Chitinase Secretion by Encysting *Entamoeba invadens* and Transfected *Entamoeba histolytica* Trophozoites: Localization of Secretory Vesicles, Endoplasmic Reticulum, and Golgi Apparatus

SUDIP K. GHOSH, JESSICA FIELD, MARTA FRISARDI, BENJAMIN ROSENTHAL, ZHIMING MAI, RICK ROGERS, AND JOHN SAMUELSON

Department of Immunology and Infectious Diseases and BioMedical Imaging Institute, Harvard School of Public Health, Boston, Massachusetts 02115

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*Entamoeba histolytica*, the protozoan parasite that phagocytoses bacteria and host cells, has a vesicle/vacuole-filled cytosol like that of macrophages. In contrast, the infectious cyst form has four nuclei and a chitin wall. Here, anti-chitinase antibodies identified hundreds of small secretory vesicles in encysting *E. invadens* parasites and in *E. histolytica* trophozoites overexpressing chitinase under an actin gene promoter. Abundant small secretory vesicles were also identified with antibodies to the surface antigen Ariel and with BiP fluorescence. Removal of an N-terminal signal sequence directed chitinase to the cytosol. Addition of a C-terminal KDEL peptide, identified on amebic BiP, retained chitinase in a putative endoplasmic reticulum, which was composed of a few vesicles of mixed sizes. A putative Golgi apparatus, which was Brefeldin A sensitive and composed of a few large, perinuclear vesicles, was identified with antibodies to ADP-ribosylating factor and to ε-COP. We conclude that the amebic secretory pathway is similar to those of other eukaryotic cells, even if its appearance is somewhat different.

*Entamoeba histolytica* is a protozoan parasite that causes dysentery and liver abscess in developing countries which cannot prevent its fecal-oral spread (56). Amebae have three virulence-associated properties, which are of interest to cell biologists (43). First, amebae survive anaerobic conditions in the lumen of the colon and tissue abscesses by means of fermentation enzymes that resemble those of anaerobic bacteria (31, 48, 57, 60). Indeed, amebae lack enzymes of oxidative phosphorylation and have an atrophic mitochondrion-derived organelle, which resembles the petite mitochondria of yeast cells grown under anaerobic conditions (11, 20, 41). Second, amebae have a vesicle/vacuole-filled cytosol like that of macrophages (63). Amebae phagocytose bacteria in the colonic lumen and epithelial cells and erythrocytes (RBC) when parasites invade tissues and cause dysentery (46, 56). Third, amebae form a chitin-walled cyst, which is the infectious form of the parasite, because cysts are resistant to stomach acids (5, 15).

Phagocytosis is the best studied of the amebic virulence mechanisms. Parasites attach to bacteria, epithelial cells, and RBC via amebic lectins, which recognize Gal or GalNAc sugars on the surface of target cells (42, 45). Amebae kill bacteria or lyse host cells within their phagolysosomes via oxygen-independent mechanisms including lysozyme, cysteine proteases, and amebapores (also known as pore-forming peptides) (8, 34, 64). Lysosomal proteins, which have been identified in supernatants of cultured trophozoites, include cysteine proteases, acid phosphatase, collagenases, glycosidases, and esterases but not pore-forming peptides (1, 35, 68). One cysteine proteinase (CP-5) is present on the surface *E. histolytica* trophozoites but is absent from the surface of avirulent *E. dispar* (26). Amebic phagocytosis is disrupted by wortmannin and by overexpression of hyperactive amebic p21rac, a ras-family protein involved in the site selection of actin polymerization (18, 23, 36). In animal models, phagocytosis mutants of amebae, selected by consumption of bromodeoxyuridine-loaded bacteria, are less virulent than wild-type parasites (58).

Secretion by amebae is much less well understood than phagocytosis. Amebae have on their surface vaccine candidates, including the Ser-rich *E. histolytica* protein (SREHP) and Gal or GalNAc lectin, which presumably get there when secretory vesicles fuse with the plasma membrane (42, 45, 51, 52, 61, 67, 73). Small and large subunits of the Gal or GalNAc lectin have signal sequences that are cleaved at sites predicted by the “−3, −1” rule of von Heijne (Table 1) (50). Signal sequences as well as propeptide sequences are cleaved from pore-forming peptides and cysteine proteases (Table 1) (8, 34). An *E. histolytica* gene has been cloned that encodes a 54-kDa peptide of the signal recognition particle, a ribonuclear protein that binds N-terminal signal sequences on secreted proteins (55). An amebic gene has also been cloned that encodes an endoplasmic reticulum (ER) retention receptor (ERD2), a cis-Golgi-associated transmembrane protein (62). ERD2 binds C-terminal KDEL peptides on proteins such as the 70-kDa heat shock protein BiP, which is a chaperonin in the lumen of the ER (10, 22). We recently cloned the *E. histolytica* bip gene and demonstrated that the protein contains a C-terminal KDEL peptide (16a). Although amebae lack a Golgi with tight lamellae, a putative Golgi was identified by confocal microscopy with NBD-ceramide and by transmission electron microscopy with thiamine-pyrophosphatase (44).

We recently used molecular cloning methods to identify amebic chitinases, which are secretory proteins expressed by encysting parasites (14). Each amebic chitinase contains a series of acidic and hydrophilic repeats between an N-terminal signal sequence and a C-half catalytic domain (50). As *E. histolytica* trophozoites are difficult to encyst in axenic culture, cyst formation has for the most part been studied by using the reptilian pathogen *E. invadens*, which converts to chitin-walled...
cysts within 2 days when deprived of glucose (15). During *E. invadens* cyst formation, chitin synthase and chitinase are both expressed, and cyst formation is inhibited by the chitin synthase inhibitors polyoxin D and Nikkomycin and by the chitinase inhibitor allosamidin (6, 13, 72).

The goal of the present studies was to visualize structures involved in secretion (secretory vesicles, ER, and Golgi) in amebic trophozoites (motile forms) are very difficult to visualize. In contrast, secretory vesicles, which contain cyst wall proteins, are so prominent in encysting giardia that they were given a special name, expression-specific vesicles or ESV (47). Similarly, encysting *G. lamblia* show increased expression of Golgi proteins and the ER-associated protein BiP (22, 37, 38). Here amebic secretory vesicles, putative ER, and Golgi apparatus of *E. invadens* were patterned after similar studies of *Giardia* parasites (secretory vesicles and Golgi). Furthermore, the chitin synthase genes, and the same sense and antisense cDNAs (14).

**TABLE 2. Antibodies and transformation constructs used to localize amebic organelles**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Antibodies to:</th>
<th>Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretory Vesicles</td>
<td>Chitinase*</td>
<td>ER BiP-myc, mammalian ARF, mammalian e-COP</td>
</tr>
<tr>
<td>Cytosol</td>
<td>ADH1</td>
<td>Chitinase (+) KDEL, mammalian e-COP</td>
</tr>
<tr>
<td>Golgi</td>
<td>Mammalian ARF, mammalian e-COP</td>
<td>Chitinase (-) signal</td>
</tr>
</tbody>
</table>

*Encytation-specific chitinase in *E. invadens*. All constructs were in the pJST4 vector and transfected into *E. histolytica* and *E. invadens* trophozoites. Anti-Ariel antibodies were localized with FITC-dextran, phagosomes with GFP-labeled bacteria, and lysosomes with the fluorescent substrate (Arg4–methoxy-2-naphthylamide).

**Materials and Methods**

**Parasites used and conditions for pinocytosis, phagocytosis, and encystation.**

The HM-1 strain of *E. histolytica*, from which chitinase, ariI, ariII, and bip genes were identified, was used to study secretion in amebic trophozoites (14, 40). The IP-1 strain of *E. invadens*, a generous gift of Dan Eichinger of New York University, was used to study secretion during encystation (15). *E. histolytica* and *E. invadens* were grown at 37 and 25°C, respectively, in the same axenic TYI-S33 medium.

**Pinocytosis by *E. histolytica* and *E. invadens* was observed by incubating trophozoites for 30 min with fluorescein isothiocyanate (FITC)-dextran (1 mg/ml) in culture medium (18). Amebae were washed in phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde, and passed through a fluorescence-activated cell sorter (Beckton Dickinson). Negative controls included paraformaldehyde-fixed parasites not exposed to FITC-dextran.

**Phagocytosis by *E. histolytica* and *E. invadens* was studied by using Escherichia coli expressing recombinant green fluorescent protein (GFP) under an isopropyl-β-D-thiogalactopyranoside-inducible promoter (70).** Tropheozoites (10^6/ml) were incubated in culture medium with GFP-labeled bacteria (10^9/ml) for 30 min and then fixed with 2% paraformaldehyde in PBS (18).

**E. invadens** encystation was induced by simultaneously reducing the osmolarity, glucose, and serum in the cultures (15). Encysting organisms after 1 to 3 days induction were identified in three ways: (i) observation of rounded organisms with a refractile wall, which were resistant to lysis in 1% Triton X-100 (ii) staining parasites with Calcofluor; or (iii) staining organisms with anti-chitinase antibodies (see below). To determine the effects of various inhibitors on cyst formation, we incubated *E. invadens* in encysting medium containing 100 μg of Brefeldin A per ml, 0.3 μM okadaic acid, or 100 nM wortmannin (18, 25, 29).

**Cloning of *E. histolytica* and *E. invadens* arf genes.** Segments of the *E. invadens* and *E. histolytica* arf genes were isolated from DNA of *E. invadens* IP-1 strain and *E. histolytica* HM-1 strain genomic DNAs by using the PCR and degenerate primers to conserved peptides in ADP-ribosylating factor (ARF) of other eukaryotes (3, 21, 28, 32). A degenerate sense primer [AGAAT(CT)(CT)(CT)ATGG(TAT)G] was used with an antisense primer [GG(A)AT(A)G(A)]CTGTG(TT)(AT)ATG(A)CG was used to PCR amplification on 100 bootstrap resamplings of each alignment. The SREHP and Ariel proteins are genetically related.

**The SREHP** and Ariel proteins are genetically related.

**The pore-forming peptide A** is representative of two other pore-forming peptides, while cysteine protease 1 is representative of five other cysteine proteases.

**The pore-forming peptide B** is represented by two other pore-forming peptides, while cysteine protease 1 is representative of five other cysteine proteases.

**TABLE 1. N-terminal signal sequences of *E. histolytica* plasma membrane, secretary, ER, or lysosomal proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane proteins</td>
<td>Lectin small subunit--milililisfg KY51611, Lectin large subunit--mllklmivlcll DLK1</td>
</tr>
<tr>
<td><strong>SREHP</strong></td>
<td>mllililisfgTNYV</td>
</tr>
<tr>
<td>Ariel</td>
<td>mllililisfgTNYV</td>
</tr>
</tbody>
</table>

**Secretary or ER proteins**

<table>
<thead>
<tr>
<th>Chitinase</th>
<th>mannalylarana HNC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. invadens</strong> chitinase</td>
<td>mannalylarana HNC</td>
</tr>
<tr>
<td>Bip</td>
<td>mllililisfgTNYV</td>
</tr>
<tr>
<td>Aldh1</td>
<td>mceayvlksdvlnilvgeclvlishalKY</td>
</tr>
</tbody>
</table>

**Lysosomal proteins**

**Pore-forming peptide A’** mkaiv45libavanaa eGIL |

Cytochrome probe f = mllililisfgTNYV |

**RRAIFNMNARIVAENNRKETFKLSVDG** was observed by incubating trophozoites with GFP-labeled bacteria (10^7/ml) for 30 min and then fixed with 2% paraformaldehyde in PBS (18). Encystation of *E. invadens* was induced by simultaneously reducing the osmolarity, glucose, and serum in the cultures (15). Encysting organisms after 1 to 3 days incubation were identified in three ways: (i) observation of rounded organisms with a refractile wall, which were resistant to lysis in 1% Triton X-100 (ii) staining parasites with Calcofluor; or (iii) staining organisms with anti-chitinase antibodies (see below). To determine the effects of various inhibitors on cyst formation, we incubated *E. invadens* in encysting medium containing 100 μg of Brefeldin A per ml, 0.3 μM okadaic acid, or 100 nM wortmannin (18, 25, 29).
Expression of chitinase genes under an actin promoter in transfected E. histolytica trophozoites. E. histolytica trophozoites, which do not encyst in axenic culture, do not express their chitinase gene (14). To identify secretory vesicles in E. histolytica trophozoites, we expressed the E. histolytica chitinase gene in transfected amebae under an E. histolytica actin 1 gene promoter (Table 2) (17, 18). Briefly, the coding region of the E. histolytica chitinase gene was isolated from genomic DNA by PCR. The sense primer (S1 = GCGGATCCATGCTACACT CAGCCATTAAT) contained a KpnI site (italics) and encoded the first six amino acids at the amino terminus of the parasite's chitinase (MSLFAA [underlined]). The antisense primer (A1 = GCGGATCCCTTGAACCTTGAATTAG) contained a BamHI site (italics) and encoded five amino acids at the C terminus of the organism's chitinase (LIEEK [underlined]). The chitinase gene was cloned into the pJST4 amebic transformation vector and electroporated into E. histolytica HM-1 strain amebae (17). Parasites were step selected in 10 to 100 μg/ml of G418 per ml (4 to 6 weeks), when the expression of chitinase mRNA was checked by RT-PCR. Overexpression of intact chitinase or modified chitinases (described below) had no effect on amebic viability, growth rate, pinocytosis, or phagocytosis.

N-terminal signal sequences on amebic secretory, ER, or plasma membrane proteins were identified by using the SignalP algorithm at the website of the Center for Biological Sequence Analysis at the Technical University of Denmark (Table 1) (50). A truncated E. histolytica chitinase gene encoding a chitinase lacking its 12-amino-acid signal sequence was made with the PCR (14). The antisense primer was A1 (described above), while the sense primer (GGGGTA CATTGCTAACACAACTGGTGAAG) contained a BamHI site (italics) and encoded Met and Ala12 to Glu17. To determine whether a C-terminal KDEL peptide would cause chitinase to be retained in the lumen of the ER of transfected parasites, we made a modified chitinase gene by using PCR. The sense primer was S1 (described above), while the antisense primer (GGGGATCCCTT AAAATCACTTTTAGACTCTTAATGATTTTG) contained a BamHI site (italics) and encoded KYIKSLKDEL (underlined) at the C terminus of the chitinase instead of the wild-type C-terminal sequence (KYIKSLIEKC) (14). The truncated chitinase and the chitinase-KDEL PCR products were cloned into pJST4 and expressed in amebae as described above. As a control for localization of the ER, amebic BiP was overexpressed with a myc tag in E. histolytica trophozoites (10, 16, 22, 38). The coding region of the E. histolytica bip gene was isolated from genomic DNA by using PCR. A sense primer (GGGGATCCATGCTAACACTTGAATTAG) contained a KpnI site (italics) and encoded the first six amino acids at the amino terminus of the parasite's BiP (MLFFLF [underlined]). An antisense primer (GGGGATCCCTTGAACCTTGAATTAG) contained a BamHI site (italics) and encoded the C-terminal KDEL (underlined), the myc epitope EQKISSELD (boldface), and the BiP hexapeptide NYEEYE adjacent to the C terminus (not underlined). The BiP constructs were cloned into the pJST4 amebic transformation vector and electroporated into E. histolytica HM-1 strain amebae as described above.

Confocal microscopy. To immunolocalize secretory proteins on the surface of E. histolytica and E. invadens, we fixed parasites with 2% paraformaldehyde for 10 min at 4°C. To visualize secretory vesicles or Golgi-associated proteins, we permeabilized amebae by incubation with 0.1% Triton X-100 for 5 min at room temperature. Similar results were obtained when parasites were permeabilized with 0.1% saponin. Amebae were immunostained for 60 min with monoclonal rabbit antibodies to chitinases, Ariel, or α-COP, diluted 1:100 or 1:200 in PBS with 1 mg of bovine serum albumin per ml (24). Organisms were washed four times and immunodecorated for 60 min with a Texas red-conjugated goat anti-rabbit antisera. Controls included parasites stained with preimmune rabbit serum. Similar methods were performed with mouse monoclonal anti-ARF and anti-myc antibodies, each diluted 1:200. Lysozymes were visualized by incubating living parasites in Arg-Arg-4-methoxy-2-naphthylamide, a substrate of cysteine proteinases that fluoresces when it is cleaved (64). Fluorescently labeled parasites were observed with a Leica NT-TCS confocal microscope fitted with argon and krypton lasers. Images of amebae were recorded in 512 image size format with a ×40 or ×100 Planapo objective. The number of vesicles per ameba labeled with anti-ARF, anti-α-COP, or anti-chitinase antibodies in parasites transfected with the chitinase-KDEL construct was determined by making serial optical sections through the parasites with the confocal microscope. To illustrate some of these structures, composite figures were made that combined multiple optical sections. The number of secretory vesicles, which were identified with antibodies to intact chitinase or Ariel, was too many to count accurately.

Nucleotide sequence accession numbers. Nucleotide and derived amino acid sequences of E. histolytica and E. invadens arf gene segments have been submitted to GenBank under accession numbers AF082517 and AF082518.

RESULTS

Chitinase is an appropriate reporter protein for studying secretion in transfected E. histolytica because it is not expressed by trophozoites. GFP, which is a popular epitope tag used to localize proteins in living eukaryotic cells, does not work in transfected amebae (unpublished observations) (54). This is because GFP fluorescence is dependent upon the presence of free oxygen, which is not present in amebic cultures. Amebic chitinase was chosen here to study secretion in transfected trophozoites because chitinase contains a series of antigenic repeats and is normally secreted by encysting parasites...
Chitinase mRNAs, detected by RT-PCR, were present in extracts of encysting *E. invadens* parasites but were absent in extracts of either *E. histolytica* or *E. invadens* trophozoites (Fig. 1). In contrast, mRNAs of the Golgi-associated protein ARF were present in extracts of trophozoites of *E. histolytica* and *E. invadens* and in extracts of encysting *E. invadens* (Fig. 1 and see further discussion below) (12). ERD2 mRNAs were also present in extracts of *E. histolytica* trophozoites (Fig. 1) (62).

Chitinase-associated secretory vesicles in encysting *E. invadens* parasites are numerous and small. Antibodies to antigenic repeats of the *E. invadens* chitinase were used to demonstrate hundreds of mostly small secretory vesicles in encysting *E. invadens* parasites (Fig. 2 and Table 2) (14, 61). In most cells, these chitinase-associated secretory vesicles were so abundant that their exact number could not be determined. Chitinase was also present in patches on the surface of encysting parasites. Chitinase was absent from trophozoites, which lack detectable messages for this gene (as above). Chitinase-associated vesicles of encysting amebae are similar in their appearance to ESV previously described in encysting *G. lamblia* (19, 39, 47).

When encysting parasites were incubated with FITC-dextran or GFP-labeled bacteria prior to fixation and staining with anti-chitinase antibodies, two results were apparent. First, parasites with many chitinase-associated secretory vesicles, which were well into encystation, pinocytosed much less FITC-dextran and phagocytosed many fewer bacteria than trophozoites. For example, after 24 h, most encysting parasites pinocytosed the same amount of FITC-dextran as the control parasites fixed before incubation with FITC-dextran (data not shown). After 24 h, most encysting parasites no longer phagocytosed bacteria. Second, chitinase-associated secretory vesicles were not overlapping with pinosomes and phagosomes, so double-staining (orange vesicles) was rare or absent (data not shown).

Secretory vesicles of *E. histolytica* trophozoites, marked by anti-chitinase antibodies in transfected parasites, are numerous and small like vesicles containing Ariel antigens or cysteine proteinases. Axenic amebae, which were transfected with an unmodified chitinase gene under an *actin* gene promoter, had numerous small chitinase-associated vesicles (Fig. 3 and Table 2). Again, these secretory vesicles in most cells were too abundant to make an accurate count. Double-labeling studies, which were performed with Texas red-labeled chitinase and FITC-dextran or GFP-labeled *E. coli*, showed that chitinase-
associated vesicles did not fuse with pinocytotic or phagocytotic vacuoles. Chitinase-associated secretory vesicles were about the same size as lysosomes (identified with Arg–Arg–4-methoxy-2-naphthylamide, a fluorescent substrate of the cysteine proteases) (64). Because the signal from the cysteine proteinase was present in both FITC and Texas red channels, it was not possible to colocalize the lysosomes and the chitinase-containing vesicles. Chitinase was absent from the surface of transfected parasites (data not shown), suggesting that the surface of trophozoites lacked chitin-binding activity present on the surface of encysting parasites.

Vesicles containing the *E. histolytica* surface antigen Ariel were small and numerous and so most closely resembled chitinase-associated vesicles of encysting *E. invadens* (Fig. 4 and Table 2) (40). Ariel antigens were also present in phagocytotic vacuoles and coated the surface of nonpermeabilized *E. histolytica* trophozoites. A similar appearance has been described for the vaccine candidate SREHP, which is encoded by a gene that belongs to the same superfamily of antigen-encoding genes as Ariel (67).

Targeting of chitinase in transfected *E. histolytica* parasites is changed when an N-terminal signal sequence is removed or a C-terminal ER retention signal is added. Amebic chitinase and other secretory or plasma membrane proteins have at their
N-termini signal sequences, which fit the \(-3,-1\) rule of von Heijne (Table 1) (50). To test the necessity of the 12-amino-acid signal sequence of the \(E.\ histolytica\) chitinase, we transfected parasites with chitinase genes that encoded an intact chitinase and a truncated chitinase lacking the signal sequence (Fig. 5 and Table 2). Anti-chitinase antibodies demonstrated a vesicle-bright, cytosol-dark distribution of the intact chitinase that was similar to that of Ariel. In contrast, anti-chitinase antibodies demonstrated a reverse image of the truncated chitinase, in which vesicles were dark and the cytosol was bright. This cytosolic distribution, in which vesicles appear dark, like the holes in Swiss cheese, was also demonstrated with antibodies to ADH1, an abundant amebic fermentation enzyme (31, 41).

To localize the amebic ER, \(E.\ histolytica\) trophozoites were transfected with construct which overexpressed BiP with a myc epitope tag (Fig. 5 and Table 2). Amebic BiP, which has a C-terminal KDEL peptide, should bind ERD2 receptors in the proximal Golgi and be retained in the ER (10, 22, 62). myc-labeled BiP was present in a putative ER, which was composed of much fewer (\(\approx 20\) per parasite) vesicles than the secretory vesicles marked by intact chitinase or Ariel. To determine whether the KDEL peptide is sufficient to retain amebic proteins in the ER, we overexpressed chitinase with a KDEL peptide at its C terminus in \(E.\ histolytica\) trophozoites. Anti-chitinase antibodies demonstrated putative ER-associated vesicles in these parasites, which resembled those identified with myc-labeled BiP. The ER was not visualized in encysting \(E.\ invadens\) because transfection methods have not been developed for these parasites.

\(E.\ histolytica\) and \(E.\ invadens\) arf genes encode a conserved Golgi-associated coatomer protein (COP) called ARF. To better understand the role of Golgi apparatus in secretion by amebic trophozoites and encysting parasites, we used the PCR and degenerate primers to conserve peptides in other ARF to clone segments of \(E.\ invadens\) and \(E.\ histolytica\) arf genes (Fig. 6) (3, 21, 28, 32). The predicted 113-amino-acid open reading frame (ORF) of the amebic arf genes, which encode about two-thirds of the expected 20-kDa ARF proteins, showed 96% positional identity with each other and 76 to 88% positional identities with other eukaryotic ARFs. Perfectly conserved in the amebic ARFs were guanine nucleotide-binding domains, including the pyrophosphate-binding loop (GLDAAGKT), switch region (DVGG), and guanine recognition motif (NKQD) (3, 21). In NJ trees, amebic ARF was easily distinguished from ARF-like proteins of parasites or humans (Fig. 7) (71).

The amebic Golgi and encystation are disrupted by Brefeldin A and okadaic acid. As discussed above, RT-PCR showed that mRNAs that encode the Golgi-associated protein ARF and the ER-retention receptor (ERD2) are expressed by \(E.\ histolytica\) trophozoites (Fig. 1) (12, 62). ARF was also expressed by \(E.\ invadens\) trophozoites and encysting \(E.\ invadens\) (Fig. 1). Anti-ARF antibodies identified a stage-independent, putative Golgi apparatus, which was composed of a few large perinuclear vesicles (\(5\) to \(20\) per cell) (Fig. 8). Similar Golgi-associated vesicles were identified with antibodies to e-COP (Fig. 9). This putative Golgi apparatus was disrupted into tiny vesicles Brefeldin A, which targets the GTPase of ARF (29). Brefeldin A also reduced the number of \(E.\ invadens\) parasites that encyst by 60%. Okadaic acid, which targets protein phosphatases and disrupts \(gels\) of eukaryotic cells, disrupted the amebic Golgi and eliminated encystation completely (25). Wortmannin, which targets phosphoinositide 3-kinases and eliminates amebic pinocytosis and phagocytosis, also eliminated encystation (18). Previously, an amebic Golgi was seen with NBD-ceramide (as seen by fluorescence microscopy) and thiamine-pyrophosphatase (as seen by electron microscopy) (44).

![FIG. 6. ARF alignment. Predicted ORF of a segment of the \(E.\ histolytica\) (\(Eh\)) arf gene aligned with ARF segments of \(E.\ invadens\) (\(Ei\)), \(G.\ lamblia\) (\(Gl\); GenBank accession number S29008), \(P.\ falciparum\) (\( Pf\); U57370), \(D.\ discoideum\) (\(Dd\); AJ800683), \(S.\ cerevisiae\) (\(Sc\); M51588), and \(B.\ taurus\) (\(Bt\); A454222). Dashes indicate identities with the \(E.\ histolytica\) ARF, while periods indicate gaps. Vertical boxes indicate locations of conserved motifs including the pyrophosphate-binding loop (GLDAAGKT), switch region (DVGG), and guanine recognition motif (NKQD) (3, 34).](http://iai.asm.org/resolve/10.1128/IAI.70.6.3078-3086.1998)
DISCUSSION

These are the first arf genes identified in amebae, and this is the first use of transfection methods to localize amebic proteins and the first experimental demonstration of N-terminal signal and C-terminal ER retention sequences. Roughly drawn, the amebic cytosol is composed of small vesicles, which include those of the putative ER, secretory vesicles, lysosomes, pinocytic vacuoles, and large vesicles, which include those of the putative Golgi and phagocytotic vacuoles.

Conservation of secretory apparatus and protein-targeting sequences in amebae. Perhaps because amebae have such prominent pinosomes, phagosomes, and lysosomes, secretory structures of the parasite were not obvious in the absence of the specific probes used here (Table 2) (18, 43, 63). Chitinase expression in transfected parasites was used to show that amebic N-terminal signal sequences and C-terminal ER retention signals appear to follow the same rules as those established for higher eukaryotes (50, 61, 62). Indeed the 2,3,2,1 rule appears to hold for all signal sequences of amebic secretory and plasma membrane proteins which have been identified to date (Table 1). The amebic Golgi and encystation were disrupted by Brefeldin A and okadaic acid, which disrupt the Golgi of higher eukaryotes (25). Disruption of the G. lamblia Golgi, which is encystation specific, also inhibits cyst formation (37).

Unique properties of the amebic secretory pathway. Amebic secretion is different from that of higher eukaryotes in at least four ways. First, the putative amebic Golgi apparatus does not form tightly packed lamellae but instead forms a few large vesicles, which are adjacent to the nucleus (12, 44, 79). This is similar to the Golgi of G. lamblia, whereas Tritrichomonas foetus, another luminal parasite, has a Golgi with tight lamellae (19, 37). Second, an amebic protein disulfide isomerase (PDI) lacks a C-terminal KDEL peptide, which usually retains other PDI in the ER (27). Third, a sharp distinction between amebic lysosomes and secretory vesicles has not yet been made (63). Fourth, signals that direct amebic proteins to the lysosomes cannot be the same as those of higher eukaryotes. Amebic lysosomal proteins lack signals for Asn-linked glycosylation, which is necessary for the addition of mannose-6-phosphates that might bind to lysosomal receptors (30, 61, 69). We are presently performing experiments to distinguish secretory vesicles from lysosomes and to identify signals that target amebic proteins to lysosomes. We plan to use immunoelectron microscopy to visualize amebic secretory vesicles, putative ER, and putative Golgi.

Implications of these findings for our understanding of amebic virulence. Four implications of these data may be important in understanding amebic virulence. First, the secretory

FIG. 8. Confocal micrographs of E. invadens parasites stained with heterologous anti-ARF antibodies. (A) ARF was present in large vesicles (presumed Golgi apparatus) surrounding the nucleus of trophozoites. (B) The ARF-stained vesicles of trophozoites were disrupted by Brefeldin A. (C) The Golgi apparatus of encysting parasites was similar when stained with anti-ARF. Micrographs shown in panels A and B represent a single section each, while the micrograph in panel C is a composite of multiple sections. Bars, 5 μm.

FIG. 9. Confocal micrographs of E. histolytica and E. invadens parasites stained with antibodies to Golgi-associated coatamer protein ε-COP. Vesicles that contain ε-COP were relatively few and large in trophozoites of E. histolytica (A), trophozoites of E. invadens (B), or encysting E. invadens (C). The micrograph shown in panel A represents a single section, while the micrographs in panels B and C are each composed of multiple sections. Bars, 5 μm.
apparatus, which targets proteins to the plasma membrane of amebic trophozoites, likely releases numerous proteins into the medium, even though to date only lysosomal enzymes have been identified in amebic supernatants (1, 35, 68). Second, the −3,−1 algorithm might be used to identify secretory proteins in EST databases of amebic trophozoites or encysting parasites (50). Third, ARF in the cytosol of amebae may activate cholera toxin in individuals who are coinfected with *Vibrio cholerae* and amebae (49). Other targets for bacterial toxins previously identified in amebae include elongation factor 2 (diphtheria and pseudomonas toxins) and Ras-superfamily GTPases (clostridial toxins) (36, 53). Fourth, stage-independent Golgi and ER in amebae suggest that these parasites may be susceptible to bacterial toxins, which enter the cytosol through the Golgi or ER (49).

**Changing image of amebae and other luminal parasites: from primitive to divergently evolved.** Lumenal parasites (*E. histolytica*, *G. lamblia*, and *Trichomonas vaginalis*) branched early from the main eukaryotic tree, lack mitochondria and enzymes of oxidative phosphorylation, and have fermentation enzymes like those of bacteria (31, 33, 48). These observations led to the idea that these microaerophilic parasites are “primitive” or living fossils from a time before eukaryotic cells (9, 48). The function of an atrophic mitochondrial genome (*E. histolytica* (7, 20, 57). The experiments described here show the similarity of the amebic secretory pathway to those of other eukaryotic cells, even if its appearance is somewhat different. Further, amebae, giardias, and trichomonads each have a gene encoding a homologue of the mitochondrial 60-kDa heat shock protein (Hsp60) (9, 11, 59). The presence of Hsp60 genes in eukaryotic cells, even if its appearance is somewhat different. Further, amebae, giardias, and trichomonads each have a gene encoding a homologue of the mitochondrial 60-kDa heat shock protein (Hsp60) (9, 11, 59). The presence of Hsp60 genes. Bacterial 60-kDa heat shock protein HSP70 homologs: implications regarding the origin of eukaryotic cells and of endosymbiotic. Nat. Struct. Biol. 27:99–106.

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