences, as well as sites of palmitoylation, myristoylation, or prenylation. An additional 25% of the identified proteins likely represent non-classical secreted proteins. Surprisingly, no membrane-association sites could be predicted for the remaining 49% of the identified proteins. To verify surface localization, 23 proteins were randomly selected and analyzed by immunofluorescence microscopy. Of these 23 proteins, 20 (87%) showed definite surface localization. These findings indicate that a far greater number of *E. histolytica* proteins than previously supposed are surface-associated, a phenomenon that may be based on the high membrane turnover of *E. histolytica*. *Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.031393, 132–144, 2014.*

The intestinal protozoan *Entamoeba histolytica* is an important human parasite. Its life cycle is relatively simple, consisting of infectious cysts that can survive outside the host and vegetative trophozoites that proliferate in the human gut. After infection, *E. histolytica* trophozoites are normally present in the intestine where they asymptomatically persist for months in the lumen. *E. histolytica* can become a pathogen by penetrating the intestinal mucosa and inducing colitis, or by disseminating to other organs, most commonly to the liver, where it induces abscess formation.

The factors that determine the clinical outcomes of *E. histolytica* infections have not been well defined. Decisive factors may include genetic aspects of the host and/or parasite, the type of immune response mounted by the host, the presence of concomitant infections, and host diet. *E. histolytica* surface proteins are regarded to be of prime importance for host-parasite interactions. Members of the galactose/N-acetyl D-galactosamine-inhibitable (Gal/GalNAc) lectin family exposed on the surface of the parasite are considered important for adherence to target cells (1, 2), with adherence necessary for killing and/or phagocytosis. In addition to their involvement in adhesion and phagocytosis, the surface molecules of *E. histolytica* are exposed to the host's immune system. To date, only about 20 proteins or protein families have been identified as exposed on the plasma membrane of the parasite. These proteins include EhADH112 and the cysteine peptidase EhCP112 (EhCP-B9), which form a 112 kDa adhesion protein (3, 4); the serine-rich *E. histolytica* protein (SREHP) (5); a calreticulin (6); an as-yet unidentified mannose binding lectin (7); transmembrane kinases, including phagosome-associated TMK96 (PATMK), TMK39 and TMK54 (8, 9); a family of Bsp-A-like molecules (10); a rhomboid protease (11); an EhRab7 molecule (12); an actinin-like protein (AAF20148) (13); the lysine (K) and glutamic acid (E) enriched proteins KERP-1 and KERP-2 (13); the cysteine peptidases EhCP-A2 and EhCP-A5 (14, 15); a peroxiredoxin (29 kDa thiol-dependent peroxidase) (16); the transcription factor URE3-BP, which localizes to the cytoplasm and inner surface of the plasma membrane (17); the ARIEL antigen (18, 19); a LIM protein (EhLimA) associated with lipid rafts in the plasma membrane (20); the M8 family surface metalloprotease (EhMSP-1) (21); alcohol dehydrogenase 3 (22); and syntaxin 1 and SNAP-25 (23).

To identify the complete set of membrane proteins that are thought to be of prime importance for host-parasite interactions, we chose a combined approach, consisting of an *in silico* analysis of predictable membrane association and bio-

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tinylation of surface proteins followed by mass spectrometric analysis. *In silico* analysis of the 8306 predicted *E. histolytica* proteins (AmoebaDB, version 1.7, http://amoebadb.org/amoeba/), using a program that predicts transmembrane protein topology, identified 1326 proteins with one or more transmembrane domains and 1079 proteins with a signal peptide with an overlap of 561 proteins. These findings support the hypothesis that the vast majority of *Entamoeba* surface proteins await identification.

Biotinylation of surface-exposed proteins using a nonpermeable reagent combined with subsequent purification and sequencing is a well-established method to characterize the cell surface proteome of various cell types and organisms (24–26). So far, the surface proteome of only one protozoan, *Trichomonas vaginalis,* was analyzed by de Miguel and colleagues using the cell surface biotinylation approach. They identified more than 400 proteins to be associated with the surface (27). Here, the surface-exposed proteins of *E. histolytica* were biotinylated, purified by affinity chromatography on Avidin agarose resin and analyzed by SDS-PAGE followed by liquid-chromatography mass spectrometry (LC-MS/MS). Of the total of 693 identified proteins, ~50% showed no predictable membrane association. Nevertheless, localization analysis indicated that about 85% of these proteins were found on the plasma membrane surface.

EXPERIMENTAL PROCEDURES

E. histolytica Cell Culture—*E. histolytica* trophozoites of strain HM-1:IMSS, obtained in 2001 from the American Type Culture Collection (ATCC; Manassas, VA, USA; Catalogue No 30459), were cultured axenically in TYI-S-33 medium supplemented with 10% adult bovine serum (ABS) at 36 °C in plastic tissue culture flasks (28).

Biotinylation of Cell Surface Molecules and Purification of Biotinylated Proteins—The experiments were performed three times independently. Cell surface proteins were biotinylated with Thermo Scientific EZ-Link Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions with some modifications. Briefly, trophozoites were grown in six T75 culture flasks until cells reached 90–95% confluence ($\leq 4 \times 10^7$ trophozoites/flask). The culture medium was removed and the trophozoites were washed carefully with 8 ml phosphate-buffered saline (PBS)[1] (6.7 mM $NaHPO_4$, 3.3 mM NaH_2PO_4, 140 mM NaCl, pH 7.2) at room temperature. One aliquot of EZ-Link Sulfo-NHS-SS-Biotin (12 mg) was dissolved in 48 ml of ice-cold PBS, and 10 ml of biotin solution was added to each flask. The solutions were incubated for 30 min at 4 °C using an orbital shaker, and 500 μl of quenching solution was added to each flask. The cells from each flask were transferred to a 50 ml reaction tube and centrifuged (400 × *g*, 3 min, 4 °C), and the trophozites were resuspended in 5 ml TBS, centrifuged again, and lysed by resuspension in 500 μl lysis buffer containing 10 μM cysteine peptidase inhibitor E-64 and 0.5% SDS. Before lysis of the cells the viability of the labeled trophozoites was quantified by trypan blue exclusion. The viability has to be >98% to proceed in the experiments. After sonication of the cell lysate for 10 s, samples were incubated for 30 min on ice, vortexed every 5 min and sonicated twice more every 10 min. The lysed cells were centrifuged at 10000 × *g* for 2 min at 4 °C and the supernatants were transferred to new tubes. The biotin-labeled proteins were isolated using NeutrAvidin Agarose and eluted as described by the manufacturer (Pierce Cell surface Protein Isolation Kit, Instructions, Thermo Scientific). Summarized, the cell lysate was incubated in a column containing NeutrAvidin Agarose for 60 min at RT using an end-over-end rotator. Before elution the agarose was washed four times using Wash Buffer. Next 400 μl of SDS-PAGE sample buffer, containing 50 mM dithiothreitol, was added to the column, incubated for 60 min at room temperature with an end-over-end rotator. Afterward the proteins were eluted by centrifugation.

As a negative control, trophozoites were treated in the same manner as described, without the addition of biotin.

One-dimensional SDS-PAGE and In-gel Digestion—The eluted protein fractions were separated on 12% SDS-PAGE gels, which were stained with Coomassie brilliant blue R-250. Each lane was cut into 36–40 slices, and each slice was washed with 50% (v/v) acetonitrile in 50 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile and dried in a vacuum centrifuge. Each gel piece was reswollen in 10 μl of 50 mM ammonium bicarbonate containing 50 ng trypsin (sequencing grade modified, Promega). After incubation for 17 h at 37 °C, the enzymatic reaction was terminated by addition of 10 μl of 0.5% (v/v) trifluoroacetic acid in acetonitrile, and the separated liquid was dried under vacuum. Each sample was reconstituted in 6 μl of 0.1% (v/v) trifluoroacetic acid and 5% (v/v) acetonitrile in water.

Mass Spectrometric Identification of Proteins—NanoLC-MS/MS experiments were performed using a reversed-phase capillary liquid chromatography system (Eksigent 2D nanoflow LC; Axel Semrau GmbH, Germany) connected to an LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). Peptides were separated on a capillary column (Atlantis dC18, 3 μm, 100 Å, 150 mm × 75 μm i.d., Waters) at an eluent flow rate of 250 nL/min using a linear gradient of 2–50% B in 60 min. Mobile phase A was 0.1% formic acid (v/v) in water; mobile phase B was 0.1% formic acid in acetonitrile. Mass spectra were acquired in a data-dependent mode with one MS survey scan in the Orbitrap and MS/MS scans of the five most intense precursor ions in the LTQ. The MS survey range was *m/z* 350–1500. The dynamic exclusion time (for precursor ions) was set at 120 s, and automatic gain control was set at 3×10^6 and 10,000 for Orbitrap-MS and LTQ-MS/MS scans, respectively.

For database searching, raw data files were processed with the Mascot Distiller (version 2.2.1; Matrix Science) to generate Mascot generic files (mgf files). The MASCOT server (version 2.2; Matrix Science, London, UK) was used to search in-house against the NCBI nonredundant database (NCBInr, date 02/02/2009, 7787617 sequences, 2685418921 residues). A maximum of two missed cleavages was allowed, and the mass tolerances of precursor and sequence ions were set at 10 ppm and 0.35 Da, respectively. Acrylamide modifications of cysteine and methionine oxidation were considered possible modifications. Scaffold (version 2.01; Proteome Software, Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if their probability was established at >70.0%, as specified by the Peptide Prophet algorithm. Protein identifications were accepted if their probability was established at >99% and if they contained at least two identified tryptic peptides. If the coverage of the protein is ≥ 5%, one identified tryptic peptide is sufficient.

In silico Analysis—The following programs were used for functional annotations as well as for identification of membrane-association sites or secretion: AmoebaDB, version 1.7, http://amoebadb.org/amoeba/; SignalP 3.0 Server, http://www.cbs.dtu.dk/services/SignalP/; NMT - The MYR Predictor, http://mendel.imp.ac.at/myristate/SUPLpredictor.htm;

[1] The abbreviations used are: PBS, phosphate-buffered saline; TBS, Tris-buffered saline; TFA trifluoacetic acid; IPTG, isopropyl-beta-D-thiogalactoside; GST, glutathione-S-transferase; ECL, enhanced chemiluminescence.

SecretomeP 2.0 Server, http://www.cbs.dtu.dk/services/SecretomeP/; TMHMM Server v. 2.0, http://www.cbs.dtu.dk/services/TMHMM-2.0/; Predotor, a prediction service for identifying putative N-terminal targeting sequences, http://urgi.versailles.inra.fr/predotar/predotar.html; PrePS - Prenylation Prediction Suite, http://mendel.imp.ac.at/sat/PrePS/index.html; Big-PI Prediction, http://mendel.imp.ac.at/gpi/cgi-bin/gpi_pred.cgi.

Isolation of Genomic DNA and RNA and cDNA Synthesis—*E. histolytica* trophozoites (1×10^6) were cultivated in 75 ml culture flasks for 24 h, harvested by chilling on ice for 5 min, and sedimented at $200 \times g$ for 5 min at 4 °C. The cell pellet was washed twice with PBS. Genomic DNA was isolated using the Easy-DNA™ kit (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Expression and Purification of Recombinant Proteins—Fragments of genes encoding putative surface localized proteins of *E. histolytica* were amplified by PCR. The sequences of the oligonucleotide primers as well as the respective restriction sites allowed the amplified DNA to be cloned in a predicted orientation into the prokaryotic expression plasmid pJC45 (supplemental Table S1), a derivative of pJC40 that also encodes a histidine leader of ten residues attached to the N terminus of the gene product (29). The following plasmids were generated: pJCrEhAct1 (Activator 1 40 kDa subunit, XM_646064.1), pJCrEhC2–1 (C2 domain-containing protein, XM_650207), pJCrEhC2–3 (C2 domain protein, XM_649407), pJCrEhDnaJ (DnaJ family protein, XM_648397), pJCrEhFeHyd (Fe-hydrogenase, XM_647747), pJCrEhGrainin1 (grainin 1, XM_645280), pJCrEhGNBP (guanine nucleotide-binding protein subunit beta 2-like 1, XM_651958), pJCrEhHypP (hypothetical protein, XM_647328), pJCrEhP120 (proliferating-cell nucleolar antigen p120, XM_649901.1), pJCrEhHydA (putative iron hydrogenase HydA, AF262400), pJCrEhRho (Rho family GTPase, XM_649396), pJCrEhRub (rubrerythrin, XM_647039.2), and pJCrEhURE3-BP (URE3-BP sequence specific DNA binding protein, AF291721). After transformation of *E. coli* BL21(DE3) pAPlacI^Q with each recombinant plasmid, the bacteria were transferred into 25 ml LB medium supplemented with 100 μg/ml ampicillin and 50 μl kanamycin, and grown overnight at 37 °C. An aliquot was transferred to 500 ml LB medium containing ampicillin and kanamycin, and grown at 37 °C until OD_{600} reached 0.6. Recombinant expression was induced by addition of isopropyl-beta-D- thiogalactoside (IPTG) at a final concentration of 0.1 mM, and culturing was continued for 3 h. Subsequently, bacteria were sedimented (6000 × *g*, 30 min), and the pellet was resuspended in purification buffer A (10 mM Tris-HCl, 100 mM Na_3PO_4, 4 M guanidinium hydrochloride, pH 8.0), treated by ultrasonification, and centrifuged for 30 min at 10000 × *g*. The supernatant was applied to a Ni^{2+}-NTA-column in binding buffer (8 M urea, 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and eluted with 0.5 M imidazole in the same buffer. PCR-amplified fragments encoding EhRab7D (XM_646823.1), EhRab7E (XM_646110.2), EhRab7G (XM_651385), EhRab7H (XM_648322), or EhRab11A (XM_642856.2) were introduced into BamHI and XhoI restriction enzyme sites of pGEX6P-1 vector (GE Healthcare) to produce N-terminal GST-fusion recombinant proteins. Expression and purification of recombinant proteins of EhRab GTPases were performed according to the manufacturer's instructions.

Generation of Polyclonal Antibodies—Antibodies were generated by injecting 100 μg of recombinant protein in complete Freund's adjuvant into BALB/c mice, followed by two booster injections, at 2 week intervals, with 100 μg of recombinant protein in incomplete Freund's adjuvant. Thirteen antisera were generated: to αAct1, αEhC2–1, αEhC2–3, αFhDnaj, αEhFeHyd, αEhGrainin1, αEhGNBP, αEhHypP, αEhP120, αEhHydA, αEhRho, αEhRub and αURE3-BP. αEhRab7D, αEhRab7E, αEhRab7G, αEhRab7H, and αEhRab11A antibodies were raised in rabbit. Additional antibodies were generated in rabbits against αEhCP-A1 (XP_650156), αEhCP-A2 (XP_650642) (30, 31), αEhCoronin (XP_654419, provided by Frank Ebert), αEhFeSOD (XP_648827) (32), αEhPrx (XP_647907) (33), αEhTR (XP_655748), αEhLectin (XP_655415) (34), whereas an antibody against αEhCP-A5 (XP_650937, (30)) was generated in chickens.

Protein Analyses—To prepare amoebic extracts, trophozoites were washed twice with PBS (6.7 mM $NaHPO_4$, 3.3 mM NaH_2PO_4, 140 mM NaCl, pH 6.8) and sedimented by centrifugation at 400 × *g* for 2 min at 4 °C. To minimize proteolysis, 20 μM trans-epoxysuccinyl-L-leucylamino-(4-guanodino)butane (E64, Sigma-Aldrich) was added. Cells were alternately flash frozen in liquid nitrogen, thawed at room temperature, and vortexed five times. Lysates were centrifuged at 40000 × *g* for 1 h at 4 °C. The supernatants contained PBS-soluble proteins. The pellets were washed twice in ice-cold PBS and solubilized in PBS supplemented with 1% Triton X-100. Extracts (50 μg/lane) were separated under reducing conditions on 12% SDS-PAGE gels. The proteins were transferred to nitrocellulose membranes using the wet blotting technique, with 25 mM Tris-HCl, 192 mM glycine, 1.3 mM SDS, pH 8.3, and 20% methanol as the transfer buffer. For Western blot analyses, primary antibodies were used at 1:500 dilution and secondary antibodies (anti-mouse HRP and anti-rabbit HRP, DAKO A/S; Glostrup, Denmark; anti-chicken IgY HRP, Sigma) at 1:5000 dilution. Blots were developed using ECL (Amersham Biosciences, ECL plus Western blotting detection reagents; GE Healthcare).

Immunofluorescence Assays (IFA)—Freshly harvested and washed amoebae (5×10^5) were fixed in 3% paraformaldehyde in PBS. Cells were permeabilized with PBS containing 0.2% saponin. Free aldehyde groups were blocked by subsequent incubation with 50 mM ammonium chloride in PBS (± saponin), followed by incubation with blocking buffer (PBS supplemented with 2% fetal calf serum) for 10 min. The trophozoites were subsequently incubated with specific antiserum (diluted 1:200 in PBS ± saponin), washed three times with PBS, and finally incubated in the dark with 1:400 dilutions of Alexa Fluor®488 goat anti-mouse or anti-rabbit antibody or Alexa Fluor®598 goat anti-chicken (Invitrogen, Molecular Probes, Eugene, OR, USA). After additional three washes with PBS, the amoebae were analyzed by fluorescence microscopy (Leica, DM BR, Wetzlar, Germany). Confocal fluorescence microscopy was performed using the Olympus IX81 microscope with FluoView Version 1.7b software (Olympus, Hamburg, Germany).

Biotin-surface labeling was validated using a 1:100 dilution of mouse monoclonal anti-biotin antibody (Sigma-Aldrich, München, Germany).

Live immunofluorescence staining was performed as described (22), with slight modifications. Briefly, amoebae (~3×10^5) were sedimented by centrifugation at 400 × *g* for 2 min at 4 °C and resuspended in 2 ml resuspension/blocking buffer, consisting of 50% v/v TYI-S-33 (without serum or antibiotics), 50% v/v PBS with 20% heat-inactivated fetal calf serum (Sigma-Aldrich) and 20 μM E64 for 10 min at 4 °C. The amoebae were again sedimented, resuspended in 400 μl resuspension buffer containing a 1:100 dilution of primary antibody, and incubated for 20 min at 4 °C. The cells were washed three times, incubated with fluorescence-labeled secondary antibody for 20 min at 4 °C, washed three times in resuspension buffer and once in PBS, and fixed in 4% paraformaldehyde for 30 min at room temperature, followed by Hoechst staining of the nuclei.

RESULTS

Preparation of E. histolytica Surface Protein-Enriched Fractions—To identify the surface-associated proteins of *E. histolytica*, live trophozoites were labeled with sulfo-NHS-SS-biotin, a thiol-cleavable amine-reactive biotinylation reagent. To reduce the possibility that this reagent would be internalized

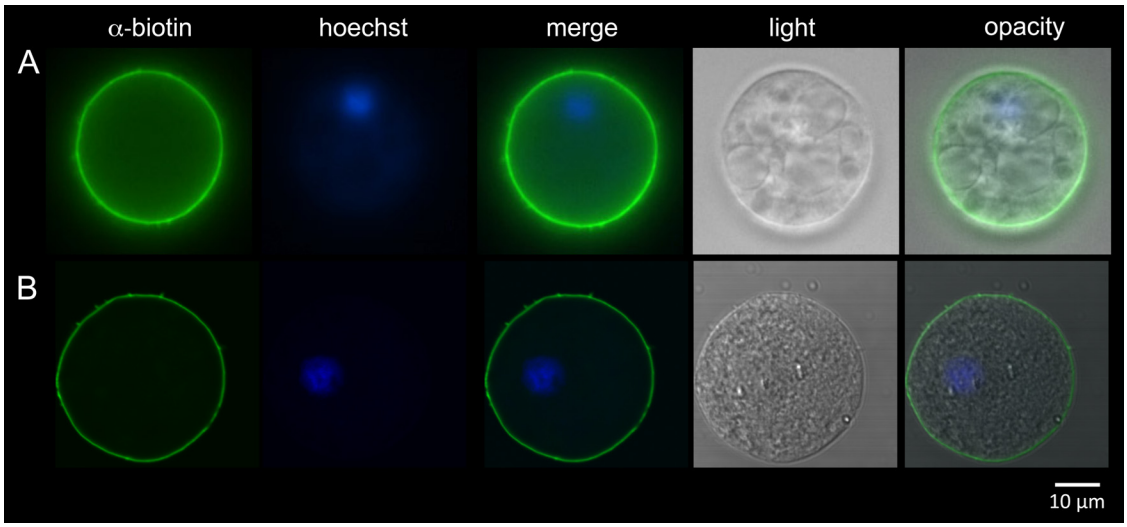

FIG. 1. **Biotinylation of *E. histolytica* surface proteins using sulfo-NHS-SS-biotin.** *E. histolytica* trophozoites were incubated with sulfo-NHS-SS-Biotin, fixed, and treated with saponin. Afterward, the biotin labeling of surface molecules was visualized with a monoclonal anti-biotin antibody and an Alexa488 conjugated secondary antibody, followed by Hoechst staining of nuclei. Fluorescence microscopy was performed using (*A*) a fluorescence microscope (Leica, DM BR, Wetzlar, Germany) or (*B*) a confocal microscope (Olympus IX81 microscope with the FluoView Version 1.7b software; Olympus, Hamburg, Germany).

by phagocytosis, labeling was performed at 4 °C for 30 min. As a control, amoebae were biotinylated and exposed to an anti-biotin antibody for immunofluorescence microscopy. The results clearly showed that biotin labeling was confined to the trophozoite surface (Figs. 1*A*, 1*B*). Nevertheless, confocal microscopy indicated that the surface of the trophozoites is not always uniformly labeled. Instead some bead-like protrusions are visible (Fig. 1*B*). The viability of the amoebae after the labeling procedure was always greater than 98%.

Cells that had been washed to remove unbound biotin were lysed, and membrane proteins were solubilized and loaded onto a streptavidin column. The column was washed to remove contaminating proteins, and the biotin-labeled proteins remaining on the column were eluted with SDS-PAGE sample buffer. The biotin-labeled protein fractions from three independent experiments were separated on a 12% SDS-PAGE gel (Fig. 2). After Coomassie blue staining, each lane was cut into 1 mm slices and the separated proteins were analyzed by mass spectrometry.

Analysis of the Surface Protein-Enriched Fractions—Out of the total of 8306 proteins encoded by the *E. histolytica* genome, 1326 (16.0%) contain one or more transmembrane domains, 1079 (13.0%) contain a signal peptide, and 561 (6.75%) contain both (AmoebaDB, version 1.7). The supplemental Table S2 shows 693 putatively surface-associated proteins. 70% (488 proteins) of them could be detected in all three independent experiments performed and 30% of them (205 proteins) could be detected in two out of three experiments.

Of these 693 surface associated proteins, 38 (5.5%) contain one or more transmembrane domains, 42 (6%) contain a signal peptide, and 14 (2%) contain both (Figs. 3*A*, 3*B*). Therefore, only 2.9% of the 1326 proteins containing one or more transmembrane domains, 3.9% of the 1079 proteins containing a predicted signal peptide and 2.5% of the 561 proteins containing both domains were identified in the membrane fraction. However, out of the 693 putative surface-associated proteins, 175 (25.2%) were predicted by the SecretomeP 2.0 server for mammalian sequences (35) to be nonclassically secreted (*i.e.* not triggered by signal peptide) proteins (Figs. 3*A*, 3*B*). So far, it has not been determined whether this type of protein secretion occurs in *E. histolytica*. Furthermore, pro-

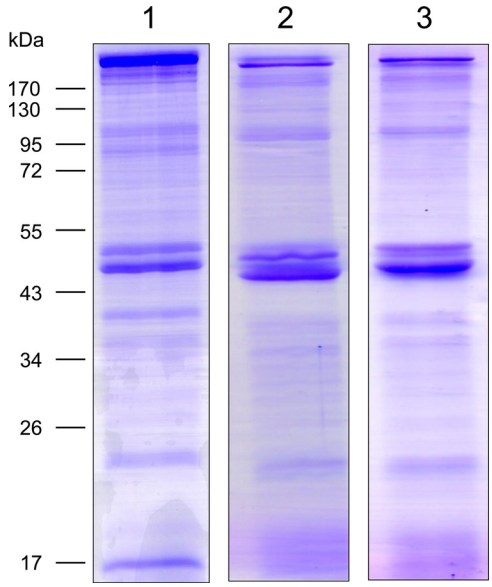

FIG. 2. **SDS-PAGE analysis of proteome preparations from three independent experiments.** Proteins were separated on a 12% SDS-PAGE gels and visualized by staining with Coomassie brilliant blue R-250.

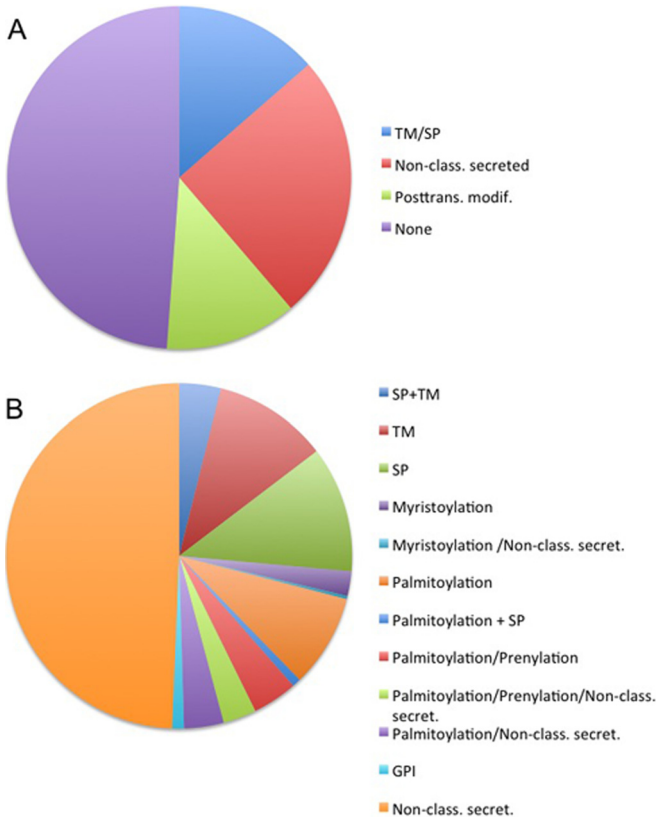

FIG. 3. **Proteins identified in the surface proteome of *E. histolytica*.** A total of 693 proteins were identified. *A*, *In silico* analysis showing that 13.5% of the identified proteins contain one or more transmembrane domains (TM) and/or a signal peptide (SP); that 12.4% contain various post-translational modifications (*e.g.* myristylation, palmitylation, farnesylation, or anchoring to GPI); and that 25.2% were nonclassically secreted. Membrane association could not be predicted for the remaining 48.8%. *B*, Pie chart showing the detailed analysis of the domains found in the 355 putative domain-containing proteins.

teins may be covalently modified post translationally by a variety of lipids, including myristate, palmitate, farnesyl, geranylgeranyl, and glycosylphosphatidylinositol (GPI). Of the 693 identified proteins, 86 (12.4%) contained at least one of these special post-translational modification sites (Figs. 3*A*, 3*B*). By contrast, none of the mentioned membrane-association domains were found in ~49% of the identified proteins (Fig. 3*A*).

BLAST analysis sorted the putative surface-associated proteins into several groups (Fig. 4). The largest group (159 of the 693 proteins, or 23%) consisted of hypothetical proteins. In addition, 11% of the identified proteins were found to be ribosomal proteins, 8.8% were nucleotide-binding/small GTPases, 7.3% were found to be involved in membrane trafficking, 3.9% were chaperones, 8.2% were involved in cytoskeletal organization, 5.5% were involved in protein processing and degradation, 12.2% were involved in general metabolic processes, 3.2% were classical membrane proteins involved in adhesion or as transporters, 4.9% were involved in nucleic acid metabolism, and 4% were involved in protein synthesis.

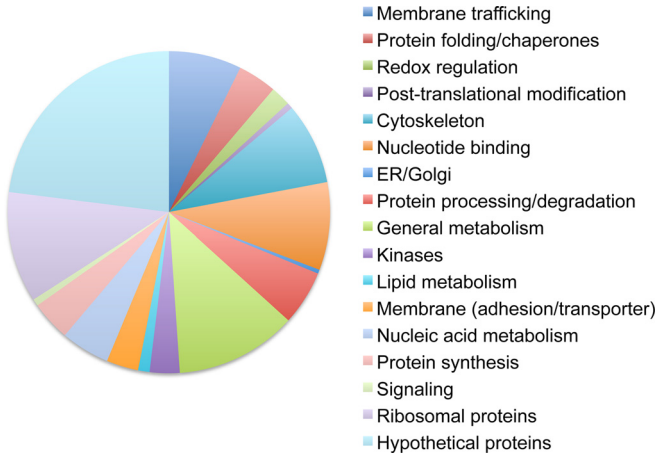

FIG. 4. **Distribution of functional annotations of the identified *E. histolytica* surface-associated proteins.** Functional groups were classified by BLAST homology analysis.

Only a few of the identified proteins (between 0.4 to 3%) were found to be involved in redox regulation, post-translational modifications, lipid metabolism, or signaling, or were ER/Golgi proteins or kinases (Fig. 4, supplemental Table S2).

Verification of Candidate Surface Proteins—To estimate the relative number of proteins that are actually surface-associated and not contaminants of membrane preparations, the location of 27 proteins was analyzed by Western blotting and/or immunofluorescence microscopy. Following random selection of a set of 23 putative surface-associated proteins, we generated antibodies against each. *In silico* analyses predicted that 11 of these proteins had no membrane-association domains, seven had a palmitoylation or prenylation domain, five had a signal peptide, one had a signal peptide and a transmembrane domain, and three were nonclassically secreted proteins. As control we also utilized antibodies against four proteins (Fe-hydrogenase, rubrerythrin, Rab7G, Rab7H) that were not detected in our proteomic study (Table I).

A solubility assay of 19 of these 27 proteins showed that DnaJ and Rab7E were present only in the membrane fraction, whereas EhC2–1, EhC2–3, EhCP-A1, EhCP-A2, EhCP-A5, EhFeSOD, EhGNBP, EhGrainin1, EhHydA, EhHypP, EhP120, EhPrx, EhRab7D, and EhRho were found in both the PBS-soluble and -insoluble fractions, and Fe-hydrogenase, rubrerythrin and EhURE3-BP were present only in the PBS-soluble fraction (Fig. 5, Table I).

To determine which of the proteins are associated with the surface live trophozoites were labeled using the antisera against these recombinant proteins. Twenty of these proteins were present on the surface, whereas seven were not, including EhAct1, EhDnaJ, EhFeHyd, EhGrainin1, EhHydA, EhHypP, and EhURE3-BP (Fig. 6). Nevertheless, immunofluorescence analyses of paraformaldehyde-fixed cells indicated that EhAct1, EhGrainin1, EhRub and EhURE3-BP were present both inside and on the surface of these cells (Fig. 7). The antibody against EhDNAJ was apparently inadequate for im-

TABLE I

Verification of candidate surface proteins. Nd: Not determined; IFA: Immunofluorescence analyses; Sap: Saponin; Pre: Prenylation Palm: Palmitoylation domain; SP: Signal peptide; TM: Transmembrane domain

Name abbr.	Gene name	Accession number	Domains/putative localization	Surface proteome	Western blot		IFA–live trophozoites	IFA–fixed trophozoites	
					PBS-soluble	PBS non-soluble		+ Sap.	− Sap.
EhAct1	Activator 1 40 kDa subunit	XP_651156, XM_646064.1	Palm	+	nd	nd	−	+	+
EhC2–1	C2 domain containing protein	XP_655299, XM_650207.2	None	+	+	+	+	+	+
EhC2–3	C2 domain protein	XP_654499, XM_649407.2	None	+	+	+	+	+	+
EhCoronin	Coronin	XP_654419, XM_649327.1	Non-class. secreted	+	nd	nd	+	nd	nd
EhCP-A1	Cysteine proteinase	XP_650156, XM_645064.2	SP	+	+	+	+	nd	nd
EhCP-A2	Cysteine proteinase 2	XP_650642, XM_645550.2	SP	+	+	+	+	nd	nd
EhCP-A5	Cysteine proteinase	XP_650937, XM_645845.2	SP	+	+	+	+	nd	nd
EhDnaJ	DnaJ family protein	XP_653489, XM_648397.1	SP	+	−	+	−	−	−
EhFeHyd	Fe hydrogenase	XP_652839, XM_647747.2	None	−	+	−	−	+	−
EhFeSOD	Iron-containing superoxide dismutase	XP_648827, XM_643735.2	None	+	+	+	+	+	+
EhGNBP	Guanine nucleotide-binding protein subunit beta 2-like 1	XP_657050, XM_651958	None	+	+	+	+	+	-
EhGrainin1	Grainin 1	XP_650372, XM_645280.2	SP	+	+	+	−	+	+
EhHydA	Putative iron hydrogenase	AAG31036, AF262400.1	None	+	+	+	−	nd	nd
EhHypP	Hypothetical protein	XP_652420, XM_647328.1	None	+	+	+	−	nd	nd
EhLectin	Galactose-specific adhesin 170kD subunit	XP_655415, XM_650323.2	SP/TM	+	nd	nd	+	+	+
EhP120	Proliferating-cell nucleolar antigen p120	XP_654993, XM_649901.1	None	+	+	+	+	+	+
EhPP2A	Serine/threonine-protein phosphatase 2A	XP_656214, XM_651122.2	Non-class. secreted	+	nd	nd	+	nd	nd
EhPrx	Peripheral membrane protein peroxiredoxin	XP_647907, XM_642815.2	Palm	+	+	+	+	+	+
EhRab7D	Rab family GTPase	XP_651915, XM_646823.1	Pre/Palm	+	+	+	+	nd	nd
EhRab7E	Rab family GTPase	XP_651202, XM_646110.2	Pre/Palm	+	−	+	+	+	+
EhRab7G	Rab family GTPase	XP_656477, XM_651385.1	Pre/Palm	−	nd	nd	+	+	+
EhRab7H	Rab family GTPase	XP_653414, XM_648322.1	Pre/Palm	−	nd	nd	+	nd	nd
EhRab11A	Rab family GTPase	XP_653051, XM_647959.2	Pre/Palm	+	nd	nd	+	nd	nd
EhRho	Rho family GTPase	XP_654488, XM_649396.2	None	+	+	+	+	+	+
EhRub	Rubrerythrin	XP_652131, XM_647039.2	None	−	+	−	+	+	+
EhTR	Thioredoxin reductase	XP_655748, XM_650656.2	None	+	nd	nd	+	+	+
EhURE3-BP	URE3-BP sequence specific DNA binding protein	AAG18423, AF291721.1	Non-class. secreted	+	+	−	−	+	+

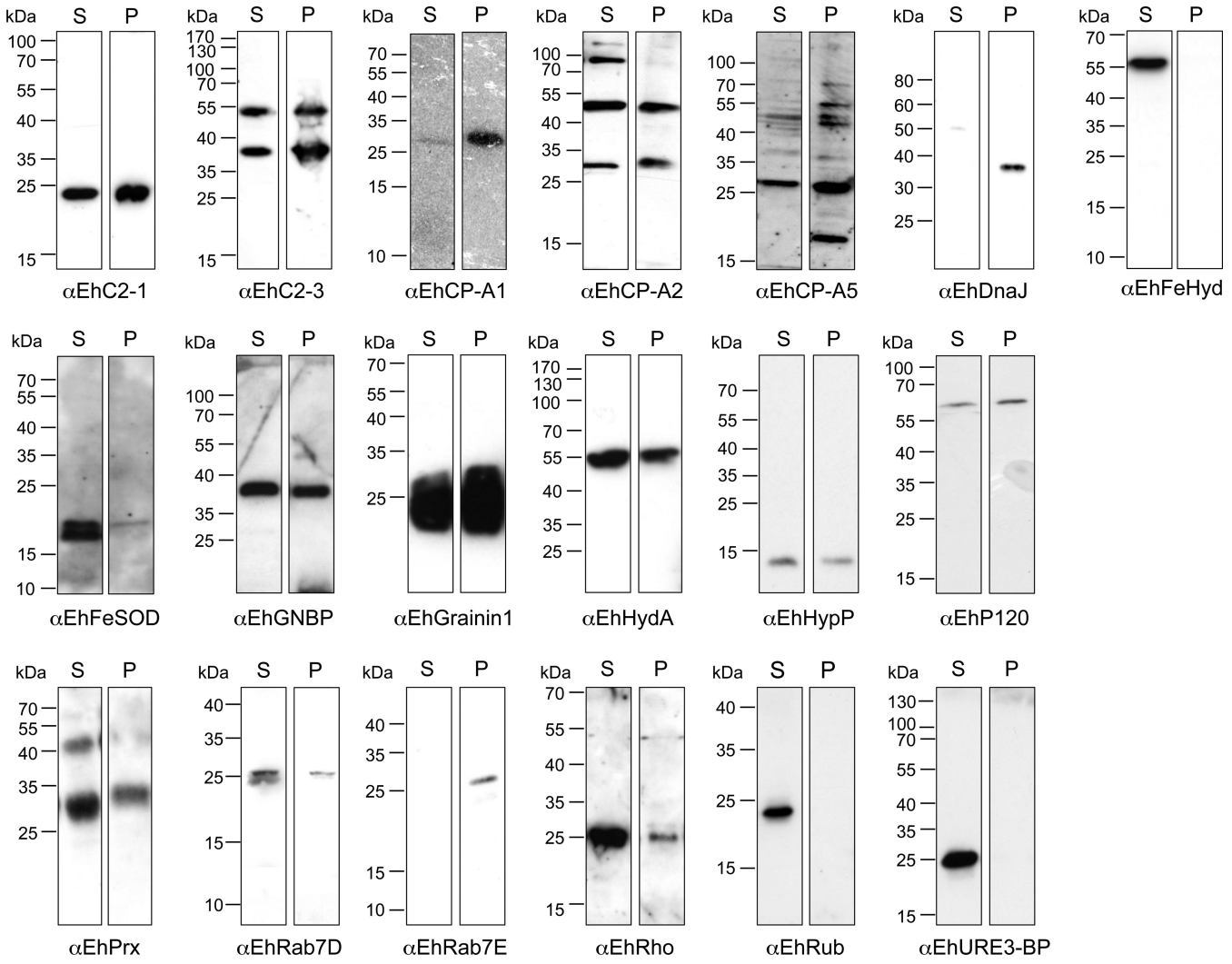

FIG. 5. **Detection of various proteins in the soluble and membrane fractions of *E. histolytica* extracts.** After lysis of the amoebae, the extracts were separated by centrifugation. The supernatant (S) contains the PBS-soluble proteins, whereas the pellet (P) contains the PBS-insoluble proteins of *E. histolytica.* The extracts were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. These Western blots were incubated with antibodies against various *E. histolytica* proteins and specific secondary antibodies, and were developed using ECL.

munofluorescence analysis because no signal was present in cells that were not treated with saponin (Fig. 7). Antibody against EhFeHyd was used as a control because, to date, the protein has only been detected in the PBS-soluble extract of *E. histolytica* (Fig. 5; (30)). This localization was confirmed as the protein was exclusively found in the cytoplasm of *E. histolytica* (Fig. 7). Interestingly, the bead-like protrusion observed on the surface of biotin-labeled amoebae, could also be observed for the majority of the live trophozoites labeled with the various antibodies (Fig. 7).

DISCUSSION

Surface-associated molecules are important for interactions of parasites with their hosts. To date, ~20 surface-exposed proteins or protein families of *E. histolytica* have been described (1–4, 6–15, 17–23, 36) (5, 16). To identify the entire set of plasma membrane-associated proteins, the surface molecules of *E. histolytica* were labeled with biotin, purified, and analyzed by mass spectrometry. Use of this non-membrane soluble biotin reagent preferentially labeled plasma membrane and plasma membrane-associated proteins. Of the total of 693 putative surface-exposed proteins identified in the *E. histolytica* strain HM-1:IMSS, 94 were found to possess a predicted transmembrane domain and/or signal peptide, whereas 86 were associated with the plasma membrane by palmitoylation, prenylation, or myristoylation, or were GPI anchored. Nevertheless, *in silico* analyses indicated that 175 of these 693 proteins were nonclassically secreted, whereas no prognosis for a plasma membrane association could be made for the remaining 339 proteins.

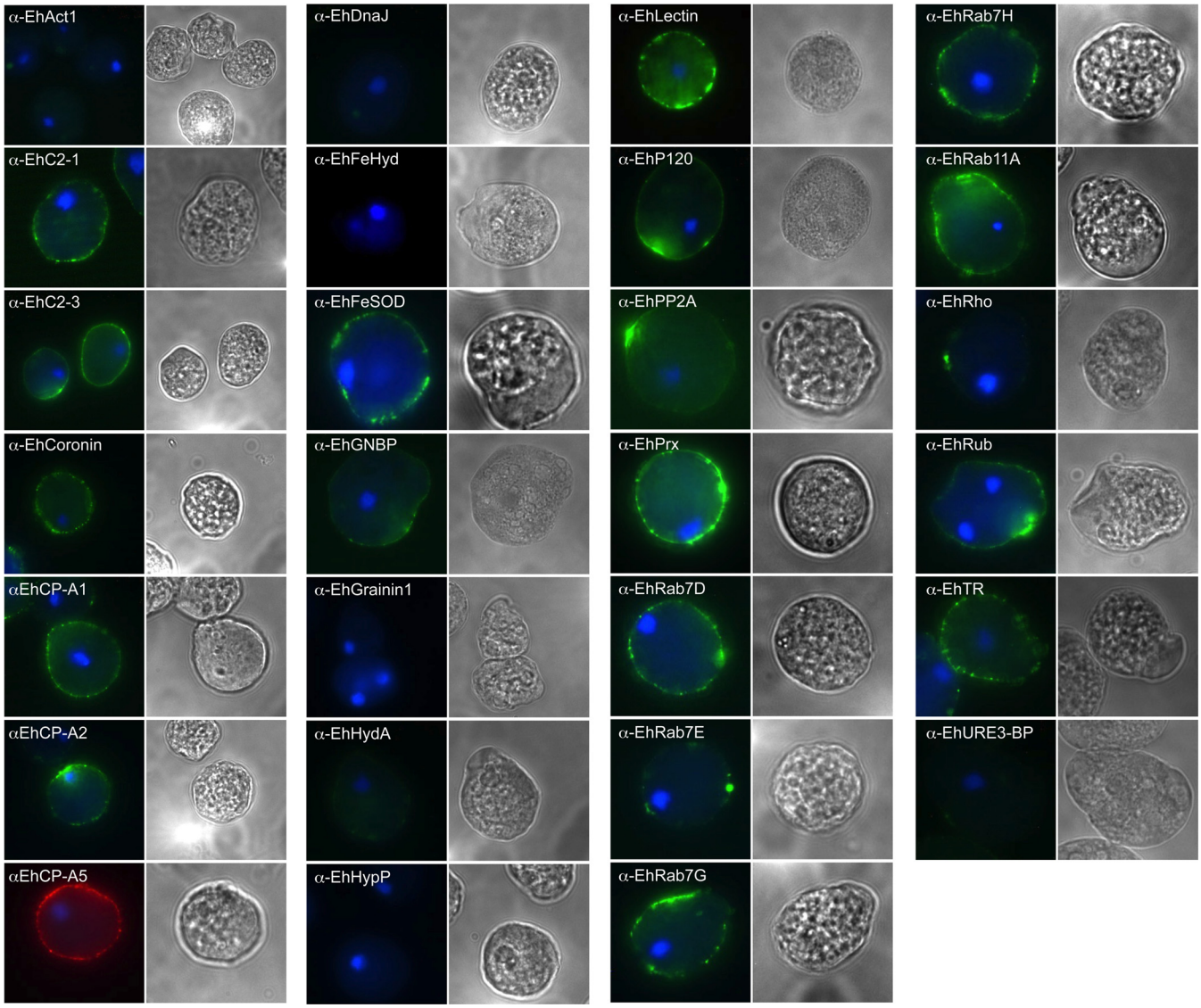

FIG. 6. **Surface association of various *E. histolytica* proteins by immunofluorescent staining of live cells.** Trophozoites were harvested at 4 °C and blocked with FCS prior to incubation with antibodies against various *E. histolytica* proteins. After washing, the amoebae were incubated with Alexa Fluor®488 goat anti-mouse or anti-rabbit antibody (green) or Alexa Fluor®598 goat anti-chicken antibody (red), fixed with paraformaldehyde, and visualized by fluorescence microscopy (Leica, DM BR, Wetzlar, Germany). Hoechst staining was used to visualize nuclei.

Twelve of the ~20 surface-exposed molecules/protein families previously identified in *E. histolytica* were identified in this study. These include the Gal/GalNAc lectin (1, 2), calreticulin (6), transmembrane kinases (8, 9), a Bsp-A like molecule (10), some Rab7 molecules (12), an actinin-like protein, KERP-1 (13), EhCP-A5 (14), EhCP-A2 (15), peroxiredoxin (37, 38), EhMSP-1 (21), and ADH3 (22).

We could not identify EhCP-B9, which previously was shown to be exposed on the *E. histolytica* cell surface (3, 4). However, the gene encoding EhCP-B9 is expressed at very low levels in *E. histolytica* strain HM-1:IMSS (39), which may explain our inability to detect the protein on the surface of these cells. In addition, we were unable to identify the serine-rich *E. histolytica* protein (SREHP) (40), rhomboid protease (11), ARIEL antigen (18, 19), and a LIM protein of *E. histolytica* (EhLimA) (20). Furthermore, we found that the protein URE3-BP, which was previously shown to be localized to the cytoplasm and inner surface of the plasma membrane (17), was present on the outer surface. By contrast, we did not detect syntaxin 1 and SNAP-25, both of which were shown to be surface-associated (23). Nevertheless, other proteins thought to be part of the SNARE machinery, including the v-SNARE protein VampF and the alpha-soluble NSF attachment protein, were detected.

In addition to transmembrane proteins localized to the plasma membrane, many transmembrane proteins were pre-

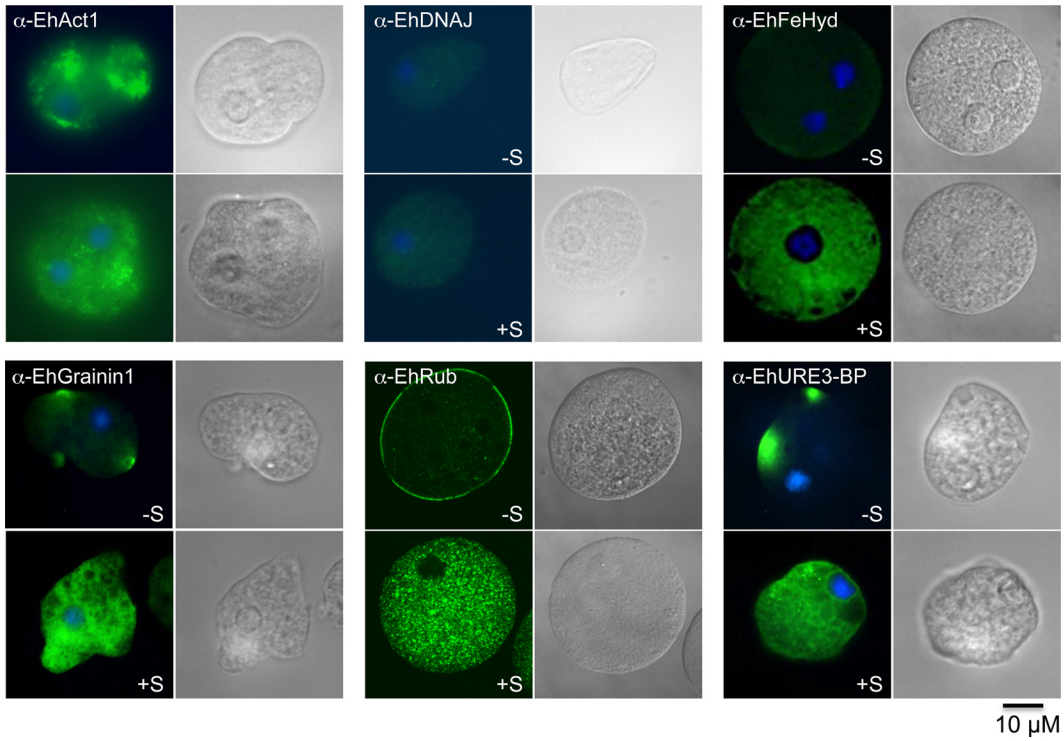

FIG. 7. **Localization of proteins identified as membrane-associated by immunofluorescence microscopy.** Amoebae were fixed with paraformaldehyde and treated with (+) or without (−) saponin. The trophozoites were incubated with specific antiserum (diluted 1:200 in PBS ± saponin), followed by AlexaFluor secondary antibody and Hoechst staining of nuclei. Localization was assessed by fluorescence microscopy.

viously reported to be localized to the ER in other organisms, such as Sec61 alpha subunit (AAU43735) (41), CAAX prenyl protease (XP_648770) (42), alpha-1,3 mannosyltransferase ALG2 (XP_653222) (43), and dolichol monophosphate mannose synthase (XP_651399) (44) (Table S2). Although many ER transmembrane proteins were identified, other ER membrane proteins were not identified in this study. For example, none of the highly expressed members of the cysteine protease binding family (CPBF), which are involved in the transport of cysteine proteases and carbohydrate digesting enzymes from the ER to downstream organelles was identified in the present study (45–47). In addition, only nineteen out of 102 Rab GTPases, most of which were expressed in the trophozoites and possessed the C-terminal isoprenylation motif were isolated from the cell surface (48). The subcellular localization of these 19 Rab GTPases is currently investigated to determine key Rab proteins that mediate the transport from the internal membrane to the cell surface.

A recent large-scale study analyzed the proteins in isolated *Entamoeba* uropods, which are formed and released from amoebae following the incubation of trophozoites with ConA (49). These uropods consist of folded membranes and therefore are composed primarily of surface-associated proteins with small amounts of cytoplasm. Similar to our findings, several of the proteins identified in the uropod proteome are not obviously plasma membrane associated, as they do not contain a transmembrane domain or signal sequence. Interestingly, ~75% of the uropod-associated proteins were also identified in the present study, including proteins involved in trafficking, several Rab family proteins and ribosomal proteins. The major difference between the two studies was the identification of several peptidases in the uropods, including four cysteine peptidases belonging to the C family, two dipeptidyl-peptidases and a serine carboxypeptidase, none of which were present in the surface-associated proteome (49). Most of the surface proteins present in the uropod proteome are likely not uropod specific, but may be localized in general to the outer plasma membrane of the trophozoite.

An analysis of the surface proteome of *Trichomonas vaginalis* identified a total of 411 proteins, of which 23% are present in other subcellular compartments, suggesting that these proteins are contaminants of membrane fractions (27, 49). These putative contaminants included the mentioned above ribosomal proteins as well as Rab proteins, all of which were present in the surface as well as in the uropod proteome of *E. histolytica* (27, 49).

To solve these uncertainties, we used immunofluorescence microscopy to analyze the surface expression on live trophozoites of 23 representative proteins. *In silico* analysis indicated that 12 of these proteins were not membrane associated. The remaining proteins were putatively prenylated or palmitoylated, were nonclassically secreted, or contained a signal peptide and/or transmembrane domain. Interestingly, immunofluorescence analyses of live trophozoites unequivo-

cally indicated that 17 of these 23 proteins were present on the cell surface. An additional three proteins showed surface association on paraformaldehyde-fixed but nonpermeabilized amoebae. Therefore, only three of these 23 proteins, a DnaJ family protein, iron hydrogenase, and a hypothetical protein XP_652420, can be classified as contaminants. In addition, four additional proteins, none of which were identified using our proteomic approach, were localized on the plasma membrane. Similar to EhRab7D and EhRab7E, which were identified as surface-associated proteins, EhRab 7G and Rab7H were found at the trophozoite surface. Rubrerythrin was also associated with the plasma membrane, whereas Fe-hydrogenase showed a cytoplasmic localization. Most of the proteins analyzed show no uniform surface stain. Instead, bead-like protrusion could be observed. One explanation may be that vesicles containing the respective proteins fuse at these areas with the plasma membrane. Nevertheless, this cannot explain, why a similar but less distinct staining was observed in biotin-labeled cells.

Immunelectron microscopy may help in future experiments to analyze if the proteins are localized on the inner or outer cell membrane. In summary, these results indicate that more proteins than suggested are surface-associated, including several molecules lacking an identifiable secretion signal or membrane binding domain. However, despite all controls included, it cannot be ruled out that the list of putative surface proteins contains also some contaminants.

Studies have shown that some proteins are indirectly associated with the extracellular surface. These peripheral membrane proteins bind temporarily, either to the lipid bilayer or to integral membrane proteins, through a combination of noncovalent interactions, including hydrophobic and electrostatic interactions. Among the proteins indirectly associated with the plasma membrane are disulfide isomerase, enolase, heat shock proteins, malate dehydrogenase, triosephosphate isomerase, thioredoxin and superoxide dismutase, all of which were also identified in the current study (25, 50–53). Interestingly, similar to *E. histolytica*, various ribosomal proteins of mycobacteria were identified as being surface-exposed (51).

Proteins lacking a signal sequence likely do not utilize the classical secretory pathway. These molecules of *Entamoeba* may reach the surface by undergoing unconventional secretion. For example, proteins may be secreted through translocation processes that convey these proteins across plasma membranes. These processes may be nonvesicular, or may involve the generation of exo- or ectosomes (54–57). The SNARE machinery, which is highly conserved in all eukaryotes and is present in the surface proteome of *E. histolytica*, may be involved. The SNARE machinery is responsible for membrane fusion as well as for the fusion of secretory vesicles with the plasma membrane. Using this pathway, secreted enzymes, membrane proteins and lipids can reach the cell surface (58, 59). In addition, the focal exocytosis of endomembranes including the ER may be crucial in compensating for the loss of cell surface area during phagocytosis (60, 61).

Studies in *Acanthamoeba* suggested that secreted peptidases may be trapped in vesicles that return membranes from the digestive vacuole to the plasma membrane (62). Some of these peptidases have a high affinity for membranes and are bound to the vesicle membrane. After fusion with the plasma membrane, the contents of these vesicles are released and their internal surface is exposed to a more alkaline pH, decreasing the affinity of these peptidases for the membrane and resulting in their release (63). Cysteine peptidases, along with additional proteins of *E. histolytica* detected at the plasma membrane, may be released by a similar mechanism.

Interestingly, six out of nine core glycolytic enzymes were found in the putative surface proteome (hexokinase, phosphofructokinase, fructose-1,6-biphosphate aldolase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase (GAPDH), enolase). One can speculate that the lysis of trophozoites during the labeling process and therefore the release of these highly expressed glycolytic enzymes may be the reason for their occurrence in the membrane fraction. However, less than 2% of amoebae died during the biotin labeling procedure, which almost excludes this possibility. For enolase, fructose-1,6-biphosphate aldolase, triosephosphate isomerase and GAPDH a surface association was described in several organisms including *Paracoccidioides brasiliensis*, *Streptococcus suis, Trichomonas vaginalis, Lactobacillus plantarum* 299v*, Mycobacterium tuberculosis, Candida albicans,* and *Staphylococcus aureus* (64–70). Interestingly, like also seen for *E. histolytica* these proteins are anchorless and the mechanism by which they reach the cell surface is unknown. It was shown that the extracellular matrix components fibronectin, laminin as well as mucin act as ligands for aldolase, enolase, triosephosphate isomerase, and GAPDH (64–66, 71, 72). Furthermore, these glycolytic enzymes have been identified to act as plasminogen receptors (70, 73). A nine residue plasminogen binding motif, which was found within the enolase sequence of several organisms, including *P. brasiliensis* and *S. pneumoniaea* was also found within the enolase of *E. histolytica* (64, 74). It was shown that the interaction of the different microorganisms with plasminogen is important for invasion of the host and establishment of an infection. For *Trichomonas vaginalis* it was described, that the binding to plasminogen mediates the penetration of the basement membrane and the adhesion to extracellular matrix proteins, like laminin and fibronection (67, 72, 75). It is hypothesized that *T. vaginalis* can adhere to the basement membrane after disruption of the vaginal epithelium (72, 76). Among others, the interaction of extracellular components including fibronectin, laminin, and mucin are also known as important factors for adhesion of *E. histolytica* of the human intestine (77, 78). Therefore it can be postulated, that enolase, aldolase, triosephosphate isomerase, and GAPDH, which were identified in the surface proteome of *E. histolytica* may be important

for the colonization and establishment of the infection. In addition, it was shown that the surface-exposed GAPDH of *Streptococcus pneumoniae* can act as ligand for the complement component C1q. It is postulated that this interaction led to modulation and evasion of the immune system (79). Hexokinase, which was also identified in the surface proteome of *E. histolytica* is normally cytoplasmic. Nevertheless, a phosphotyrosine-containing hexokinase was found to be associated with the plasma membrane fraction of sperms (80, 81). Surface biotinylation of intact mouse sperm followed by precipitation with either hexokinase antiserum or with avidin-Sepharose suggests that this hexokinase possesses an extracellular domain (81). Phosphofructokinase was also found in the bull sperm surface proteome. However, it is not known if this molecule is localized at the surface. In general, an association of glycolytic enzymes with the *E. histolytica* membrane makes sense, because of the high membrane turnover and to drive membrane fusion events.

In conclusion, we have identified the almost complete surface proteome of *E. histolytica*. Most notably, about 50% of the identified proteins lacked the conventional characteristics associated with membrane localization. Nevertheless, the vast majority is indeed membrane-associated. This unexpected finding suggests that the plasma membrane is not a static cellular compartment, but rather part of a highly interconnected cellular machinery, in which intracellular membrane systems are in constant exchange with the plasma membrane. This can result in the presence of proteins outside of the cell that have no conventional signs of plasma membrane-association.

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S This article contains supplemental Fig. S1 and Tables S1 and S2.

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