A Glycosylphosphatidylinositol (GPI)-Negative Phenotype Produced In Leishmania major by GPI Phospholipase C from Trypanosoma brucei: Topography of Two GPI Pathways

Kojo Mensa-Wilmot,* Jonathan H. LeBowitz,† Kwang-Poo Chang,§ Ahmed Al-Qahtani,* Bradford S. McGwire,§ Samantha Tucker,* and James C. Morris*

*Department of Zoology, University of Georgia, Athens, Georgia 30602; †Department of Biochemistry, Purdue University, Lafayette, Indiana 47907; and §Department of Microbiology and Immunology, UHS/Chicago Medical School, N. Chicago, Illinois

Abstract. The major surface macromolecules of the protozoan parasite Leishmania major, gp63 (a metalloprotease), and lipophosphoglycan (a polysaccharide), are glycosylphosphatidylinositol (GPI) anchored. We expressed a cytoplasmic glycosylphosphatidylinositol phospholipase C (GPI-PLC) in L. major in order to examine the topography of the protein-GPI and polysaccharide-GPI pathways. In L. major cells expressing GPI-PLC, cell-associated gp63 could not be detected in immunoblots. Pulse-chase analysis revealed that gp63 was secreted into the culture medium with a half-time of 5.5 h. Secreted gp63 lacked anti-cross reacting determinant epitopes, and was not metabolically labeled with [3H]ethanolamine, indicating that it never received a GPI anchor. Further, the quantity of putative protein-GPI intermediates decreased ~10-fold. In striking contrast, lipophosphoglycan levels were unaltered. However, GPI-PLC cleaved polysaccharide-GPI intermediates (glycoinositol phospholipids) in vitro. Thus, reactions specific to the polysaccharide-GPI pathway are compartmentalized in vivo within the endoplasmic reticulum, thereby sequestering polysaccharide-GPI intermediates from GPI-PLC cleavage. On the contrary, protein-GPI synthesis at least up to production of Man(1α6)Man(1α4)GlcN-(1α6)-myo-inositol-l-phospholipid is cytosolic. To our knowledge this represents the first use of a catabolic enzyme in vivo to elucidate the topography of biosynthetic pathways.

GPI-PLC causes a protein-GPI-negative phenotype in L. major, even when genes for GPI biosynthesis are functional. This phenotype is remarkably similar to that of some GPI mutants of mammalian cells: implications for paroxysmal nocturnal hemoglobinuria and Thy-1-negative T-lymphoma are discussed.

Please address all correspondence to Dr. K. Mensa-Wilmot, Department of Zoology, University of Georgia, Athens, Georgia 30602.

1. Abbreviations used in this paper: CRD, cross-reacting determinant; dol-P-Man, dolichol-phosphoryl-mannose; eGPI-PLC, recombinant glycosylphosphatidylinositol phospholipase C; EtN, ethanolamine; gp63, 63-kD GPI-anchored glycoprotein of Leishmania parasites; GIPL, glycoinositol phospholipid; GPI, glycosylphosphatidylinositol; GPI-PLC, glycosylphosphatidylinositol phospholipase C; Gal, Galactofuranose; GlcN, glucosamine; GlcNAC, N-acetyl glucosamine; GP1, glycosylphosphatidylinositol; GIPL, glycoinositol phospholipid; C2, Gal, Galactofuranose; GIPL, glucosamine; GlcNAC, N-acetyl glucosamine; LP-1, putative protein-GPI precursor (possibly EtN-phospho-Man-GlcN-P1); LP-2, putative protein-GPI precursor; LPG, lipophosphoglycan; Man, mannose; mVSG, membrane-form VSG; PI, phosphatidylinositol; PNH, paroxysmal nocturnal hemoglobinuria; UDP-GlcNAC, uridine 5'-diphospho N-acetylglucosamine; VSG, variant surface glycoprotein of Trypanosoma brucei.

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Despite the wealth of information on biosynthesis, there is no report on the topography of the GPI pathway in *T. brucei*. In this work we present evidence from the related trypanosomatid *Leishmania major* indicating that GPI biosynthesis is initiated on the cytoplasmic side of the endoplasmic reticulum.

*Leishmania major*, a member of the family trypanosomatidae, is a causative agent of human leishmaniasis. *L. major* has copious amounts of GPI bands termed glycoinositol phospholipids (GIPls) which are unattached to macromolecules (45). GPIs that are covalently bound either to polysaccharides (lipopolysaccharide [LPS] [46]) or proteins (gp63, PSA-2, gp46/M-2 [6, 20, 55, 59]) are also found in *Leishmania*. The core glycan of the gp63 GPI anchor is identical to the core glycan of the VSG GPI (62). The two GPI anchors differ in the kinds of fatty acids and the nature of their linkage to the glycerol of PI; instead of dimyristoyl glycerol, the *L. major* protein-GPI contains 1-O-alkyl-2-O-acetyl glycerol. A putative protein-GPI anchor precursor, LP-1, has been identified in *Leishmania mexicana* (25). Polysaccharide-GPIs of *L. major* contain a "phosphosaccharide core" consisting of a glucosaminyl, two mannosyl, and a galactofuranosyl residue, Gal(1,3)Man(1,3)Man(1,4)GlcN, attached to myo-inositol-1-phospho-p-haplid. Hence, the Man(1,4)GlcN(1,6)-myo-inositol-1-phospho-p-haplid moiety is found both in the core glycan of protein-GPIs and in the phosphosaccharide core of polysaccharide-GPIs. Consequently, it has been suggested that steps leading to the biosynthesis of Man(1,4)GlcN(1,6)-myo-inositol-1-phospho-p-haplid are common to both polysaccharide and protein-GPI pathways. Further, Man(1,4)GlcN(1,6)-myo-inositol-1-phospho-p-haplid is inferred to be a branch point for the protein-GPI and polysaccharide-GPI pathways (43). Nevertheless, neither the site of synthesis of Man(1,4)GlcN(1,6)-myo-inositol-1-phospho-p-haplid nor the topography of the subsequent PI-linked glycosylation reactions is known for either pathway.

*T. brucei* contains a glycosylphosphatidylinositol phospholipase C (GPI-PLC) which can cleave dimyristoyl glycerol from the VSG GPI anchor (8, 12, 27, 31). The enzyme also cleaves protein-GPI precursors in vitro (39, 42). GPI-PLC is an integral membrane protein (8, 27, 31, 50) that has been localized to the cytoplasmic side of intracellular vesicles (9). We report the use of GPI-PLC to delineate the topography of the two GPI pathways in *L. major*, and demonstrate compartmentalization of the protein and polysaccharide-GPI pathways. Our observations establish GPI-PLC as a valuable tool for studying the topography of GPI biosynthesis in vivo. To our knowledge this represents the first use of a catabolic enzyme in vivo to elucidate the topography of biosynthetic pathways.

Intriguingly, *L. major* cells expressing GPI-PLC have the phenotype of protein-GPI deficient cells, although they have no defects in genes required for protein-GPI biosynthesis. This result has implications on GPI-negative mammalian cells. Paroxysmal nocturnal hemoglobinuria (PNH) is a defect in affected hematopoietic stem cells resulting from a deficiency of GPI-anchored proteins (reviewed in 60). Lack of GlcNAc-PI is a hallmark of affected cells (2, 32, 68). The GPI-negative phenotype of *L. major* expressing GPI-PLC suggests that catabolism of GPI intermediates could explain the phenotype of some PNH and Thy-1-negative T-lymphoma mutants (29, 70).

**Materials and Methods**

**Construction of pX63NEO.GPI-PLC (pGPI-PLC)**

A 1.4-kb EcoRI fragment containing *T. brucei* GPI-PLC cDNA was purified from pDH4 (30). EcoRI overlaps were filled with Klenow, and the fragment was digested with HaeIII to remove a partial miniexon from the 5' end of the gene (63). The resultant HaeIII-EcoRI fragment was blunt-end ligated into *BamHI* digested, Klenow treated, pX63NEO (40). A recombinant, pX63NEO.GPI-PLC (pGPI-PLC), with the GPI-PLC gene cloned in the same orientation as the dihydrofolate reductase-thymidylate synthase transcriptional control region of pX63NEO was selected.

**Culture of Parasites, Transfection, and Selection of Recombinants**

Promastigote (insect) stages of *L. major* strain L7252 CC-1 clone (40) and the *L. major* HOM/IQ/73/LCR-L32 strain (14) were studied. Cells were maintained at 25°C in M9 medium supplemented with 10% FBS (Hyclone Labs., Logan, UT) (complete medium). Parasites were transfected by electroporation and plating (36). LCR-L32 cells were selected in complete medium containing 50 µg/ml G418, and used without further cloning after adaptation to growth in 200 µg/ml G418.

**Cell Lysis, Partial Fractionation, and GPI-PLC Assay**

A pellet of 10⁹ cells was lysed in 1 ml hypotonic buffer (10 mM Tris-HCl pH 8, 2 mM EDTA) containing a protease inhibitor cocktail, consisting of phosphoramidon, leupeptin, aprotinin, antipain, EDTA, and (4-amidinoephthoph)-methane sulfonfyl fluoride (APMSF) (Boehringer Mannheim Corp., Indianapolis, IN). The cell suspension was incubated on ice for 30 min, and centrifuged at 14,000 g for 15 min (4°C). The membranous pellet was extracted with 500 µl of 1X AB (50 mM Tris-HCl, pH 8.5, 5 mM EDTA, 1% NP-40). Protein solubilized in the detergent extraction was assayed for GPI-PLC activity using [3H]myristate-labeled VSG as substrate (8, 27, 31). Detergent extracts were diluted with 1X AB to obtain values within the linear range of the assay (0.1-1.5 U).

**Metabolic Labeling, Immunoprecipitation, and Kinetic Analysis**

Promastigotes seeded at 10⁶/ml and grown to a density of 10⁷/ml (5 x 10⁸ total cells) were harvested by low speed centrifugation, washed in 10 ml of PBS (10 mM NaCl, 2 mM KH₂PO₄, 137 mM NaCl, 8 mM KCl, pH 7.4), and washed twice in 10 ml of methionine-free RPMI 1640 (GIBCO BRL, Gaithersburg, MD). Parasites were resuspended in 5 ml of methionine-free RPMI (25) supplemented with 10% dialyzed FBS (Hyclone). Cells and media were harvested in 1-ml cultures (25, 30) in glucose-free RPMI (25) supplemented with 100 µCi/ml of [³H]methionine (1,322 Ci/mmol, Amersham Corp., Arlington Heights, IL) (63). Cells were harvested, washed in 10 ml PBS, and placed in 7 ml of prewarmed complete medium supplemented with 100 µCi/ml non-radioactive methionine (17). A 1-ml aliquot of culture was withdrawn at 0, 1, 2, 4, 6, and 18 h (unless otherwise stated), centrifuged at 14,000 g for 5 min, and 980 µl of medium carefully withdrawn into a prelabeled tube. After removal of the remaining supernatant, pellet and media were both stored at --20°C until use.

Parasites cultured in complete medium to a density of 10⁷/ml were washed as described previously, and resuspended at a density of 2 x 10⁹/ml in 5 ml of ethanolamine labeling medium (M999 supplemented with 20 mM L-glutamine, non-essential amino acids (Sigma Chem. Co., St. Louis, MO), 40 mM Hepes, pH 7.5, 20 mM NaOH, and 10% labeled FBS (Hyclone)). Cells were labeled overnight at 26°C with 100 µCi/ml of [³H]ethanolamine hydrochloride (50 Ci/mmoll, American Radiolabeled Chemicals Inc., St. Louis, MO). Cells and media were harvested in 1-ml portions (see below).

Glycosinolipid phospholipids (GIPls) were labeled by incubating cells (4 x 10⁶/ml) with [³H]galactose (40 Ci/mmoll, Amersham) at 50 µCi/ml in glucose-free RPMI (25) supplemented with 10% (10³Ci/ml) of 100 µCi/ml of 0.5 mg/ml of fatty acid free BSA (Sigma Chem. Co.) and 25 mM Hepes pH 7.4. Label-
ing was performed at 25°C for 16 h. Media and cells were processed as described earlier.

A pellet of 7 x 10^7 [35S]methionine-labeled cells was lysed by thorough resuspension in 1 ml of ice-cold immunoprecipitation dilution buffer (IDB) (1.25% Triton X-100, 150 mM NaCl, 60 mM Tris-HCl, pH 7.5, 6 mM EDTA, 10 U/ml Trasylol) (3), followed by incubation on ice for 30 min. A 250-μl aliquot of cell lysate or culture supernatant, about 2 x 10^7 cell equivalents, was analyzed. To the cell lysate 750 μl of 1.33× IDB and 5 μl of anti-gp63 (or anti-cross reacting determinant, CRD) polyclonal antibody was added (4). The solution was incubated at 4°C overnight with continuous mixing by inversion. A 100-μl portion of a 1:1 suspension of protein A-Sepharose beads (Sigma Chem. Co.) was added and the incubation continued for 2 h at room temperature. Immune complexes adsorbed to protein A-Sepharose were washed three times, each for 10 min, with 1 ml of immunoprecipitation wash buffer (0.1% Triton X-100, 0.02% SDS, 150 mM Tris-HCl pH 7.5, 5 mM EDTA, 10 U/ml Trasylol). The beads received a final wash in 1× TBS (3) after which 50 μl of 2.5X Laemmli (SDS-PAGE sample) buffer was added. Beads were vortexed briefly and heated at 90°C for 5 min. Protein in 25 μl of eluate was analyzed by SDS-PAGE (12%). The gel was soaked in Enatensify™ (Dupont), and radiolabeled proteins detected by fluorography with preflashed Hyperfilm-MP (Amersham). 35C-Labeled proteins (Sigma Chem. Co.) were used as molecular weight standards. For kinetic analysis 10 μl of eluate was quantitated in a liquid scintillation counter (Beckman LS6000).

**Immunoblotting**

Promastigotes of *Leishmania major* LT252 CC-1 clone adapted to growth in 32 μg/ml G418 were harvested at a density of 10^7/ml. An aliquot of detergent extract (10^7 cell equivalents) prepared as described above was concentrated (74), proteins were resolved by 12% SDS-PAGE and transferred to Immobilon-P membranes (Amicon, Bedford, MA). Cell-associated gp63 was detected with anti-gp63 polyclonal antibody (14) and alkaline phosphatase-conjugated secondary antibody (Boehringer Mannheim Corp.). Color development was achieved with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium chloride (BioRad Labs., Hercules, CA). To detect lipophosphoglycan (LPG), 10^7 cell equivalents of total cell lysate was analyzed by Western blotting with monoclonal WIC 79.3 (38) (a gift from Dr. David Russell, Washington University). Color development was with alkaline phosphatase-conjugated secondary antibody.

**Immunofluorescence Assays**

Cells (10^4) were mounted on heavy teflon-coated slides (Cell Line, Newfield, NJ), air-dried for 1.5 h, and fixed with 2% formaldehyde in PBS for 30 min at room temperature. Cells were washed three times in PBS, permeabilized in 0.5% Triton X-100 for 30 min at 4°C, and blocked with 10% FBS in PBS for 1 h at 25°C (37). After washing three times in PBS, a 1:10 dilution of polyclonal antibody directed against the 20 amino-terminal residues of GPI-PLC polypeptide was added. (In control experiments, preimmune serum or antiserum against the T. brucei homologue of BiP (5), a gift from Dr. Jay Bangs, University of Wisconsin, was used as primary antibodies.) Adsorption was allowed for 1 h, and cells were washed in PBS. Fluoresein isothiocyanate-conjugated anti-rabbit secondary antibody (1:500 dilution) was then incubated with the cells for 1 h. Cells were washed with PBS, covered with 10% glycerol, and visualized by UV fluorescence microscopy using a Zeiss inverted microscope (ICM 405).

**Isolation of Glycolipids and Thin Layer Chromatography**

A washed pellet of 2 x 10^7 cells that had been labeled separately with [3H]methanamine, [3H]galactose, or [3H]myristate was extracted twice with 500 μl of freshly mixed ice-cold chloroform/methanol (2:1, vol/vol) (CM). The partially delipidated cellular debris was then extracted twice with 500 μl of water-saturated butanol. The butanol phases were pooled (about 500 μl) and back-extracted twice with 500 μl of water. Butanol phases from the latter extraction were dried under a stream of nitrogen gas and resuspended in 20 μl of freshly mixed CMW for TLC.

**Results**

**GPI-PLC Cleavage of GPIs**

GPIs extracted with CMW (see above) from [3H]galactose or [3H]myristate-labeled pX63NEO/L. major cells were dried and carefully resuspended in 1X AB by repeated pipeting (100 μl per 2 x 10^7 cells, about 25 x 10^4 CPM). Recombinant GPI-PLC (200 ng) (50) was added and the mixture incubated at 37°C for 3 h. Reaction was terminated by extracting twice with 250 μl of water-saturated butanol. The butanol phases were pooled (about 500 μl) and back-extracted twice with 500 μl of water. Butanol phases from the latter extraction were dried under a stream of nitrogen gas and resuspended in 20 μl of freshly mixed CMW for TLC.

**GPI-PLC Decreases the Growth Rate of Leishmania major**

Cells transfected with either pX63NEO or pX63NEO/GPI-PLC (pGPI-PLC) grew well in medium containing 7 μg/ml G418. However, with increasing drug concentration, which is expected to raise copy number of the episomal expression vectors (and with it GPI-PLC levels), the growth rate of cells expressing GPI-PLC (pGPI-PLC/L. major cells) decreased relative to cells harboring the vector pX63NEO (pX63NEO/L. major cells). At 32 μg/ml G418 the growth rate of pGPI-PLC/L. major is half that of control cells (Fig. 1 A). At a higher drug concentration (128 μg/ml) pGPI-PLC/L. major cells which were seeded at 10^7/ml appear to have growth arrested, one week after inoculation, at a density of 10^6/ml. Control cells reached a density of 10^7/ml in the same period (Fig. 1 B).

To determine whether the slow growth phenotype was directly related to GPI-PLC expression levels, we quantitated GPI-PLC produced and compared it to the amount of enzyme found in *T. brucei* TREU 667 (51). No GPI-PLC-like activity was detected in wild-type *L. major*, or in pX63NEO/L. major cells (Table I). In pGPI-PLC/L. major clone #9 about the same quantity of GPI-PLC was produced as was found in *T. brucei* (Table I); clone #4 produced 16-fold less GPI-PLC. However, both clones displayed the slow growth phenotype (Fig. 1, and data not shown), and both clones appear to be devoid of cell-associated gp63 in the steady state (Fig. 2). We conclude that we had expressed GPI-PLC in *L. major* to the same level (or less) as present per cell in *T. brucei*, and that overexpression is not necessary for display of the slow growth phenotype, or for cellular gp63 depletion (Fig. 2).

**GPI-PLC Is Associated with Cytoplasmic Membranous Structures**

The subcellular location of GPI-PLC in pGPI-PLC/L. major was investigated. In indirect immunofluorescence studies, specific staining occurred on cytoplasmic structures which emanated from the perinuclear region and frequently filled most of the cell. However, the plasma membrane, kinetoplast, and nucleus were not stained. This staining pattern is reminiscent of the endoplasmic reticulum (ER) network. In control experiments, a similar pattern of weaker intensity was observed when antibody against the *T. brucei* homologue of BiP (5), an ER-resident protein, was used on these pGPI-PLC/L. major cells. Control pX63NEO/L. major cells showed the ER-like staining pattern with anti-BiP antibody, but not with anti-GPI-PLC antibody.
Figure 1. Effect of GPI-PLC on growth of L. major. Cells selected on M199 plates containing 7 μg/ml G418 were adapted to grow in different drug concentrations by step-wise increases in drug level. Growth rates were measured at the different G418 concentrations, by seeding cells at the indicated density and monitoring growth at stated intervals.

Table I. Levels of GPI-PLC in Cloned L. major CC-1 and T. brucei TREU 667

| Clone                     | GPI-PLC Activity (×10^-3)/10^9 cells |
|---------------------------|--------------------------------------|
| pX63NEO/L. major          | Not detectable                       |
| pGPI-PLC/L. major clone 4 | 1.2                                  |
| pGPI-PLC/L. major clone 9 | 20.0                                 |
| T. brucei TREU 667        | 20.0                                 |

A pellet of 10^8 cells was lysed in 1 ml hypotonic buffer and centrifuged at 14,000 g for 15 min. The membranous pellet from the centrifugation was extracted with 1X AB and assayed for GPI-PLC.

Figure 2. GPI-PLC expression in L. major depletes cell-associated gp63. Cells adapted to growth in 32 μg/ml G418 were harvested at a density of 10^7/ml, lysed hypotonically, and a detergent fraction prepared (Materials and Methods). An aliquot (10^7 cell equivalents) was concentrated by organic solvent precipitation (74), and proteins resolved by 12% SDS-PAGE. Cell-associated gp63 was detected with anti-gp63 polyclonal antibody by immunoblotting. (Lane 1) pX63NEO/L. major; (lane 2) pGPI-PLC/L. major clone #4; (lane 3) pGPI-PLC/L. major clone #9.

40). The remaining 20% of the enzyme activity might be associated with lipid micelles that were not pelleted at 14,000 g. Together with data from the immunofluorescence studies, these observations strongly suggest that GPI-PLC is associated with cytoplasmic membranous structures in L. major.

Lastly, we tested whether GPI-PLC entered the lumen of the ER. The enzyme was immunoprecipitated from [35S]-methionine-labeled cells and digested with N-glycanase. A 40-kD protein was specifically immunoprecipitated which was insensitive to N-glycanase. Thus, the four potential N-glycosylation sites of GPI-PLC are not available for glycosylation in the ER lumen. Controls with gp63 showed it to be N-glycosylated. These results suggest strongly that GPI-PLC does not enter the ER lumen.

L. major Cells Expressing GPI-PLC Have Reduced Levels of gp63

The major surface protein of L. major is a 63-kD GPI-anchored glycoprotein, gp63 (20). We examined the transfected L. major cells for cell-associated gp63 as an indicator of the state of GPI metabolism. In Western blots using polyclonal antibody against gp63 we detected very little gp63 in membrane fractions of pGPI-PLC/L. major clones (Fig. 2, lanes 2 and 3). The protein was detectable in control cells containing the vector, pX63NEO (Fig. 2, lane 1). We conclude that GPI-PLC causes a decrease in cell-associated gp63.

Rapid Secretion of gp63 Caused by GPI-PLC

Several hypotheses, including the following, could explain
Figure 3. Kinetics of gp63 secretion. Cells grown to a density of 10^7/ml in complete medium were labeled with [35S]methionine and "chased" (Materials and Methods). Cell lysates or media (2 x 10^7 cell equivalents each) were immunoprecipitated with anti-gp63 polyclonal antibody. [3S]Methionine-labeled protein eluted from antibody-protein A complex was analyzed by SDS-PAGE and fluorography. (A) L. major LCR-L32: (lane 1) total cell lysate (0 h chase), not immunoprecipitated; (lane 2) cells 0 h; (lane 3) medium 0 h; (lane 4) medium 1 h; (lane 5) medium 2 h; (lane 6) medium 4 h; (lane 7) medium 7 h; (lane 8) medium 16 h. (B) pGPI-PLC/L. major LCR-L32: (lane 1) cells 0 h; (lane 2) medium 0 h; (lane 3) medium 1 h; (lane 4) medium 2 h; (lane 5) medium 4 h; (lane 6) medium 7 h; (lane 7) medium 16 h. Indicated time refers to hours of chase.

The absence of gp63 in pGPI-PLC/L. major cells: (a) GPI-PLC inhibited synthesis of gp63; (b) GPI-PLC caused release of gp63 from the plasma membrane; (c) GPI-PLC promoted gp63 secretion, or (d) GPI-PLC caused degradation of gp63. We tested these hypotheses and distinguished between them by metabolically labeling L. major with [35S]methionine followed by an examination of both cells and culture medium for gp63 (Fig. 3). Determination of the biochemical properties of released gp63 (product analysis) was particularly informative.

L. major wild-type cells were efficiently labeled with [35S]methionine (Fig. 3 A, lane 1), as were pGPI-PLC/L. major LCR-L32 cells (not shown). In both cell types we could immunoprecipitate cell-associated gp63 after a 3-h pulse with [35S]methionine (Fig. 3 A, lane 2; Fig. 3 B, lane 1). In the ensuing "chase" gp63 appeared in the medium of both cell types (Fig. 3 A, lanes 3-8; Fig. 3 B, lanes 2-7), but with different kinetics. Compared to the total amount of gp63 present initially within cells, a significant amount of gp63 was released in just 2 h of chase from pGPI-PLC/L. major cells: it took 7 h for a significant quantity of gp63 to accumulate in the medium of L. major wild-type cells.

Immunoprecipitated gp63 was quantitated by liquid scintillation spectrometry of equal volumes of material eluted from the protein A-Sepharose column. For kinetic analyses, the quantity of gp63 present in the medium was expressed as a fraction of the amount of gp63 that was present within cells at the beginning of the chase (Fig. 4). Gp63 in L. major had an apparent half-time (time required for release of 50% of gp63 present at t = 0 h into medium) of at least 18 h (Fig. 4). Remarkably, in pGPI-PLC/L. major cells the apparent half-time of gp63 cell-association decreased to 5.5 h (Fig. 4). Thus, for the latter cells which double every 24 h, 94% of gp63 that was initially labeled is expected to be released within the generation time of each cell. The latter observation is consistent with the apparent absence of gp63 from pGPI-PLC/L. major cells (Fig. 2) in steady-state analysis (Western blots).

We conclude that pGPI-PLC/L. major cells appear to have no cell-associated gp63 because the bulk of the protein (~70%, Fig. 4) is secreted into the culture medium within 7 h. Electrophoresis of an aliquot of total culture medium followed by fluorography revealed three other proteins secreted into the medium with the same kinetics as gp63 (data not shown). The identity of these proteins, which we presumed to be GPI-anchored, is unknown, however the observation indicates that GPI-PLC effects are not limited to gp63.

Gp63 Is Secreted Without Prior Addition of a GPI Anchor

Release of a GPI-anchored protein into culture medium can
occur before or after acquisition of a GPI anchor. To distinguish these possibilities, protein released from [35S]methionine-labeled L. major cells was immunoprecipitated with polyclonal antibody against either gp63 or against the cross-reacting determinant epitopes of cleaved GPls (anti-CRD). Secreted gp63 was immunoprecipitable with anti-gp63 but not with anti-CRD (Fig. 5 A). The data suggests strongly that secreted gp63 did not initially acquire a GPI anchor and then lose it by GPI-PLC cleavage, because when cleaved in vitro by PLC, gp63 reacts with anti-CRD (6, 62).

We then tested whether cells labeled with [35S]methionine possessed anti-CRD epitopes. Total cell lysates were preincubated at 37°C for 1 h to allow endogenous phospholipases C to cleave [35S]methionine-labeled GPI moieties, and then immunoprecipitated with anti-CRD antibody. As shown in Fig. 5 B, columns 1 and 2, cellular anti-CRD epitopes can be immunoprecipitated, indicating that the anti-CRD antibody was functional. Consistent with the extra phospholipase C activity in pGPI-PLC/L. major cells, due to expression of GPI-PLC, the quantity of immunoprecipitable anti-CRD antigens doubled (Fig. 5 B, column 2).

The possibility remained that gp63 dissociated from the plasma membrane with its GPI anchor intact, in which case the released gp63 will not be detected by anti-CRD antibody. To test this hypothesis, released [35S]methionine-labeled gp63 was incubated with an excess of recombinant GPI-PLC (eGPI-PLC) to cleave the presumably intact GPI anchor. The anti-CRD epitopes should be exposed after this cleavage, and the solubilized gp63 ought to be immunoprecipitable by anti-CRD antibody (a buffer containing 5 mM EDTA was used so as to protect GPI-PLC from the proteolytic activity of gp63). Data presented in Fig. 5 B, columns 3 and 4, indicate that anti-CRD reactive epitopes are absent on the released gp63. The [35S]methionine-labeled gp63 could not be immunoprecipitated with anti-CRD even after in vitro treatment with eGPI-PLC. We conclude that released gp63 does not have an intact GPI anchor.

Lastly, the cells were metabolically labeled with [3H]ethanolamine to test for the presence or absence of GPI core glycan components on released gp63. This was necessary because gp63 could initially have received a GPI anchor that was subsequently cleaved by GPI-PLC. However, the anti-CRD epitopes could have been destroyed by the action of a phosphodiesterase present in the secretory pathway, or in the medium. Then, the results of the anti-CRD immunoprecipitations will be negative for entirely different reasons.

Figure 5. Secreted gp63 never received a GPI anchor. (A) Secreted gp63 lacks anti-CRD Epitopes. Medium collected from [35S]methionine-labeled pGPI-PLC/L. major cells was immunoprecipitated with polyclonal antibody against either gp63 (lane 1) or against the cross-reacting determinant (lane 2). Immune complexes were analyzed as described in Fig. 4 legend. (B) [35S]Methionine-labeled anti-CRD epitopes are immunoprecipitable from cell lysates. Cells were lysed (Materials and Methods), and then incubated at 37°C for 1 h to enhance cleavage of GPls by endogenous phospholipases. Lysates were immunoprecipitated with anti-CRD antibody. Material eluted from the protein-A Sepharose beads was quantitated by liquid scintillation spectrometry. A background of 40 CPM is subtracted from the data presented. Media was adjusted to IX AB, purified recombinant GPI-PLC (eGPI-PLC, 40 U) was added, and the mixture incubated at 37°C for 1 h. Anti-CRD epitopes were assayed as described above. Column 1, L. major; column 2, pGPI-PLC/L. major; column 3 (height same as background), L. major medium plus eGPI-PLC; column 4 (height same as background), pGPI-PLC/L. major medium plus eGPI-PLC. (C) Secreted gp63 cannot be metabolically labeled with [3H]ethanolamine. Cells (pGPIPLC/L. major) were labeled with [3H]ethanolamine, and media was harvested. Cells and media were immunoprecipitated with anti-gp63 antibody. Immunoprecipitated protein was eluted, resolved by SDS-PAGE, and detected by fluorography. (Lane 1) Immunoprecipitate from 2 x 10⁷ cells; (lane 2) immunoprecipitate of spent culture medium (from 2 x 10⁷ cells).
creted gp63 could not be metabolically labeled with [3H]ethanolamine (Fig. 5 C, lane 2), but the residual cellular gp63 (Fig. 4) could be labeled (Fig 5 C, lane 1). This result confirms our earlier conclusion that secreted gp63 never received a GPI anchor. Interestingly, similar analysis of gp63 released by wild-type \textit{L. major} indicated that it also never received a GPI anchor (not shown). Surprisingly, in \textit{Leishmania amazonensis} barely any gp63 is released from wild-type cells (not shown). Thus, in some \textit{Leishmania} species (e.g., \textit{L. major}) the requirement for GPIs might exceed the capacity of the biosynthetic pathway.

Cleavage of the gp63 carboxyl terminal hydrophobic tail might not have occurred in \textit{pGPI-PLC/L. major}, because we did not detect the expected increase in mobility of secreted gp63 on SDS-PAGE (compared to cell-associated gp63). However, we have not ruled out the possibility that the cleaved peptide was too small to produce a shift in the mobility of secreted gp63. Lastly, we note reports that \textit{L. donovani chagasi} and \textit{L. mexicana} express gp63 transcripts that do not have GPI addition signals (47, 58, 73). If such proteins were present in \textit{L. major} and were membrane bound, their cell association will not be expected to be affected by GPI-PLC.

**GPI-PLC Causes a Decrease of Protein-GPI Intermediates**

To test the hypothesis that GPI-PLC affected the biosynthesis of protein-GPI intermediates, glycolipids were isolated from [3H]ethanolamine-labeled cells (10⁷ equivalents) and analyzed by TLC. A putative protein-GPI precursor, LP-1, is diminished in \textit{pGPI-PLC/L. major} cells (Fig. 6 A, lane 2), as compared to \textit{pX63NEO/L. major} cells (Fig. 6 A, lane 1). Quantitation of LP-1 indicated that its level was reduced 10-fold in \textit{pGPI-PLC/L. major} cells. LP-1 was confirmed as a GPI by cleavage with eGPI-PLC in vitro (not shown). A second putative GPI intermediate LP-2 (Fig. 6), less polar than LP-1, was decreased about sevenfold. Amounts of other [3H]ethanolamine-labeled lipids of higher relative mobility were also reduced. However, other lipids (marked with asterisk) were present at similar levels in the two cell types. Some lipids (Fig. 6 A, marked with diamond) were slightly more abundant in \textit{pGPI-PLC/L. major} cells as compared to the \textit{pX63NEO/L. major} cells. We conclude that GPI-PLC causes a decrease of protein-GPI anchor precursors in \textit{pGPI-PLC/L. major} cells. The decrease in other ethanolamine-labeled lipids could be an indirect effect of LP-1 depletion (see Discussion), because those other lipids were not sensitive to eGPI-PLC cleavage.

**GPI-PLC Cleaves Polysaccharide-GPI Intermediates In Vitro, Yet Lipophosphoglycan Level Is Unaffected In Vivo**

The major surface macromolecule of \textit{L. major} is LPG, a

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**Figure 6.** GPI-PLC decreases levels of putative protein-GPI precursors. (A) Cells were labeled with [3H]ethanolamine and extracted sequentially with chloroform/methanol (CM) and chloroform/methanol/water (CMW). CMW extract (10⁷ cell equivalents) was analyzed by TLC (silica gel 60) in chloroform/methanol/water (10:10:3). Radioactive species were detected by fluorography. (Lane 1) pX63NEO/L. major; (lane 2) pGPI-PLC/L. major. Migration positions of putative protein-GPI precursors, LP-1 and LP-2, are indicated. Representative lipids that are present in roughly equal quantities in each lane, per set of experiments, are indicated by an asterisk. Lipids that appear to be enhanced in \textit{pGPI-PLC/L. major} cells are marked with filled diamond. (B) LP-1 and LP-2 from Fig. 6 A were quantitated by integrating the volume under an elliptical zone encompassing the respective fluorographic images using ImageQuant™ ver3.0 software (Molecular Dynamics). The results of two separate experiments are presented. Stippled column, pX63NEO/L. major; black column, pGPI-PLC/L. major.
GPI-anchored polysaccharide (46). Surprisingly, in \textit{L. major} cells expressing GPI-PLC, LPG levels did not decrease (Fig. 7 A), in marked contrast to the results obtained with cellular gp63 (Fig. 2). This last result could be explained by a resistance of polysaccharide-GPI intermediates to GPI-PLC cleavage, or by sequestration of polysaccharide-GPI intermediates away from GPI-PLC in vivo.

We therefore investigated the susceptibility of polysaccharide-GPI precursors (glycoinositol phospholipids, GIPLs) to GPI-PLC, after their metabolic labeling with \[^{3}H\]galactose (Fig. 7 B, lane 1). GIPL-I, GIPL-II, GIPL-III, and GIPL-IV (presumably GIPL-I, GIPL-2, GIPL-3, and lyso-GIPL-3, respectively (18), see Fig. 8 for structures) were cleaved by eGPI-PLC in vitro (Fig. 7 B, lane 2). The least polar of the \[^{3}H\]galactose-labeled glycolipids GIPL-X (possibly GlcNAc-GIPL formed after epimerization of galactose to glucose (26)) was not cleaved (Fig. 7 B, lane 2), in accordance with the specificity of GPI-PLC (50, see Fig. 8 legend). Similar results were obtained with \[^{3}H\]myristate-labeled GIPLs (not shown). We conclude that the in vivo effects of GPI-PLC are specific to the protein-GPI pathway, despite the susceptibility of polysaccharide-GPI intermediates to GPI-PLC.

**Discussion**

\textit{GPI-PLC Causes Rapid Secretion of GPI-Anchorless gp63: GPI Biosynthesis Is Initiated on the Cytoplasmic Side of the Endoplasmic Reticulum}

The GPI biosynthetic pathway has been studied extensively in many eukaryotes ranging from protozoan to mammalian cells. The topography of the pathway remains one of the intriguing but unanswered questions in almost all of these cells. Do all the biosynthetic reactions take place in the ER cisternae where prefabricated GPI anchors are transferred to nascent protein? Or, does part of the pathway occur on the cytoplasmic leaflet of the ER? To address this issue in the trypanosomatid \textit{Leishmania major} we employed a novel approach based on the heterologous expression of a cytoplasmic membrane protein, GPI-PLC, which is capable of degrading GPI intermediates. Metabolism of a GPI-anchored protein, gp63, was monitored vis-à-vis GPI addition. Putative GPI anchor intermediates were quantitated. Results from these studies, in the context of the cytoplasmic localization of the catabolic enzyme, indicate that GPI synthesis is initiated on the cytoplasmic side of the ER. The principles applied in this work could be extended to topological studies of other membrane bound biosynthetic pathways. For example, expression of a cytoplastically targeted β-hexosaminidase or mannosidase could contribute to analysis of the topography of dolichol-linked oligosaccharide biosynthesis.
Intermediates of GPI biosynthesis and GPI-anchored macromolecules are membrane bound (see 19, 22 for recent reviews). Localization of GPI-PLC to the cytoplasmic side of intracellular membranes (see below) and the enzyme's ability to cleave GPI biosynthetic intermediates in vitro (42, Fig. 7 B) suggested that GPI-PLC might cleave GPI intermediates in vivo if it were to colocalize with them. Such a situation could arise in any cell in which GPI-PLC is expressed. \textit{L. major} was particularly attractive for these studies because: (a) The cells lack GPI-PLC-like activity (Table I); (b) intermediates of both the protein-GPI and the polysaccharide-GPI pathways can be cleaved by GPI-PLC in vitro (42, Fig. 7 B), and (c) topography of the two GPI pathways is unknown; a comparative analysis had the potential of being very informative.

Two models could explain the rapid release of gp63 in pGPI-PLC/\textit{L. major} cells (Fig. 4). The basic tenet of both models is that GPI-anchored proteins are committed to the secretory pathway by default, and are retained in the plasma membrane only by GPI addition (11, 13, 21, 67). In the first, the GPI-addition-cleavage model, gp63 was presumed to initially acquire a GPI anchor in the ER. Subsequently, the protein encountered GPI-PLC in transit to, or at, the plasma membrane, had the GPI anchor cleaved and was released.

This model requires GPI-PLC to enter the secretory pathway. Further, since gp63 is presumed to receive a GPI anchor before losing it, the model predicts that released gp63 will have CRD epitopes. When cleaved by GPI-PLC, GPIs display epitopes on GlcN(la6)inositol 1,2 cyclic monophosphate which are recognizable by anti-CRD antibodies (7, 75).

The second model is the GPI precursor depletion hypothesis. In this scenario, GPI-PLC is assumed to be associated with the cytoplasmic side of cellular membranes (including the ER). There, it encounters intermediates of GPI biosynthesis which it cleaves. Degradation of GPI anchor intermediates creates a shortage of fully assembled GPI anchors in the ER lumen. Consequently, proteins with GPI addition signals (reviewed in 19) do not receive GPI anchors, and are secreted. Important distinctions between this model and the GPI-addition-cleavage model are (a) secreted proteins are predicted not to have anti-CRD epitopes, (b) residual components of the GPI core glycan [EtN-phospho-6Man(la2)-Man(la6)Man(la4)GlcN] cannot be found on the secreted protein, and (c) GPI-PLC is not required to enter the ER lumen.

Our data supports the GPI precursor depletion model. First, GPI-PLC in \textit{L. major} is membrane bound, cytoplas-
mic, and does not enter the ER lumen (see Results section).
Therefore, any effects on GPI metabolism are exerted by
GPI-PLC on the cytoplasmic side of cellular membranes.
In addition, secreted gp63 never received a GPI anchor (Fig. 5 A, B, and C).
Moreover, when compared to control
pX63NEO/L. major cells, pGPI-PLC/L. major cells have a
tenth of the amount of a putative protein-GPI precursor,
LP-1, presumably EtN-phospho-Man5-GlcN-PI (Fig. 6).
Together, these observations strongly support the concept
that protein-GPI biosynthesis is initiated on the cytoplasmic
leaflet of the ER. In agreement with this conclusion, over
70% of GlcN-PI (possibly the earliest glycolipid committed
to the GPI biosynthetic pathway) is found on the outer leaflet
of microsomes in the thymoma cell line BW5147.3 (72).
In addition, three genes cloned by complementation of GPI-
deficient mammalian cells, Fig-A, GPI-H, and Fig-F, all
appear to encode cytoplasmic membrane proteins (33, 34, 52).

GPI-PLC Does Not Inhibit the Polysaccharide-GPI
Pathway: The First Two Mannosylation Reactions of the
Protein-GPI Pathway Are Cytoplasmic

LPG, a major surface macromolecule of L. major, is a GPI-
anchored polysaccharide. LPG consists of a 27-mer (on
the average) of the disaccharide-phosphate backbone PO4-
6Gal(1β4)Man(α1), attached by a phosphosaccharide core,
PO4-6Gal(1α6)Gal(1α3)Gal(1β3)Man(1α3)Man(1α4)GlcN,
to a lysO-1-O-alkylphosphatidylinositol anchor (46, 71). L.
major also contains copious quantities of glycoinositol phos-
pholipids (GIPLs), GIPs that are not attached to protein or
polysaccharide. GPI-PLC-2, Gal(1α3)Gal(1β3)Man(1α3)Man-
(1α4)GlcN-Pl, is the most abundant of them. GIPL-3 con-
tains a 1-O-alkyl-2-acyl phosphatidylinositol linked to the
identical phosphosaccharide core found on LPG. Lyso-
GIPL-3 is presumed to be an intermediate in LPG biosyn-
thesis (44).

The polysaccharide-GPI and protein-GPI pathways appear
to share several steps culminating in the production of Man-
(1α4)GlcN-PI (Fig. 8) (43, 45). Thereafter, the protein-GPI
pathway branches off from the polysaccharide-GPI pathway.
The next mannosyl residue added to Man(1α4)GlcN-PI is in
the α3 configuration for polysaccharide-GPIs, but in the
α6 configuration for protein-GPs. If inhibition of GPI met-
abolism in pGPI-PLC/L. major occurred before Man(1α4)-
GlcN-PI production one would expect a reduction in the
amount of cellular LPG. Contrary to that prediction, LPG
levels were not reduced (Fig. 7 A). Thus, the GPI-PLC inhibi-
tory effects (Figs. 2 and 6) are limited to the protein-GPI
pathway. This specificity exists in spite of the susceptibility
of polysaccharide-GP- intermediates to GPI-PLC in vitro
(Fig. 7 B).

To account for the in vivo specificity of GPI-PLC, we
propose that the target of GPI-PLC occurs after Man(1α4)-
GlcN-PI synthesis. Thus, Man(1α6)Man(1α4)GlcN-PI, or
an intermediate occurring after it in the protein-GPI path-
way, is the susceptible precursor. Hence, the first manno-
sylation specific to the protein-GPI pathway is cytoplasmic
(Fig. 8). In contrast, the first mannosylation specific to the
polysaccharide-GPI pathway, which generates Man(1α3-
Man(1α4)GlcN-PI, occurs inside the ER, after "flipping" of
Man(1α4)GlcN-PI into the lumen. Consequently, Man-
(1α3)Man(1α4)GlcN-PI is sequestered from GPI-PLC,
which remains on the cytoplasmic side of the ER. We sur-
mise that reactions specific to the polysaccharide-GPI path-
way are compartmentalized inside the ER, away from the
protein-GPI pathway which is chiefly cytosolic (Fig. 8).
Man(1α4)GlcN-PI, the common intermediate, might not be
cleaved in vivo for kinetic reasons: it may flip rapidly into the
ER lumen, or might be quickly mannosylated cytoplasmically
to Man(1α6)Man(1α4)GlcN-PI, which is then cleaved.
Alternatively, a pool of Man(1α4)GlcN-PI synthesized within
the ER might be used for polysaccharide-GPI synthesis. How-
ever, we prefer the former model for reasons of "cellular
economy."

In effect, the topography of the GPI pathway is similar to
that of dolichol-linked oligosaccharide biosynthesis. Both
pathways are initiated on the cytoplasmic side of the ER (1,
72), yet the fully assembled precursors are transferred to
protein inside the ER (1, 19, 22). Intriguingly, dol-P-mann
might serve as a mannosyl donor in GPI synthesis on the
cytoplasmic side of the ER, but flips into the ER for use in the
dolichol-linked oligosaccharide pathway (57).

T. brucei GPI-PLC, an In Vivo Probe of the
Topography of GPI Biosynthesis

Several properties of T. brucei GPI-PLC suggested its poten-
tial use as a membrane-impermeable probe of the topog-
raphy of GPI biosynthesis. First, the enzyme is highly
specific for GPIS (10, 27, 31); it cleaves GPIs from proteins,
and will cleave biosynthetic intermediates occurring
after GlcNAc-PI if the inositol moiety is not acylated
(16, 42, 49). Second, GPI-PLC is an integral membrane pro-
ten even though Kyte-Doolittle analysis does not reveal a
"typical" transmembrane domain (8, 27, 31, 50). GPI-PLC
membrane insertion requires a 27-amino acid sequence from
the carboxyl terminal half of the protein (Al-Qahtani, A.,
and K. Mensa-Wilmot, unpublished observations). Third,
GPI-PLC does not enter the signal sequence-directed secre-
tory pathway. GPI-PLC entry into the secretory pathway
would make product analysis uninterpretable, as the enzyme
could encounter its substrate while both are in transit to the
plasma membrane.

In T. brucei, there is ample evidence indicating that GPI-
PLC does not enter the secretory pathway: (a) GPI-PLC has
been localized by immuno-electron microscopy of cryo-
sections to the cytoplasmic side of intracellular membranes
(9); (b) GPI-PLC lacks a signal sequence needed to direct
it into the lumen of the ER (12, 30); (c) GPI-PLC is not
glycosylated, yet it has four potential N-linked glycosylation
sites at positions 82, 101, 315, and 353 of the 358-amino acid
protein (16, 30, 50); and (d) GPI-PLC (by subcellular
fractionation) is not found on the plasma membrane. It is
associated with intracellular organelles including nuclear
dense and microsomal pellets (8, 28). Similarly, GPI-PLC in L. major
is cytoplasmic, membrane bound, and unglycosylated.

Conceptually, GPI-PLC could affect GPI synthesis in any
cell in which it is expressed, if the cleavable GPI intermedi-
ates (beginning with GlcN-PI) colocalized with it on the
outer leaflet of the ER. In L. major, this prediction is borne
out. GPI-PLC cleaves GPI intermediates, causing a protein-
GPI negative phenotype (Figs. 2, 3, and 6).

Interestingly, the GPI-negative phenotype observed in
pGPI-PLC/L. major is not evident in bloodstream form T.
brucei where GPI-PLC is endogenous. Each T. brucei cell
has ~10^7 copies of a GPI-anchored VSG, implying that a
large amount of GPI anchors is synthesized within the generation time (~6–8 h) of this trypanosomatid. The cell-association half-time of VSG is about 34 h (9), unlike gp63 which has a cell-association half-time of 5.5 h in pGPI-PLC/L. major cells. Why does GPI-PLC in T. brucei not cause a rapid release of VSG, similar to the secretion of gp63 in pGPI-PLC/L. major? Previous explanations relied on compartmentalization of GPI-PLC (which is cytoplasmic) from VSG (found at plasma membrane); those arguments might explain why VSG itself is not cleaved. However, recognizing now that protein-GPI anchor precursors can be cleaved by GPI-PLC in vivo, as demonstrated for the Leishmania spp., an explanation other than compartmentalization is needed to rationalize why GPI precursors in T. brucei are not degraded. Our preliminary investigations indicate that GPI-PLC is posttranslationally modified in T. brucei (Mensa-Wilmot, K., unpublished observations). We speculate that the modification (the nature of which is under investigation) regulates GPI-PLC action by keeping the enzyme quiescent, to prevent it from degrading GPI precursors under normal physiological conditions. An extracellular signal might trigger removal of the modification from the GPI-PLC, enabling it to become enzymatically active. During hypotonic lysis (a possible source of such an extracellular signal) almost all the VSG GPI is cleaved within 15 min, presumably by GPI-PLC (12). Possibly, the inhibitory effect of GPI-PLC on protein-GPI metabolism in pGPI-PLC/L. major occurs because GPI-PLC is constitutively active. Leishmania species might not be capable of the posttranslational modification necessary to keep GPI-PLC quiescent.

**GPI-PLC Can Induce a GPI-Negative Phenotype: Implications for Paroxysmal Nocturnal Hemoglobinuria and Thy-1-negative T-lymphoma**

PNH is a defect in a hematopoietic stem cell progenitor, which after expansion usually manifests as hemolysis of the abnormal red cells. GPI-anchored complement regulatory proteins, e.g., C8 binding protein and CD55 (decay accelerating factor) (reviewed in 60), are markedly deficient in hematopoietic cells from PNH patients. Increased plasma levels of CD55, suggestive of CD55 secretion, is also observed (15, 48, 56). GlcNAc-PI is deficient in lines of affected cells (2, 32, 68). PNH cells are class A mutants in the T-lymphoma nomenclature (2, 66) (see below).

Several GPI-defective complementation groups of Thy-1-negative murine T-lymphoma cells have been established (reviewed in 69). The phenotype of the class E (defective in dol-P-mann synthase [13]) and B (accumulates Man$_2$-GlcN-PI [35]) mutants bear a dramatic resemblance to pGPI-PLC/L. major cells. Both class E and B mutants secrete Thy-1 protein (21), similar to the secretion of gp63 by pGPI-PLC/L. major. Further, both mutants lack EtN-phospho-Man$_2$-GlcN-PI, akin to the depletion of LP-1 (EtN-phospho-Man$_2$-GlcN-PI) in pGPI-PLC/L. major. Given that pGPI-PLC/L. major cells are not mutants in the GPI pathway, our observations provide evidence that a GPI-negative phenotype is inducible by catabolism of intermediates. Thus, defects in genes whose products regulate the balance between biosynthesis and breakdown of intermediates could produce a GPI-negative phenotype similar to that of pGPI-PLC/L. major. In support of this notion, treatment of the class B mutants with the aminoglycoside G418 can induce surface expression of Thy-1 protein, indicating that the class B defect is probably not in a gene of the GPI biosynthetic pathway (29). Instead, a hyperactive GPI mannosidase that degrades Man$_2$-GlcN-PI may be responsible for the class B phenotype, similar to the postulated cleavage of Man$_2$-GlcN-PI by GPI-PLC in pGPI-PLC/L. major.

The ideas above can be extended to the class A, C, and H mutants all of which, similar to PNH cells, lack GlcNAc-PI (64). One possibility for the existence of three complementation groups at the GlcNAc-PI synthesis step is a requirement for three different gene products for the GlcNAc transfer reaction (64). However, most glycosyl transferases and hydrolases are single subunit enzymes (53, 65). Therefore, the GlcNAc transferase required for GPI biosynthesis might well be a single subunit enzyme. If so, some of the class A, C, or H mutations might be in factors that regulate GlcNAc-PI metabolism. Some mutants might synthesize GlcNAc-PI which is then rapidly degraded by a hyperactive catabolic activity, e.g., a "Labile Regulator" of GPI biosynthesis (LR) (Fig. 9), similar to the degradation of protein-GPI precursors by GPI-PLC in pGPI-PLC/L. major. LR activity might be controlled normally by an "Inhibitor of the Labile Regulator" (ILR), possibly composed of at least two subunits (ILRA and ILRB, [Fig. 9]). Mutations in ILR might produce hyperactive LR leading to depletion of GlcNAc-PI, even when all genes for GPI biosynthesis are functional. The surprising finding that sodium butyrate causes reexpression of cell surface Thy-1 in class H mutants (29, 70) is compatible with this model; the compound might directly suppress LR activity (or stimulate ILR activity). Interestingly, in agreement with experimental observations, ILR mutants will not be dominant in cell fusion analysis.

Consistent with this ILR hypothesis, the human Pig-A gene complements PNH and class A T-lymphoma cells, but fails to complement class C and H mutants (52). Further, Pig-A has no sequence similarity with GlcNAc transferases, raising the possibility that Pig-A encodes an ILR. Mutations in Pig-A (as an ILR), as reported in PNH (68), might cause rapid degradation of GlcNAc-PI. However, the effect will be recessive, as observed in GlcNAc-PI biosynthesis studies in vitro (64). These observations suggest that kinetic analysis of GlcNAc-PI metabolism in PNH cells, and in some T-lymphoma mutants, might be appropriate to rule out the possibility that absence of GlcNAc-PI in the steady state is the result of very swift catabolism.

**Consequences of the Protein-GPI-Negative Phenotype in L. major**

GPI-anchored molecules serve many different functions in cells, as suggested by the diverse groups of macromolecules represented. Therefore, the consequences of a GPI-negative phenotype are expected to be diverse, and manifested differently, in a cell type-specific fashion. In L. major the protein-GPI-negative phenotype causes "slow growth" (Fig. 1), at least. This slow growth might be due to a deficiency in a GPI-anchored ectoprotein that serves as a growth factor receptor. Candidate molecules for such an ectoprotein include the L. major equivalents of the folic acid receptor, and a cyclic-AMP-binding protein (41, 54). Three other proteins were rapidly secreted, in addition to gp63, from pGPI-PLC/L. major cells; one of them might be the deficient molecule.
Figure 9. On the control of GPI biosynthesis by a labile regulator (The labile regulator hypothesis). It is assumed, as observed in some GPI-negative murine T-lymphoma cells (70), that all genes for GPI biosynthesis are functional. We propose that GlcNAcPI is subject to degradation by a factor, the "Labile Regulator" (LR), that is unstable during cell lysis. High activity of LR might prevent detection of GlcNAcPI. LR activity might normally be suppressed by an inhibitor, termed the "Inhibitor of the Labile Regulator" (ILR). ILR might be composed of two gene products ILRA and ILRB (at least). Mutations in ILRA and/or ILRB might permit hyperactivity of LR, which results in depletion of GlcNAcPI. The possibility that the two layers of regulation are actually exerted on LR directly, or by stimulating ILR activity.

Degraded GlcNAc-PI

GPI Precursor

Inhibitor of Labile Regulator (ILR)

(-)

GlcNAc transferase

GlcNAc-PI

Labile Regulator, LR

(-)

Sodium butyrate Aminoglutethamide G418

Other effects of this protein-GPI-negative phenotype on the biology of Leishmania are under investigation.

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