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

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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 [^{3}H]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-1-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.

GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)[1] anchors attach a diverse group of macromolecules to membranes in eukaryotes (see 19, 22 for recent reviews). The plasma membrane of the protozoan parasite *Trypanosoma brucei* is covered with about 10^7 molecules of the variant surface glycoprotein (VSG), a GPI-anchored molecule. The VSG GPI consists of dimyristoylphosphatidylinositol linked to a linear tetrasaccharide "core glycan." The core glycan contains a glucosaminyl (GlcN) and three mannosyl (Man) residues with phosphoethanolamine (EtN-phospho) at the non-reducing end, EtN-phospho-6Man(1α2)-Man(1α6)Man(1α4)GlcN. The amino group of ethanolamine (EtN) is attached to the α-carboxyl of the COOH-terminal residue of the mature protein by an amide bond (23).

Biosynthesis of GPIs in *T. brucei* begins with the transfer of N-acetylglucosamine (GlcNAc) from uridine 5′-diphospho N-acetylglucosamine (UDP-GlcNAc) to phosphatidylinositol (PI), forming GlcNAc-PI which is deacetylated to glucosaminyl-PI (GlcN-PI). Sequential transfer of mannosyl residues from dolichol-phosphoryl-mannose (dol-P-man) yields Man_3-GlcN-PI. Phosphoethanolamine is then added to Man_3-GlcN-PI, producing glycolipid A′ (EtN-phospho-Man_3-GlcN-PI). Replacement of the fatty acids on glycolipid A′ with myristate produces glycolipid A (also termed P2 [39, 42]) which can be transferred to nascent proteins in the endoplasmic reticulum (consult 19, 22 for reviews).

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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_f, Galactofuranose; GlcN, glucosamine; GlcNAc, N-acetyl glucosamine; LP-1, putative protein-GPI precursor (possibly EtN-phospho-Man_3-GlcN-PI); LP-2, putative protein-GPI precursor; LPG, lipophosphoglycan; Man, mannose; mfVSG, 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 GPIs termed glycoinositol phospholipids (GIPLs) which are unattached to macromolecules (45). GPIs that are covalently bound either to polysaccharides (lipophosphoglycan [LPG] [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-acyl 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$_f$(1β3)Man(1α3)Man(1α4)GlcN, attached to *myo*-inositol-1-phospho-lipid. Hence, the Man(1α4)GlcN(1α6)-*myo*-inositol-1-phospho-lipid 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(1α4)-GlcN(1α)6-*myo*-inositol-1-phospho-lipid are common to both polysaccharide and protein-GPI pathways. Further, Man(1α4)GlcN(1α)6-*myo*-inositol-1-phospho-lipid is inferred to be a branch point for the protein-GPI and polysaccharide-GPI pathways (43). Nevertheless, neither the site of synthesis of Man(1α4)GlcN(1α)6-*myo*-inositol-1-phospho-lipid 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 dimyristoylglycerol 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 *Eco*RI fragment containing *T. brucei* GPI-PLC cDNA was purified from pDH4 (30). *Eco*RI overhangs were filled with Klenow, and the fragment was digested with *Hae*III to remove a partial miniexon from the 5' end of the gene (61). The resultant *Hae*III–*Eco*RI fragment was blunt-end ligated into *BamH*I 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 LT252 CC-1 clone (40) and the *L. major* HOM/IQ/73/LCR-L32 strain (14) were studied. Cells were maintained at 25°C in M199 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 10^8 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-amidinophenyl)-methane sulfonyl 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 mM EDTA, 1% NP-40). Protein solubilized in the detergent extraction was assayed for GPI-PLC activity using [^{3}H]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 10^6/ml and grown to a density of 10^7/ml (5×10^8 total cells) were harvested by low speed centrifugation, washed in 10 ml of PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 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 (prewarmed to room temperature) to which 500 μl of FBS (dialyzed against methionine-free RPMI 1640) and 100 μl of 1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.4 had been added. Parasites were labeled for 3 h with 250 μCi of [^{35}S]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 μg/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 10^7/ml were washed as described previously, and resuspended at a density of 2×10^7/ml in 5 ml of ethanolamine labeling medium (M199 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% dialyzed FBS (Hyclone). Cells were labeled overnight at 26°C with 100 μCi/ml of [1-^{3}H]ethanolamine hydrochloride (50 Ci/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO). Cells and media were harvested in 1-ml portions (see above).

Glycoinositol phospholipids (GIPLs) were labeled by incubating cells (4×10^7/ml) with [6-^{3}H]galactose (40 Ci/mmol, Amersham) at 50 μCi/ml in glucose-free RPMI (25) supplemented with 10% dialyzed FBS (Hyclone). Cells were labeled with [9,10-^{3}H]myristate (40 Ci/mmol, Amersham) at 100 μCi/ml (17) in serum-free M199 supplemented with 0.5 mg/ml of fatty acid free BSA (Sigma Chem. Co.) and 25 mM Hepes pH 7.4. Label-

ing was performed at 26°C for 16 h. Media and cells were processed as described earlier.

A pellet of 7×10^7 [^{35}S]methionine-labeled cells was lysed by thorough resuspension in 1 ml of ice-cold immunoprecipitation dilution buffer (IDB) (1.25% Triton X-100, 190 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 2×10^7 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 Entensify™ (Dupont), and radiolabeled proteins detected by fluorography with preflashed Hyperfilm-MP (Amersham). ^{14}C-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 *p*-toluidine 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 (10^5) 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], were used as primary antibodies.) Adsorption was allowed for 1 h, and cells were washed in PBS. Fluorescein 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 2×10^7 cells that had been labeled separately with [^{3}H]ethanolamine, [^{3}H]galactose, or [^{3}H]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 freshly mixed ice-cold chloroform/methanol/water (10:10:3) (CMW) (24). CM and CMW extracts were pooled separately, and dried under a stream of nitrogen gas. Dried extracts were partitioned in 500 μl each of water and *n*-butanol. Pooled butanol phases were dried under a stream of nitrogen gas, and resuspended in 20 μl of freshly mixed chloroform/methanol/water (10:10:3). Lipids were resolved by TLC on Silica gel 60 plates using CMW, and detected by fluorography with preflashed Hyperfilm™ (Amersham). Quantitation of the fluorograph was performed on a Computing Densitometer Model 300A (Molecular Dynamics, Sunnyvale, CA).

GPI-PLC Cleavage of GIPLs

GIPLs extracted with CMW (see above) from [^{3}H]galactose or [^{3}H]myristate-labeled pX63NEO/*L. major* cells were dried and carefully resuspended in 1X AB by repeated pipeting (100 μl per 2×10^7 cells, about 25×10^3 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.

Results

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 10^5/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 pGPIPLC/*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.

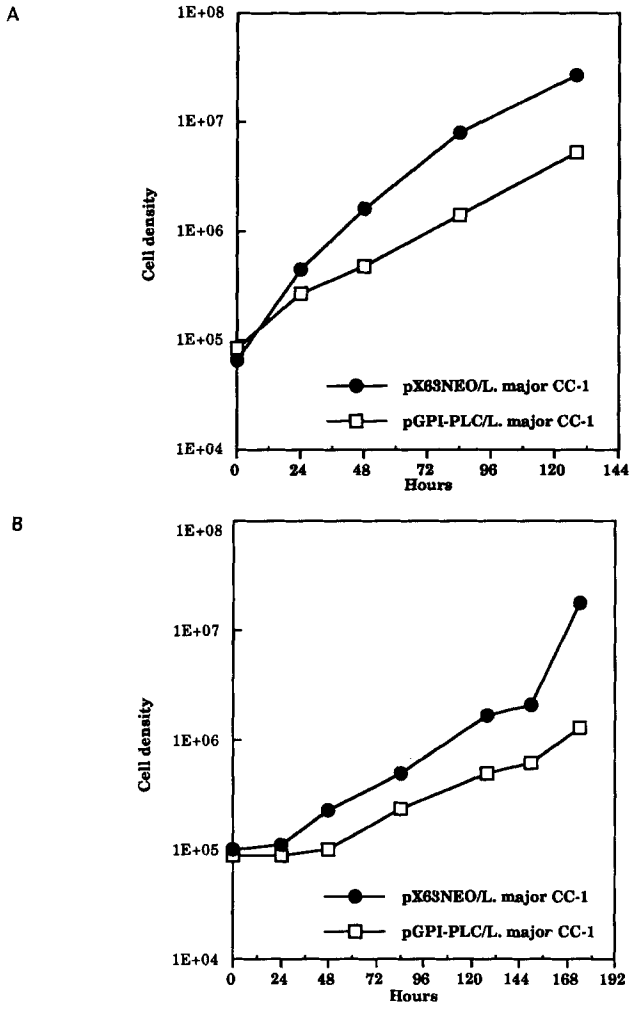

Figure 1. Effect of GPI-PLC on growth of *L. major*. Cells selected on M199 plates containing 7 μg/ml G418 were adapted to grow in different drug concentrations by step-wise increases in drug level. Growth rates were measured at the different G418 concentrations, by seeding cells at the indicated density and monitoring growth at stated intervals.

Next, we determined whether GPI-PLC was soluble or associated with particulate material. Cells were lysed in 1 ml of hypotonic buffer (Materials and Methods) and centrifuged for 15 min at 14,000 *g* (4°C). Over 80% of the GPI-PLC activity present in the total lysate was associated with the pellet from this centrifugation (70 μl vol), and could be extracted with 1X AB (50 mM Tris-HCl, pH 8, 5 mM EDTA, 1% NP-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*.

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

Clone	GPI-PLC Activity Units ($\times 10^{-3}$)/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.

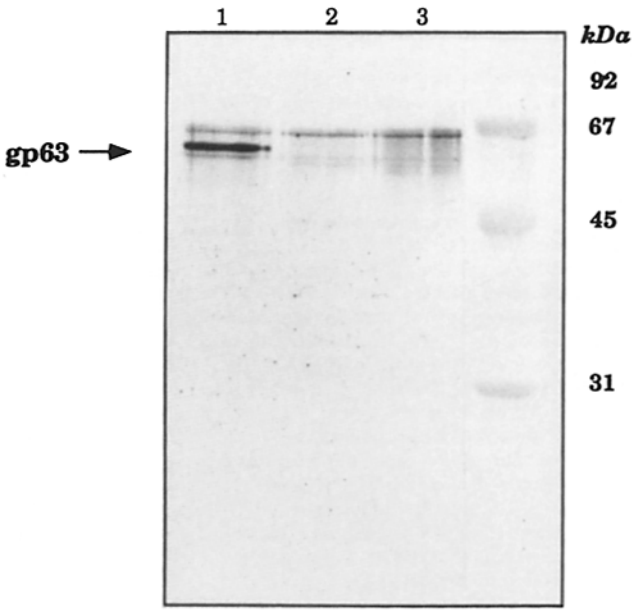

Figure 2. GPI-PLC expression in *L. major* depletes cell-associated gp63. Cells adapted to growth in 32 μg/ml G418 were harvested at a density of 10^7/ml, lysed hypotonically, and a detergent fraction prepared (Materials and Methods). An aliquot (10^7 cell equivalents) was concentrated by organic solvent 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.

Lastly, we tested whether GPI-PLC entered the lumen of the ER. The enzyme was immunoprecipitated from [^{35}S]-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

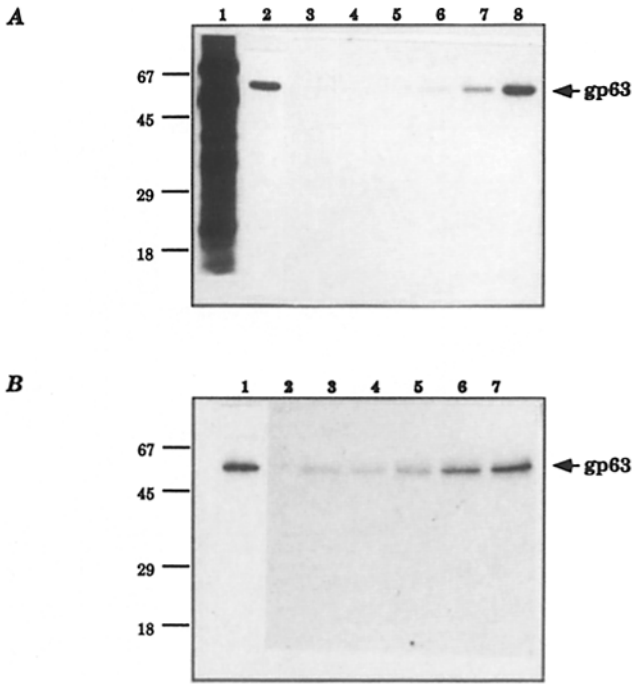

Figure 3. Kinetics of gp63 secretion. Cells grown to a density of 10^7/ml in complete medium were labeled with [^{35}S]methionine and "chased" (Materials and Methods). Cell lysates or media (2×10^7 cell equivalents each) were immunoprecipitated with anti-gp63 polyclonal antibody. [^{35}S]Methionine-labeled 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.

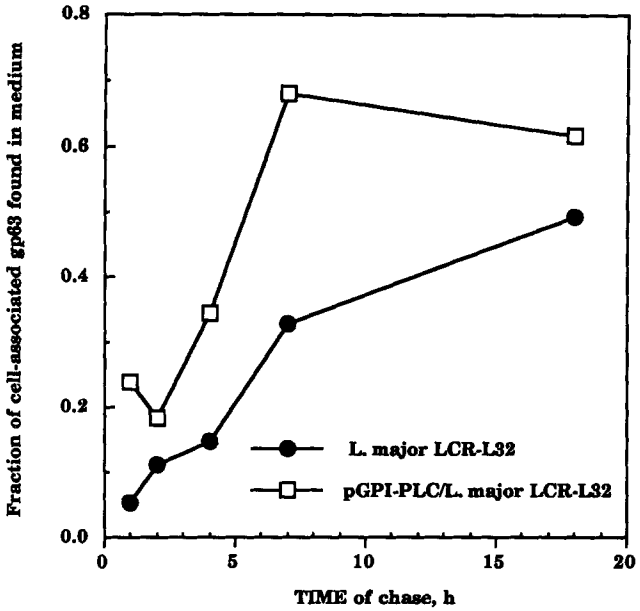

Figure 4. Effect of GPI-PLC on the cell-association half-time of gp63. Gp63 was immunoprecipitated from cells or culture medium at specified times. A 10-μl aliquot of material eluted from the protein A-Sepharose column (Fig. 3) was quantitated by liquid scintillation counting. Amount of labeled gp63 detected in the medium was normalized to the quantity of gp63 immunoprecipitated from cells at time zero (i.e., at initiation of chase, see Fig. 3) to obtain information presented on the y-axis. Data presented is a mean of duplicate determinations. A background of 50 CPM is subtracted. 100% immunoprecipitated material corresponds to 795 CPM for *L. major*, and 528 CPM for pGPI-PLC/*L. major* cells.

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 [^{35}S]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 [^{35}S]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 [^{35}S]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 [^{35}S]methionine-labeled *L. major* cells was immunoprecipitated with polyclonal antibody against either gp63 or against the cross-reacting determinant epitopes of cleaved GPIs (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 [^{35}S]methionine possessed anti-CRD epitopes. Total cell lysates were preincubated at 37°C for 1 h to allow endogenous phospholipases C to cleave [^{35}S]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 [^{35}S]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 [^{35}S]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 [^{3}H]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. Se-

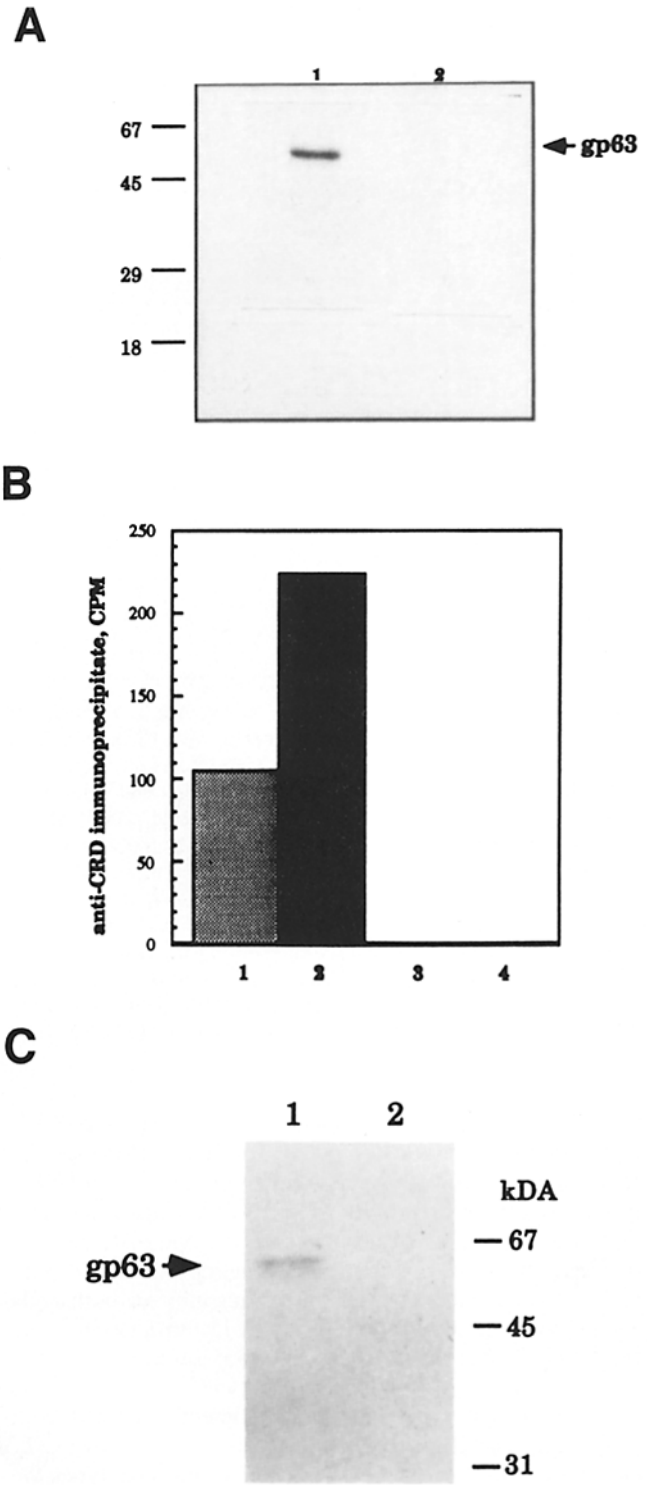

Figure 5. Secreted gp63 never received a GPI anchor. (*A*) Secreted gp63 lacks anti-CRD Epitopes. Medium collected from [^{35}S]methionine-labeled pGPI-PLC/*L. major* cells was immunoprecipitated with polyclonal antibody against either gp63 (lane *1*) or against the cross-reacting determinant (lane *2*). Immune complexes were analyzed as described in Fig. 4 legend. (*B*) [^{35}S]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 GPIs 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 1X 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 [^{3}H]ethanolamine. Cells (pGPIPLC/*L. major*) were labeled with [^{3}H]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 2×10^7 cells; (lane *2*) immunoprecipitate of spent culture medium (from 2×10^7 cells).

creted gp63 could not be metabolically labeled with [^{3}H]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 *L. major* indicated that it also never received a GPI anchor (not shown). Surprisingly, in *Leishmania amazonensis* barely any gp63 is released from wild-type cells (not shown). Thus, in some *Leishmania* species (e.g., *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 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 *L. donovani chagasi* and *L. mexicana* express gp63 transcripts that do not have GPI addition signals (47, 58, 73). If such proteins were present in *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 [^{3}H]ethanolamine-labeled cells (10^7 equivalents) and analyzed by TLC. A putative protein-GPI precursor, LP-1, is diminished in pGPI-PLC/*L. major* cells (Fig. 6 *A*, lane 2), as compared to pX63NEO/*L. major* cells (Fig. 6 *A*, lane *1*). Quantitation of LP-1 indicated that its level was reduced 10-fold in 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 [^{3}H]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 pGPI-PLC/*L. major* cells as compared to the pX63NEO/*L. major* cells. We conclude that GPI-PLC causes a decrease of protein-GPI anchor precursors in 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 *L. major* is LPG, a

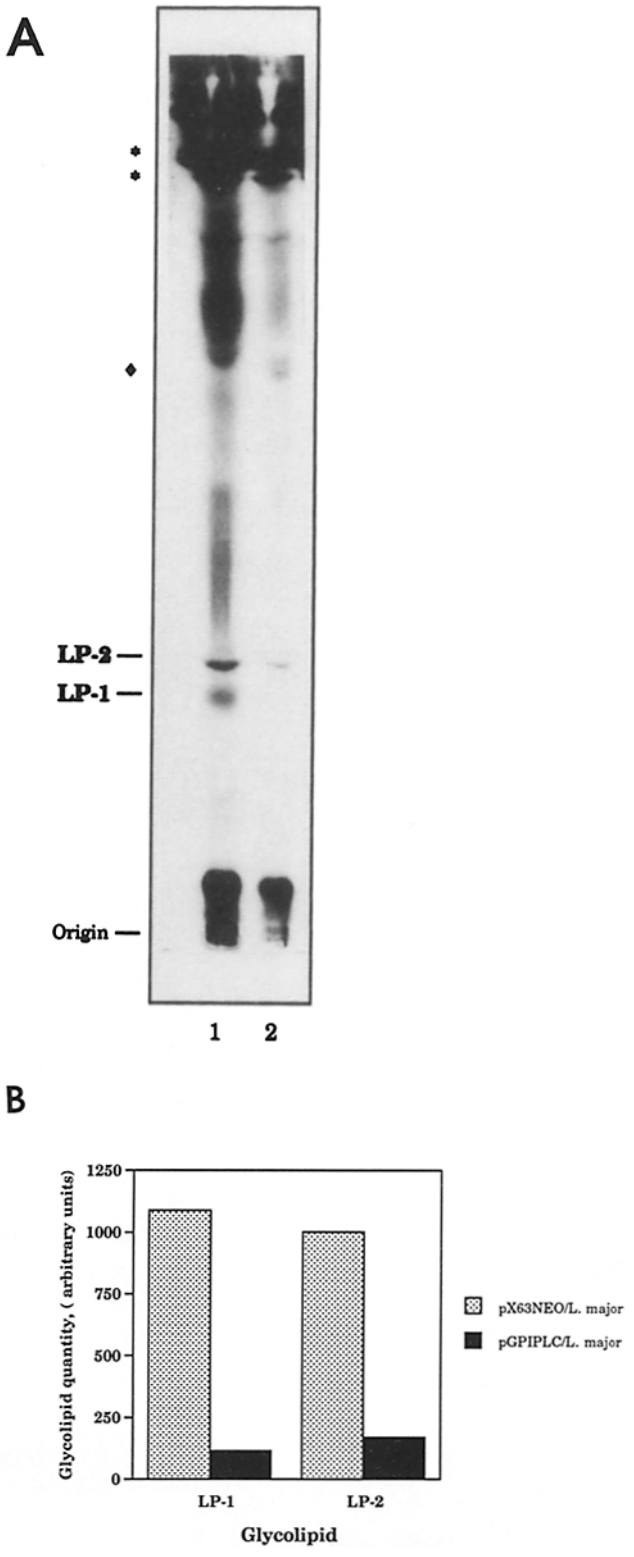

Figure 6. GPI-PLC decreases levels of putative protein-GPI precursors. (*A*) Cells were labeled with [^{3}H]ethanolamine and extracted sequentially with chloroform/methanol (*CM*) and chloroform/methanol/water (*CMW*). CMW extract (10^7 cell equivalents) was analyzed by TLC (silica gel 60) in chloroform/methanol/water (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 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*.

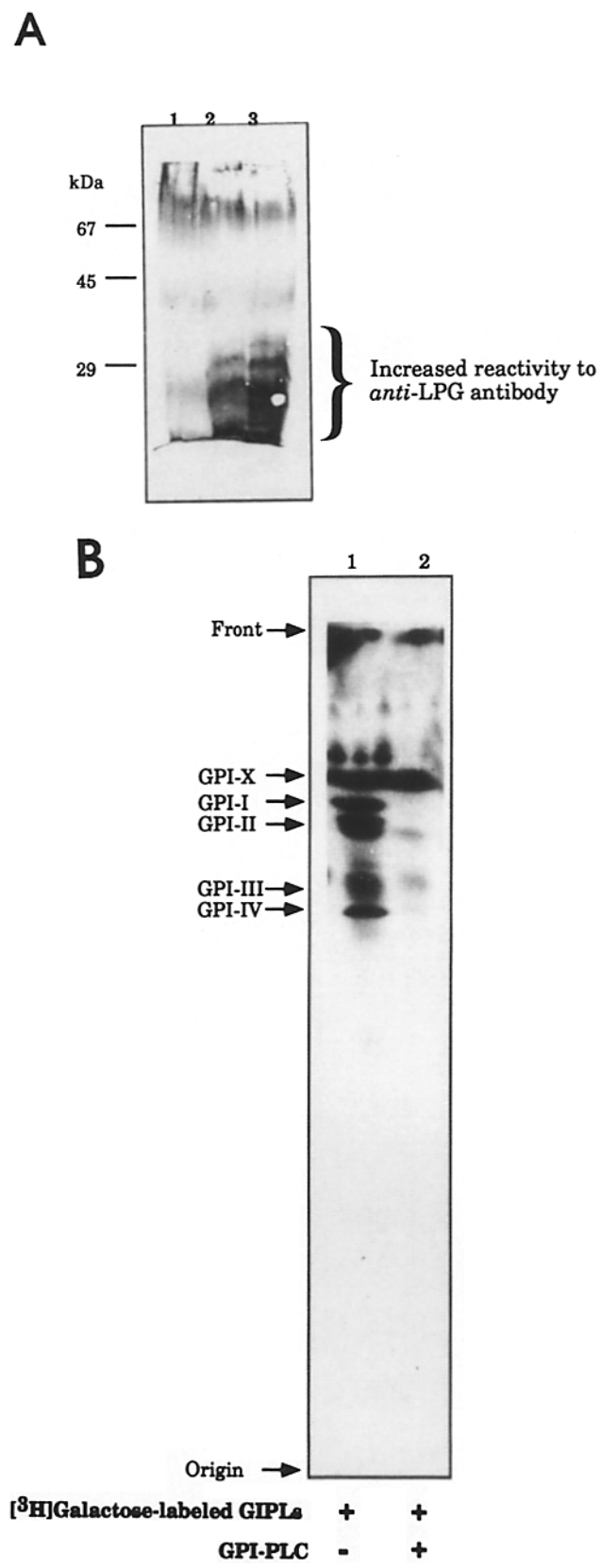

Figure 7. (*A*) Cellular lipophosphoglycan is not decreased by GPI-PLC. Promastigotes adapted to growth in 32 μg/ml G418 were lysed by suspension in Laemmli buffer. An aliquot (10^7 cell equivalents) was resolved by 12% SDS-PAGE, and probed with monoclonal WIC 79.3. (Lane *1*) pX63NEO/*L. major*; (lane *2*) pGPI-PLC/*L. major* clone 4; (lane *3*) pGPI-PLC/*L. major* clone 9. (*B*) GPI-PLC cleaves polysaccharide-GPI intermediates. Glycoinositol phospholipids obtained by chloroform/methanol/water extraction of [^{3}H]galactose-labeled pX63NEO/*L. major* (2×10^7 cells) were dried and resuspended in 100 μl of 1X AB. Recombinant GPI-PLC (200 ng) was either omitted (lane *1*) or added (lane 2). The mixture was incubated at 37°C for 3 h. Butanol-extractable material was analyzed by TLC and fluorography.

GPI-anchored polysaccharide (46). Surprisingly, in *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*). GPI-I, GPI-II, GPI-III, and GPI-IV (presumably GIPL-1, 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 GPI-X (possibly GlcNAc-PI 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

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 *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 cytoplasmically targeted β-hexosaminidase or mannosidase could contribute to analysis of the topography of dolichol-linked oligosaccharide biosynthesis.

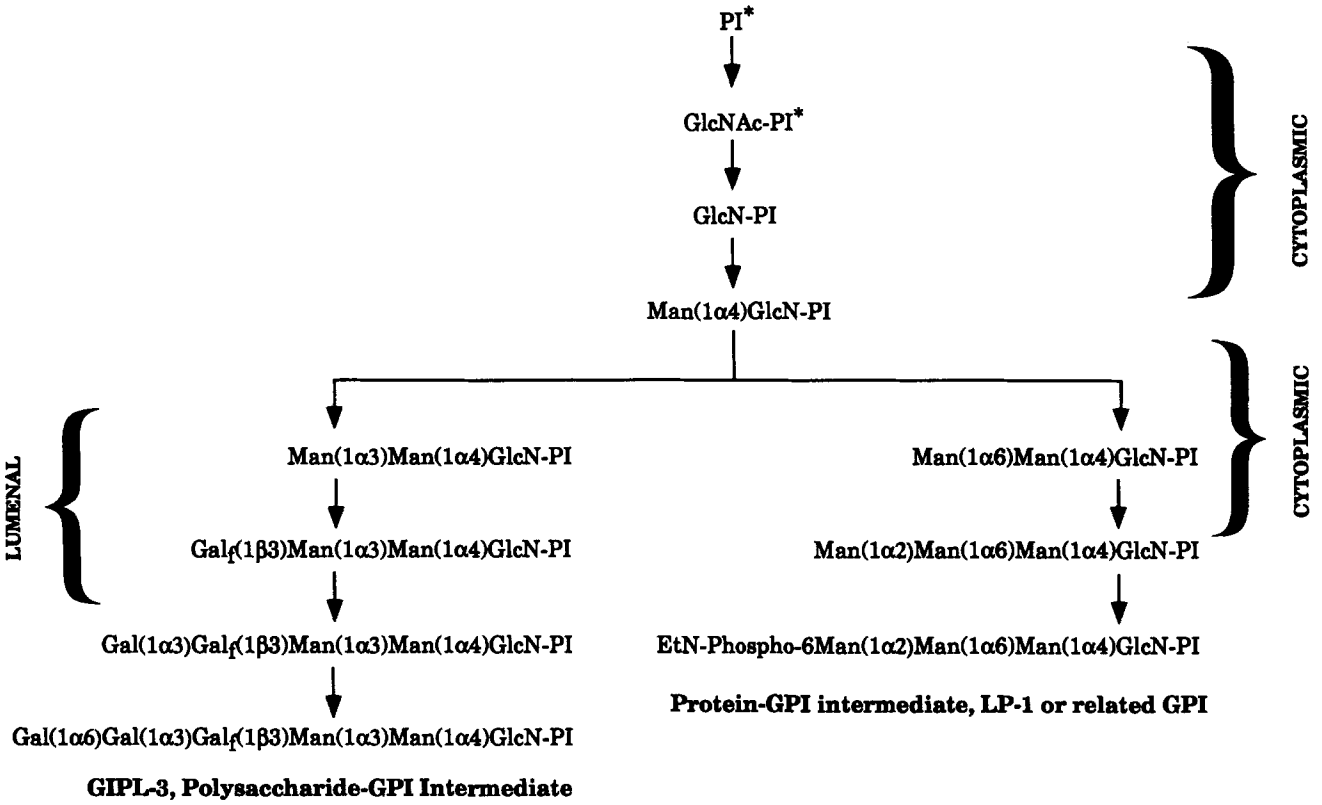

Figure 8. Topography of polysaccharide-GPI and protein-GPI pathways. It is proposed that reactions leading to the synthesis of Man(1α4)GlcN-PI are membrane bound, and occur on the cytoplasmic side of the ER. In addition, the reactions are common to the protein-GPI and polysaccharide-GPI pathways as previously suggested (43). A proportion of Man(1α4)GlcN-PI flips into the ER lumen where it might be mannosylated by an α1,3 transferase; it then enters the polysaccharide-GPI pathway. (The possibility that the complete polysaccharide-GPI pathway is lumenal can also be accommodated by our data, consult text for details.) Man(1α4)GlcN-PI which remains on the cytoplasmic leaflet of the ER might be mannosylated by a cytosolic α1,6 transferase: the product of the latter reaction is committed to the protein-GPI pathway. Consistent with the shortage of LP-1 (Fig. 6 *B*), it is possible that the entire protein-GPI pathway occurs cytoplasmically. Our data provides evidence for the first two mannosylations, at least, of the protein-GPI pathway occurring in the cytosol. *, denotes lipids that are not GPI-PLC substrates in vitro (Fig. 7 *B*, [8, 16, 27, 31, 50]).

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. *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/*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(1α6)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(1α2)-Man(1α6)Man(1α4)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 *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-Man_3-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, *Pig-A*, *GPI-H*, and *Pig-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 PO_4-6Gal(1β4)Man(1α), attached by a phosphosaccharide core, PO_4-6Gal(1α6)Gal(1α3)Gal_f(1β3)Man(1α3)Man(1α4)GlcN, to a *lyso*-1-O-alkylphosphatidylinositol anchor (46, 71). *L. major* also contains copious quantities of glycoinositol phospholipids (GIPLs), GPIs that are not attached to protein or polysaccharide. GIPL-2, Gal(1α3)Gal_f(1β3)Man(1α3)Man(1α4)GlcN-PI, is the most abundant of them. GIPL-3 contains 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 biosynthesis (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 α1,3 configuration for polysaccharide-GPIs, but in the α1,6 configuration for protein-GPIs. If inhibition of GPI metabolism 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 inhibitory effects (Figs. 2 and 6) are limited to the protein-GPI pathway. This specificity exists in spite of the susceptibility of polysaccharide-GPI 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 pathway, is the susceptible precursor. Hence, the first mannosylation 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 surmise that reactions specific to the polysaccharide-GPI pathway 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. However, 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-man 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 potential use as a membrane-impermeable probe of the topography of GPI biosynthesis. First, the enzyme is highly specific for GPIs (10, 27, 31); it cleaves GPI anchors 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 protein 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 secretory 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 cryosections 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 long protein (12, 30, 50); and, (*d*) GPI-PLC (by subcellular fractionation) is not found on the plasma membrane. It is associated with intracellular organelles including nuclear 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 intermediates (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 $\sim 10^7$ 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 activity 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-man 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_3-GlcN-PI, akin to the depletion of LP-1 (EtN-phospho-Man_3-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_3-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.

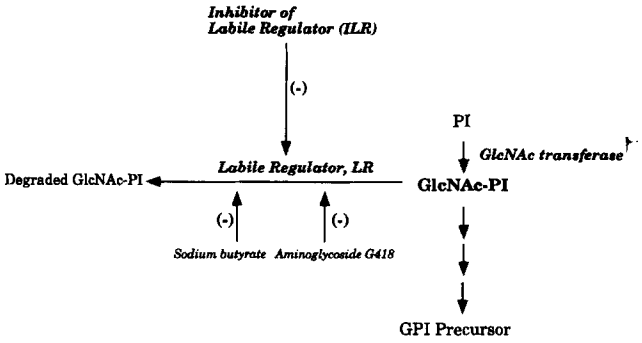

Figure 9. On the control of GPI biosynthesis by a labile regulator (The labile regulator hypothesis). It is assumed, as observed in some GPI-negative murine T-lymphoma cells (70), that all genes for GPI biosynthesis are functional. We propose that GlcNAc-PI is subject to degradation by a factor, the "Labile Regulator" (LR), that is unstable during cell lysis. High activity of LR might prevent detection of GlcNAc-PI. 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 GlcNAc-PI. The possibility that the two layers of regulation are actually exerted on GlcNAc transferase itself, making the LR/ILR complex a regulatory component of the enzyme, is compatible with this hypothesis. We propose that sodium butyrate (70) and G418 (29) cause reexpression of GPI-anchored proteins by either suppressing activity of LR directly, or by stimulating ILR activity.

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

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References

1. Abeijon, C., and C. B. Hirschberg. 1992. Topography of glycosylation reactions in the endoplasmic reticulum. *Trends. Biochem. Sci.* 17:32–36.
2. Armstrong, C., J. Schubert, E. Ueda, J. J. Knez, D. Gelperin, S. Hirose, R. Silber, S. Hollan, R. E. Schmidt, and M. E. Medof. 1992. Affected paroxysmal nocturnal hemoglobinuria T lymphocytes harbor a common defect in assembly of N-acetyl-D-glucosamine inositol phospholipid corresponding to that in class A Thy-1- murine lymphoma mutants. *J. Biol. Chem.* 267:25347–25351.
3. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1988. Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York. 10.16.6–10.16.7.
4. Bangs, J. D., D. Hereld, J. L. Krakow, G. W. Hart, and P. T. Englund. 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. *Proc. Natl. Acad. Sci. USA.* 82:3207–3211.
5. Bangs, J. D., L. Uyetake, M. J. Brickman, A. E. Balber, and J. C. Boothroyd. 1993. Molecular cloning and cellular localization of a BiP homologue in Trypanosoma brucei. Divergent ER retention signals in a lower eukaryote. *J. Cell Sci.* 105:1101–1113.
6. Bordier, C., R. J. Etges, M. J. Turner, and M. L. Cardoso de Almeida. 1986. *Leishmania* and *Trypanosoma* surface glycoproteins have a common glycophospholipid membrane anchor. *Proc. Natl. Acad. Sci. USA.* 83:5988–5991.
7. Broomfield, S. J., and N. M. Hooper. 1993. Characterization of an antibody to the cross-reacting determinant of the glycosyl-phosphatidylinositol anchor of human membrane dipeptidase. *Biochim. Biophys. Acta.* 1145:212–218.
8. Bulow, R., and P. Overath. 1986. Purification and characterization of the membrane-form variant surface glycoprotein hydrolase of *Trypanosoma brucei. J. Biol. Chem.* 261:11918–11923.
9. Bulow, R., G. Griffiths, P. Webster, Y.-D. Stierhof, F. R. Opperdoes, and P. Overath. 1989. Intracellular localization of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei. J. Cell. Sci.* 93:233–240.
10. Bulow, R., C. Nonnengasser, and P. Overath. 1989. Release of the variant surface glycoprotein during differentiation of bloodstream to procyclic forms of *Trypanosoma brucei. Mol. Biochem. Parasitol.* 32:85–92.
11. Caras, I. W., G. N. Weddell, M. A. Davitz, V. Nussenzweig, and D. W. Martin, Jr. 1987. Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. *Science (Wash. DC).* 238:1280–1283.
12. Cardoso de Almeida, M. L., and M. J. Turner. 1983. The membrane form of variant surface glycoproteins of *Trypanosoma brucei. Nature (Lond.).* 302:349–352.
13. Chapman, A., K. Fujimoto, and S. Kornfeld. 1980. The primary glycosylation defect in class E Thy-1-negative mutant mouse lymphoma cells is an ability to synthesize dolichol-P-mannose. *J. Biol. Chem.* 255:4441–4446.
14. Chaudhuri, G., M. Chaudhuri, A. Pan, and K. P. Chang. 1989. Surface acid proteinase (gp63) of *Leishmania mexicana. J. Biol. Chem.* 264: 7483–7489.
15. Davitz, M. A., M. G. Low, and V. Nussenzweig. 1986. Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C. *J. Exp. Med.* 163:1150–1161.
16. Doering, T. L., W. J. Masterson, P. T. Englund, and G. W. Hart. 1989. Biosynthesis of the glycosyl phosphatidylinositol membrane anchor of the trypanosome variant surface glycoprotein. Origin of the non-acetylated glucosamine. *J. Biol. Chem.* 264:11168–11173.
17. Doering, T. L., J. Raper, L. U. Buxbaum, G. W. Hart, and P. T. Englund. 1990. Biosynthesis of glycosyl phosphatidylinositol protein anchors. *Methods: A Companion to Methods in Enzymology.* 1:288–296.
18. Elhay, M. J., M. J. McConville, J. M. Curtis, A. Bacic, and E. Handman. 1993. Identification of truncated forms of lipophosphoglycan in mutant cloned lines of *Leishmania major* that are deficient in mature lipophosphoglycan. *Parasitol. Res.* 79:435–438.
19. Englund, P. T. 1993. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annu. Rev. Biochem.* 62:121–138.
20. Etges, R., J. Bouvier, and C. Bordier. 1986. The major surface protein of *Leishmania* promastigotes is anchored in the membrane by a myristic acid-labeled phospholipid. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:597–601.
21. Fatemi, S. H., and A. M. Tartakoff. 1986. Hydrophilic anchor-deficient Thy-1 is secreted by a class E mutant T lymphoma. *Cell.* 46:4441–4446.
22. Ferguson, M. A. J. 1992. Lipid anchors on membrane proteins. *Curr. Opin. Struct. Biol.* 1:522–529.
23. Ferguson, M. A. J., S. W. Homans, R. A. Dwek, and T. W. Rademacher. 1988. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. *Science (Wash. DC).* 239:753–759.
24. Field, M. C., and A. K. Menon. 1991. Biosynthesis of glycosylphosphatidylinositol membrane protein anchors. *In* Lipid Modification of Proteins. N. M. Hooper, and A. J. Turner, editors. IRL Press, Oxford. 155–190.
25. Field, M. C., E. Medina Acosta, and G. A. Cross. 1991. Characterization of a glycosylphosphatidylinositol membrane protein anchor precursor in *Leishmania mexicana. Mol. Biochem. Parasitol.* 48:227–229.
26. Field, M. C., E. Medina-Acosta, and G. A. M. Cross. 1993. Inhibition of glycosylphosphatidylinositol biosynthesis in *Leishmania mexicana* by mannosamine. *J. Biol. Chem.* 268:9570–9577.
27. Fox, J. A., M. Duszenko, M. A. Ferguson, M. G. Low, and G. A. M. Cross. 1986. Purification and characterization of a novel glycan-phosphatidylinositol-specific phospholipase C from *Trypanosoma brucei. J. Biol. Chem.* 261:15767–15771.
28. Grab, D. J., P. Webster, S. Ito, W. R. Fish, Y. Verjee, and J. D. Lonsdale-Eccles. 1987. Subcellular localization of a variable surface glycoprotein phosphatidylinositol-specific phospholipase C in African trypanosomes. *J. Cell Biol.* 105:737–746.
29. Gupta, D., A. Tartakoff, and E. Tisdale. 1988. Metabolic correction of defects in the lipid anchoring of Thy-1 in lymphoma mutants. *Science (Wash. DC).* 242:1446–1448.
30. Hereld, D., G. W. Hart, and P. T. Englund. 1988. cDNA encoding the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei. Proc. Natl. Acd. Sci. USA.* 85:8914–8918.
31. Hereld, D., J. L. Krakow, J. D. Bangs, G. W. Hart, and P. T. Englund. 1986. A phospholipase C from *Trypanosoma brucei* which selectively cleaves the glycolipid on the variant surface glycoprotein. *J. Biol. Chem.* 261:13813–13819.
32. Hillmen, P., M. Bessler, P. J. Mason, W. M. Watkins, and L. Luzzatto.

1993. Specific defect in N-acetylglucosamine incorporation in the biosynthesis of the glycosylphosphatidylinositol anchor in cloned cell lines from patients with paroxysmal nocturnal hemoglobinuria. *Proc. Natl. Acad. Sci. USA.* 90:5272–5276.

33. Inoue, N., T. Kinoshita, T. Orii, and J. Takeda. 1993. Cloning of a human gene, Pig-F, a component of glycosyl-phosphatidylinositol anchor biosynthesis, by a novel expression cloning strategy. *J. Biol. Chem.* 268: 6882–6885.
34. Kamitani, T., H. M. Chang, C. Rollins, G. L. Waneck, and E. T. H. Yeh. 1993. Correction of the class H defect in glycosylphosphatidylinositol anchor biosynthesis in Ltk$^-$ cells by a human cDNA clone. *J. Biol. Chem.* 268:20733–20736.
35. Kamitani, T., A. K. Menon, Y. Hallaq, C. D. Warren, and E. T. Yeh. 1992. Complexity of ethanolamine phosphate addition in the biosynthesis of glycosylphosphatidylinositol anchors in mammalian cells. *J. Biol. Chem.* 267:24611–24619.
36. Kapler, G. M., C. M. Coburn, and S. M. Beverley. 1990. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. *Mol. Cell. Biol.* 10:1084–1094.
37. Kardami, E., T. W. Pearson, R. P. Beecroft, and R. R. Fandrich. 1992. Identification of basic fibroblast factor-like proteins in African trypanosomes and *Leishmania. Mol. Biochem. Parasitol.* 51:171–182.
38. Kelleher, M., A. Bacic, and E. Handman. 1992. Identification of a macrophage-binding determinant on lipophosphoglycan from *Leishmania major* promastigotes. *Proc. Natl. Acad. Sci. USA.* 89:6–10.
39. Krakow, J. L., D. Hereld, J. D. Bangs, G. W. Hart, and P. T. Englund. 1986. Identification of a glycolipid precursor of the *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 261:12147–12153.
40. LeBowitz, J. H., H. Q. Smith, L. Rusche, and S. M. Beverley. 1993. Coupling of poly(A) site selection and trans-splicing in *Leishmania. Genes Dev.* 7:996–1007.
41. Lee, H. C., R. Shoda, J. A. Krall, J. D. Foster, J. Selhub, and T. L. Rosenberry. 1992. Folate binding protein from kidney brush border membranes contains components characteristic of a glycoinositol phospholipid anchor. *Biochemistry.* 31:3236–3243.
42. Masterson, W. J., T. L. Doering, G. W. Hart, and P. T. Englund. 1989. A novel pathway for glycan assembly: biosynthesis of the glycosylphosphatidylinositol anchor of the trypanosome variant surface glycoprotein. *Cell.* 56:793–800.
43. McConville, M. J. 1991. Glycosylated phosphatidylinositols of the trypanosomatidae. *In* Biochemical Protozoology. G. Coombs and M. North, editors. Taylor and Francis, Washington, D.C. 286–303.
44. McConville, M. J., and A. Bacic. 1990. The glycoinositolphospholipid profiles of two *Leishmania major* strains that differ in lipophosphoglycan expression. *Mol. Biochem. Parasitol.* 38:57–67.
45. McConville, M. J., S. W. Homans, J. E. Thomas Oates, A. Dell, and A. Bacic. 1990. Structures of the glycoinositolphospholipids from *Leishmania major.* A family of novel galactofuranose-containing glycolipids. *J. Biol. Chem.* 265:7385–7394.
46. McConville, M. J., J. E. Thomas Oates, M. A. Ferguson, and S. W. Homans. 1990. Structure of the lipophosphoglycan from *Leishmania major. J. Biol. Chem.* 265:19611–19623.
47. Medina-Acosta, E., R. E. Karess, and D. G. Russell. 1993. Structurally distinct genes for the surface protease of *Leishmania mexicana* are developmentally regulated. *Mol. Biochem. Parasitol.* 57:31–46.
48. Medof, M. E., E. I. Walter, W. L. Roberts, R. Haas, and T. L. Rosenberry. 1986. Decay accelerating factor of complement is anchored to cells by a C-terminal glycolipid. *Biochemistry.* 25:6740–6747.
49. Menon, A. K., S. Mayor, M. A. Ferguson, M. Duszenko, and G. A. M. Cross. 1988. Candidate glycophospholipid precursor for the glycosylphosphatidylinositol membrane anchor of *Trypanosoma brucei* variant surface glycoproteins. *J. Biol. Chem.* 263:1970–1977.
50. Mensa-Wilmot, K., and P. T. Englund. 1992. Glycosyl phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*: expression in *Escherichia coli. Mol. Biochem. Parasitol.* 56:311–322.
51. Mensa-Wilmot, K., D. Hereld, and P. T. Englund. 1990. Genomic organization, chromosomal localization, and developmentally regulated expression of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei. Mol. Cell. Biol.* 10:720–726.
52. Miyata, T., J. Takeda, Y. Lida, N. Yamada, N. Inoue, M. Takahashi, K. Maeda, T. Kitani, and T. Kinoshita. 1993. The cloning of Pig-A, a component in the early step of GPI-anchor biosynthesis. *Science (Wash. DC).* 259:1318–1320.
53. Mooser, G. 1992. Glycosidases and Glycosyltransferases. *In* The Enzymes XX. P. Boyer, editor. Academic Press, New York. 187–233.
54. Muller, G., K. Schubert, F. Fiedler, and W. Bandlow. 1992. The cAMP-binding ectoprotein from *Saccharomyces cerevisiae* is membrane-anchored by glycosyl-phosphatidylinositol. *J. Biol. Chem.* 267:25337–25346.
55. Murray, P. J., T. W. Spithill, and E. Handman. 1989. The PSA-2 glycoprotein complex of *Leishmania major* is a glycosylphosphatidylinositol-linked promastigote surface antigen. *J. Immunol.* 143:4221–4226.
56. Nakakuma, H., S. Nagakura, T. Kawaguchi, K. Horikawa, T. Kagimoto, M. Kawakita, M. Tomita, and K. Takatsuki. 1992. Increased plasma decay-accelerating factor levels in paroxysmal nocturnal hemoglobinuria. *Int. J. Hematol.* 55:121–125.
57. Orlean, P. 1992. Enzymes that recognize dolichols participate in three glycosylation pathways and are required for protein secretion. *Biochem. Cell Biol.* 70:438–447.
58. Ramamoorthy, R., J. E. Donelson, K. E. Paetz, M. Maybodi, S. C. Roberts, and M. E. Wilson. 1992. Three distinct RNAs for the surface protease gp63 are differentially expressed during development of *Leishmania donovani chagasi* promastigotes to an infectious form. *J. Biol. Chem.* 267:1888–1895.
59. Rivas, L., L. Kahl, K. Manson, and D. McMahon-Pratt. 1991. Biochemical characterization of the protective membrane glycoprotein GP46/M-2 of *Leishmania amazonensis. Mol. Biochem. Parasitol.* 47:235–243.
60. Rosse, W. F. 1992. Paroxysmal nocturnal hemoglobinuria. *Curr. Top. Microbiol. Immunol.* 178:163–173.
61. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 162–171.
62. Schneider, P., M. A. J. Ferguson, M. J. McConville, A. Mehlert, S. W. Homans, and C. Bordier. 1990. Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease. *J. Biol. Chem.* 265:16955–16964.
63. Schneider, P., J. Rosat, J. Bouvier, J. Louis, and C. Bordier. 1992. *Leishmania major*: differential regulation of the surface metalloprotease in amastigote and promastigote stages. *Exp. Parasitol.* 75:196–206.
64. Stevens, V., and C. R. H. Raetz. 1991. Defective glycosyl phosphatidylinositol biosynthesis in extracts of three *thy-1* negative lymphoma cell mutants. *J. Biol. Chem.* 266:10039–10042.
65. Svensson, B., and M. Sogaard. 1993. Mutational analysis of glycosylase function. *J. Biotech.* 29:1–37.
66. Takahashi, M., J. Takeda, S. Hirose, R. Hyman, N. Inoue, T. Miyata, E. Ueda, T. Kitani, M. E. Medof, and T. Kinoshita. 1993. Deficient biosynthesis of N-acetylglucosaminyl-phosphatidylinositol, the first intermediate of glycosyl phosphatidylinositol anchor biosynthesis, in cell lines established from patients with paroxysmal nocturnal hemoglobinuria. *J. Exp. Med.* 177:517–521.
67. Takami, N., K. Oda, and Y. Ikehara. 1992. Aberrant processing of alkaline phosphatase precursor caused by blocking the synthesis of glycosylphosphatidylinositol. *J. Biol. Chem.* 267:1042–1047.
68. Takeda, J., T. Miyata, K. Kawagoe, Y. Iida, Y. Endo, T. Fujita, M. Takahashi, T. Kitani, and T. Kinoshita. 1993. Deficiency of the GPI anchor caused by a somatic mutation of the *Pig-A* gene in paroxysmal nocturnal hemoglobinuria. *Cell.* 73:703–711.
69. Tartakoff, A. M., and N. Singh. 1992. How to make a glycoinositol phospholipid anchor. *Trends. Biochem. Sci.* 17:470–473.
70. Tisdale, E. J., J. C. Schimenti, and A. M. Tartakoff. 1991. Sodium butyrate causes reexpression of three membrane proteins on glycolipid-anchoring mutants. *Somat. Cell Mol. Genet.* 17:349–357.
71. Turco, S. J., and A. Descoteaux. 1992. The lipophosphoglycan of *Leishmania* parasites. *Annu. Rev. Microbiol.* 46:65–94.
72. Vidugiriene, J., and A. K. Menon. 1993. Early lipid intermediates in glycosyl-phosphatidylinositol anchor assembly are synthesized in the ER and located in the cytoplasmic leaflet of the ER membrane bilayer. *J. Cell Biol.* 121:987–996.
73. Webb, J. R., L. L. Button, and W. R. McMaster. 1991. Heterogeneity of the genes encoding the major surface glycoprotein of *Leishmania donovani. Mol. Biochem. Parasitol.* 48:173–184.
74. Wessel, D., and U. I. Flugge. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* 138:141–143.
75. Zamze, S. E., M. A. J. Ferguson, R. Collins, R. A. Dwek, and T. W. Rademacher. 1988. Characterization of the cross-reacting determinant (CRD) of the glycosyl-phosphatidylinositol membrane anchor of *Trypanosoma brucei* variant surface glycoprotein. *Eur. J. Biochem.* 176: 527–534.