Golgi and Endoplasmic Reticulum Functions Take Place in Different Subcellular Compartments of *Entamoeba histolytica*#*

Received for publication, June 28, 2005, and in revised form, July 18, 2005 Published, JBC Papers in Press, July 18, 2005, DOI 10.1074/jbc.M507035200

Luis M. Bredeston, Carolina E. Caffaro, John Samuelson, and Carlos B. Hirschberg

From the Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, Boston, Massachusetts 02118-2492

*Entamoeba histolytica* is a protozoan parasite that causes dysentery in developing countries of Africa, Asia, and Latin America. The lack of a defined Golgi apparatus in *E. histolytica* as well as in other protists led to the hypothesis that they had evolved prior to the acquisition of such organelle even though glycoproteins, glycolipids, and antigens have been detected, the latter of which react with antibodies against Golgi apparatus proteins of higher eukaryotes. We here provide direct evidence for Golgi apparatus-like functions in *E. histolytica* as well as for components of glycoprotein folding quality control. Using a combination of bioinformatic, cell biological, and biochemical approaches we have (a) cloned and expressed the *E. histolytica* UDP-galactose transporter in *Saccharomyces cerevisiae*; its 

\[ \text{K}_m \] for UDP-galactose is 2.9 \text{mM}; (b) characterized vesicles in an extract of the above protist, which transport UDP-galactose into their lumen with a \text{K}_m of 2.7 \text{mM}; (c) detected galactosyltransferase activity(ies) in the lumen of the above vesicles with the \text{K}_m for UDP-galactose, using endogenous acceptors, being 93 \text{mM}; (d) measured latent apprayer activities in the above vesicles, suggesting they are in the lumen; (e) characterized UDP-glucose transport activities in *E. histolytica* and endoplasmic reticulum-like vesicles with \text{K}_m for UDP-glucose of \text{K}_m = 2.4 \text{mM}. Although the endoplasmic reticulum-like fraction showed UDP-glucose: glucoprotein glycosyltransferase activity, the Golgi apparatus-like fraction did not. This fraction contained other glucosyltransferases. Together, these studies demonstrate that *E. histolytica* has different vesicles that play a role in protein glycosylation and folding quality control, analogous to the above organelar functions of higher eukaryotes.

*Entamoeba histolytica* (*Eh*), a protozoan parasite, causes dysentery and liver abscesses in developing countries of Africa, Asia, and Latin America. Infection occurs by ingestion of the cyst form of the parasite, which then differentiates to trophozoites that colonize the intestine and other organs, including the liver (1). Glycoproteins, glycolipids, and lectins on the surface of trophozoites are thought to play pivotal roles in regulating host cell adhesion and invasion and may be potential targets for drugs and vaccines (2, 3). To date, limited information is available on the structures of these glycoconjugates as well as the subcellular sites and pathways leading to their biosynthesis (4–6). The lack of a morphologically defined Golgi apparatus (GA) led until recently to the hypothesis that these protists had evolved prior to the acquisition of this organelle (7, 8), raising important biosynthetic questions about glycoconjugates, many of which are known to be synthesized in the endoplasmic reticulum (ER) and GA of eukaryotes (9, 10).

Very recent ultrastructural evidence as well as immunolocalization studies with trophozoites suggest that GA-like elements do occur in this organism. Cryofixation and cryosubstitution studies showed flattened cisternae in the cytoplasm that resemble GA of higher eukaryotes (11). Antibodies against mammalian GA proteins such as ECOP and ARF labeled vesicles of trophozoites that could be disrupted by Brefeldin A and okadaic acid, both known disrupters of GA structures of higher eukaryotes (3, 12). Previously, a combination of cytotoxic and transmission electron microscopy studies identified *Eh* vesicles with glucose-6-phosphatase and thiamine pyrophosphatase activities as well as vesicles fluorescently labeled with C6–12(α-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ceramide. These approaches have been previously used to identify ER and GA elements of higher eukaryotes (13).

The cytosolic and luminal side of the ER membrane and the luminal side of the GA cisternae of higher and lower eukaryotes are the principal subcellular organelar sites where glycosylation of proteins and lipids occur (9, 10). The nucleotide substrates for the luminal reactions, nucleotide sugars, which are mostly synthesized in the cytosol, must first be translocated into the lumens of the above organelles. Translocation is mediated by specific transporters in a nucleotide sugar transport/antiport cycle with the corresponding nucleoside monophosphate (14). The physiological relevance of this cycle is well documented as mutants in nucleotide sugar transporters as well as in antiporter-generating enzymes have biochemical and developmental phenotypes described in humans, cows, yeast, nematodes, *Drosophila, Leishmania*, and plants (14, 15).

Very recently the entire genome of *Eh* was published (16). Examination of this genome suggested the presence of genes encoding three putative nucleotide sugar transporters as well as other putative GA and ER proteins of the nucleotide sugar transport/antiport cycle including nucleotide phosphatases and the UDP-glucosyl:glycoprotein glucosyltransferase. We have used a combination of bioinformatics as well as biochemical and cell biological approaches to obtain evidence for GA- and ER-like functions of the above proteins suggesting that glycoconjugates may be synthesized in this organism in a manner broadly analogous to these macromolecules in higher eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Radioactive Substrates**—The following radioactive substrates used were all from PerkinElmer Life Sciences except as indicated: UDP-[3H]Glc (11.5 Ci/mmol), UDP-[3H]GlcNAc (39.7 Ci/mmol), UDP-[3H]GalNAc (5.8 Ci/mmol), GDP-[14C]mannose (260 mCi/mmol), GDP-[3H]mannose (10 Ci/mmol).
Nucleotide Sugar Transporters

GDP-[3H]fucose (17.5 Ci/mmol), CMP-[3H]sialic acid (33.6 Ci/mmol). UDP-[3H]Gal (5.8 Ci/mmol) and UDP-[14C]GlcA (300 mCi/mmol) were from American Radiolabeled Chemicals (St. Louis, MO).

Molecular Biology—Standard molecular biology protocols were used as described by Sambrook et al. (17) unless otherwise noted. The EhNST1 gene (locus 425.t00006) was amplified from total DNA isolated from Eh HM1 (a generous gift of Dr. Suchismita Das) by PCR using puRETaq DNA polymerase (Amersham Biosciences) and primers XbaIVS (forward) and XhoIrev (reverse). The sequence of oligonucleotide primers was CTCTCTAGAAGATCACAATGGTACGAA-ATGAAACCCTGGGTAGAAGCTAGTGACCTGTAAT- TTTCTCTTTTTTTGTTGTGTXc (XbaIVS) and GAGCTCG-AGTATGTTTTGCAGCTATCTACTTTAATATTGAC- AATTGAAATATGAC(XhoIrev).

The isolated PCR product (EhNST1) sequence fused with an 11 amino acid VSV-G tag coding sequence was digested with Xbal/XhoI and ligated to the p426GPD vector (digested with Spel/XhoI) to obtain plasmid pEhNST1 for the expression in yeast (18). The identity of the cloned fragment was confirmed by DNA sequencing.

Yeast Strain, Transformation, and Growth Media—Saccharomyces cerevisiae strain PRY225 (ura3-52, lys2-801am, ade2-1020c, his3, leu2, trplΔ1) was used to express EhNST1. For transformation with pEhNST1 a lithium acetate/polyethylene glycol method was utilized (19). Cells were grown in complete medium (0.75% yeast extract, 1.13% tryptone, 2.2% dextrose), and transformants were selected for their ability to grow in the absence of uracil on plates containing 6.7% yeast-nitrogen base without amino acids (YNB), 0.67% complete supplemented medium minus Ura (Ura-), 2.2% dextrose, and 1.5% agar.

Western Blotting Analysis—Total membrane fractions, from 3 ml of liquid yeast cultures (A600 = 3.0), were prepared by glass bead disruption of the cells in membrane buffer (0.8 m sorbitol, 1 mM EDTA, protease inhibitor mixture (Roche Applied Science), and 10 mM triethanolamine/acetic acid, pH 7.2, at 4 °C) plus 2 mM dithiothreitol followed by centrifugation at 134,000 × g for 1 h. Total membranes were resuspended in membrane buffer, and proteins were electrophoresed on 12% SDS acrylamide gels according to Laemmli (20) and subsequently electropherographed onto Millipore Immobilon P membranes. Nonspecific binding was blocked by incubating the membranes for 1 h at room temperature in 0.05% Tween and 1% nonfat dry milk in phosphate-buffered saline buffer. The membranes were incubated 1 h with 1 μg/ml mouse anti-VSV-G antibody (Roche Applied Science) in phosphate-buffered saline. Detection was performed by using horseradish peroxidase-conjugated mouse IgG (Promega) followed by chemiluminescence using ECL™ Western blotting detection reagents (Amersham Biosciences).

Yeast Subcellular Fractionation—Typically 3 liters of S. cerevisiae PYR225 transformed with p426GPD or pEhNST1 were grown in SC-URA liquid medium to an A600 of 3.0. The culture was chilled and centrifuged, and cells (30 g) were converted to spheroplasts as described previously (21). Briefly, cells were incubated for 30 min at 37 °C, using 1 mg of Zymolyase 100T (Seikagaku America, Rockville, MD)/g of cells in spheroplast buffer (1.4 m sorbitol, 20 mM NaPO₄, 48 mM 2-mercaptoethanol, and 50 mM potassium phosphate, pH 7.5, at 37 °C). The spheroplast suspension was centrifuged at 2000 × g for 10 min. Cells were broken by suspending the pellet in 1.5× volume of membrane buffer and by drawing the cells rapidly several times into a narrow bore serological pipette. The suspension was centrifuged successively 10 min at 2000 × g, 8 min at 5000 × g, and finally 45 min at 125,000 × g to obtain the pellet fraction P125 enriched in ER- and GA-derived vesicles (21).

Nucleotide Sugar Translocation Assays—The theoretical basis for the translocation assay of the nucleotide derivatives into vesicles has been described previously (22). Briefly, the yeast P125 fraction (1.0 mg of protein/ml) was incubated at 0 or 30 °C in 1 ml of 0.5 mM sucrose, 30 mM triethanolamine, pH 7.20, 5 mM MgCl₂ and 5 mM MnCl₂ with the radioactive nucleotide sugar to be tested. After 3 min, the reaction was stopped by addition of 50 ml of cold stop solution (1 mM EDTA), and the vesicles were separated from the incubation medium by centrifugation at 134,000 × g for 30 min. The pellet was washed four times with 4 ml of stop solution and resuspended in 1 ml of 4% perchloric acid. After 15 min on ice the acid-insoluble fraction was separated by centrifugation at 16,000 × g for 30 min and the supernatant, containing radioactive nucleotide sugar within and in between the vesicles, was counted in 9 ml of scintillation solution (Ecoscin™ A, National Diagnostics). Transport activity is defined as total radioactive solutes in the vesicle pellet after incubation at 30 °C–0 °C.

E. histolytica Cell Culture and Membrane Isolation—Axenic Eh strain HM1:1MSS was obtained from the American Type Tissue Culture Collection (Rockville, MD). Trophozoites were cultured in TYI-S33 medium at 37 °C in 50-cm² flasks. Crude membranes were prepared by a modification of the procedure described for COS-1 cells by Enyedi et al. (23). Briefly, trophozoites from six flasks were chilled, collected by centrifugation (2000 × g, 15 min), and washed once with 100 ml of phosphate-buffered saline. The cells (0.5 ml) were resuspended in 5 ml of cold hypotonic solution containing 10 mM MgCl₂, 25 mM KCl, 10 mM Hepes-KOH, pH 7.20, at 4 °C and complete protease inhibitor mixture. After 10 min on ice, cells were homogenized with 40 strokes in a Dounce homogenizer with a B pestle. The homogenate was diluted with an equal volume of the same buffer containing 0.5 mM sucrose, homogenized again with 25 strokes, and centrifuged at 5000 × g for 15 min. The pellet (P5K) was frozen. The supernatant was centrifuged at 130,000 × g for 60 min, and the pellet (P130K) was resuspended in a solution containing 0.5 mM sucrose, 10 mM MgCl₂, 25 mM KCl, 10 mM Hepes-KOH, pH 7.2, and complete protease inhibitor mixture at a protein concentration of 0.5 mg/ml. The resuspended P130K fraction was frozen in liquid nitrogen and stored at −80 °C.

UDP-Gal Transport into Eh130K (“Light”) Vesicles—A Eh130K vesicle fraction (960 μg of membrane protein) was isolated as described above and incubated with 0.6 μM UDP-[3H]Gal for 4 min at 0 or 37 °C in a reaction medium containing 0.5 mM sucrose, 30 mM TEA, pH 7.2, and 10 mM MnCl₂. Transport activity, UDP-[3H]Gal within vesicles, is defined as soluble radioactivity remaining after perchloric acid precipitation of washed vesicles.
**Nucleotidase Activity**—The assay was essentially as described previously (25). Briefly, incubation mixtures contained, in a final volume of 100 μl, 10 μg of membrane protein (P130K), 2 mM CaCl₂ or EDTA, 200 mM imidazole, pH 7.5, 2 mM nucleosides, diphosphates, or nucleoside triphosphates, and with or without 0.1% Triton X-100. After incubation at 30 °C for 30 min, the reaction was stopped by adding 200 μl of 7.5% SDS. Inorganic phosphate released was determined by adding 700 μl of Ames reagent (0.42% ammonium molybdate in 1 N sulfuric acid:10% ascorbic acid, 6:1), followed by incubation for 20 min at 45 °C. Absorbance was measured at 660 nm.

**Protein Assay**—Protein concentration was estimated by the method of Bradford (26) using bovine serum albumin as a standard.

**RESULTS**

**Yeast Vesicles Expressing EhNST1 Transport UDP-Galactose**—Previous studies from our and other laboratories showed that the amino acid sequence of nucleotide sugar transporters cannot predict the substrate specificity of an unknown transporter and that transporters of very different amino acid sequences can mediate transport of the same nucleotide sugar in the same organism (27, 28). Based on this we queried the genome of *Eh* with the amino acid sequence of several nucleotide sugar transporters including that for the UDP-galactose transporter of *Schizosaccharomyces pombe*.

As can be seen in Fig. 1A, *Eh* has three putative proteins with relatively high amino acid sequence identity to that of the *S. pombe* UDP-Gal transporter. Kyte-Doolittle hydrophobicity plots of these putative transporters suggested very hydrophobic proteins with multiple transmembrane domains (Fig. 1B).

To determine the substrate specificity of the above putative nucleotide sugar transporters we used a previously described approach developed in our laboratory (29, 30). It consists of transforming *S. cerevisiae* with the transporter encoding gene as well as the plasmid without insert followed by isolation of ER- and GA-enriched vesicles from both yeasts (29, 30). Nucleotide sugar transport was then measured into vesicles from both yeasts. Because *S. cerevisiae* has only endogenous transport activities for GDP-mannose and UDP-glucose this heterologous expression system provides a relatively low background system to measure transport of other nucleotide sugars than the above. We successfully expressed the VSV-tagged version of *Eh* NST1 (Fig. 2A). The mobility was somewhat lower than that predicted for a protein of 36.5 kDa (Fig. 1A). The same had been previously observed by us for another transporter (29). So far we have not expressed the other two putative transporters.

Golgi apparatus-enriched vesicles from *S. cerevisiae* transformed with empty vector or vector encoding the VSV-*Eh* NST1 fusion protein were assayed for their ability to transport different nucleotide sugars. Results obtained are shown in Fig. 2B. Transport of GDP-mannose and UDP-glucose were comparable in both vesicle preparations suggesting that the insert gene does not encode a transporter for these substrates. Transport of UDP-galactose, however, was ~10-fold higher in vesicles with the *Eh*NST1 gene product suggesting this nucleotide sugar is a substrate for this putative transporter. Other nucleotide sugars, as shown in Fig. 2B, appeared not be substrates for this putative transporter.

---

**FIGURE 1.** Sequence comparison of putative *Eh* nucleotide sugar transporters and *S. pombe* UDP-Gal transporter (SpUGT). **A**, primary sequence of predicted proteins for EhNST1 (EAL43205), EhNST2 (EAL49481), and EhNST3 (EAL48786) were compared with the SpUGT (NP_58804) using ClustalW and then depicted using Boxshade. **B**, Kyte-Doolittle hydrophobicity plots of EhNST1 (top), EhNST2 (middle) and EhNST3 (bottom). The protein identification numbers of the sequences are shown in parenthesis.
FIGURE 2. Functional expression in *S. cerevisiae* of VSV-G-tagged EhNST1. A, Western blot analysis. 100 μg of membrane protein from *S. cerevisiae* transformed with the empty vector (lane 1) or the vector encoding VSV-EhNST1 (lane 2) was applied to each lane of a 12% SDS-polyacrylamide gel and subjected to immunoblot analysis with anti-VSV-G monoclonal antibody as was described under “Experimental Procedures.” B, nucleotide sugar transport into *S. cerevisiae* vesicles transformed with empty (white bars) or VSV-EhNST1 (black bars) vector. A vesicle fraction was isolated as described under “Experimental Procedures” and assayed for transport of different nucleotide sugars at 0.1 μM for 3 min at 0 and 30 °C. Transport activity, solutes within vesicles, is defined as total solutes associated with the vesicle pellet after incubation at 30 °C minus total solutes associated with the vesicle pellet after incubation at 0 °C. Results shown are the average of three independent assays and the standard error. C, rate of UDP-Gal transport versus UDP-Gal concentration of vesicles from yeast transformed with VSV-EhNST1. Incubations were for 2 min at 0 and 20 °C with different concentrations of UDP-[3H]Gal. Transport activity is defined as above. The line represents the best fit of the data given by the Michaelis-Menten equation using Sigma Plot for Windows. The data points are the average of two experiments with two different vesicle preparations.
We next determined whether the kinetics for UDP-galactose transport mediated by the EhNST1 gene product expressed in S. cerevisiae, were similar to those previously observed for other nucleotide sugar transporters. As shown in Fig. 2C this was indeed the case; transport of UDP-Gal was saturable with an apparent \( K_m \) of 2.9 \( \mu M \). Other nucleotide sugar transporters have \( K_m \) values for their nucleotide sugar substrates between 1 and 10 \( \mu M \) (14). These results therefore suggest that \( E. hystosum \) has a nucleotide sugar transporter for UDP-galactose with kinetics similar to those of other GA transporters in eukaryotes.

Golgi-like Functions Occur in Eh130K Vesicles—Does \( E. hystosum \) have GA-like functions consistent with the above-described gene encoding the UDP-galactose transporter? Previously we had described that GA and ER vesicles from higher and lower eukaryotes contain luminal nucleoside di- and triphosphatases whose role is to give rise to nucleoside monophosphates, which are necessary for mediating the nucleotide sugar transport/antiport cycle (14, 31). To answer the above question, a cell suspension of \( E. hystosum \) trophozoites was subjected to Dounce homogenization as previously described (22). Following centrifugation at 130,000 \( \times g \) a membrane pellet was obtained, resuspended, and assayed for latency of different nucleoside di- and triphosphatases, which had been shown to be luminal enzymatic activities of the GA and ER (31). As shown in Fig. 3, the high latency toward nucleoside di- and triphosphatases strongly suggests that the extract contained vesicles with luminal ectonucleoside triphosphate diphosphohydrolase activities as previously observed in mammalian cells as well as Caenorhabditis elegans (14, 31). This result also suggests that the above vesicles have the same membrane orientation as in vivo.

Examination of the \( E. hystosum \) genome showed five putative proteins with appyrase-conserved regions (31) and high homology to the crystallized human appyrase 151DB (Fig. 4). Homologies to other appyrases from humans, insects, nematodes, Toxoplasma, and Cryptosporidium were also found (not shown). No homologs to the \( S. cerevisiae \) nucleoside diphosphatase GDA1 gene were found. Although we are unable to determine at this stage which of the above \( E. hystosum \) appyrases are responsible for the activities shown in Fig. 3 the results strongly suggest that \( E. hystosum \) contains vesicles, presumably derived from ER- and GA-like organelles, with the same topographic orientation as vesicles from other eukaryotes. Evidence further confirming this hypothesis was obtained from experiments in which we measured transport of UDP-galactose into vesicles of the above extract. As shown in Fig. 5A, radioactive solutes within vesicles were significantly higher at 37 than at 0 °C suggesting that transport of the nucleotide sugar had indeed occurred. This assay, where mostly solutes within vesicles are measured, requires a relatively large amount of protein and therefore is not conducive for multiple measurements. In most cases with GA- and ER-derived vesicles one measures a combination of transport of nucleotide sugars into the vesicles coupled with subsequent transfer of the sugar to luminal endogenous acceptors. The latter reactions are catalyzed by glycosyltransferases, known to occur in the lumens of these vesicles. In these instances incubations containing 1–10 \( \mu M \) nucleotide sugars were used; subsequent to entry into the vesicles these substrates are concentrated ~30–50-fold and can therefore attain the \( K_m \) values of ~100 \( \mu M \) of many glycosyltransferases, before transfer of the sugar to the corresponding endogenous acceptor can occur. Thus an important control in this transport/transfer-coupled assay is that the amount of sugar covalently transferred to endogenous acceptors can be considerably higher when intact compared with permeabilized vesicles are used; the latter can neither transport nor concentrate the nucleotide sugar in their lumen for efficient transfer when low micromolar concentrations of nucleotide sugars are used. In a preliminary experiment, using 5 \( \mu M \) UDP-galactose, with an extract of intact and Triton-permeabilized vesicles, we found significantly higher transfer of galactose into endogenous acceptor macromolecules using intact vesicles than into disrupted ones, strongly suggesting the presence of a transport/transfer system in these vesicles (Fig. 5B). Further evidence for this hypothesis was obtained by studying the kinetics of this combined transport/transfer reaction. As shown in Fig. 5C the reaction was saturable with an apparent \( K_m \) for UDP-Gal of 2.7 \( \mu M \). This was very similar to that of transport of UDP-galactose when EhNST1 was expressed in \( S. cerevisiae \) (Fig. 2C). In this latter system there is no transfer of galactose to endogenous macromolecules subsequent to transport. Final proof that this nucleotide sugar transport/transfer was indeed occurring in \( E. hystosum \) was obtained by direct measurement of the \( K_m \) for UDP-Gal of glycosyltransferase(s) using endogenous acceptors as substrates. For this, disrupted vesicles were incubated with different concentrations of UDP-galactose and the \( K_m \) for the reaction(s) was determined to be ~93 \( \mu M \) (Fig. 6). This is similar to the \( K_m \) values for other glycosyltransferases measured in other systems and demonstrates, as described previously, that the overall \( K_m \) measured for UDP-Gal transport/transfer reactions into sealed vesicles represents the affinity of the nucleotide sugar for its transporter and not the glycosyltransferase(s) (14).

We were also interested whether transport activities for other nucleotide sugars could be measured in 130K vesicles from \( E. hystosum \). For this, we compared incorporation of radiolabeled sugar, derived from low micromolar concentrations of nucleotide sugars, into macromolecules of sealed and permeabilized vesicles in a manner analogous to the experiment shown in Fig. 5B. As can be seen in Fig. 7, incorporation of radioactivity from UDP-glucose and perhaps UDP-N-acetylglucosamine was significantly higher into sealed vesicles, compared with permeabilized ones. The absolute values for the latter nucleotide sugar were rather low. Further evidence for UDP-glucose transport was obtained by showing that it was saturable with a \( K_m \) of 2.1 \( \mu M \) (Fig. 8).

Components of Glycoprotein Folding Quality Control Are Present in EhSK Vesicles—What is the role of UDP-glucose transport in \( E. hystosum \)? Among the best described functions for transport of UDP-glucose into a luminal compartment of the GA and the ER is to serve as substrate for the UDP-glucose:glycoprotein glucosyltransferase, the so-called Parodi enzyme (32). This enzyme serves as a glycoprotein folding sensor in the ER by transferring glucose from UDP-glucose to N-linked glycans of misfolded proteins or folding intermediates (32, 33). Three independent lines of evidence had also suggested that such an enzyme might occur in \( E. hystosum \). In a previous study it was shown that \( E. hystosum \) synthesized the substrate for the above enzyme, Man\(_5\)GlcNAc\(_2\)-N-linked to proteins (34). Examination of the \( E. hystosum \) genome suggested the existence of a protein with strong homology to the \( S. pombe \) UDP-glucose:glycoprotein glucosyl-
transferase (16). N-Glycans from *Eh* are capped with one glucose unit at the nonreducing end of the lower arm.3

To obtain direct evidence for the UDP-glucose:glycoprotein glucosyltransferase activity, vesicles from the above 130K fraction were permeabilized and assayed for their ability to glucosylate native versus denatured thyroglobulin, the latter a known substrate for the above enzymatic activity (24). As shown in Fig. 9A, to our surprise, the glucosylation of both substrates was rather similar and 4–5-fold higher compared with endogenous acceptors (not shown) suggesting the occurrence of glucosyltransferases of unknown endogenous acceptors. On the assumption that the glucosyltransferase activity in the assayed fraction was not mainly the result of the Parodi enzyme we assayed for the latter activity in a membrane fraction obtained, by analogy to ER membranes from rat liver, at lower speeds of centrifugation (5,000 g). As seen in Fig. 9A, this fraction showed a marked difference in its ability to glucosylate denatured thyroglobulin compared with native protein. Together these experiments suggest that although the heavier membranes (5K) are markedly enriched in the Parodi enzyme activity, the lighter ones (130K) must also have other glucosyltransferases.

Additional evidence supporting the existence of the Parodi enzyme in the 5K membrane fraction was obtained by comparing the role of Triton and cations in the glucosylation activity of membranes toward native and denatured thyroglobulin. As can be seen in Fig. 9B no significant glucosylation was observed without Triton in the reaction medium, suggesting that only a small portion of these membranes were in the form of broken vesicles. Addition of Triton to the reaction, however, showed a marked increase in glucosylation of denatured thyroglobulin, with Mn2+ and Ca2+ being more active than Mg2+. This is consistent with glucosylation being mediated by a luminal enzyme, as previously determined to be the case for the Parodi enzyme (24).

Virtually no glycosylation was seen with 5K membranes compared with 130K ones, when using 5 mM UDP-galactose (not shown). In other studies, using high pressure liquid chromatography, we found only a low level of epimerization of UDP-Gal to UDP-glucose in the reaction medium (not shown).

The glucosyltransferase activities present in 130K and 5K vesicle fractions also showed marked differences in their *Km*’s for UDP-glucose with that of the latter fraction toward denatured thyroglobulin being ~19 μM (Fig. 10A), whereas that of the former toward endogenous acceptors was 81 μM (Fig. 10B). The *Km* of transport/transfer of UDP-glucose into vesicles from the 5K fraction was 3.7 μM (Fig. 11), significantly lower than the *Km* for UDP-glucose:glycoprotein glucosyltransferase using denatured thyroglobulin as the acceptor. The difference in *Km* for transport and transferase, however, was not nearly as dramatic as for GA transporters/transferases.

**DISCUSSION**

Although *Eh* is a protozoan parasite that causes dysentery in millions of people, fundamental questions regarding its pathways for the biosynthesis and secretion of glycoproteins and glycoconjugates in general remain unknown. In this study we have obtained, to our knowledge, the

---

3 P. Magnelli, personal communication.
first evidence for GA-like functions such as glycosylation in this organism. Although previous studies had detected antigens using antibodies against GA proteins such as COP and ARF in vesicles of trophozoites that could be disrupted by Brefeldin A, GA functions per se had not (3, 12). We now demonstrate that *E. histolytica* has GA transporters for UDP-galactose and UDP-glucose. These activities are membrane bound and are part of vesicles that have luminal ectonucleoside triphosphate diphosphohydrolase, similar to those previously described for Golgi apparatuses of other eukaryotes such as mammals and *C. elegans* (14, 31). The GA-like vesicles we isolated and characterized, following centrifugation of an *E. histolytica* trophozoite extract, appeared to be sealed and of the same topographical orientation as *in vivo* based on their similar behavior to other GA previously characterized in other eukaryotes (14). With these vesicles we have demonstrated directly solute transport into their lumen (Fig. 5A) and indirectly concentration of solutes in their lumen and transfer to insoluble macromolecular acceptors following transport (Fig. 5B). Evidence for this was obtained by showing that transfer of radiolabel, from low micromolar concentrations of nucleotide sugars in the reaction medium, was significantly higher into intact rather than disrupted vesicles. In the former, based on analogy with other well-characterized nucleotide sugar transporter systems, subsequent to transport of the intact nucleotide sugar into the vesicle lumen, the concentration...
of the solutes is 30–50-fold higher than the reaction medium (14). This and the fact that the $K_m$ for most glycosyltransferases are near 100 μM, allows more efficient transfer of the sugars to macromolecules within the vesicle lumen than using disrupted vesicles where no transport and therefore no concentration of solutes can occur.

As shown in Fig. 7, we have demonstrated transport of UDP-galactose and UDP-glucose into Golgi-like vesicles from *Eh*. We have also cloned and expressed a transporter for UDP-galactose from this organism (*EhNST1*). Although the apparent $K_m$ of *EhNST1* expressed in *S. cerevisiae* was very similar to that obtained when measuring transport of UDP-Gal into *Eh* vesicles (2.7 versus 2.9 μM), we cannot be certain that all of the UDP-Gal transport measured into these vesicles is mediated by *EhNST1*. It is possible that part of the transport is also mediated by at least one of the two other putative transporters for nucleotide sugars identified in the *Eh* genome (*EhNST2* and -3, Fig. 1A). Another GA-like function identified in this study is that of galactosyltransferases, which appear to have a luminal active site in the vesicles and have an apparent $K_m$ for galactosyltransferases of 91 μM, similar to other glycosyltransferases in other organisms. The biochemical characteristic of the radiolabeled endogenous acceptors, studied by extraction in chloroform/methanol/water showed that the radioactivity was essentially in the interphase, with very low counts in the organic phase, suggesting that the acceptors were proteins (not shown). In a separate study where N- and O-glycans of *Eh* were examined galactose and glucose were found. This strongly suggests that at least some of the role for the above UDP-Gal transporter and galactosyltransferases is to give rise to the above glycoconjugates.

An unexpected finding was the apparent existence of two separate UDP-glucose transport systems; the one enriched in GA-like vesicles appears to have kinetic parameters similar to that of ER-like vesicles. We do not know whether transport of the above nucleotide sugar is mediated by the same transporter or a different one. *EhNST3* has a KKXX motif in its carboxyl terminus, consistent with it being an ER protein (35), whereas *EhNST2* does not and therefore might have a role in UDP-glucose transport into the GA. *EhNST1* and -2 also have 44% identity as shown in Fig. 1A, whereas *EhNST3* is much less homologous.
to either of the above two other transporters (Fig. 1A). However, the luminal glucosyltransferases appear to be rather different. Although the ones in GA-like vesicles do not discriminate between native and denatured thyroglobulin, the ones in the ER-like vesicles very much do. This strongly suggests that the ER-like vesicles contain the UDP-glucose:glycoprotein glucosyltransferase activity (32), whereas the lighter, GA-like vesicles, do not and presumably have other glucosyltransferases. Two additional lines of evidence support the occurrence of this glucosyltransferase, in one study analyses of N-glycans showed glucose capping and examination of the Eh genome shows a protein with strong similarity to the S. pombe UDP-glucose:glycoprotein glucosyltransferase.

This study also suggests that Eh has a glycoprotein folding quality control system at least broadly analogous to that found in the ER of higher eukaryotes, including mammals. This consists of a UDP-glucose transporter, the UDP-glucose:glycoprotein glucosyltransferase, a nucleoside diphosphatase, and the calreticulin/calnexin cycle (32, 33). Some higher eukaryotes, including mammals. This consists of a UDP-glucose:glycoprotein glucosyltransferase.

In a recent study it was found that Eh can synthesize dolichol-P-\(\text{GlcNAc}\_5\text{Man}_5\) and transfer this oligosaccharide to proteins. Genes encoding some of the above enzymes were also identified in its genome including Alg7, -1, -2, -11, and STT3 (34).

Among the previously described glycoconjugates of Eh are the serine-rich protein terminal \(\text{O}\)-linked \(\text{N\text{-acetylglucosamine}}\) (5) and a glycosylphosphatidylinositol-anchored proteophosphoglycan with an acidic polypeptide to which probably a polymer containing glucose and galactose is attached via a serine phosphate (4). It is tempting to hypothesize that the above-described transporters for UDP-galactose and UDP-glucose of the GA-like vesicles play a role in the biosynthesis of this polymer. Further studies will be required to determine whether this is indeed the case as well as the site(s) of biosynthesis for other known glycoconjugates of this parasite.

Acknowledgments—We thank Drs. Claudia Abeijon and Patricia M. Berninose for helpful discussions.

REFERENCES

1. Stanley, S. L. (2003) Lancet 361, 1025–1034
2. Stanley, S. L., Blanchard, J. L., Johnson, N., Foster, L., Kunz-Jenkins, C., Zhang, T., Tian, K., and Cogswell, F. B. (1995) Vaccine 13, 947–951
3. Ghosh, S. K., Field, J., Frisardi, M., Rosenthal, B., Mai, Z., Rogers, R., and Samuelson, J. (1999) J. Infect. Immun. 67, 3073–3081
4. Moody-Haupt, S., Patterson, J. H., Mirelman, D., and McConville, M. J. (2000) J. Mol. Biol. 297, 409–420
5. Stanley, S. L., Tian, K., and Li, E. (1995) J. Biol. Chem. 270, 4121–4126
6. Ayya, R., Mehra, A., Bhattacharyya, S., Vishwakarma, R. A., and Bhattacharya, A. (2003) Mol. Biochem. Parasitol. 126, 1–8
7. Bakker-Grunwald, T., and Westmond, C. (1993) Parasitol. Today 9, 27–31
8. Dacks, J. B., Davis, L. A., Sjogren, A. M., Andersson, J. O., Roger, A. J., and Doolittle, W. F. (2003) Proc. R. Soc. Lond. B Biol. Sci. 270, (suppl.) 168–171
9. Hirschberg, C. B., and Snider, M. D. (1987) Annu. Rev. Biochem. 56, 63–87
10. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–634
11. Chavez-Munguia, B., Espinosa-Castellano, M., Castanon, G., and Martinez-Palomo, A. (2000) Arch. Med. Res. 31, (suppl.) 165–167
12. Manning-Cela, R., Marquez, C., Franco, E., Talamas-Rohana, P., and Meza, I. (2003) Cell Microbiol. 5, 921–932
13. Mazzuco, A., Benchimol, M., and De Souza, W. (1997) Microen 28, 241–247
14. Hirschberg, C. B., Robbins, P. W., and Abeijon, C. (1998) Annu. Rev. Biochem. 67, 71–98
15. Hirschberg, C. B. (2001) J. Clin. Investig. 108, 3–6
16. Loftus, B., Anderson, I., Davies, R., Almark, U. C. M. Samuelson, J., Amedeo, P., Roncaglia, R., Berriman, M., Hirt, R. P., Mann, B. J. et al. (2005) Nature 433, 865–873
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Mumberg, D., Muller, R., and Funk, M. (1995) Gene (Amst.) 156, 119–122
19. Elble, R. (1992) BioTechnology 10, 18–20
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Abeijon, C., Orlean, P., Robbins, P. W., and Hirschberg, C. B. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6935–6939
22. Perez, M., and Hirschberg, C. B. (1987) Methods Enzymol. 138, 709–715
23. Enyedi, A., Verma, A. K., Filoteo, A. G., and Penniston, J. T. (1993) J. Biol. Chem. 268, 10621–10626
24. Trombetta, S. E., Bosch, M., and Parodi, A. J. (1989) Biochemistry 28, 8108–8116
25. Yanagisawa, K., Resnick, D., Abeijon, C., Robbins, P. W., and Hirschberg, C. B. (1990) J. Biol. Chem. 265, 19351–19355
26. Bradford, M. (1976) Anal. Biochem. 72, 248–256
27. Guillen, E., Abeijon, C., and Hirschberg, C. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7888–7892
28. Berninose, P. M., and Hirschberg, C. B. (2000) Curr. Opin. Struct. Biol. 10, 542–547
29. Berninose, P., Eckhardt, M., Gerardy-Schahn, R., and Hirschberg, C. B. (1997) J. Biol. Chem. 272, 12616–12619
30. Berninose, P., Wang, H. Y., Zemtseva I., Horvitz, H. R., and Hirschberg, C. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3738–3743
31. Uccellotto, D., O’Callahan, C., Berninose, P., Zemtseva, I., Abeijon, C., and Hirschberg, C. B. (2004) J. Biol. Chem. 279, 27390–27398
32. Trombetta, S. E., and Parodi, A. J. (2003) Annu. Rev. Cell Dev. Biol. 19, 649–676
33. Helenius, A., and Aebi, M. (2004) Annu. Rev. Biochem. 73, 1019–1049
34. Samuelson, J., Banerjee, S., Magnelli, P., Cui, J., Kelleher, D. J., Gilmore, R., and Robbins, P. W. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1548–1552
35. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1990) EMBO J. 9, 3153–3162
36. D’Alessio, C., Trombetta, E. S., and Parodi, A. J. (2003) J. Biol. Chem. 278, 22379–22387
37. Dai, J., Liu, J., Deng, Y., Smith, T., and Lu, M. (2004) Cell 116, 649–659
Golgi and Endoplasmic Reticulum Functions Take Place in Different Subcellular Compartments of *Entamoeba histolytica*

Luis M. Bredeston, Carolina E. Caffaro, John Samuelson and Carlos B. Hirschberg

_**J. Biol. Chem.** 2005, 280:32168-32176; doi: 10.1074/jbc.M507035200 originally published online July 18, 2005_

Access the most updated version of this article at doi: 10.1074/jbc.M507035200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 11 of which can be accessed free at [http://www.jbc.org/content/280/37/32168.full.html#ref-list-1](http://www.jbc.org/content/280/37/32168.full.html#ref-list-1)