Reduction of Cell Surface Glycosylphosphatidylinositol Conjugates in *Entamoeba histolytica* by Antisense Blocking of *E. histolytica* GlcNAc-Phosphatidylinositol Deacytlylase Expression: Effect on Cell Proliferation, Endocytosis, and Adhesion to Target Cells

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Received 23 May 2005/Returned for modification 2 July 2005/Accepted 6 August 2005

Glycosylphosphatidylinositol (GPI)-anchored molecules such as cell surface Gal/GalNAc lectin and proteophosphoglycans of the protozoan parasite *Entamoeba histolytica* are thought to be involved in pathogenesis. Here, we report the identification of genes that may be involved in the GPI biosynthetic pathway of *E. histolytica* by use of bioinformatic tools applied to the recently published genome sequence. Of the genes identified, one of the early genes, GlcNAc-phosphatidylinositol deacetylase (PIG-L), was partially characterized. Cell lines deficient in *E. histolytica* PIG-L (EhPL-AS) or overproducing it (EhPL-S) were generated by expressing the gene in the antisense or sense orientation, respectively, in a tetracycline-inducible system. The overexpressing cells showed higher EhPig-L activity and increased production of GlcNP-PI. Conversely, cells expressing the antisense RNA displayed reduced GlcP-PI production. The total number of GPI-containing molecules was also reduced in these cells, as demonstrated by Alexa 488 fluorescently labeled proaerolysin labeling. The distribution of GPI-linked PPG and Gal/GalNAc lectin was altered in the tetracycline-induced EhPL-AS cell lines. Further, the antisense-blocked cells showed 36% suppression of cell growth, 50 to 60% inhibition of fluid phase endocytosis, and about 50% inhibition of adhesion to target cells. Therefore, our data suggest the importance of GPI anchors in regulating some of the events in amoebic pathogenesis. They also demonstrated the use of antisense RNA-mediated inhibition of GPI biosynthetic enzymes as an approach to decrease the amount of GPI conjugates in *E. histolytica*.

The intestinal protozoan parasite *Entamoeba histolytica* is the causative agent of invasive amebiasis, responsible for about 40,000 deaths every year (22, 40). Glycoconjugates and carbohydrate-binding proteins are involved in various disease pathologies, including amebiasis. Attachment of *E. histolytica* trophozoites to target cells is required for cytolysis, and the process is thought to be mediated by the cell surface Gal/GalNAc lectin (44, 55). The glyccalx layer on the surface of pathogenic *E. histolytica* trophozoites is predominantly made of proteophosphoglycan (PPG) (26, 41). No significant glyccalx layer has been found on the surface of the nonpathogenic *Entamoeba dispers* (34). This correlates with the absence of PPG in *E. dispers* and suggests that PPG may have a role in pathogenesis (2, 3). Both Gal/GalNAc lectin and PPG are anchored to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors and have been suggested to be good vaccine targets (19, 26). A number of reports emphasize the role of glycoconjugates in pathogenesis of other protozoan parasites (23, 36). For example, lipophosphoglycan (LPG) is the predominant molecule on the surface of *Leishmania* promastigotes and is known to be involved in pathogenesis (17, 32). Promastigotes deficient in GPI-anchored gp63 and LPG molecules displayed reduced virulence in terms of macrophage infectivity (24, 28, 51, 52). LPG facilitated promastigote attachment to the sandfly insect vector and provided resistance to complement-mediated lysis of metacyclic forms and may be essential for viability, replication, and/or virulence (24, 28, 33). GPI-deficient *Trypanosoma cruzi* amastigotes differentiated poorly to promastigotes and were inhibited in cellular growth (1). Malarial GPI molecules were found to be potent toxins closely associated with clinically severe malaria and capable of inducing cytokine and adhesin expression in macrophages and vascular endothelium (48). The glycan chain in the GPI was suggested to be a good vaccine candidate (47).

The Gal/GalNAc lectin of *E. histolytica* consists of two subunits (heavy and light), of which the light subunit is GPI anchored (29). There is also an intermediate form (IGL) of the lectin that colocalizes with the heavy and light subunits (10). The IGL is also anchored to the membrane through a GPI linkage. Cells expressing a dominant-negative form of the light subunit of Gal/GalNAc lectin (LGL) showed reduced cytolysis (21). Moreover, expression of truncated LGL molecules lacking the capability to have GPI anchors in *E. histolytica* cells also led to a reduction in virulence (30), suggesting that GPI anchors are important for lectin function and that the enzymes involved in GPI biosynthesis may be potential drug targets.

GPIs share a common core structure (28). Typically phosphatidylinositol (PI) is glycosidically linked to the reducing end of a nonacetylated glucosamine moiety, followed by three man-
nose residues which, in turn, are attached to ethanolamine linking the anchor to the protein destined to be GPI anchored (28). However, the GPI anchors in *E. histolytica* have a unique glycan backbone, that is, Gal-Man-Man-GlcN-myoinositol, with the terminal mannose being replaced by α-Gal, which is not found in any other eukaryotic GPI core structures reported so far (35). The biosynthesis of GPI anchors occurs in the endoplasmic reticulum and involves the stepwise assembly of the anchor on PI in the endoplasmic reticulum membrane (28). Though there are a few species-specific modifications in terms of the sequence of events, transfer of GlcNAc and deacetylation to form GlcN-PI appear to be the first two universal steps in the GPI biosynthesis (12, 18). The GlcN-PI transferase (GPI-Gnt) complex catalyzes the first step of the pathway, that is, transfer of GlcNAc from UDP-N-acetylglucosamine to PI. There are at least six subunits that make up the complex (58, 59, 61). On the other hand, a single polypeptide, PIG-L or PGI12 (*Saccharomyces cerevisiae*), carries out the deacetylation reaction (37, 55).

The enzymes of GPI anchor biosynthesis are thought to be excellent targets for chemotherapy in many parasite infections, and GPI anchors have also been targets of immunoprophylaxis (6, 26, 34, 47). Nothing is known about the GPI anchor biosynthesis. There are at least six subunits that make up the complex (58, 59, 61). Nothing is known about the GPI anchor biosynthesis. In this report, we have identified the various components of the pathway from sequence analysis of the *E. histolytica* genome database. Further, we have characterized GlcNAC-PI-deacetylase, the enzyme catalyzing the second step of GPI biosynthetic pathway, and generated cell lines that are partially blocked in this enzyme. These cells had an overall reduction of total GPI-containing molecules and were altered in many biological parameters, such as growth, adhesion, and endocytosis.

**MATERIALS AND METHODS**

**Antibodies, strains and cell growth.** Monoclonal antibody 2D7.10 was raised against the PPG of *E. histolytica* strain HM-1:IMSS (5). Monoclonal anti-Gal/GalNAc lectin 1D4 antibody (anti-LGL) was a kind gift of W. A. Petri (31).

All experiments were carried out with *E. histolytica* strain HM-1:IMSS clone 6, which was obtained from William A. Petri (University of Virginia). The cells were grown and maintained in TYI-S-33 medium supplemented with 15% adult bovine serum, 2% Diamond’s vitamin mix, and antibiotic (0.3 units/ml penicillin and 0.25 mg/ml streptomycin). Hygromycin (Sigma) was added at 10 μg/ml for maintaining the transformed cell lines. Cell viability was determined by microscopy using a trypan blue dye exclusion test. Experiments were conducted with cells that showed >90% viability.

**Sequence analysis.** Amino acid sequences of genes involved in GPI anchor biosynthesis from various organisms were retrieved from the “PIGs Main” server ([http://mendel.imp.univie.ac.at/sequences/gpi-biosynthesis](http://mendel.imp.univie.ac.at/sequences/gpi-biosynthesis)). Sequences that were not available at “PIGs Main” were identified using the NCBI database (http://www.ncbi.nlm.nih.gov). These were then used for BLAST analysis for a search of the *E. histolytica* genome database (12.5× coverage, that is, the final assembled genome sequence) at The Institute of Genome Research (http://www.tigr.org/dbb/ezki/eha1), the Welocyte Trust’s Sanger Institute (http://www.sanger.ac.uk/Projects/E_histolytica), and NCBI. The identified genes from the *E. histolytica* database were then further analyzed and confirmed by a number of search tools using a variety of databases such as reverse BLAST, PSI-BLAST, CD-search (http://www.ncbi.nlm.nih.gov/BLAST), Pfam (http://pfam.wustl.edu/hmmsearch.shtml), MPSearch (http://www.ebi.ac.uk/MPSearch), and ProDom (http://www.sanger.ac.uk/software/Pfam). The transmembrane prediction of the protein was made using various programs available at Bioinformatics.Net (http://www.bioinformatics.org/ genomes/transmembran%252Ddomains.shtml). Determination of amino acid composition, translation of a given DNA sequence, and multiple alignments (CLUSTALW) were performed using the BioEdit sequence alignment editor (Tom Hall, version 7.0).

**Metabolic labeling of cells.** Mid-log-phase cells (2.5 × 10⁷ cells/ml) were incubated with radiolabeled precursor N-acetyl-t-[¹⁴C]glucosamine (Amer sham Biosciences) (100 μCi/ml) in TYI-S-33 medium for indicated time periods. After incubation, the cells were harvested and washed twice with phosphate-buffered saline (PBS).

**Preparation of amebic crude membranes and total cell lysate.** Membranes were prepared from *E. histolytica* as described previously by Carver and Turco (9). Briefly, 4 × 10⁷ cells were suspended in 10 ml of HEPES buffer (100 mM HEPES [pH 7.5], 50 mM KCl, 10 mM MnCl₂, 10 mM MgCl₂, 2 mM PMSF [phenylmethylsulfonyl fluoride], 5 to 10 μg/ml leupeptin) containing 10% glycerol and lysed by glass Dounce tissue homogenizer at 4°C. The debris and unbroken cells were removed by centrifugation at 250 × g for 5 min. The membrane fraction was collected by ultracentrifugation at 100,000 × g for 1 h at 4°C. The membrane pellet was washed once with reaction buffer (50 mM HEPES [pH 7.5], 5 mM EDTA, 1 μg/ml leupeptin, 2 mM PMSF) without glycerol and finally resuspended in 1% NP-40 (vol/vol) as the solvent system.

Total cell lysate was prepared as described previously (4). Briefly, the cells were harvested and washed thrice with PBS. The cells were then lysed by hypotonic shock (10 mM Tris-Cl [pH 7.5], 2 mM PMSF, 1 mM PHEMB [p-hydroxymercuribenzoate], 4 μg/ml leupeptin) for 10 min. After three cycles of freezing and thawing at −70°C and 37°C, respectively, DNA was sheared by sonication thrice for 15 s. The lysate was stored as aliquots at −20°C.

**Preparation of GPI pathway intermediates.** Radiolabeled GPI intermediates were synthesized in vitro by incubating *E. histolytica* crude membrane preparation (typically 1 mg protein) with UDP-[¹⁴C]GlcNAc (0.05 μCi) (Amer sham Biosciences) in reaction buffer in a total volume of 200 μl. After incubation at 37°C for 2 h, the reaction was stopped by incubating on ice. The labeled products were extracted first by shaking with 600 μl water and 3 ml ice-cold chloroform-methanol (1:2 [vol/vol]) followed by addition of 1 ml each of chloroform and water. The mixture was centrifuged at 1,000 × g for 10 min at 4°C in order to separate the phases. The lipid containing the chloroform-rich lower phase was washed twice with 0.5 ml mock upper phase to remove any contaminating UDP-[¹⁴C]GlcNAc or breakdown products. Then, 10% of the lipid-containing phase was taken for liquid scintillation counting. The remainder of the sample was dried, dissolved in 20 μl water-saturated butanol (w-butanol), and analyzed by high-performance thin-layer chromatography (HPTLC). HPTLC plates (Merck) were developed using chloroform-methanol-1 M ammonium hydroxide (10:10:2 [vol/vol/vol]) as the solvent system. The plates were air dried to remove any traces of ammonia followed by spraying with EN’HANCE (NEN Life Science) for fluorography.

**Characterization of GPI pathway intermediates.** (i) Phenyl Sepharose chromatography. The extracted glycolipids were loaded on a 1 ml phenyl Sepharose column equilibrated with 100 mM ammonium acetate–5% 1-propanol in water (solvent A). A gradient was set up with various concentrations of solvent A and solvent B (60% 1-propanol in water) at a flow rate of 1 ml/min. The mixture was centrifuged at 1,000 × g for 10 min at 4°C in order to separate the phases. The lipid containing the chloroform-rich lower phase was washed twice with 0.5 ml mock upper phase to remove any contaminating UDP-[¹⁴C]GlcNAc or breakdown products. Then, 10% of the lipid-containing phase was taken for liquid scintillation counting. The remainder of the sample was dried, dissolved in 20 μl water-saturated butanol (w-butanol), and analyzed by high-performance thin-layer chromatography (HPTLC). HPTLC plates (Merck) were developed using chloroform-methanol-1 M ammonium hydroxide (10:10:2 [vol/vol/vol]) as the solvent system. The plates were air dried to remove any traces of ammonia followed by spraying with EN’HANCE (NEN Life Science) for fluorography.

(ii) Enzymatic cleavage of glycolipids by PI-PLC treatment. Purified phosophatidylinositol-specific phospholipase C (PI-PLC) was obtained from Martin Low (Columbia University College of Physicians and Surgeons, New York, N.Y.). Radiolabeled products were treated with 0.1 μM of PI-PLC at 37°C for indicated time periods, and the lipids were extracted as described above and analyzed by HPTLC followed by fluorography.

(iii) Acetylation. The dried sample (10⁻¹⁴C]-labeled glycolipids) was resuspended in 50 μl of saturated sodium bicarbonate, and 2 μl of acetic anhydride was then added. After 30 min of incubation on ice another 5 μl of acetic anhydride was added. The reaction mixture was further incubated at 20°C for 30 min. Acetylated glycolipids were extracted using w-butanol. The reaction mixture was again washed with butanol, and the butanol phases were pooled and dried. The dried acetylated product was resuspended in w-butanol and analyzed by HPTLC as mentioned above.

(iv) Nitrous acid deamination. A total of 250 μl of freshly prepared 0.4 M NaNO₂ (pH 5.5) was added to the dried [¹⁴C]-labeled glycolipids. The pH of the reaction was adjusted to 3.5 to 4.0 using acetic acid, and then the reaction mixture was incubated for 4 h. Further, 50 μl of freshly prepared 1 M NaNO₂ was added and incubation was continued for another 8 h at room temperature. The reaction was terminated by adding 250 μl of w-butanol, and the reaction mixture was mixed and then centrifuged for phase separation. The upper butanol phase was removed, and the lower phase was reextracted with w-butanol. All the butanol phases were pooled and dried using vacuum and then resuspended in 20 μl of the same solvent. Deaminated glycolipids were subjected to HPTLC analysis.
The PIG-L gene was inserted in either the sense or the antisense orientation. Plasmids containing PIG-L gene in sense and antisense orientations were constructed and named pEHPL-S and pEHPL-AS, respectively. The cell lines generated with parental vector (pEHHYG-tetR-O-CAT), pEHPL-S, and pEHPL-AS constructs are referred to as vector, pEHPL-S, and pEHPL-AS, respectively. PLS-F and PLS-R primers were used to amplify reverse-transcribed products obtained from total HM1:IMSS RNA (5 μg) under similar conditions, using standard molecular biology protocols (46).

**Transfection of* E. histolytica* trophozoites by electroporation.** Transfection was performed by electroporation as described previously (15, 60). Two consecutive pulses were applied instead of one pulse, each with an exponential discharge of 3,000 V/cm at a capacitance of 25 μF (Gene Pulser II; Bio-Rad). For each transfection 1 x 10³ trophozoites and 300 μg DNA were used. Drug selection with 10 μg/ml hygromycin (Sigma) was started 48 h after transfection.

**Immunostaining.** *E. histolytica* cells (2 x 10⁵) suspended in incomplete TYI-33 medium at 36°C were transferred onto coverslips placed in a petri dish and allowed to adhere for 10 min at 36°C, and cells were fixed with 3.7% prewarmed paraformaldehyde for 30 min at 36°C. After fixation, the cells were permeabilized with 0.1% Triton X-100–PBS for 1 min and then quenched with 50 mM NH₄Cl–PBS for 30 min. The coverslips were blocked with 1% BSA–PBS before incubation with secondary antibodies at a 1:200 dilution for 30 min. The preparations were further washed with PBS and mounted on a glass slide using DABCO [1,4-diazabicyclo(2,2,2)octane] (Sigma) (10 mg/ml) in 80% glycerol and sealed. Cy3-labeled and Alexa-488-labeled cells were visualized using a laser scanning confocal microscope (FV300 Olympus) after excitation at 543 nm and 488 nm, respectively.
FIG. 1. The EhPIG-L gene of *E. histolytica*. ClustalW alignment of EhPIG-L protein (Eh) with those of *Leishmania* (Lm), *Trypanosoma* (Tb), and human (Hs) homologs, respectively. The boxed regions show a conserved Pfam domain (PF02585) common to all PIG-L proteins. Black- and gray-shaded regions indicate identical and similar amino acids. Amino acid positions are indicated on the left.

**RESULTS**

GPI anchor biosynthetic pathway genes in *E. histolytica*. The biosynthesis of GPI anchors is a multistep process involving many intermediates. A number of enzymes and enzyme complexes participate in these steps leading to the synthesis of GPI-anchored molecules. Data from other biological systems suggest the presence of at least 10 reaction steps for the GPI core biosynthesis, and more than 20 genes may be involved in this pathway (13).

In an effort to understand and elucidate the GPI biosynthesis pathway in *E. histolytica* we undertook a detailed computational analysis of the *E. histolytica* genome (12.5× coverage) (25). Using the amino acid sequences of known genes of GPI biosynthesis pathway from other organisms such as yeast and humans and *Leishmania* and *Trypanosoma* spp. as queries, we searched for their corresponding homologues in *E. histolytica*. The hits were considered significant when the maximum e-value obtained in the BLAST analysis was 0.00001. Some of the hits were also confirmed by the presence of motifs of demonstrated similarity with protein families by use of “Prosite” and “Pfam.” Out of a total of 22 genes in yeast and 23 in humans only 15 genes were identified in *E. histolytica* (Table 1). It is difficult to say at present whether homologs of proteins present in other organisms and not found in *E. histolytica* are missing or have diverged significantly so that these are not detectable in spite of the use of sensitive search methods. Interestingly, the proteins that were found to be absent in *E. histolytica* by database search were not the catalytic subunits of multienzyme complexes (Table 1). It appears from these data that the GPI biosynthetic pathway of *E. histolytica* is likely to be substantially different from that present in other organisms. Since the deacetylase activity is carried out by a single subunit.
encoded by PIG-L, we subsequently focused our studies on this gene.

**Sequence analysis of EhPIG-L gene.** The putative *E. histolytica* EhPIG-L gene (EAL49111.1) showed 33% identity and 47.5% similarity with the human PIG-L gene. *E. histolytica* PIG-L encodes a putative protein of 237 amino acids, with a predicted size of about 27 kDa. ClustalW alignment with the proteins from human and other protozoan parasites showed significant regions of conservation dispersed over the length of the protein (Fig. 1). A conserved Pfam domain (accession number PF02585) common to all PIG-L orthologs was also found in EhPIG-L spanning amino acid positions 39 to 154.

**GlcNAc-PI-deacetylase activity in *E. histolytica* membrane vesicles.** Data presented in the previous section showed the presence of a putative GlcNAc-PI-deacetylase gene in the *E. histolytica* genome. In order to show whether such an activity exists in these cells, biochemical assays were performed for the first two enzymes, namely, GPI-N-acetylglicosamine trans-ferase (GPI-Gnt complex) and GlcNAc-PI-deacetylase (PIG-L), in a cell-free system containing microsomal membranes (9). The cell-free system was incubated with UDP-[14C]GlcNAc, and the products were analyzed by HPTLC as described in Materials and Methods. The products resolved into two major radiosabeled products, X and Y, which migrated slower than PI (Fig. 2A). The products (X and Y) migrated with Rf values of 0.76 and 0.72, respectively. The values were found to be similar to those of GlcNAc-PI and GlcN-PI as reported for other organisms, such as mammals, yeast, and *Leishmania* spp.

In order to show that the reaction products were indeed GlcNAc-PI and GlcN-PI, further analysis was carried out. The radiolabeled reaction products bound phenyl Sepharose and were found to elute at 30% n-propanol, a property typical of many GPI-anchored molecules (references 9 and 14 and data not shown). The reaction products were susceptible to PI-PLC hydrolysis (Fig. 2B). About 85% of the material was digested within 1 h when incubated with 0.1 U/ml of PI-PLC (data not shown). Since the bulk of the radioactivity released was in the aqueous phase migrating near the origin (Fig. 2B, Lane 2), it suggested that a glycan group may have been generated after
the digestion. Both the products were susceptible to digestion by the enzyme (Fig. 2B, lane 3).

The susceptibility to nitrous acid deamination is also a diagnostic feature of a GPI-anchored molecule. After nitrous acid deamination, the glycolipids labeled in vitro were hydrolyzed, as revealed by loss of product Y only, unlike the results seen with PI-PLC treatment (Fig. 2C). This showed that one of the products (Y) contains a PI anchor with a free amine, which may have been generated through a deacetylation reaction.

In order to conclusively show the identity of the deacetylated intermediate, reaction products were subjected to N-acetylation by acetic anhydride. After nitrous acid deamination, the glycolipids labeled in vitro were hydrolyzed, as revealed by loss of product Y only, unlike the results seen with PI-PLC treatment (Fig. 2C). This showed that one of the products (Y) contains a PI anchor with a free amine, which may have been generated through a deacetylation reaction.

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Molecular characterization of EhPIG-L gene. The nucleotide sequence of the predicted gene, EhPIG-L, was used to design PCR primers PLS-F (forward) and PLS-R (reverse), and the PCR amplicon was cloned and sequenced. The endogenous expression of the gene in *E. histolytica* was examined by Northern blotting. A single transcript of about 0.9 kb was observed in Northern blot analysis (Fig. 3A). The extra nucleotides in the transcript compared with the coding region may be contributed by untranslated regions and a poly(A) tail. Reverse transcription-PCR also showed the presence of a band of expected size (data not shown). These results suggested that the putative PIG-L of *E. histolytica* is expressed in trophozoites.

Inducible expression of PIG-L. A tetracycline-inducible gene expression system for *E. histolytica* was reported previously (45). Molecular cloning of the PIG-L gene in pEh-HYG-tetR-O-CAT vector has been described in Materials and Methods and is schematically shown in Fig. 3B.
In order to determine expression levels of EhPIG-L in over-expressing (EhPL-S) and antisense-blocked (EhPL-AS) cells, Northern hybridization of EhPIG-L was carried out using the entire EhPIG-L coding region in the presence and the absence of tetracycline. In EhPL-AS cells a high level of expression of antisense PIG-L was observed in the presence of tet (Fig. 3C; ss). However, there was a twofold reduction in EhPIG-L transcripts in EhPL-AS cells when the double-stranded gene was used in Northern analysis (Fig. 3C; ds). There was a fivefold increase in the expression of the PIG-L gene upon addition of $10^6$/H9262 g/ml tet for 72 h in EhPL-S cells (Fig. 3D; ds). The results demonstrated the usefulness of the tet-regulated system for conditional expression of a gene in E. histolytica and showed that EhPIG-L expression can be down regulated in EhPL-AS cells upon addition of tet.

The transformed cell lines were further characterized biochemically by use of the cell-free system described earlier (58) to analyze the activities of GPI-Gnt complex and PIG-L. Equal amounts of extracted radiolabeled glycolipids (20,000 to 30,000 for different experiments) were subjected to HPTLC analysis. The ratio of the two products, namely, GlcNAc-PI and GlcN-PI, is indicative of the amount of PIG-L present in the cells. Extracts from EhPL-S cells grown in the presence of tet showed an accumulation of radioactive GlcN-PI and a reduction in GlcNAc-PI levels (Fig. 4A). The ratio of GlcN-PI to GlcNAc-PI was found to be 1.14 and 2.18 in the absence and the presence of tet, respectively, in a typical experiment (Fig. 4B). These experiments were repeated a number of times ($n$ = 4); there was a statistically significant difference ($P$ value < 0.05) in the ratio of GlcN-PI to GlcNAc-PI in the presence and the absence of the inducer. This result is expected if the putative gene codes for PIG-L enzyme whose levels rise in cells in the presence of tet. The converse is expected from EhPL-AS cells, where tet induction would lead to production of antisense RNA and thus inhibition of gene expression (Fig. 4C). In these cells the ratios of GlcN-PI to GlcNAc-PI were found to be 0.882 and 0.445 before and after tet induction, respectively, suggesting an increase in acetylated product (Fig. 4D).

The level of GPI-containing molecules. Aerolysin and proaerolysin have been shown to bind with high affinity to GPI-anchored molecules on the cell surface and therefore can be used to map GPI anchors quantitatively (8). The binding of proaerolysin to lipid bilayer was studied with FLAER, and the labeled cells were analyzed by confocal microscopy as described in Materials and Methods. This analysis indicated that there was a reduction of 85 to 90% in the number of fluorescent foci upon tet induction of EhPL-AS cells (Fig. 5A). The cells containing the parental vector showed no statistically significant change in labeling in the presence of tet (Fig. 5B). This indicated that the antisense RNA-mediated blocking of EhPIG-L gene expression led to a reduction in the amount of GPI-containing molecules on the amoebic cell surface.
The expression of GPI-containing PPG and the small subunit of Gal/GalNAc lectin were studied in EhPL-AS cells by confocal fluorescence microscopy after immunostaining. The images are shown in Fig. 6. PPG was found mainly on the cell surface, and a uniform distribution was observed on EhPL-AS cells in the absence of tet (Fig. 6A, PPG). However, after induction most of the stained material was in the intracellular vacuoles and not on the cell surface (Fig. 6B, PPG). The specific staining of Gal/GalNAc lectin revealed a pattern of granular distribution spread over the cell as observed previously for the lectin (21). After tet induction the level of staining was also reduced significantly, but its distribution seemed to be unaffected (Fig. 6C and 6D; LGL). It appears from these results that there is a reduction in the number of GPI molecules in EhPIG-L expression-blocked cells.

Functional characterization of transformed cells. Cell proliferation is one of the key indicators of an altered cell physiology. Cell proliferation was determined in relation to antisense PIG-L expression. Cell number of EhPL-AS cells was found to decrease by 31 to 36% (P value / \text{H11001} \text{H11021} 0.01) in different experiments in the presence of tet for 72 h (Fig. 7A). No significant change was observed in cell proliferation when a control cell line carrying the parental vector was used.

In order to investigate the role of GPI-anchors in cytoadhesion, an assay involving binding of transformed E. histolytica trophozoites to CHO and HRBCs was performed. There was a 40% reduction in adherence of both CHO cells and HRBCs to EhPL-AS cells in the presence of tet compared to the results seen with cells grown without tet or the cells carrying the parental vector in the presence of the inducer (Fig. 7B). The quantum of reduction differed from experiment to experiment, ranging from 40 to 60%. This result suggests that GPI anchors are important for amoebic adhesion, as already known for one of the GPI conjugates in E. histolytica (Gal/GalNAc lectin).

The level of endocytosis was measured by determining the uptake of FITC-dextran. The level of FITC-dextran uptake in tet-induced EhPL-AS cells was found to be reduced by 50 to 60% compared to uninduced cell results. The cells containing vector alone did not show any significant change in uptake in the presence or the absence of the inducer (Fig. 7C). Fluorescent microscopic analysis of the cells showed similar results. The number of fluorescent pinocytic vesicles was reduced in EhPL-AS cells in the presence of tet (Fig. 7C). The results suggested that the level of endocytosis is reduced by antisense inhibition of PIG-L gene expression.

DISCUSSION

GPI-anchored molecules are involved in important processes in a variety of cell types and organisms. The amount and type of such molecules differ in different organisms, being less abundant in mammalian cells than in yeast and protozoa. Interestingly, GPI-anchored molecules in some of these organisms are thought to be involved in parasitism and pathogenesis (23, 36). The two major GPI-anchored cell surface molecules of E. histolytica, PPG and Gal/GalNAc lectin, are thought to be involved in amoebic adhesion and pathogenesis.
FIG. 7. Functional characterization of pEhPL-AS-transformed cells. (A) Growth of cell lines transfected with pEhPL-AS and vector constructs. All cells were grown in the presence of 10 μg/ml hygromycin B, and growth was measured in the absence and the presence of tet for 72 h. (B) Target cell adherence. An adherence assay was performed as described in Materials and Methods. *E. histolytica* cells were incubated with target cells, and the numbers of amoebae with three or more adherent target cells (CHO cells and HRBCs) were estimated. (C) Fluid-phase endocytosis. EhPL-AS- and vector-transformed cells were incubated with 2 mg/ml FITC-dextran in PBS for 30 min and subjected to quantitative estimation by spectrofluorimeter as described in Materials and Methods. The cells (right panels) were also mounted on glass slides for viewing under a fluorescent microscope (Axioscope plus; Zeiss) at 40× magnification. Some of the error bars are not visible due to small values. (*, $P$ value < 0.05; **, $P$ value < 0.01; ***, $P$ value < 0.001).
key molecules involved in amoebic pathogenesis (3, 26, 34, 41, 53, 54). In this study an attempt was made to generate cell lines that have reduced levels of overall GPI-containing molecules and to correlate this reduction with functional characteristics. The approach used was to block expression of one of the early enzymes in the pathway.

At the outset the presence of GPI-Gnt complex and PIG-L activities in E. histolytica was demonstrated using a cell-free system. The products of these enzyme activities were susceptible to PI-PLC hydrolysis. Nitrous acid deamination revealed that one of the products (Y) contains a PI anchor with a free amine, which may have been generated through a deacetylation reaction. These products were interconvertible by acetylation and deacetylation. Thus, the data put together confirmed that products X and Y were GlcNAc-PI and GlcN-PI, respectively.

One of the ways to decipher the function of a molecule is to generate a knockout cell line or block expression of specific genes. Generating gene knockouts in E. histolytica has not been possible because of the high ploidy of this organism (57). Therefore, expression blocking appears to be a better option for functional studies. Since some of the genes may be essential for proliferation, an inducible antisense approach was adopted. Such an approach utilizing the inducible tet repressor-operator system was previously shown to be useful in generating conditional expression blocking of a calcium binding protein gene of E. histolytica (45). In this study, in silico approaches were used to identify genes encoding the enzymes involved in the biosynthetic pathway of GPI. Since some of these genes were conserved, it was possible to find these enzymes, and one of the first enzymes, PIG-L, was used for expression blocking of the pathway by the antisense approach. The rationale for targeting one of the first enzymes is to block all types of GPI anchors and not necessarily some specific class of GPI-containing molecules.

The data presented here show that the putative PIG-L of E. histolytica is indeed a GlcNAc-PI-deacetylase by the following criteria. (i) EhPIG-L has 33% overall sequence identity with human PIG-L. A conserved domain is also present in EhPIG-L. (ii) E. histolytica cells partially blocked for PIG-L expression, when subjected to conditional tet induction, showed lower levels of deacetylation activity. Conversely, when EhPIG-L was overexpressed, the deacetylase activity was found to increase. These results demonstrated the usefulness of a system regulated by tet for conditional expression of the PIG-L gene in E. histolytica. (iii) The total repertoire of GPI-anchored molecules in the PIG-L-blocked cells was reduced by 85 to 90% as visualized by FLAER labeling. This was also confirmed by measuring the amount of PPG and Gal/GalNAc lectin light subunit in these cells. The antisense-inhibited cells showed a reduction in the levels of both molecules. Expression blocking of EhPIG-L resulted in partial inhibition of E. histolytica cell proliferation. This observation was similar to results seen with yeast and trypomonomes, where genes known to be involved in GPI anchor biosynthesis were essential for viability (16, 36).

GPI-anchored molecules are thought to participate in endocytosis (11, 27, 38, 49). Loss of endocytosis and an inhibition in adherence would reduce the pathogenic potential of E. histolytica. Here we showed that partial blocking of the EhPIG-L gene leads to reduction in the amoebic cell surface adhesion to target cells and a decrease in fluid phase pinocytosis. These observations are consistent with the previous finding that over-expression of a mutant form of the small subunit of Gal/GalNAc lectin lacking the GPI anchor reduced the pathogenic properties of the cells (42).

Blocking of a complete biosynthetic pathway by inhibiting a key enzyme has been achieved in many organisms (20, 39, 56). This is one of the first examples of such an approach in the protozoan parasite E. histolytica. This is also the first report describing some of the events in the biosynthesis of GPI anchors in E. histolytica.

ACKNOWLEDGMENTS

This work was partially supported by grants from the Department of Biotechnology and Department of Science & Technology, Government of India. D.V. thanks the Council for Scientific and Industrial Research and University Grants Commission, India, for a predoctoral fellowship.

We thank M.G. Low (Columbia University College of Physicians and Surgeons, New York, N.Y.) for the generous gift of bacterial PI-PLC enzyme.

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Editor: W. A. Petri, Jr.