KIMBERLY DEAN BUTLER
Control of Glycosylphosphatidylinositol Metabolism by a Phospholipase C
(Under the Direction of KOJO MENSA-WILMOT)

Glycosylphosphatidylinositol (GPI) cleavage by a GPI phospholipase C (GPI-PLCp) is suppressed in *Trypanosoma brucei*. However, heterologous expression of GPI-PLCp in *Leishmania* results in a GPI deficiency, indicating an absence of regulation of the phospholipase activity against GPIs.

The purpose of this study was to determine possible mechanisms governing regulation of GPI-PLCp activity against GPIs *in vivo* using *Leishmania* as a model system. We identified several criteria that affect the ability of GPI-PLCp to cleave GPIs *in vivo*: i) Cys80 or ii) at least two Cys within a C-terminal cluster (positions 269,270, 273) must be present. A specific intracellular location and activity of GPI-PLCp were needed for the enzyme to cause the GPI-negative phenotype.

GPI-PLCp is an integral membrane protein. The affinity of the enzyme for biological membranes allows it to gain access to GPIs. Therefore, it is important to understand the mechanism by which GPI-PLCp binds to membranes. We report the presence of two regions capable of post-translationally targeting a soluble reporter to *T. brucei* membranes. We propose a model in which GPI-PLCp is anchored in the membrane by “hydrophobic patches” created by the amphipathic helices in the protein.

INDEX WORDS: GPI-PLC, *Trypanosoma brucei*, *Leishmania*, phenotype, integral membrane protein, post-translationally, hydrophobic, amphipathic, α-helix.

CONTROL OF GLYCOSYLPHOSPHATIDYLINOSITOL METABOLISM IN TRYPANOSOMES BY A PHOSPHOLIPASE C

by

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DEDICATION

I dedicate this thesis to my family, who have supported me, unconditionally, in every aspect of my life. I will love you always.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

TRYPANOSOMA BRUCEI

The protozoan *Trypanosoma brucei* causes African trypanosomiasis (sleeping sickness) in humans and *nagana* in cattle. Found in sub-Saharan Africa, the parasite is transmitted between vertebrate hosts by the tsetse fly. Three sub-species of *Trypanosoma brucei* exist; *T. brucei rhodesiense*, *T. brucei gambiense* (both infective to humans) and *T. brucei brucei* (infects cattle).

An inflammatory lesion known as the trypanosomal chancre is formed at the site where an infected tsetse fly bites a human and the lesion becomes the initial site of parasite division. From here, parasites spread into the lymphatic system and blood stream causing hemolymphatic trypanosomiasis. This acute stage of the disease is associated with fever, headache, joint and muscle pain, and a rash. Parasites can be found in the blood, lymphs, and bone marrow during this first stage of the infection. The chronic form of sleeping sickness is marked by invasion of the central nervous system (CNS), and can be diagnosed by presence of parasites in the spinal fluid. During this period, headaches and fever become more pronounced, accompanied by severe impairment of CNS function (i.e. mental retardation, lethargy, low and tremulous speech, and altered reflexes) [49].

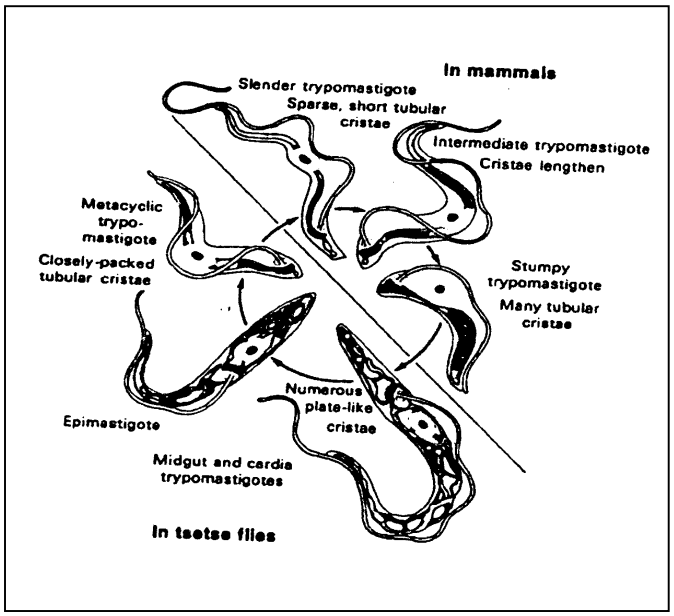
Of the two sub-species which infect humans, *T.b. rhodesiense* is the more virulent, resulting in faster progression of the disease. A *T.b. gambiense* infection is more drawn out and can take up to two years to run its course. Treatment for this disease is by

chemotherapy (melarsoprol or tryparsamide). Drug cytotoxicity to patients aside, resistant parasite strains are becoming more prevalent, making treatment of this disease increasingly difficult [49].

Life Cycle

T. brucei alternate between two distinct stages: i) blood stream forms (BSF) found in the mammalian circulatory system, and ii) procyclics which reside in the tsetse fly (Figure 1) [58]. BSF parasites take up glucose from the mammalian blood stream and derive all their ATP from glycolysis without using the Kreb's cycle [6]. In contrast, upon differentiation to procyclics in the insect vector, mitochondrial function is restored. Another difference between BSF and procyclics is the protein coat on the extracellular surface of the plasma membrane. BSF express a variant surface glycoprotein (VSG) [11], whereas procyclic acidic repetitive protein (PARP) covers procyclics [53]. Each of these proteins is vital to the survival of each developmental stage of the parasite in their respective environments and will be discussed in greater detail later.

Figure 1: *T. brucei* life cycle [58].



When a tsetse fly takes a blood meal from an infected vertebrate, the BSF differentiate into procyclic trypomastigotes in the midgut of the insect (Figure 1). The trypomastigotes develop into epimastigotes, which migrate to the salivary glands where they transform into infective metacyclic trypomastigotes. At the next blood meal, a tsetse fly transmits metacyclic parasites into the bloodstream of a mammalian host, where they differentiate into BSF trypomastigotes. After a series of rapid cell divisions, a percentage of these parasites become a non-dividing form, termed the "short stumpies," which are pre-adapted to differentiate into procyclics in an invertebrate host [49].

Antigenic Variation

In order to maintain an infection in a vertebrate, *T. brucei* must escape and survive the host immune (defense) system. This task is accomplished by a process known as "antigenic variation" [12]. The plasma membrane of BSF *T. brucei* is covered with 10^7 molecules of a variant surface glycoprotein (VSG) [11]. Each parasite is estimated to possess over 1000 VSG genes [68] encoding antigenically distinct proteins that are expressed one at a time. Naturally, the host mounts an immune response against the dominant VSG, and eliminates the parasites expressing this protein. However, a small percentage spontaneously switch their VSG coat. This antigenically distinct population survives antibody-dependent parasite killing, and are expanded within the mammalian host (reviewed in [13, 50]). This repeated fluctuation in peak parasitemia is characteristic of *T. brucei* infections.

Glycosylphosphatidylinositol (GPI) Anchors: Structures and Function

Trypanosomes are great over-producers of glycosylphosphatidylinositols (GPIs), which make them a good system in which to study these glycolipids. The structure of the

VSG GPI was the first to be solved (Figure 2A) [22]. This glycolipid consists of three general regions including an evolutionarily conserved protein-GPI 'core', phosphoethanolamine-mannoseα1-2mannoseα1-6mannoseα1-4glucosamineα1-6myo-inositol-phospho (EtN-*P* -Manα1-2Manα1-6Manα1-4GlcNα1-6myo-Ins-*P*). The core is decorated with galactose (Gal) residues in either α1-2, α1-3, or α1-6 linkages to the first mannose from the reducing end [22]. Finally, unique to VSG, the fatty acids on the phosphatidylinositol (PI) moiety of the GPI are myristate [22] (Figure 2A).

The GPI structure for the procyclic acidic repetitive protein (PARP) (Figure 2B), the major surface protein on the insect form of *T. brucei*, is different from that of the VSG GPI. The PARP GPI core is substituted with a complex side chain consisting of N-acetylglucosamine (GlcNAc), galactose (Gal), and sialic acid (NANA) ($NANA_4$-$GlcNAc_7$-Gal_7) [21]. Additionally, the PARP GPI contains a *lyso*-1-O-stearoylglycerol moiety (Figure 2B). Finally, the C2 or C3 position of the Ins ring is substituted with palmitate or stearate [23].

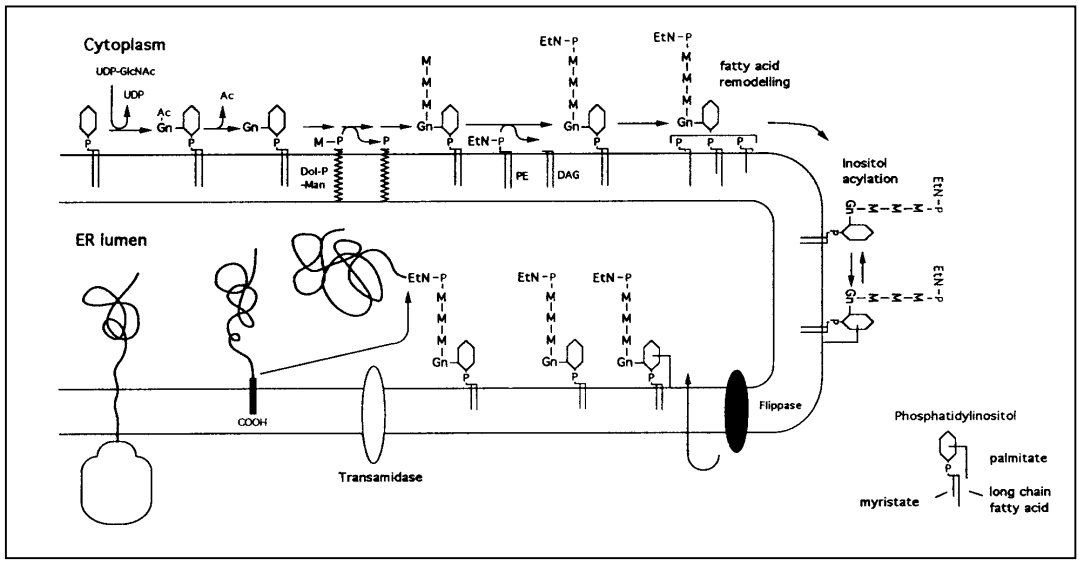
In addition to the protein linked GPIs (Type I), there are two other types of GPIs found in trypanosomatids. Polysaccharide-linked or Type II GPIs (e.g. the GPI of lipophosphoglycan (LPG) in *Leishmania*) are based on Manα1-3Manα1-4GlcNα1-6Ins-1-phospho-glycerolipid (reviewed in [20]). Type III GPIs (e.g. glycoinositolphospholipids (GIPLs)), also known as free GPIs, can be based on a Manα1-6(Manα1-3)Manα1-4GlcNα motif attached to either alkylacyl-PI or *lyso*alkyl-PI [35]. GPIs function in such diverse processes as membrane attachment (i.e. VSG [22] and PARP [65] of *T. brucei*), developmental regulation of vector-host interactions (as with LPG-dependent *Leishmania*-sand fly interactions [36]), growth of parasites within

mammalian cells (i.e. GIPLs of *Leishmania* [42]), and protein targeting to cellular microdomains such as caveolae [5, 52].

Biosynthesis of GPIs

Cellular synthesis of GPIs (Figure 3) begins on the cytosolic face of the endoplasmic reticulum (ER) (reviewed in [20, 47]) with the addition of GlcNAc, from UDP-GlcNAc, to PI [16]. GlcNAc-PI formed from this reaction is deacetylated to GlcN-PI [16]. The next steps are the sequential addition of three Man residues from dolichol-phosphoryl mannose (Dol-P-Man) yielding Man_3-GlcN-P [40, 41, 41], to which a phosphoethanolamine (*P*-EtN) is donated from phosphatidylethanolamine (PE) [39]. The

Figure 3: GPI biosynthesis [34].



resulting GPI, known as glycolipid A' (EtN-*P*-Man_3-GlcN-PI), undergoes a series of "fatty acid remodeling reactions" in which the longer chain fatty acids of PI (usually stearate and arachidonate [64]) are exchanged for myristate, yielding glycolipid A [35]. Glycolipid C, an inositol-palmitoylated version of glycolipid A [29], is also formed

during GPI biosynthesis in *T. brucei*, although its precise function has yet to be determined.

Two signals within the polypeptide sequence are required in order for a protein to acquire a GPI anchor. The first is an N-terminal signal peptide targeting the nascent polypeptide to the endoplasmic reticulum for translocation into the ER lumen [72]. The C-terminus of the signal peptide contains a second signal (Figure 4) (reviewed in [63]) for addition of the GPI in a transamidation reaction within the lumen of the ER [19, 67]. This GPI addition signal sequence is composed of three regions: i) a C-terminal hydrophobic region; ii) a hydrophilic spacer; and iii) a cleavage/GPI addition (ω) site located N-terminal to the hydrophilic spacer [67].

Figure 4: C-terminal GPI addition signal [46].

Construction of GPI anchors occurs at the endoplasmic reticulum (ER) [20] and has been shown to begin on the cytosolic leaflet [69]. GPI addition to protein is a post-translational event that takes place inside the ER [69], since synthesis of GPI anchored proteins is completed in the ER lumen. This topology dictates that at some point in the biosynthetic pathway one of the GPI intermediates must be "flipped" into the ER lumen from the cytoplasmic leaflet for the transamidation reaction.

GPI-anchored proteins are vital, both to procyclic forms, which express GPI-linked procyclin (PARP), and to bloodstream *T. brucei*, which express GPI-anchored VSG. Other proteins are GPI anchored as well (e.g. transferin receptor [55]). Disruption

of the pathway could be deleterious to the survival of the parasite in the insect vector as well as the mammalian host [34]. Therefore, GPI biosynthesis may represent an avenue for therapeutic drug design [34].

GPI-specific Phospholipase C (GPI-PLCP)

T. brucei express a GPI-specific phospholipase C (GPI-PLCp) [9, 30], which is apparently localized to the cytoplasmic leaflet of intracellular membranes [8]. GPI-PLCp can cleave protein-linked GPIs and the various biosynthetic intermediates *in vitro* [45]. However, in *T. brucei* significant cleavage is not detectable, evidenced by the fact GPIs are successfully added to VSG. Therefore, GPI-PLCp activity against GPIs appears to be suppressed in *T. brucei*.

There are several lines of evidence that support the hypothesis that GPI-PLCp activity against GPIs is limited in *T. brucei*. First of all, GPI-PLCp is localized to the cytosolic face of intracellular membranes [8], where GPI intermediates are surmised to be [69]. Despite this apparent co-localization with its putative substrates, GPI-PLCp does not cause a GPI deficiency in *T. brucei*. Secondly, heterologous expression of GPI-PLCp in two distantly related kinetoplastids, *Leishmania major* [43] and *Trypanosoma cruzi* [28], results in the depletion of GPIs, evidenced by the secretion of major GPI-linked surface proteins (gp63 in *Leishmania* and Ssp-4 in *T. cruzi*). In both of these organisms this GPI-negative phenotype results in a decreased parasite virulence in mammals [27, 42]. This evidence suggests that in *T. brucei*, dysregulation of GPI-PLCp activity might cause in a GPI deficiency. One possible result of this GPI deficiency would be failure to attach VSG to the plasma membrane. Prevention of VSG membrane attachment would

result in a loss in the ability of BSF parasites to undergo “antigenic variation”. It has been speculated that VSG deficient BSF *T. brucei* will be killed by the complement system.

There are two possible ways in which GPI-PLCp activity could be regulated in *T. brucei*; i) by suppression of activity, and/or ii) localization of GPI-PLCp away from GPIs. The former is supported by the fact that activation of GPI-PLCp enzyme activity is associated with formation of (protein) tetramers [4]. In *T. brucei* GPI-PLCp exists in a less active monomeric state, whereas in *Leishmania major*, the enzyme is considered active, GPI-PLCP forms tetramers. A second model for regulation of GPI-PLCp activity in *T. brucei* is supported by preliminary localization studies of a GPI-PLC· GFP (green fluorescent protein) fusion in the parasite. GPI-PLCp appears to be targeted to the flagellar membrane of *T. brucei*, potentially away from GPI intermediates (M. Rashid and K. Mensa-Wilmot, unpublished).

Post-translational modification is one possible mechanism by which GPI-PLCp may be limited to specific regions of *T. brucei*. The enzyme is covalently modified by the fatty acids myristate and palmitate on cysteine residues [2]. Palmitoylation can target some integral membrane proteins, such as influenza hemagglutinin, to microdomains in cell membranes [38]. Therefore, it is possible that lipid modification plays a role in targeting GPI-PLCp away from GPIs in *T. brucei*.

LEISHMANIA

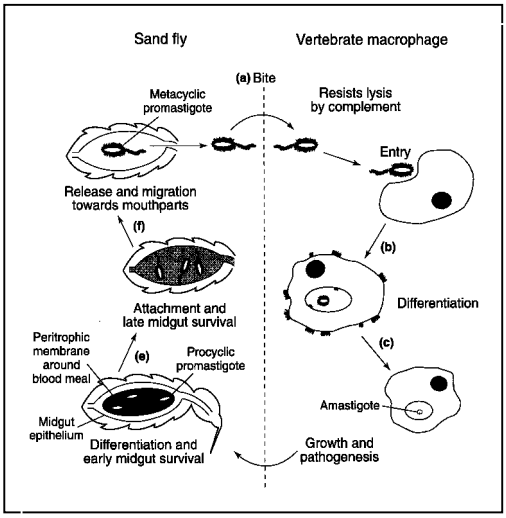
Leishmania comprise several subspecies (e.g. *L. major, L. donovani, L. chagasi, L. braziliensis, L. mexicana*). Together they infect an estimated twenty million people worldwide. The parasite is found in Asia, Europe, South and Central America, and Africa. There are three forms of the human leishmaniasis (causative subspecies are

named in parenthesis): cutaneous (*L. major*), mucocutaneous (*L. braziliensis, L. mexicana*), and visceral (*L. donovani and L. chagasi*). Cutaneous leishmaniasis is limited to the dermal tissues, resulting in either wet or dry ulcerous lesions on the skin. The mucocutaneous form of the disease is similar to the cutaneous form, with greater involvement of mucous membranes. Visceral leishmaniasis is characterized by infection of the liver, spleen, and lymph nodes, resulting in enlargement of these organs as well as fever and cachexia. A pentavalent antimonial (Pentostam) is the chemotherapeudic agent of choice. An additional antibiotic regimen is recommended to avoid secondary bacterial infections which are frequently the cause of death [49].

Life Cycle

Leishmania alternate between a flagellated, extracellular (promastigote) stage which is found in *Phlebotomus* flies (sand flies) and an intracellular stage (amastigote) found within mammalian macrophages [66]. When a sand fly laden with parasites takes a blood meal, infective metacyclic promastigotes are injected into the skin of a mammalian host (Figure 5). These parasites invade mononuclear phagocytes (macrophages), taking up residence in a phagolysosome inside the host cell. Here they differentiate into amastigotes and divide intracellularly. After several rounds of replication the parasites rupture the infected cell releasing amastigotes, which can either

Figure 5: *Leishmania* life cycle [7].



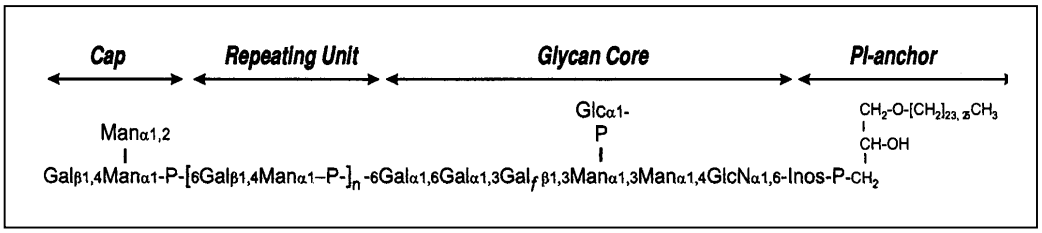
invade surrounding vertebrate cells or can be taken up by a sand fly during a blood meal. Once inside the fly, amastigotes differentiate into procyclic promastigotes, which attach to the midgut wall to avoid excretion with the blood meal. Transformation into non-dividing metacyclic promastigotes, which are infective to mammals, is accompanied by detachment of the parasites from the gut wall. Migration of the parasite to anterior regions of the fly facilitates infection of a vertebrate at the next blood meal [49].

Glycosylphosphatidylinositols (GPIs) in Leishmania

Glycoconjugates and proteins appear to be essential for the survival and infectivity of *Leishmania* in both the insect vector and in the mammalian macrophage. Some of these molecules are important for parasite-host interactions in the insect [37] as well as the vertebrate [31]. Most of these cell surface proteins and oligo/polysaccharides are attached to parasite membranes by glycosylphosphatidylinositol (GPI) anchors.

The major polysaccharide-GPI on the surface of *Leishmania* promastigotes is lipophosphoglycan (LPG) [14]. This polysaccharide is the most abundant component of a

Figure 6: Structure of LPG from *Leishmania* [14].



densely packed glycocalyx coat, which covers the plasma membrane. LPG (from *L. donovani*) is made up of four regions (Figure 6); i) a 1-O-alkyl-2-lyso-phosphatidyl(myo)inositol; ii) a glycan core Galα1-6Galα1-3Gal_fβ1-3Manα1-6(Glcα1-

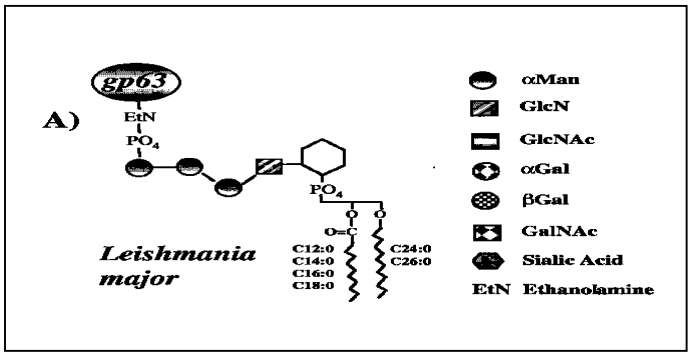
P)Manα1-4GlcN [36,67]; iii) repeating disaccharide phosphate units (6Galβ1-4Manα1-*P*); and iv) an oligosaccharide cap (Galβ1-4(Manα1-2)Manα1-*P*).

LPG is involved in numerous parasite-host interactions (review in [7]). It has been documented that in the mammalian host LPG is important for invasion of and maturation in macrophages [15, 18], as well as resistance to complement lysis within the blood stream [36, 62]. Furthermore, in the insect vector LPG is essential for protection against the hydrolytic environment within the midgut and attachment to the midgut wall in a species-specific manner [36, 62].

Glycoinositolphospholipids (GIPLs) are “free” GPIs (i.e. unattached by macromolecules) which share a common Manα1-6(Manα1-3)Manα1-4GlcNα motif, and are linked to either alkylacyl-PI or *lyso*alkyl-PI [35]. GIPLs are important for amastigote replication and viability within mammalian macrophages [42].

Gp63 is the major GPI-linked protein on the plasma membrane of *Leishmania* promastigotes [54]. The glycan core of the gp63 GPI (Figure 7) is similar to VSG-GPI of *T. brucei*. However, this GPI lacks Gal side chains and the glycerol moiety contains a 1-O-alkyl-2-O-acyl group instead of dimyristoylglycerol [56]. The glycerol moiety is acylated with one of several saturated fatty acids (laurate, myristate, palmitate, or stearate) at the *sn*-2 position, and one of two long chain fatty acids (C24 or C26) at the *sn*-1 position

Figure 7: Structure of gp63 GPI [47].



(Figure 7A) [56]. Gp63 is important to parasite survival within the mammalian blood stream by protecting the parasite from complement mediated lysis, and facilitating parasite entry into and survival within macrophages [10, 31].

Leishmania as a Model System

Leishmania is a good model system in which to study the regulation of GPI-PLCp. First of all, *Leishmania* express gp63 (~10^6/cell) on its plasma membrane. The extent of GPI anchoring of gp63 can be used as an indirect measure of the amount of GPIs in the cell. Secondly, *Leishmania* has no detectable GPI-PLCp activity [44]. This allows analysis of altered proteins without an interfering background of wild type GPI-PLCp activity. Finally, expressing GPI-PLCp in *Leishmania* leads to a GPI-negative phenotype manifested by constitutive secretion of gp63 [43]. It is clear that activity of GPI-PLCp against GPIs is not suppressed in *Leishmania*, as it is in *T. brucei*, which makes this an ideal system to study *in vivo* regulation of the enzyme in a trypanosomatid.

LIPID MODIFICATIONS OF PROTEINS

There are three classes of lipid modifications found on proteins: i) isoprenylation; ii) N-myristoylation; and iii) S-acylation (reviewed in [71]). Covalent modification of proteins by lipids occurs in all eukaryotes and has multiple functions. In addition to the attachment of some soluble proteins to biological membranes, protein lipidation enhances protein-protein interaction.

Isoprenylation

Isoprenylation is the addition of the isoprenoids farnesyl (C15) or geranylgeranyl (C20) group to C-terminal cysteine residues in a thioether bond [70]. The most common consensus sequence for this type of modification is Cys-a-a-X, where the cysteine is

lipidated, “a” must be an aliphatic residue (e.g. Gly, Ala, Val, Leu, and Ile), and “X” is any amino acid [33]. Cleavage and esterification of the residues C-terminal to the lipid modification follows isoprenylation. Some isoprenylated proteins are involved in cellular signaling and vesicular transport, (e.g. Ras superfamily of small GTPases [32]) and some γ-subunits of heterotrimeric G proteins.

N-myristoylation

N-myristoylation, catalyzed by N-myristoyl transferase, is the covalent linkage of myristate (C14) to an N-terminal glycine residue in an amide bond at the consensus sequence M-G-X-X-X-A/T (“X” is any amino acid) (reviewed in [71]). This reaction is followed by cleavage of the initiator methionine by a peptidase. Many, but not all, myristoylated proteins are membrane bound. The hydrophobicity of myristate is insufficient to integrate proteins stably onto membranes [51], suggesting the need for additional signals for permanent membrane association. Such signals include a polybasic amino acid cluster adjacent to the acylation site (as in Src family of non-receptor tyrosine kinases) [32], as well as S-palmitoylation [26]. In fact, myristoylation is often a requirement for palmitoylation of Gα subunits [25] and non-receptor tyrosine kinases [52]. Myristoylation and other protein modifications allow a reversible association with the plasma membrane, possibly in response to cellular signals, and targeting of proteins to specific membrane compartments [71].

S-acylation

S-acylation is most commonly the addition of palmitate (C16) to a cysteine residue *via* an ester linkage (reviewed in [17]). Recently S-myristoylation has been described in trypanosomatids [2, 3]. Palmitoylated proteins can be classified into three

groups depending on the location of the acylation site. The first group are those proteins, including the Gα subunits and Src family members, which are modified at the N-terminus. Myristoylation at G2 in the sequence M-G-X-X-X-A/T often accompanies this type of palmitoylation, as in the $G\alpha_i$ protein family [52]. The second group of proteins are palmitoylated at the C-terminus and are exemplified by the Ras family. The consensus sequence is not clear but palmitoylation of these proteins often occurs proximal and N-terminal to a prenylation site [17]. The final class of palmitoylated proteins are trans-membrane proteins that are usually modified near membrane spanning domains [17].

Palmitoylation is involved in the stable association of proteins with lipid bilayers by increasing the hydrophobicity of soluble proteins. This function of acylation is evident in the palmitoylated α subunits of heterotrimeric G proteins (except for α_t which is not palmitoylated). In the case of $G\alpha_i$, both myristate and palmitate are required for stable membrane association [59]. The reversible nature of palmitoylation allows proteins to bind reversibly to intracellular membranes, lending itself to a possible role in regulation of protein localization within the cell.

Palmitate targets proteins to cellular subdomains known as “lipid rafts,” which are regions of membranes that are enriched in sphingolipids and cholesterol as well as fatty acylated proteins (e.g. GPI anchored proteins) [60]. Lipid rafts are thought to play a role in the structure and function of caveolae, invaginations of the plasma membrane implicated in such processes as signal transduction, cholesterol trafficking, endocytosis and transcytosis [1]. Unlike conventional membranes, those derived from lipid rafts are insoluble in some cold detergents and are referred to as detergent resistant membranes

(DRMs). Proteins recruited to DRMs tend to be dually acylated by saturated fatty acid chains [57], such as with the lipid moieties on GPI anchors [5]. Furthermore, palmitoylation of the α subunits of some G-proteins is involved in targeting to membrane microdomains [24]. Targeting to membrane microdomains [61] is quite possibly due to the interaction between the lipid on the protein and lipids in the DRMs [48]. It is hypothesized that the “raft” targeting is due to the structure of the lipid rather than its hydrophobicity [46].

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CHAPTER II

CONTROL OF GLYCOSYLPHOSPHATIDYLINOSITOL (GPI) CATABOLISM IN *LEISHMANIA* BY A PHOSPHOLIPASE C[1]

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ABSTRACT

Glycosylphosphatidylinositol phospholipase C (GPI-PLCp), a membrane-bound protein in *Trypanosoma brucei*, potentially co-localizes with glycosylphosphatidylinositol (GPI) biosynthesis. However, the enzyme causes no GPI-deficiency in *T. brucei*. Unexpectedly, heterologous expression of GPI-PLCp in *Leishmania* causes a GPI-negative phenotype; the transgenic parasites are i) deficient in GPIs, and (ii) constitutively secrete the major GPI-anchored protein, gp63. GPI-PLCp is myristoylated and palmitoylated on cysteine residues in *Leishmania*. In lieu of efforts to understand why GPI-PLCp does not cause a GPI deficiency in *T. brucei*, we explored the possibility that cysteine residues influence the ability of the enzyme to cleave GPIs *in vivo*. Each of eight cysteine residues in the GPI-PLCp were changed to either alanine or serine. Stable transfectants of *L. major* expressing these mutant proteins were analyzed for the GPI-negative phenotype normally associated with expression of the native enzyme. Cys80Ala, Cys269,270Ala, Cys269,273Ala, Cys270,273Ala, and Cys269,270,273Ala rendered GPI-PLCp incapable of causing the GPI-negative phenotype. In contrast, Cys184Ala and Cys347Ala GPI-PLCp produced the expected GPI-deficiency in the parasites. Intrinsic enzyme activity was retained in all mutants, since all of them were active against purified GPIs *in vitro*. To resolve the apparent discrepancy between the *in vitro* and *in vivo* data, we hypothesized that sub-cellular location of GPI-PLCp plays a role in access to GPIs *in vivo*. In immunolocalization studies, GPI-PLCp was found predominantly at or near the Golgi. This specific anterior localization was also observed in mutants of GPI-PLCp that produced a GPI deficiency. However, in the mutants that did not cause a GPI-negative phenotype a marked decrease

of the enzyme in the Golgi zone was observed. Concurrently, the fraction of GPI-PLCp in regions posterior to the parasite nucleus increased dramatically. Importantly, an enzymatically inactive Gln81Leu mutant localized specifically to the anterior structure. We conclude that i) GPI-PLCp is targeted specifically to Golgi/lysosome of *Leishmania*, ii) cysteines C80, C269, C270, and C273 determine the Golgi/lysosome location of GPI-PLCp, and iii) GPI-PLC enzyme activity and Golgi localization are both necessary to confer a GPI-negative phenotype on *Leishmania*.

The abbreviations used are: *Ala*, alanine; *APRB*, alkaline phosphatase reaction buffer; *BSA*, bovine serum albumin; *BSN buffer*, Bjerrum Schafer-Neissen buffer; *ConA*, concanavalin A; *Cys*, cysteine; *DMSO*, dimethylsulfoxide; *ER*, endoplasmic reticulum; *EtN*, ethanolamine; *FBS*, fetal bovine serum; *G418*, geneticin; *GlcN*, glucosamine; *Gln*, glutamine; *GPI*, glycosylphosphatidylinositol; *GPI-PLC*, glycosylphosphatidylinositol phospholipase C; *HLB*, hypotonic lysis buffer; *IDB*, immunoprecipitation dilution buffer; *Leu*, leucine; *M199*, medium 199; *Man*, mannose; *MBS*, m-maleimidobenzoyl-N-hydroxysuccinimide ester; *mfVSG*, membrane form variant surface glycoprotein; *NEM*, n-ethylmaleimide; *P*, phosphate; *PAGE*, polyacrylamide gel electrophoresis; *PBS*, phosphate buffered saline; *pCMPS*, para-chloromercuriphenylsulfonic acid; *PCR*, polymerase chain reaction; *Phe*, phenylalanine; *PI*, phosphatidylinositol; *PIC*, protease inhibitor cocktail; *SDS*, sodium dodecyl sulfate; *Ser*, serine; *TLCK*, N-toysl-L-lysine chloro-methyl-ketone; *UTR*, untranslated region; *VSG*, variant surface glycoprotein.

INTRODUCTION

The African trypanosome *Trypanosoma brucei* is the causative agent of sleeping sickness in humans and *nagana* in cattle. Expression of variant surface glycoprotein (VSG), a glycosylphosphatidylinositol (GPI)-anchored protein, on the plasma membrane allows the parasite to establish and maintain an infection in a mammal. *T. brucei* express a GPI-specific phospholipase C (GPI-PLCp) [7, 12] which is able to cleave both the GPI of VSG and biosynthetic intermediates [24]. GPI-PLCp is an integral membrane protein which binds to intracellular membranes [5], possibly colocalizing with the early intermediates of GPI biosynthesis [33]. If GPI-PLCp cleaved the GPI intermediates, *T. brucei* may become GPI deficient. The later condition is potentially lethal [26]. In spite of this possibly deleterious situation, the prefabricated GPI anchor, glycolipid A (EtN-*P*-Man_3-GlcN-PI) [9, 16, 17, 18, 19], is efficiently added to VSG. Hence, the biological evidence indicates that GPI-PLCp activity against GPIs is most likely suppressed in *T. brucei*.

In sharp contrast to the situation in *T. brucei*, when GPI-PLCp is heterologously expressed in *Leishmania major* (promastigotes), a GPI deficiency, resulting from cleavage of GPI intermediates, is observed [22]. This GPI-negative phenotype is manifested by constitutive secretion of the major GPI-linked surface protein, gp63. GPI-deficient promastigotes are able to infect mammalian macrophages but show decreased virulence [20]. The apparent absence of GPI-PLCp regulation, and the ease of detecting the GPI-negative phenotype make *Leishmania* a unique system in which GPI-PLCp activity may be studied *in vivo*.

Several mechanisms may contribute to the apparent quiescence of GPI-PLCp activity against GPIs of *T. brucei*. One possibility is the ability of GPI-PLCp to form oligomers [3]. In *T. brucei*, GPI-PLCp exists predominantly as a monomer. However, under conditions where the enzyme is active (*in vitro* and in *Leishmania*), tetramers are the major form [3]. Second, GPI-PLCp is covalently modified with lipids. Acylation with myristate and palmitate in both *T. brucei* and *Leishmania* is on cysteine residues [2, 3]. From studies in *Xenopus laevis* oocytes it has been proposed that the site(s) of acylation lies within a cluster of cysteines (residues 269, 270, and 273) [27]. Lipidation increases enzyme activity 30-fold, by raising the rate at which the enzyme-substrate complex is converted to the product and free enzyme [3].

Cysteine residues might play an important role in control of GPI-PLCp activity. First, GPI-PLCp is acylated on a cysteine residue(s). Second, enzyme activity is inhibited by sulfhydryl reagents such as p-chloromercuriphenylsulfonic acid (pCMPS) and n-etheylmaleimide (NEM) [12]. Lastly, mutagenesis studies revealed that Cys80 is at or near the active site of the protein [31]; a substitution with alanine (Ala) at position Glu81 inactivated GPI-PLCp. In contrast, a Cys80Ala mutant is active. Curiously, a Cys80Phe mutant abrogated enzyme activity [31]. Therefore, we hypothesize that cysteines (and possibly S-lipidation) could regulate GPI-PLCp activity *in vivo*.

To test the potential role of cysteines in control of GPI-PLCp activity *in vivo*, Cys residues were substituted with either Ala or Ser. *In vivo* activity of the mutated enzymes was monitored by assessing the presence or absence of a GPI-negative phenotype [23]. Intrinsic ability of the mutated protein to cleave GPIs was tested with purified GPIs *in vitro* [25]. Finally, to test a hypothesis that *in vivo* activity may be modulated by targeting

of the protein to specific regions of *Leishmania*, intracellular location of GPI-PLCp was determined. Our data suggest that specific cysteines are required for *in vivo* activity of GPI-PLCp against *Leishmania* GPIs. Strikingly, these cysteines determine the subcellular localization of GPI-PLCp in the parasite.

MATERIALS AND METHODS

Materials

Restriction enzymes were from New England Biolabs (Beverly, MA); nonidet P40 was from Calbiochem (San Diego, CA); plasmid purification kits were from Qiagen (Valencia, CA); and the Gene Editor mutagenesis kit was from Promega (Madison, WI). RPMI and medium 199 (M199) were purchased from Life Technologies (Gaithersburg, MD). Sodium dodecyl sulfate (SDS), Triton X-100, 5-bromo-4-chloro-indoyl phosphate (BCIP) and p-nitroblue tetrazolium chloride (NBT) were from BioRad (Melville, NY). Fetal bovine serum and new born calf serum were from Hyclone (Logan, UT). [9,10-^{3}H]Myristate and [9,10-^{3}H]palmitate were purchased from Amersham Pharmacia (Arlington Heights, IL). Leupeptin, aprotinin, and alkaline phosphatase conjugated goat anti-rabbit secondary antibody were from Roche (Indianapolis, IN). Alexafluor 288-conjugated anti-rabbit antibody was purchased from Molecular Probes (Eugene, OR). Bicinchoninic acid protein detection kit, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), and dimethylsulfoxide (DMSO) were purchased from Pierce (Rockford, IL). Butanol and G418 (Geneticin) were from Fisher Scientific (Norcross, GA) and Immobilon P membrane was purchased from Millipore (Bedford, MA). All other reagents were from Sigma (St. Luis, MO).

Plasmid Construction

The GPI-phospholipase C expression plasmid pUTK-GPIPLC (Figure 1) was constructed by insertion of a *GPI-PLC* coding region [6, 11] and a translational enhancing 5' UTR [30] into a *Leishmania* expression plasmid pXUTE-Kana^R [29]. The *GPI-PLC* insert was generated by polymerase chain reaction (PCR) using LacZ-ACC (KCR4) (forward primer) and KCR5 (reverse primer) (Table 1 and Figure 2). The PCR product was digested with *BamH* I, ligated into the *Bgl* II site of pXUTE-Kana^R, and transfected into *E. coli* DH5α for cloning.

Mutagenesis of GPI-PLC

Alteration of the coding sequence of *GPI-PLC* was performed with the "Gene Editor" site directed mutagenesis kit (Promega; Madison, WI, USA). Figure 2A shows a schematic of this protocol. A mutagenic primer (directed GPI-PLC) was used in conjunction with an antibiotic selection primer (targeted to β-lactamase) to initiate synthesis of one complete strand of the plasmid (in two fragments) using T7 DNA polymerase and deoxynucleoside triphosphates. The two single stranded DNA fragments, presumably still base paired to the template, were joined by T7 DNA ligase. The resulting "hybrid plasmid" consists of one normal and one mutant strand. This plasmid is transformed into a strain of *E. coli* which is mutant for DNA mismatch repair (mutS). In this strain of *E. coli* the plasmid will be replicated in a semiconservative manner to produce two different plasmids; one containing the desired mutations and one with the native sequences. The mutation in the β-lactamase coding region gives resistance to an altered ampicillin resistance mix (provided by Promega), so that only the bacteria containing the mutated β-lactamase will survive. In this manner, plasmid containing the

β-lactamase mutation can be selected and separated from non-mutated plasmids. Hopefully, if the conditions were correct for production of the β-lactamase mutation, they were also favorable for production of the desired mutation in the *GPI-PLC* coding region. Base pairs (in the coding region of *GPI-PLC*) corresponding to cysteine residues Cys24, Cys80, Cys184, Cys269, Cys270, Cys273, Cys332, and Cys347, of GPI-PLCp were changed to either Ala or Ser (Figure 1) in either single, double, or triple combinations. Mutations were verified by DNA sequencing (Molecular Genetics Instrumentation Facility (University of Georgia, Athens, GA) (primers are listed in Table 1). Primers used for sequencing were as follows (Table 1): KCR4 was used to confirm the Cys24Ala, C80Ala, and Cys184Ala mutations, whereasKCR5 was used to confirm all mutations made in the cluster of cysteines (269,270,273), as well as the Cys332Ala and Cys347Ala.

Culture of Leishmania major and DNA Transfection

Stable cell lines of the HOM/IQ/73/LCR-L32 *Leishmania* strain [8] expressing either native or mutagenized GPI-PLCs were obtained as follows. Cells were grown at 27°C to a density of 10^7/ml in medium 199 (supplemented with 5% fetal bovine serum (FBS), 40 mM HEPES, 0.0005% hemin, 0.0001% biotin, 0.1 mM adenine, and 1% antibiotic-antimitotic) [35], harvested (5,000 x g, 5 min), washed once with phosphate buffered saline (PBS) (136 mM NaCl, 27 mM KCl, 5.3 mM Na_2HPO_4, and 1.7 mM KH_2PO_4), and resuspended in electroporation buffer (21 mM HEPES pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2PO_4, 6 mM glucose) [14] at 10^8/ml. To this suspension, 50 μg of plasmid DNA purified by the QIAGEN-tip 500 (Qiagen; Valencia, CA) (dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) to a final concentration of 1 μg/μl) were added. Electroporation was performed in 2 mm cuvettes with 300 V and 500 μF using a

BioRad Gene Pulser II (BioRad; Melville, CA) [14]. Twelve hours post-transfection, G418 dissolved in PBS (and filter sterilized) was added to a final concentration of 30 μg/ml. Cells were selected at this drug concentration until vigorous growth was detected, at which time the G418 concentration was increased every two weeks in a step-wise fashion beginning with 50 μg/ml G418 and progressing to a final drug concentration of 200 μg/ml.

Stable transfectants were maintained in M199 containing either 50 μg/ml or 200 μg/ml G418. During passing, cells that reached late log phase (2 x 10^7 cells/ml) were diluted to 10^6 cells/ml to reinitiate growth.

GPI-PLCp Enzyme Assay

Leishmania growing stably at 200 μg/ml G418 were harvested at a density of 10^7/ml (5,000 x g, 5 min), washed once with PBS, and resuspended in 100 μl of hypotonic lysis buffer (HLB) (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) containing a protease inhibitor cocktail (PIC) (2.1 μM leupeptin, 0.1 mM *N*-toysl-L-lysine chloro-methyl-ketone (TLCK), 0.4 U aprotinin). This lysate was incubated for 15 min on ice and centrifuged (14,000 x g, 10 min, 4°C). The pellet was solubilized in 100 μl of assay buffer AB (1% NP-40, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0). Five microliters of this detergent extract were added to 25 μl of AB containing 10 μg of [^{3}H]-membrane form variant surface glycoprotein (mfVSG) on ice [24]. The reaction (30 μl) was incubated at 37°C for 20 min, and terminated by extraction with 500 μl of H_2O-saturated butanol. After centrifugation for 1 min (room temperature, 14,000 x g), the amount of [^{3}H]dimyristoylglycerol released from mfVSG was quantified by scintillation counting of 400 μl of the organic phase [25]. One unit of GPI-PLC cleaves 500 ng of

[^{3}H]mfVSG [25]. Protein concentration of cell lysates was determined by a bicinchoninic acid assay (Pierce; Rockford, IL).

Immunoadsorption of GPI-PLC expressed in Leishmania major

Parasites (5 x 10^{8}) were lysed in 10 ml HLB containing PIC, and incubated on ice for 15 min. The lysate was centrifuged (10,000 x g) and the supernatant was discarded. The pellet (containing GPI-PLCp) was extracted with 1 ml immunoprecipitation dilution buffer (IDB) (1% Triton X-100, 200 mM NaCl, 60 mM Tris-HCl pH 7.5, 6 mM EDTA) and incubated on ice for 15 min. The detergent extract was cleared by centrifugation for 20 min (14,000 x g, 4°C); and the pellet was discarded. Two microliters of monoclonal antibody 2A6-6 (mc2A6-6) (ascites fluid) [11], was added to the supernatant, which was incubated for 1 h (4°C) with continuous inversion. Fifty microliters of a protein A sepharose CL-4B:water (1:1 v/v) suspension were added to the mixture and kept at 4°C with continuous inversion for 18 hours. The sepharose· mc2A6-6· GPI-PLC complex [1] was recovered by centrifugation for 20 seconds (14,000 x g, 4°C). The sepharose beads were washed as follows: once with 1 ml PBS, twice with 1 ml PBS containing 0.2% NP-40, and a final wash with 1 ml PBS. Each wash was for 10 min with continuous inversion at 4°C. All wash buffers contained PIC.

GPI-PLCp was eluted from the sepharose beads by addition of 20 µl of 2.5X SDS-PAGE sample buffer (25% (v/v) glycerol, 5% (w/v) SDS, 5% (v/v) β-mercapto-ethanol, 0.05 (v/v) bromophenol blue, 0.05M Tris-HCl, pH 8.0) and heating at 90°C for 3 min. Proteins were separated by SDS-PAGE (14% minigel) and transferred to an Immobilon-P membrane using a Trans-Blot semi-dry cell (BioRad) in Bjerrum and

Schafer-Niessen Buffer (BSN) (192 mM Tris, 156 mM glycine, 20% methanol, and 0.03% SDS) (BioRad; Melville, NY) at 300 mA for 2 hours [1].

Western Blotting

GPI-PLCp on Immobilon P (see above) was detected by western blotting. The membrane was soaked for 1 h at room temperature in 30 ml of blocking solution (1% (v/v) Tween 20, 10% (v/v) newborn calf serum, 13% (w/v) glycerol, 18% (w/v) D-glucose in PBS) [21]. The membrane was then incubated for 1 h with polyclonal antiserum R18B3 (1:3000 dilution in blocking solution) [21]. The membrane was washed 3 times, each for 5 min with 30 ml PBS, and incubated for 1 h with alkaline phosphatase conjugated goat anti-rabbit IgG (1:1000 dilution in blocking solution). The wash step was repeated with PBS as before, followed by two 5 min washes in alkaline phosphatase reaction buffer (APRB) (75 mM NaCl, 75 mM Tris-HCl pH 9.5, 3.8 mM $MgCl_2$). The enzyme-linked secondary antibody was detected with 5-bromo-4-chloro-indoyl phosphate (BCIP) and p-nitroblue tetrazolium chloride (NBT).

For detection of gp63, parasites (5×10^7) were suspended in 1 ml HLB containing PIC and 5 mM p-chloromercuriphenyl-sulfonic acid (pCMPS). (pCMPS was used to prevent any post-lysis cleavage of GPI-anchors by GPI-PLC [12]). This lysate was incubated on ice for 15 min. The lysate was centrifuged (14,000 x g, 4°C, 20 min) and the supernatant was discarded. The pellet was resuspended thoroughly in 1 ml high salt buffer (10 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 500 mM NaCl) containing PIC and 5 mM pCMPS. The suspension was centrifuged (14,000 x g, 4°C, for 10 min), and the pellet was washed in 1ml HLB containing PIC and 5 mM pCMPS, to remove excess salt from the previous wash. The pellet was resuspended in 80 µl of 2.5X SDS-PAGE sample

buffer, divided into two tubes (one for detection of gp63 and one for detection of conconavalin A (Con A) binding proteins) and heated at 90°C for 3 min. Solubilized proteins were separated by SDS-PAGE (14% minigel), and transferred to an Immobilon P membrane. Western blotting of the membrane with anti-gp63 was carried out as described for GPI-PLC except that a rabbit polyclonal antibody raised against gp63 (1:2000 dilution in blocking solution) was used as the primary antibody.

For detection of glycoproteins to which Con A bound, membranes were blocked in 5% non-fat milk (Carnation) in PBS for 1 h with constant shaking, and treated with Con A (5 μg/ml) in 5% non-fat milk for 1 h (27°C) with constant shaking. The membrane was washed 3 times each with 30 ml PBS. Membrane was incubated with anti-Con A (Sigma) (1:6000 dilution) in non-fat milk for 1 h at room temperature. The membrane was washed with PBS and incubated for 1 h with alkaline phosphatase conjugated goat anti-rabbit IgG (Roche) (1:1000 dilution in non-fat milk). Membranes were then processed for antigen detection with BCIP/NBT as substrates [21].

Metabolic labeling of Leishmania major with [^{3}H] Fatty Acids

Three different labeling protocols were tested in order to determine which was the most efficient. For the first protocol [2], 5 mCi of [^{3}H]myristate in ethanol were dried under a nitrogen stream in a water bath (37°C). The dried fatty acid was resuspended in 50 μl of 95% ethanol and then 450 μl of 20 mg/ml fatty acid free bovine serum albumin (BSA) in PBS was added. Twenty microliters of this [^{3}H]-myristate· BSA complex were added to 2 ml of serum-free M199 supplemented with 0.5 mg/ml BSA and 25 mM HEPES, pH 7.4. The mixture was incubated for 30 min at room temperature. Mid-log phase promastigotes (5 x 10^8 total cells) were harvested (5,000 x g, room temperature),

washed once in 10 ml PBS, and suspended in 18 ml serum-free M199 supplemented with 0.5 mg/ml BSA. To this cell suspension the [^{3}H]-myristate· BSA complex (above) was added, and metabolic labeling was allowed for 18 h at 26°C. Cells were pelleted (5,000 x g, room temperature), and washed once with PBS, and stored at -20°C. Immunoprecipitation of GPI-PLC [23] was performed and the labeled protein was detected by phosphorimaging (Personal Molecular Imager FX (BioRad)).

In protocol #2 [34], 300 μCi of [^{3}H]myristate or [^{3}H]palmitate were dried under nitrogen and resuspended in 300 μl of serum-free M199 supplemented with BSA (20 mg/ml). This suspension was incubated for at least 10 min at room temperature. Mid-log phase promastigotes (3 x 10^8) were harvested (above) and washed once in PBS. Cells were resuspended in 5 ml of serum free M199 and incubated for 1 h at room temperature. Starved cells were then harvested and resuspended in 2.7 ml serum-free M199. The [^{3}H]lipid· BSA complexes were added to the parasites and incubated at room temperature for 18 hours. *Leishmania* were harvested, and washed once with PBS, and pellets were stored at -20°C.

The third protocol was modified from the second. Three hundred μCi of dried [^{3}H]-myristate or [^{3}H]-palmitate were suspended in 300 μl of serum-free M199 containing 20 mg/ml BSA and incubated at room temperature for 10 min. Cells (3 x 10^8 mid-log phase) were harvested, washed in PBS, resuspended in 2.7 ml serum-free M199, and metabolically labeled for 18 h at room temperature by incubating with the [^{3}H]lipid· BSA complex (above). Cells were harvested and washed once in PBS and stored at -20°C.

Labeled cells (3 x 10^8) were lysed in 5 ml HLB (containing PIC). Membranes were solubilized in 40 µl 2.5X SDS-PAGE sample buffer and proteins were separated by SDS-PAGE (14%, minigel). Labeled proteins were detected by phosphorimaging (Personal Molecular Imager (BioRad)).

Chemical Crosslinking of GPI-PLCp in vivo

In vivo chemical crosslinking of GPI-PLC was performed as follows [3]. Mid-log phase *L. major* (5 x 10^8) were harvested (5,000 x g, room temperature) and washed once with PBS. The washed pellet was resuspended in 20 ml methionine-free RPMI medium and incubated at 27°C for 1 h. One hundred µCi of [^{35}S]methionine (1000 Ci/mmol) were added and the parasites were labeled at 27°C for 3 h. Labeled parasites were harvested (5,000 x g, room temperature) and washed in 2 ml PBS, which was divided into two microfuge tubes (1 ml each). Harvested parasites (2.5 x 10^8) were resuspended in 990 µl of PBS. Ten microliters of 100 mM m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (dissolved in dimethylsulfoxide (DMSO)) were added. Control cells were treated with 10 µl of DMSO. Crosslinking was carried out for 15 min at room temperature, and terminated by the addition of 100 µl of 1 M Tris-HCl, pH 7.5. Parasites were washed once with PBS and harvested (14,000 x g, 4°C).

GPI-PLCp was immunoadsorbed (see *Immunoadsorption of GPI-PLC from Leishmania major*) with the following changes [3]. The sepharose beads to which GPI-PLCp were bound was washed sequentially with i) 1 ml PBS plus 3M KCl, ii) 1 ml of 0.1% NP-40 in PBS, iii) 750 µl of a high salt buffer (3.17 M potassium glutamate, 0.59 M potassium chloride, and 0.1% (w/v) n-octyl glucoside), iv) 1 ml wash buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.02% SDS, 1% Triton X-100), v) 1 ml

0.1% sodium deoxycholate in PBS, and vi) twice with 1 ml PBS. Proteins were eluted from the beads by heating at 90°C for 3 min in 20 µl of SDS-PAGE sample buffer. Solubilized proteins were separated by SDS-PAGE (14% minigel) and visualized by phosphorimaging.

Immunostaining of GPI-PLCp

In order to reduce the background from non-specific antibodies in the anti-serum, RC300 was preincubated on cover slips containing fixed and permeabilized *Leishmania* transfected with pUTK. Parasite coated cover slips were incubated with 300 µl of RC300 diluted (1:1200) on parafilm for 20 min at room temperature. The cover slips were then carefully removed leaving as much "precleared" antibody solution behind as possible (between 150-200 µl) on the coverslip. Anti-serum recovered after this protocol was used for detection of GPI-PLCp.

Parasites growing in 50 µg/ml G418 were harvested (2000 x g, 2 min, room temperature) in mid-log phase (10^7/ml) and washed once in 1 ml PBS. The *Leishmania* pellet was resuspended in 500 µl PBS and 300 µl of this suspension were pipetted onto a poly-L-lysine-coated (Sigma; procedure #P8920) round cover slip (22 mm diameter). Parasites were allowed to settle on the cover slips for 20 min before the PBS was aspirated. Cells were fixed by adding 300 µl of 2% paraformaldehyde in PBS onto the cover slips containing the parasites, and incubating for 20 min at room temperature. Excess paraformaldehyde was removed. Fixed cells were permeabilized on the coverslips with 300 µl of 0.25% (w/v) Triton X-100 and incubation at 4°C for 8 min, after which the detergent was removed. The cover slips were transferred to a six well plate (one cover slip per well) and washed briefly 3 times with 3 ml PBS each time. Blocking was carried

out by placing the parasite coated coverslips face down onto 150 µl of 1% BSA in PBS which had been pipetted onto parafilm and incubated for 20 min at room temperature. Thereafter, parasites were incubated with about 150 µl of precleared anti-GPI-PLCp antibody RC300 (a 1:1200 dilution in blocking solution) [21] on parafilm for 45 min at room temperature. Cells were then washed as follows, once, for 5 min in 3 ml PBS; twice, 5 min each, in 3 ml high salt wash buffer (PBS plus 500 mM NaCl), and twice, 5 min each, in 3 ml PBS. Cells were then incubated in the dark with anti-rabbit Alexafluor 288-conjugated IgG (1:1000 dilution in blocking solution of a 2 mg/ml stock) for 1 hour at 4°C on parafilm. From this point on the cells were protected from light as much as possible (by covering plates with aluminum foil) to avoid bleaching of the fluorescence. Cells were washed thrice in six well plates, 5 min each, in 3 ml PBS.

The endoplasmic reticulum (ER) was localized by detection of the ER chaperonin BiP. For this purpose, fixed and permeabilized cells (described above) were incubated on parafilm with 150 µl of the anti-BiP antibody [4] (1:4000 dilution in blocking solution) for 45 min at room temperature. Cover slips were then washed as follows: once for 5 min in 3 ml PBS; twice, 5 min each, in 3 ml high salt wash buffer (PBS plus 500 mM NaCl), and twice, 5 min each, in 3 ml PBS. Detection and visualization of anti-BiP was carried out exactly as described above for the detection of GPI-PLCp.

Fluorescence microscopy

Prepared cells were viewed on a Leica microscope (model #DMIRBE). Images were captured using an interline chip cooled CCD camera (Orca 9545: Hamamatsu), and processed with Openlab 2.2 software.

RESULTS

GPI-PLC cysteine mutants are expressed in Leishmania major

pUTE-Kana^R (pUTK) was chosen as the expression vector because it has a 5' "universal translational enhancer" (*UTE*) upstream of neomyocin phosphotransferase gene (*NEO*) [30] which allows for more rapid outgrowth of *Leishmania major* transfectants (unpublished data). The vector contains 5' and 3' regions of the dihydrofolate reductase-thymidylate synthase gene [15] from *Leishmania* (Figure 1, solid black bars) which directs correct pre-mRNA maturation by addition of a spliced leader to the 5' end and polyadenylation of the 3' end of GPI-PLCp (reviewed in [28]).

Western blots were performed in order to determine the extent of GPI-PLC polypeptide (GPI-PLCp) expression (Figure 3). In the native GPI-PLCp, Cys80Ala (C80A), Cys184Ala (C184A), Cys269,270Ser (C269,270S), Cys269,273Ser (C269,273S), Cys270,273Ser (C270,273S), Cys269,270,273erS (C-Triple-S) and Cys347Ala (C347A), a 39 kDa protein corresponding to GPI-PLCp was detected (Figure 3, lanes 2-9). However, in *Leishmania* transfected with GPI-PLC encoding Cys24Ala and Cys332Ala, no GPI-PLCp or activity was detected (data not shown). Protein levels in the western blots of GPI-PLCp cysteine mutants (Figure 3) correlated with the amount of enzyme activity in the *in vitro* assays (see below). In control experiments, no GPI-PLCp was found in *Leishmania* transfected with pUTE-Kana^R which lacks *GPI-PLC* (Figure 3, lane 1).

In vitro cleavage of GPIs by cysteine mutants of GPI-PLCp

To determine the intrinsic enzymatic activity of each GPI-PLC cysteine mutant, GPI cleavage of purified membrane form VSG (mfVSG) was assessed (Figure 4A). All

of the tested cysteine mutants exhibited levels of enzyme activity comparable to that of the native enzyme (Figure 4B). This result suggests that the cysteine mutations do not cause GPI-PLCp to misfold. This observation is also consistent with the reported absence of disulfide bonds in GPI-PLC [3].

Loss of GPI cleavage in certain cysteine mutants of GPI-PLCp in vivo

Expression of native GPI-PLCp in *Leishmania* produced a GPI-negative phenotype [22] that is easily monitored by disappearance of gp63 from the membrane fraction of a *Leishmania* lysate (Figure 5, compare lanes 1 and 2). This GPI deficiency was found in cells expressing Cys184Ala and Cys347Ala (Figure 5, lanes 4 and 9). However, *Leishmania* expressing Cys80Ala, Cys269,270Ser, Cys260,273Ser, Cys270,273Ser, and C-Triple-S GPI-PLCp retained gp63 (Figure 5, lanes 3, and 5-8); indicating that the parasites were no longer GPI-negative. A faster migrating band of unknown origin cross-reacted with anti-gp63 antiserum was seen in all lanes. This protein was sometimes used as an internal protein loading control.

Western blots of Con A binding proteins revealed multiple bands on an SDS-PAGE gel. Of these proteins, one occurring at the junction between the stacking and separating gel was seen in all lanes (Figure 5, bottom panel). This unknown protein was used as a loading control, in addition to the gp63 cross-reacting band.

We surmise that mutation of GPI-PLCp at cysteine 80 or any combination (of at least two) within the cluster of cysteines (269, 270, and 273) render the protein defective for efficient digestion of GPIs *in vivo*. Retention of a single cysteine within the Cys269, Cys270, Cys273 cluster abrogates activity against GPIs in vivo. Therefore, dual Cys within the cluster, or Cys 80 (solo) are needed *in vivo* for GPI cleavage in *Leishmania*.

Oligomerization of GPI-PLC in Leishmania major

The quartenary structure of GPI-PLCp influences its enzyme activity. Tetramers of GPI-PLCp are more active than the monomers of the protein [3]. Monomeric GPI-PLCp predominates in *T. brucei* until activation of the enzyme by hypotonic lysis, when tetramers are formed. In *Leishmania*, native GPI-PLCp exists chiefly as an oligomer, even without osmotic stress [3]. To assess whether loss of GPI cleavage *in vivo* in some Cys mutants correlated with inability to tetramerize *in vivo*, protein-protein interactions in representative GPI-PLCps were examined.

Treatment of cells expressing the native protein with m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) results in the formation of a tetramer and a "GPI-PLC complex" *in vivo* (Figure 7B, compare lanes 1 and 2). The C-Triple-S mutant, which does not cause a GPIs deficiency *in vivo*, formed tetramers and the "GPI-PLC complex" in *Leishmania*. These data suggests that inability of C-Triple-S mutant to cause a GPI deficiency may not result from failure to oligomerize.

Metabolic Labeling of L. major and T. brucei with [^{3}H] fatty acids

GPI-PLCp is lipidated in both *T. brucei* and *Leishmania*, although the acylated Cys is not known. Studies in *Xenopus laevis* suggest that in vertebrate cells the site of acylation lies within the cluster of cysteines (Cys269, Cys 270, and Cys273), and that at least two of the cysteines bear a lipid [27]. To determine the acylation site of GPI-PLCp in *Leishmania*, metabolic labeling with radioactive fatty acids was attempted.

It was necessary in preliminary experiments to verify that lipidation of proteins with [^{3}H]fatty acids could be achieved in *Leishmania*. *L. major* (LH32 and CC1 strains) were metabolically labeled (protocol #2) with [^{3}H]myristate or [^{3}H]palmitate. Proteins

from cells lysed in SDS-PAGE loading buffer were separated by SDS-PAGE. Phosphorimaging revealed no labeling of protein (data not shown). However, there was an aggregate of radioactive material that failed enter the gel, indicating that label was taken up by the cells. Further attempts to label proteins in *Leishmania* using two other published protocols (#1 and #3) were not successful (data not shown). Consequently, we were unable to determine whether any or all of the Cys mutants of GPI-PLCp were lipidated. Thus, one can only hypothesize that any effects of Cys mutation on GPI-PLCp could be mediated by thio-lipidation. Further experiments are needed to resolve this issue.

Localization of GPI-PLCp in Leishmania major

GPI-PLCp has never been localized in *Leishmania*, despite the known physiological effects of the enzyme on the parasite [20, 22]. Therefore, we determined the sub-cellular location of the native GPI-PLCp by immunofluorescence. No GPI-PLCp was detectable in *L. major* cells transfected with pUTK (Figure 7A and B), whereas DAPI staining revealed both the nucleus and the kinetoplast DNA (Figure 7B, arrows). Native GPI-PLCp was found primarily at the anterior end of the parasite, frequently between the kinetoplast and the flagellum (Figure 7C and D, arrow). These results prove that GPI-PLCp does not bind indiscriminately to cell membranes but is specifically targeted to a region between the kinetoplast and the flagellum.

GPI biosynthesis is believed to occur on the endoplasmic reticulum (ER) [10, 33]. *Leishmania* ER was visualized by monitoring BiP, which is found predominantly in this organelle [4]. The distribution of the ER was distinct from that seen for the native GPI-PLCp (Figure 7, compare C and D with E and F). BiP was at the periphery of the cell

nucleus, and seemed to radiate in tubular structures toward both the anterior and posterior of the parasite, as expected for the peripheral ER [13]. From these observations we conclude that GPI-PLCp localization is distinct from the ER. This conclusion is consistent with an earlier finding that GPI-PLCp resides in the Golgi/lysosome region (Figure 7).

In order to determine if there is a correlation between the loss of activity *in vivo* and cellular location of GPI-PLCp, we compared the localization patterns of the native protein to that of the cysteine mutants. Positive controls Cys184Ala and Cys347Ala mutants, both of which retained activity *in vivo* against GPIs, are found in the same region of the cell as native GPI-PLCp (Figure 8, compare panels A-C). Additionally, the enzymatically inactive Glu81Leu mutant [31] was found in the Golgi/lysosome region (Figure 8, compare panels A and D). This last result proves that GPI-PLCp does not have to be enzymatically active to localize predominantly to this region. More importantly, these data indicate that enzyme activity is critical for GPI-PLCp to cause a GPI-negative phenotype in *Leishmania*.

In contrast, mutations of cysteines at position Cys80 as well as the double and triple mutations, relocalized GPI-PLCp throughout the cell (Figure 8, panels E-I). The proportion of total GPI-PLCp in the anterior end of *Leishmania* was significantly less in the Cys80Ala, Cys269,270Ser, Cys269,273Ser, Cys270,273Ser, and Cys-Triple-Ser when compared to the pattern obtained for native GPI-PLC, Cys184Ala, and Cys347Ala. Redistribution of the majority of GPI-PLCp from the anterior of *Leishmania* to the whole cell correlates with the loss of *in vivo* (but not intrinsic) activity against GPIs. Apparently, mistargeting of the enzyme (possibly away from GPIs), enables the parasites to retain

their GPI-positive physiology. These data indicated that cysteines are important for targeting GPI-PLCp to GPIs in *Leishmania*.

In summary, enzyme activity and location to the Golgi region of *Leishmania* are essential for GPI-PLCp to cause a GPI deficiency *in vivo*. Finally, specific Cys residues can dictate the intracellular location of GPI-PLCp in *Leishmania*.

DISCUSSION

GPI-PLCp from *T. brucei* can cleave GPIs, but enzyme activity appears to be suppressed in the protist. Previous studies have provided evidence for several possible mechanisms for regulation of GPI-PLCp activity in *T. brucei*. First, GPI-PLCp is acylated on cysteine residues. Lipid modification influences the turnover rate of the enzyme [1]. A significant amount of GPI-PLCp lipidation is co-translational. Despite acylation's role in activating the enzyme *in vitro*, the lipid modification does not necessarily activate the enzyme *in vivo*. Lipids have been shown to target proteins to specific subcellular locations. Second, GPI-PLCp can form tetramers, which are enzymatically the more active form. GPI-PLCp exists as a (less active) monomer in *T. brucei*. Suppression of GPI-PLCp activity against GPIs in *T. brucei* makes it impossible to study the suppression mechanism of enzyme activity in its native biological system. *T. brucei GPI-PLC* knockouts are available [36], however, complementation of these strains with *GPI-PLC* genes does not ensure that the regulatory mechanism will be eliminated. What one needs are *T. brucei* strains in which the mechanism(s) for suppression of GPI-PLCp activity are no longer functional. Fortunately, GPI-PLCp heterologously expressed in the distantly related kinetoplastid *Leishmania*, results in cleavage of GPIs *in vivo* [23], rendering transgenic parasites GPI deficient. Thus, the *Leishmania* system offers an

excellent opportunity to unravel possible mechanisms controlling GPI-PLCp activity *in vivo*. Some principles learned from this system might be applicable to *T. brucei*.

Importance of cysteines to subcellular localization and regulation of GPI-cleavage

Cysteines appear to be important for GPI-PLCp function and/or regulation. Lipid modification of GPI-PLCp in *T. brucei* and *Leishmania* has been documented and shown to influence catalysis *in vitro* [1, 2].

However, possible contribution of cysteines to the regulation of GPI-PLCp activity *in vivo* have not been explored. For reasons stated earlier, the effect of cysteine mutations on *in vivo* activity of GPI-PLCp was studied in *Leishmania*.

GPI-PLC is targeted to a specific region in Leishmania: mechanisms for Golgi targeting in Leishmania

This is the first report of localization of GPI-PLCp in *Leishmania*. The protein is found in a region anterior to the kinetoplast, possibly the Golgi apparatus, which is found in next to the flagellar pocket [37].

It has been suggested that GPI-PLCp colocalizes with GPI biosynthetic intermediates, which allow the enzyme to cause a GPI deficiency in *Leishmania* [24]. However, GPI biosynthesis is believed to occur on the endoplasmic reticulum [10, 33]. Our data indicate (indirectly) that, at least, *Leishmania* GPIs make an appearance on the cytoplasmic leaflet of the Golgi prior to flipping into the lumen. Alternatively, most GPI synthesis must be taking place at the Golgi, as has been proposed for the lipophosphoglycan (LPG) GPI.

GPI-negative phenotype caused by GPI-PLCp is regulated by intracellular of the enzyme

In the present work, mutations of Cys80 (singly), Cys269, Cys270, and Cys273 (doubly and triply) abrogated activity of GPI-PLCp *in vivo* (Figure 5) without significantly effecting GPI digestion *in vitro* (Figure 4). This suggests that these cysteines play an important role in regulation of GPI-PLCp *in vivo*.

Cysteine dependent targeting of GPI-PLCp to a structure similar to the Golgi [37] in *Leishmania* appears to play a role in controlling the protein's access to GPIs *in vivo* (Figure 9). Mutation of specific cysteines (C80, C269,270,273) in GPI-PLCp resulted in a different sub-cellular localization from that of the native protein in *Leishmania*. Redistribution of GPI-PLCp cysteine mutants from the Golgi region (native protein) to throughout the cell appears to coincide with a loss in the ability of the protein to cause a GPI deficiency. These results support the theory that loss of activity of Cys80Ala, Cys269,270Ser, Cys269,273Ser, Cys270,273Ser, and Cys269,270,273Ser mutants *in vivo* is due, at least in part, to a change in the subcellular local. Since lipid dependent targeting of palmitoylated proteins to biological membranes (e.g. caveolae) has been documented [32], and GPI-PLCp is acylated (by myristate and palmitate) on cysteines, it is possible that the acyl group(s) directs localization of GPI-PLCp in *Leishmania*.

We propose a "cysteine masking" model for GPI-PLCp regulation in both *Leishmania* (Figure 9A) and in *Trypanosoma brucei* (Figure 9B). GPI-PLCp cysteines important to Golgi targeting are exposed in *Leishmania*. These cysteines direct targeting of the protein to GPIs *in vivo* (top panel), resulting in a GPI deficiency, after cleavage of the biosynthetic intermediates. On the other hand, when the targeting signal is removed, GPI-PLCp is found throughout the cell (away from GPI intermediates) (Figure 8). In *T.*

brucei the Golgi targeting signals (cysteines) are “hidden” or “masked” by either an acyl group or some protein that might recognize a protein motif around the cysteine. In this case, the cysteines would be covered up and, thus, GPI-PLCp would be targeted away from GPIs. In this way, *T. brucei* could maintain its GPI positive state, even in the presence of GPI-PLCp.

Despite the convincing evidence presented above, there is a second hypothesis which cannot be eliminated. It is possible that loss in the ability of some GPI-PLCp cysteine mutants to cleave GPIs in *Leishmania* could be due to reduction in enzyme activity *in vivo*. Because acylation increases the turnover rate of GPI-PLCp [1], removal of the acylation site could reduce the quantity of GPIs cleaved. The two cysteine-dependent regulation models of GPI-PLCp activity *in vivo* need not be mutually exclusive.

Table 1: Primers used for sub-cloning and site directed mutagenesis. Primers are listed in the first column. The sequences are given: protein coding sequences are in lower case letters, *BamH* I sites are underlined (solid line), LacZ-ACC 5' UTR is underlined (dotted line). Mismatch bases in the mutagenic primers are indicated in bold. Prefixes "s" and "as" which appear before the primer name denote "sense" and "anti-sense", respectively.

Oligo Name	*Sequence (5→ 3')*
sKCR4	TAAGGATCCTTAACACAGGAGGCAGACCatgtttggtggtgtaaag
asKCR5	TATGTGGATCCTTatgaccttgcggtttggt
asC24A	ttgaccaat**cgc**tttcttctcaatgg
asC80A	agaaagattttg**cgc**acgtcccca
asC184A	ctgccagaggtt**cgc**aagtggtgt
sC269,270S	gcggtggcagcg**tcttct**ggcgcgtgtccc
sC269,273S	gcggtggcagcg**tct**tgtggcgcg**tct**cccggttcacat
sC270,273S	gcagcgtgt**tct**ggcgcg**tct**cccggttcacat
sCtripleS	gcggtggcagcg**tcttct**ggcgcg**tct**cccggttcacat
sC332A	gaaggcact**gcg**actgttaaggga
sC347A	gttgcatta**gcg**gttcatttaaacacc

Figure 1: Graphical map of the *Leishmania* expression plasmid pUTK-GPIPLC.

Figure 1

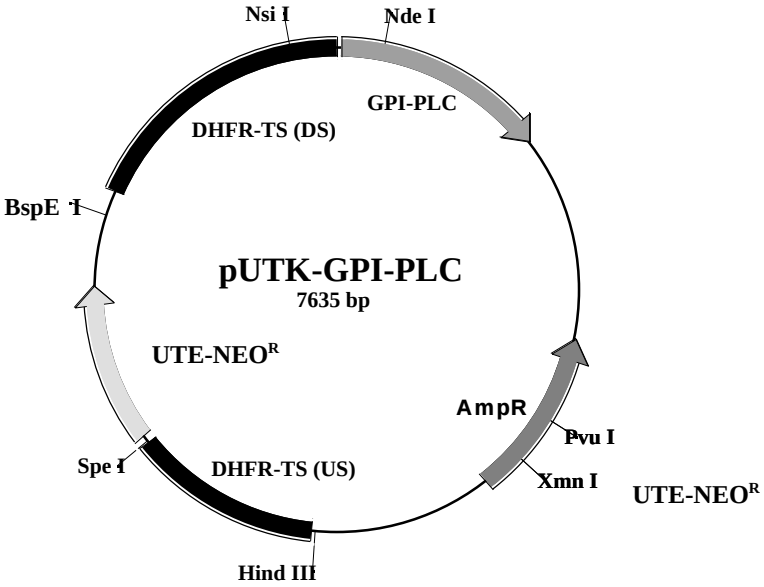
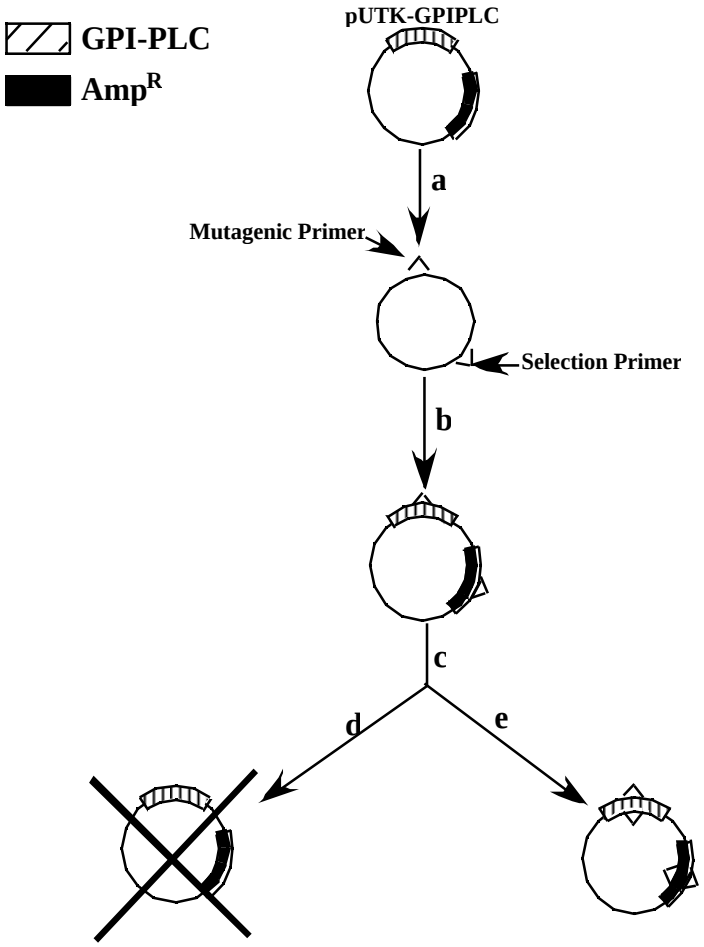

Nsi I
Nde I
GPI-PLC
DHFR-TS (DS)
BspE I
pUTK-GPI-PLC
7635 bp
UTE-NEO^R
AmpR
Pvu I
Xmn I
UTE-NEO^R
Spe I
DHFR-TS (US)
Hind III

Figure 2: Gene Editor mutagenesis of *GPI-PLC*. A) Mutagenic primers were annealed to alkali denatured pUTK-GPIPLC (a); DNA was synthesized and the strands were ligated producing the hybrid plasmid (b); hybrid plasmids were transformed into mutS *E. coli* cells (c); cells containing mutated plasmids (e) survived while cells containing the normal plasmids (d) did not. B) *GPI-PLC* cassette showing the various cysteine mutations. KCR4 and KCR5 added a *BamH* I restriction site onto the ends of the *GPI-PLC* (cross lines in the arrows) cassette allowing insertion of the unmutagenized *GPI-PLC* into the *Bgl* II site of pUTE-Kana^{R}. Cysteines and their respective mutations are noted (stars). Within the COOH-terminal cluster of cysteines (bracket) there were 4 different mutants: i) C269,270S; ii) C269,273S; iii) C270,273S, iv) C269,270,273S (C-Triple-S).

Figure 2

A


GPI-PLC
Amp^R
pUTK-GPIPLC
a
Mutagenic Primer
Selection Primer
b
c
d
e

B

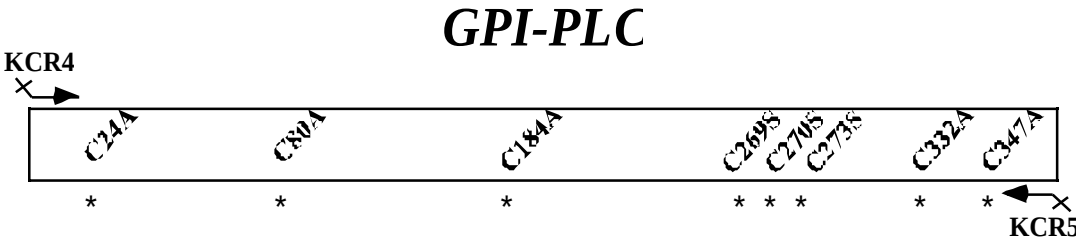

GPI-PLC
KCR4
C24A
C80A
C184A
C269S
C270S
C273S
C332A
C347A
KCR5

Figure 3: Immunoblot of GPI-PLCp expressed in *L. major*. Transfected parasites (5×10^8 mid log phase) were lysed hypotonically and GPI-PLCp was immunoprecipitated with the monoclonal antibody 2A6-6. Adsorbed proteins were separated on a 14% SDS-PAGE minigel, transferred onto an Immobilon-P membrane, and probed with the polyclonal antibody R18B3. GPI-PLCp (39 kDa) is indicated by the arrow. The heavy and light chains of mc2A6-6 are noted by asterisks (*).

Figure 3

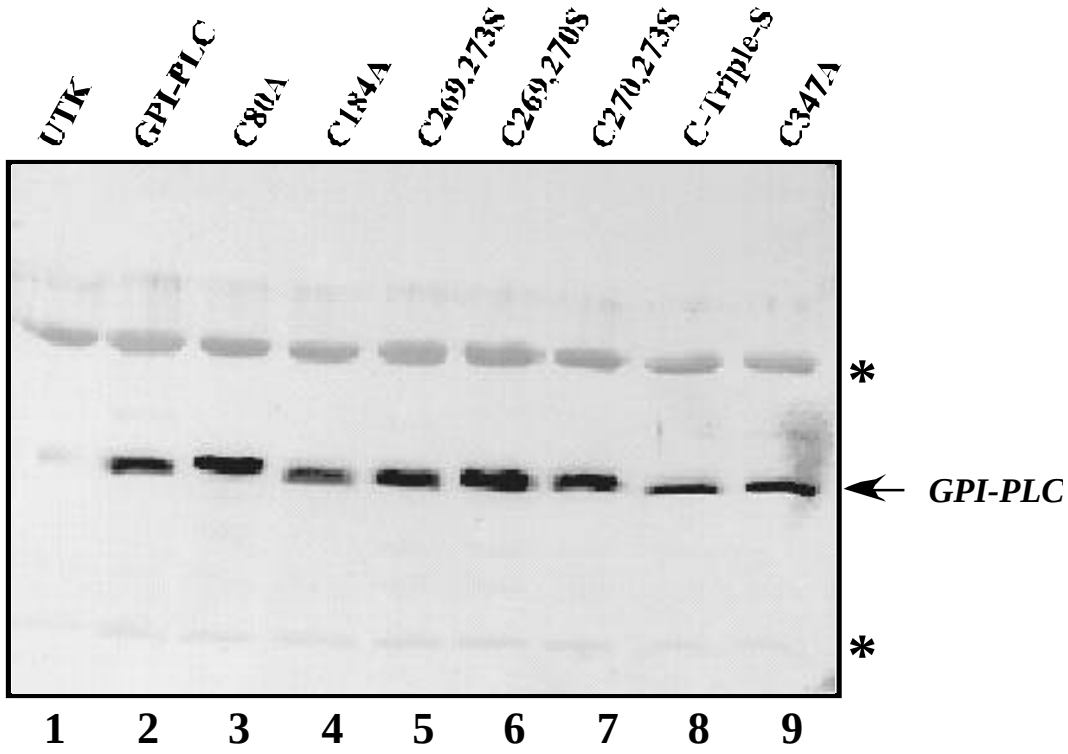
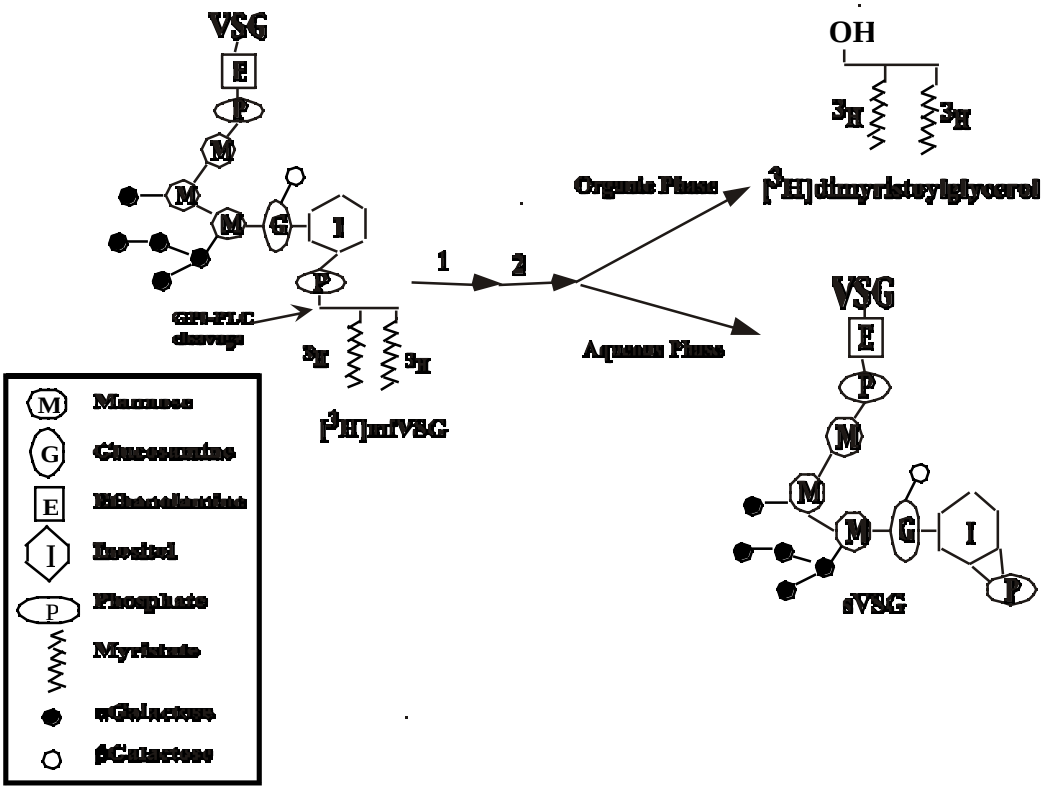

UTK
GPI-PLC
C80A
C184A
C269,273S
C269,270S
C270,273S
C-Triple-S
C347A
*
GPI-PLC
*
1
2
3
4
5
6
7
8
9

Figure 4: Intrinsic activity of GPI-PLCp cysteine mutants. A) Schematic of GPI-cleavage assay. 1) Detergent extract from *L. major* transfected with *GPI-PLC* was assayed for enzyme activity. Total protein concentrations were determined by a bicinchoninic acid protein detection kit. B) The native protein was set at 100% and cysteine mutant activities were compared as relative specific activity. 100% specific activity corresponds to 5200 dpm of released [H^3]dimyristoylglycerol. A background of 155 dpm was subtracted.

Figure 4

A


VSG
E
P
M
M
M
G
I
P
GPI-PLC cleavage
^{3}H
^{3}H
[^{3}H]myrVSG
1
2
Organic Phase
OH
^{3}H
^{3}H
[^{3}H]dimyristoylglycerol
Aqueous Phase
VSG
E
P
M
M
M
G
I
P
aVSG
M Mannose
G Glucosamine
E Ethanolamine
I Inositol
P Phosphate
Myristate
αGalactose
βGalactose

B

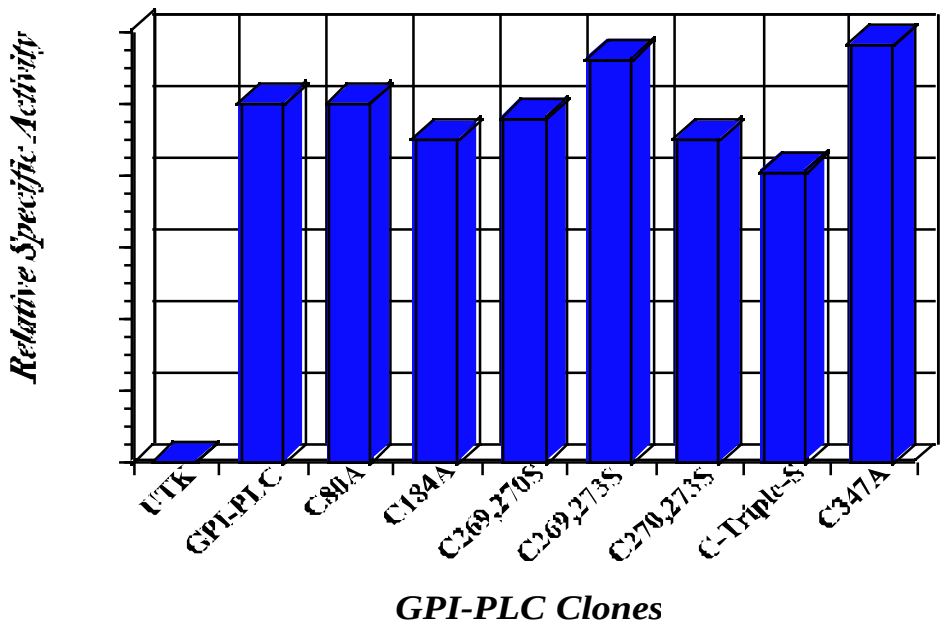

Relative Specific Activity
UTK
GPI-PLC
C80A
C184A
C269,270S
C269,273S
C270,273S
C-Triple-S
C347A
GPI-PLC Clones

Figure 5: Effect of GPI-PLCp on the presence of gp63p in *L. major*. Transfected parasites (5×10^7 mid-log phase) were lysed hypotonically. Membrane-bound proteins were separated on a 14% SDS-PAGE minigel and transferred onto an Immobilon P membrane. Proteins were detected by either anti-gp63, or Con A immunoblotting. Gp63p (63 kDa) (top panel) is indicated. Con A positive band (middle panel) and a band present due to non-specific binding of the anti-gp63 (bottom panel) were used as a loading controls for panel A.

Figure 5

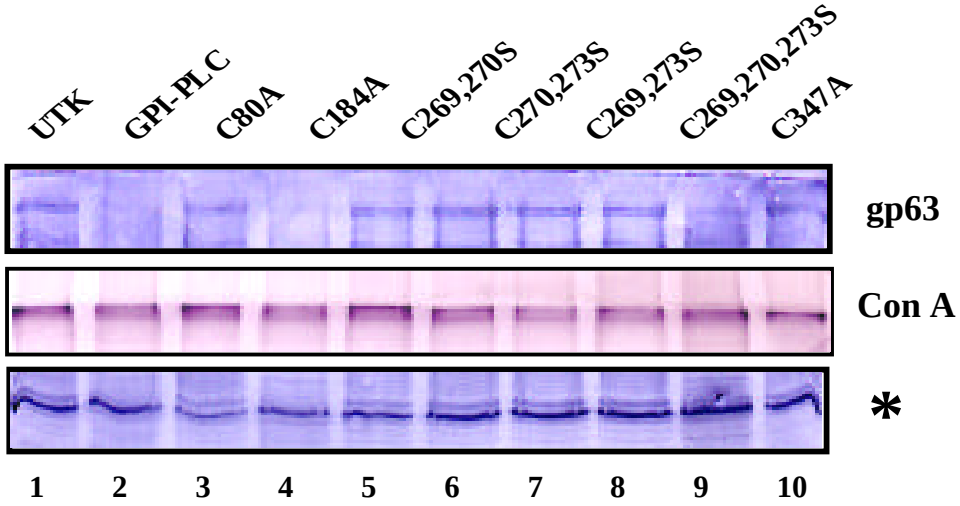
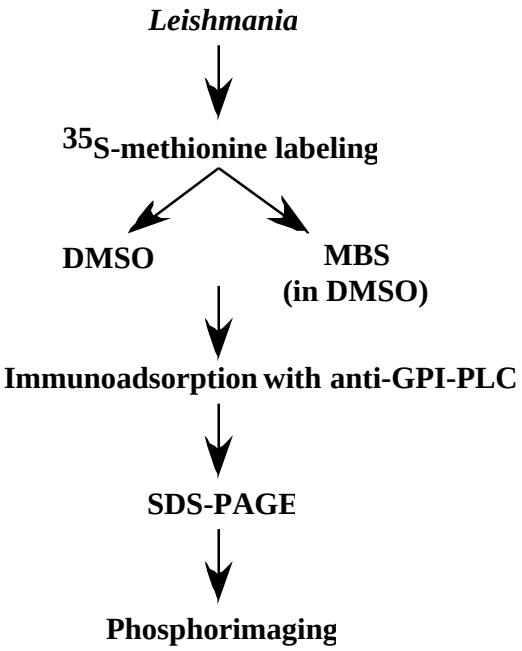

UTK
GPI-PLC
C80A
C184A
C269,270S
C270,273S
C269,273S
C269,270,273S
C347A
gp63
Con A
*
1
2
3
4
5
6
7
8
9
10

Figure 6: GPI-PLC cysteine mutants can form oligomers in *L. major*. Schematic of the experiment performed. [^{35}S]Met-labeled GPI-PLCp was either crosslinked with MBS or treated with DMSO (as a control) and immunoprecipitated using the monoclonal antibody 2A6-6. Immunoadsorbed proteins were solubilized from sepharose beads by addition of 20 µl of SDS-PAGE sample buffer and electrophoresed on an SDS minigel (14%). Proteins were visualized by phosphorimaging. The monomeric, tetrameric, and "GPI-PLC complex" are indicated by the arrows.

Figure 6


Leishmania
^{35}S-methionine labeling
DMSO
MBS
(in DMSO)
Immunoadsorption with anti-GPI-PLC
SDS-PAGE
Phosphorimaging

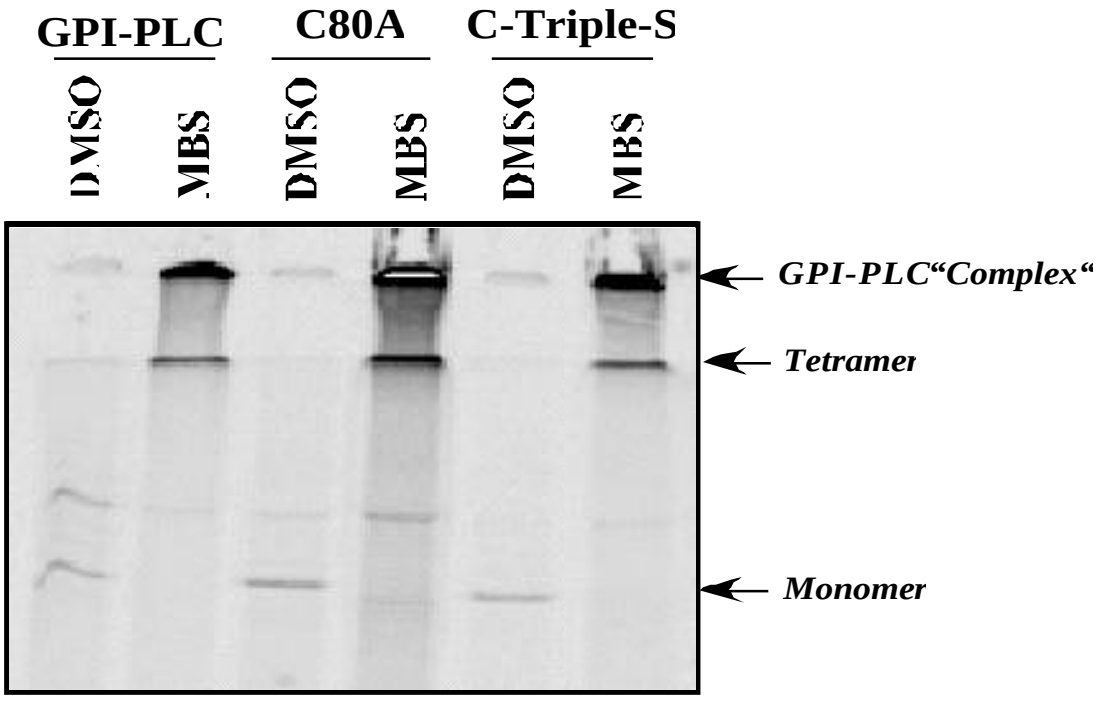

GPI-PLC
C80A
C-Triple-S
DMSO
MBS
DMSO
MBS
DMSO
MBS
GPI-PLC"Complex"
Tetramer
Monomer

Figure 7: Localization of GPI-PLC and BiP. *Leishmania* were adhered to poly-L-lysine oversleeps, fixed and permeabilized. Cells with either pUTK (A, B) or pUTK-GPIPLC (B, C, D, E) were probed with the GPI-PLC specific polyclonal antibody RC300 (A, B, C, D) or anti-BiP polyclonal antibody (E, F). Cells were mounted in anti-fade solution and DAPI. Proteins were detected with Alexafluor 288-conjugated anti-rabbit. Blue color shows DNA, green color indicates GPI-PLC, and red color reveals BiPp. Panels A, C, and E are images of only protein while panels B, D, and F show the DIC image merged with protein and DAPI staining.

Figure 7

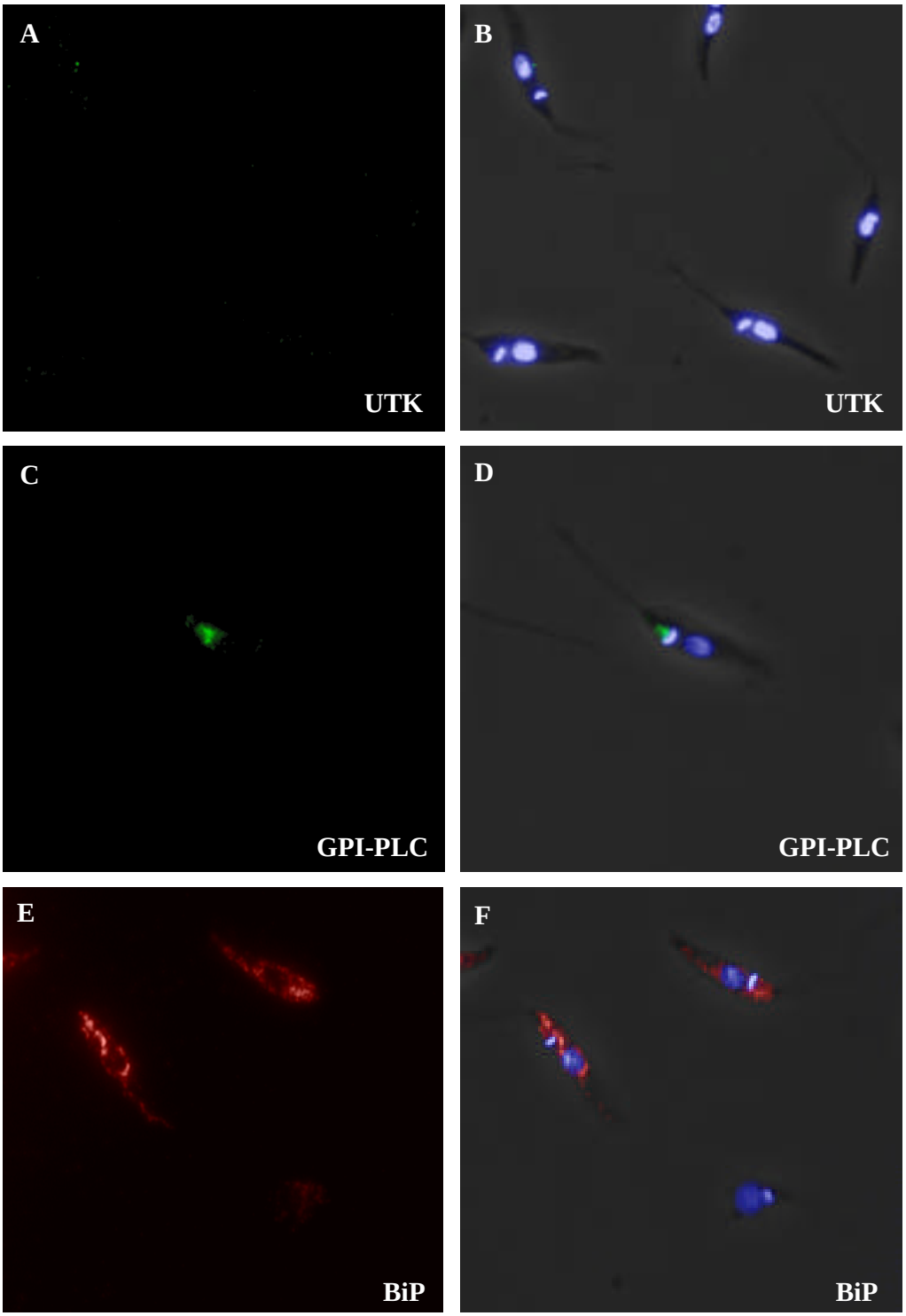


Figure 8: Localization of cysteine mutants of GPI-PLCp. Cells were prepared, fixed and permeabilized as in Figure 7. Fixed and permeabilized cells were incubated with RC300 and detected with Alexafluor 288-conjugated anti-rabbit antibody. Each panel shows a DIC image merged with the GPI-PLC (green staining) and the DAPI (blue staining) stain. A) GPI-PLCp, B) Cys184Ala, C) Cys347Ala, D) Gln81Ala, E) Cys80Ala, F) Cys269,270Ser, G) Cys269,273Ser, H) Cys270,273Ser, I) Cys26,270,273Ser.

Figure 8

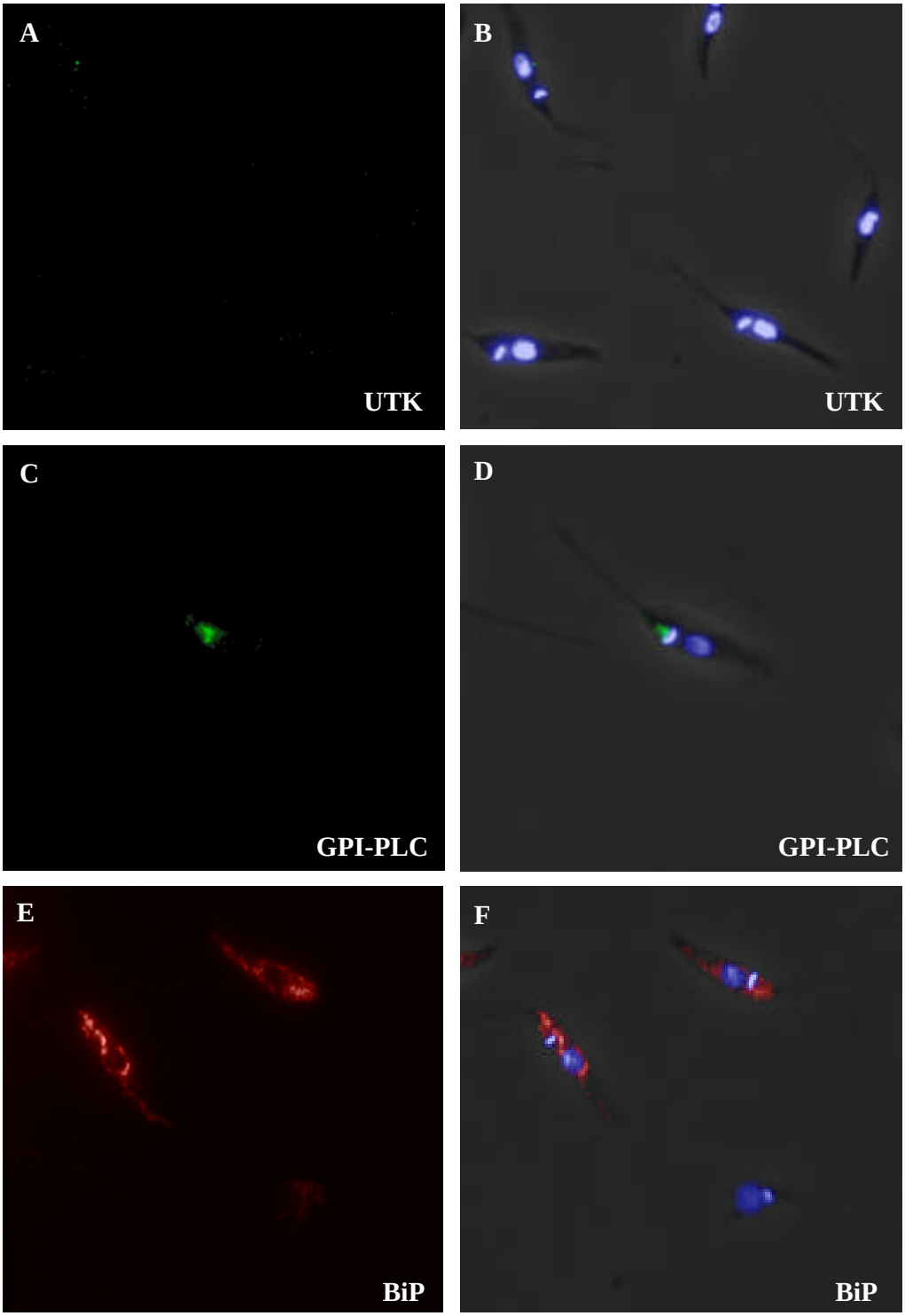
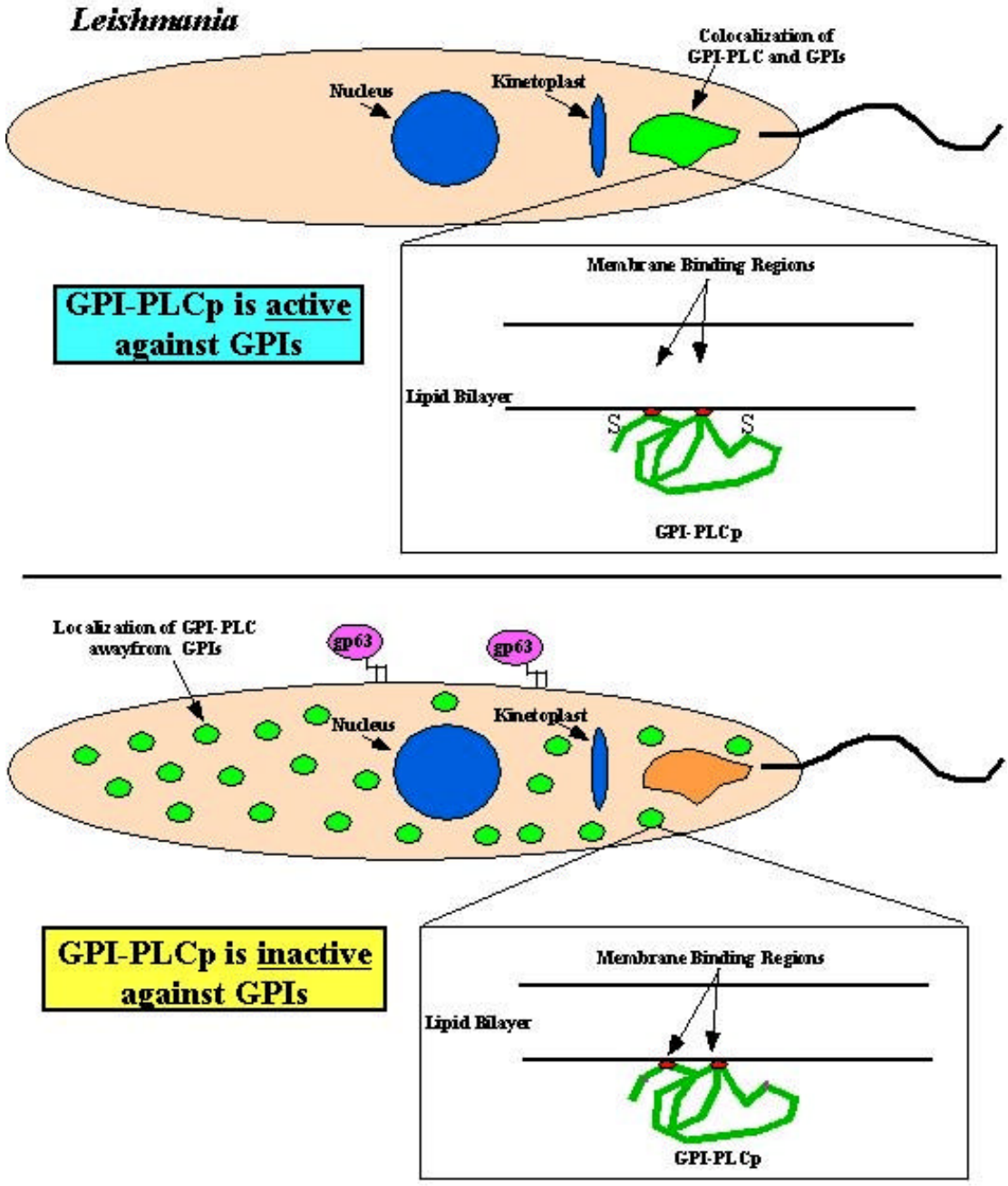
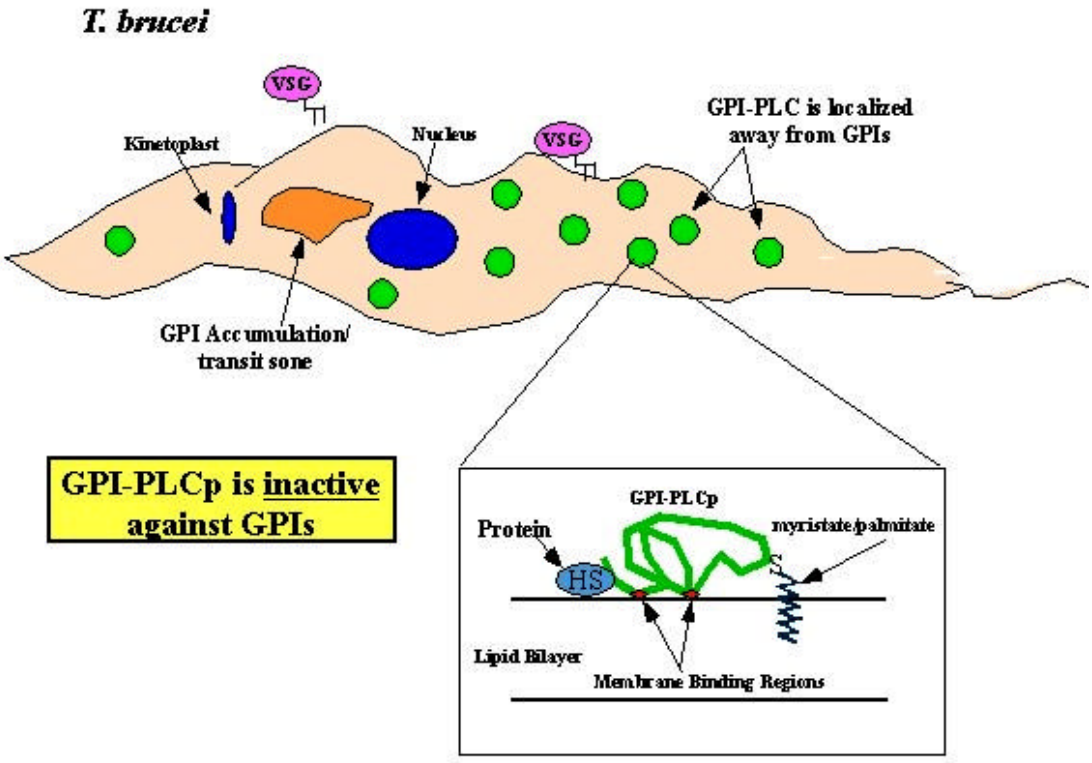


Figure 9: Model of cysteine dependent regulation of GPI-PLC in *Leishmania* (A) and *Trypanosoma brucei* (B).

Figure 9


A
Leishmania
Colocalization of GPI-PLC and GPIs
Nucleus
Kinetoplast
Membrane Binding Regions
Lipid Bilayer
S
S
GPI-PLCp
GPI-PLCp is active against GPIs
Localization of GPI-PLC away from GPIs
gp63
gp63
Nucleus
Kinetoplast
Membrane Binding Regions
Lipid Bilayer
GPI-PLCp
GPI-PLCp is inactive against GPIs

B


T. brucei
VSG
Kinetoplast
Nucleus
VSG
GPI-PLC is localized away from GPIs
GPI Accumulation/ transit zone
GPI-PLCp is inactive against GPIs
GPI-PLCp
Protein
myristate/palmitate
HS
Lipid Bilayer
Membrane Binding Regions

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CHAPTER III

IDENTIFICATION OF MEMBRANE BINDING REGIONS OF A GPI-PHOSPHOLIPASE C FROM *TRYPANOSOMA BRUCEI*[1]

[1]Butler, K.D. and Mensa-Wilmot, K. 2000. To be submitted to Molecular and Biochemical Parisitology.

ABSTRACT

The African trypanosome, *Trypanosoma brucei*, expresses a glycosylphosphatidylinositol-specific phospholipase C (GPI-PLCp). Biochemical analysis indicates that GPI-PLCp is an integral membrane protein. Surprisingly, methods commonly used to predict membrane spanning domains of proteins do not identify any such regions in GPI-PLCp. In an attempt to elucidate the mechanism by which GPI-PLCp integrates into lipid bilayers, amino and carboxy terminal fragments of GPI-PLCp were fused to green fluorescent protein (GFPp) (a soluble polypeptide), and the chimeras tested for their ability to bind *T. brucei* membranes post-translationally. Two segments, amino acid 60-120 and residues 238-298 of GPI-PLCp, termed membrane binding region 1 (MBR-1) and MBR-2, respectively, proved sufficient to direct membrane binding of GFPp. Secondary structure predictions indicate that both MBR-1 and MBR-2 contain a stretches of amino acids that could form amphipathic α-helices. We propose that GPI-PLCp binds to biological membranes by interactions between the hydrophobic faces of the amphipathic α-helices and one leaflet of a lipid bilayer. Thus, GPI-PLCp is an integral, but not transmembrane, protein.

The abbreviations used are: *BSF*, blood stream form; *dATP*, deoxyadenosine triphosphate; *dCTP*, deoxycytidine triphosphate; *dGTP*, deoxyguanosine triphosphate; *DTT*, dithiothreitol, *dUTP*, deoxyuridine triphosphate; *GFP*, green fluorescent protein; *GPI-PLC*, glycosylphosphatidylinositol phospholipase C; *NP-40*, nonidet P-40; *PAGE*, polyacrylamide gel electrophoresis; *PBS*, phosphate buffered saline; *PCR*, polymerase chain reaction; *PIC*, protease inhibitor cocktail; *PRB*, pellet resuspension buffer; *DB*, dilution buffer; *RMB*, rough microsome buffer; *SDS*, sodium dodecyl sulfate; *TLCK*, N-toysl-L-lysine chloro-methyl-ketone; *UTR*, untranslated region.

INTRODUCTION

Glycosylphosphatidylinositol-specific phospholipase C (GPI-PLCp) from *Trypanosoma brucei* is an integral membrane protein [2, 6, 8, 11, 12]. The enzyme is associated with the pellet of a hypotonic cell lysate, and the membrane bound enzyme is not extracted by reagents that solubilize peripheral membrane proteins (e.g. 1M NaCl and 20 mM EDTA) [11]. Solubilization of GPI-PLCp is not achieved by low detergent concentrations (such as 0.1% nonidet P-40 (NP-40)); high concentration of detergent of NP-40 (1%) are needed to remove GPI-PLCp from membranes [11]. Lastly, purified GPI-PLCp is found in the detergent fraction (instead of the aqueous) after Triton X-114 phase partitioning [11]. This tight association of GPI-PLCp with biological membranes and/or detergent micelles is exhibited by recombinant protein expressed in *E. coli* (R. Howard, unpublished data), suggesting that membrane binding does not require eukaryotic-specific modifications (e.g. lipidation).

Surprisingly, hydropathy plots (e.g. Kyte-Doolittle analysis) [10] show no apparent membrane spanning domain within the primary structure of GPI-PLCp [8]. This fact has led to claims that GPI-PLCp may not be a membrane protein. Preliminary evidence suggests that secondary structure of the protein is important for membrane binding (R. Howard, unpublished results). While relatively mild extraction methods (e.g. with high salt or high pH) fail to separate recombinant GPI-PLCp from *E. coli* membranes, chaotropic agents (e.g. 8M urea or 6M guanidinium hydrochloride), which are known to denature proteins, solubilize GPI-PLCp (R. Howard *et al*, unpublished data).

This work is an attempt to resolve the discrepancy between the biochemical data and the prediction from the hydropathy analysis. The immediate objective is to determine specific regions of GPI-PLCp that may be important for membrane binding. To this end, NH_2- and COOH-terminal truncations of GPI-PLCp were fused to the green fluorescent protein (GFPp) as a soluble reporter. The ability of the GPI-PLCp fragments to adsorb GFPp to *T. brucei* membranes was determined. We have found two distinct regions within GPI-PLCp that are sufficient to target GFPp to *T. brucei* membranes. We speculate how peptides within these membrane binding "domains" might mediate binding of GPI-PLCp to biological membranes.

MATERIALS AND METHODS

Plasmids

The plasmids CO64 and pCO59 were from Dr. George Cross (Rockefeller University): contain NH_2- and COOH-terminal fusions, respectively, of *GPI-PLC* to *GFP* (Fig.1, panel A). These vectors were used as templates to generate various truncated *GPI-PLC:GFP* fusions.

Generation of DNA fragments encoding GPI-PLC truncations

Polymerase chain reaction (PCR) was used to delete portions of the *GPI-PLC* gene (Figure 1A), resulting in the DNA sequences for production of NH_2- and C00H-terminal portions of the *GPI-PLC* coding region fused to *GFP* (Fig. 1B). All forward primers (Table 1) include a T7 promoter for RNA synthesis *in vitro* and a 5' untranslated region (UTR) to enhance translation [17]. All reverse primers (Table 1) introduced a stop codon downstream of the coding region.

The PCR reaction contained a buffer (10 mM Tris-HCl pH8, 2.5 mM MgCl2, 0.05% Tween 20, 0.05% nonidet-P 40, 50 mM KCl, 0.25 mM of each deoxynucleotide and 20 μM primers, forward and reverse), 50 ng of DNA template, and 5 U of *Taq* DNA polymerase [9]. Cycling conditions were i) denaturation at 95°C for 90 seconds, ii) annealing at 56°C for 90 seconds, and, iii) extension at 74°C for 2 min. Twenty-five cycles were performed, to which a final extension at 74°C for 7 min was appended. Amplified products were purified using the Wizard PCR DNA purification system (Promega) and subsequently resuspended in nuclease-free water to a final concentration of 1 μg/μl as determined at OD_{260}. Aliquots (500 ng) were electrophoresed on a 1% agarose gel to verify the product size, and ascertain the DNA concentration.

In Vitro Transcription of DNA

Purified DNA fragments were transcribed using the Ampliscribe™ T7 Kit (Epicenter Technologies, town, state). The reaction (20 μl final volume) was set up as follows: 1 μg purified DNA template, 1X T_7 reaction buffer, 7.5 mM of each nucleotide (ATP, GTP, CTP, UTP), 10 mM dithiothreitol (DTT), 2 μl of the T7 RNA polymerase, and the reaction was taken up to 20 μl with nuclease-free water. All of the reagents (except the template) were provided in the Epicenter kit. Reactions were carried out for 2 h at 37°C. RNA was purified with a protocol and reagents provided by the Ampliscribe™ T7 Kit. One unit of RNase free DNase I was added to the reaction and incubated at 37°C for 15 min. The DNase treated reaction was extracted with 20 μl of H_2O-saturated phenol/chloroform, and ethanol precipitated by adding sodium acetate (pH) to a final concentration of 0.3 M, followed by 2.5 volumes of absolute ethanol and incubating at -20°C for 30 min. The precipitated RNA was pelleted (14,000 x g, 15 min, 4°C). RNA

was resuspended in 10 µl of nuclease-free water, followed by a reading at OD_{260} to determine the concentration. Samples were diluted to a concentration of 1 µg/ml with nuclease-free water. RNA concentration was verified by electrophoresing 500 ng on a 1% agarose gel [15].

In vitro protein synthesis

The reagents for an *in vitro* translation reaction (60 µl) were added in the following order: i) 4 µg RNA (in 4 µl of nuclease free water), ii) 17.2 µl nuclease-free water, iii) 2 µl of 1 mM methionine-free amino acid mixture (Promega), iv) 2 µl of RNA inhibitor (RNAsin) (Promega), v) 4.8 µl (4.8 µCi) [^{35}S]methionine, and vi) 30 µl of rabbit reticulocyte lysate (Promega). The translation reaction was mixed thoroughly by repeated pipetting, and incubated at 30°C for 90 min.

Preparation of T. brucei microsomes

A pellet of 2 x 10^9 blood stream *T. brucei* (BSF) (donated by S. Subramanya [4]) was washed in PBS and resuspended in 1 ml of homogenization buffer (HB) (250 mM sucrose, 50 mM KOAc, 6 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM DTT) and lysed by sonication (Vibra Cell Ultrasonicator, model #VC-501, at an amplitude of 20 for 10 min). The cells were in 1.5 ml microfuge tubes that were floated in an ice water bath at 0°C). The mixture was centrifuged (2000 x g, 10 min, 4°C) to sediment large cellular debris. The supernatant was centrifuged (14,000 x g for 20 min at 4°C) and the pellet was resuspended in 25-50 µl of rough microsome buffer (RMB) (250 mM sucrose, 50 mM HEPES (pH 7.2), 50 mM KOAc, 12 mM $Mg(OAc)_2$, 1 mM DTT) supplemented with 1 µg/ml TLCK and 5 µg/ml leupeptin. The OD_{260} of a 1:50 dilution of the resuspended membranes in 0.5% SDS was determined. From this determination the microsome stock

was diluted with RMB to a final OD_{260} of 10, aliquoted in 25 µl portions, quick frozen in liquid nitrogen, and stored at -80°C. Twenty-six microliters of these microsomes (total OD_{260} of 260) were used in the GPI-PLCp cosedimentation experiments (see below).

Post-translational binding of proteins to membranes and co-sedimentation analysis

Twenty microliters of an *in vitro* translation reaction were mixed with 32 µl of RMB containing a PIC, either with or without *T. brucei* membranes (260 OD_{260} units). These samples were incubated at 30°C for 10 min to allow the protein to bind the membranes. Next, 100 µl of dilution buffer (DB) (100 mM KOAc, 20 mM HEPES-KOH pH7.2, 2.5 mM $Mg(OAc)_2$, 20 mM EDTA) was added and the samples were incubated on ice for 10-15 min. This sample was laid on top of 70 µl sucrose cushion (500 mM sucrose, 20 mM HEPES-KOH, pH 7.2) and centrifuged at 100,000 x g for 30 min at 4°C (52,000 rpm in a 100.3 rotor, (Beckman) Optimaä TL Ultracentrifuge). The supernatant (S) was transferred to a fresh microfuge tube. The pellet (P), containing membrane-bound protein, was solubilized by suspending in 70 µl of pellet resuspension buffer (PRB) (500 mM neat Tris base, 5% SDS, 10 mM DTT) and heating at 55°C for 15 min [13]. Equivalent volumes of each fraction (i.e. 2 µl of the translation reaction (T), 22 µl of S, and 7 µl of P) were separated by SDS-PAGE (14% mini gel), and radiolabeled proteins were visualized by phosphorimaging (Personal Molecular Imager FX, BioRad). Quantity One Software (BioRad) was used to quantitate amount of signal in each band.

RESULTS

Development of an assay for measuring GPI-PLCp membrane binding in vitro

GPI-PLCp, GFPp, and gene fusions whose transcripts were successfully translated *in vitro* and produced proteins of the correct size are depicted (Figure 2). The

full length GPI-PLC:GFP fusion showed a faster migrating truncated protein on the gel, is due either to proteolysis or internal initiation of protein synthesis (Figure 2, lane 7).

In a living *T. brucei*, GPI-PLCp membrane binding may have a co-translational component. Attempts were made to carry out the membrane binding assay co-translationally, but the addition of *T. brucei* membranes greatly inhibited translation in the reticulocyte system (data not shown). This technical limitation restricted our assays to post-translational membrane binding events. Given that GPI-PLCp localizes to the Golgi/lysosome region in *Leishmania* (Chapter 2), it is possible that post-translational membrane association features predominantly in GPI-PLCp interactions with cell membranes. The protein lacks and NH_2-terminal signal sequence for insertion into the ER membrane.

Unfused GFPp and GPI-PLCp proteins were necessary controls to explore the effectiveness of the co-sedimentation assay for determining the membrane binding regions of the trypanosome protein. GFPp is a soluble protein [3] and not pelletable either in the absence, or presence of *T. brucei* membranes (Figure 3B, compare lanes 1 and 2 to lanes 3 and 4). With GPI-PLCp, the majority (75%) of the protein is found in the supernatant when membranes are absent (Figure 3B, lanes 5 and 6). There is some of protein in the pelleted fraction (Figure 3B, lane 6), which is possibly due to the presence of small amounts of membranes in the rabbit reticulocyte lysate to which GPI-PLCp could be binding. Another possibility is that GPI-PLCp is forming large aggregates, which are pelleted during the analysis. More importantly, addition of *T. brucei* membranes causes a greater proportion (60%) of GPI-PLCp to pellet (Figure 3, compare lanes 5 and 6 with lanes 7 and 8). Despite the significant membrane-dependent shift in

the proportion of the fusion protein from the supernatant to the pellet in the presence of membranes, 100% of the protein was never found in the pellet. This might be because of pre-adsorption of GPI-PLCp to membranes in the reticulocyte lysate as noted earlier.

These data show GPI-PLCp, unlike GFPp, binds to *T. brucei* membranes post-translationally. Consequently, fusions of GPI-PLCp segments to GFPp can be studied in the presence of *T. brucei* membranes to identify membrane binding regions.

Amino acid residues 60-120 of GPI-PLC are sufficient to direct membrane binding

In order to determine the specific regions which are important for membrane binding, the ability of NH*2*-terminal portions of GPI-PLCp to target GFPp to *T. brucei* membranes was tested (Figure 4). The full-length fusion protein where GPI-PLCp is joined to the COOH-terminus of GFPp (Figure 1) bound to *T. brucei* membranes (Figure 4, compare lanes 1 and 2 to lanes 3 and 4). Note the reversal in the distribution of protein between the supernatant and pellet fractions upon the addition of membranes (compare lanes 1 and 2 with lanes 3 and 4). Without membranes, the protein is primarily non-sedimentable (23% in the pellet), while the addition of membrane results in pelleting of 63% of the polypeptide. An alteration in distribution between the soluble and pelletable fractions is also observed when residues 1-179 of GPI-PLCp are linked to GFPp. In the absence of membranes, 25% of the protein is in the supernatant. Addition of membranes results in 50% of the protein in the pellet. Thus, the NH_2-terminal half of GPI-PLCp is capable of targeting GFPp to membranes (Figure 4, compare lanes 5 and 6 to lanes 7 and 8). Additionally, residues 1-120 successfully targeted GFPp to membranes (Figure 4, compare lanes 9 and 10 to 11 and 12) as measured by an alteration in the amount of protein found in the pellet as compared to the supernatant. However, when fused to GFPp

the first sixty amino acids of the GPI-PLCp do not increase the fraction of GFPp that is pelleted upon the addition of *T. brucei* membranes. Therefore, amino acids 1-60 of GPI-PLCp are not sufficient to target the reporter protein (GFPp) to membranes (Figure 4, lanes 13-16).

Together, these data indicate residues 60-120 of GPI-PLCp are sufficient to target GFPp to *T. brucei* membranes.

A second membrane binding region is present in the COOH-terminal half of GPI-PLC

Previous studies performed in *E. coli* indicated the possible existence of a membrane binding region in the COOH-terminal half of GPI-PLCp (Al-Qahtani, unpublished). Therefore, we were interested in I) determining whether the COOH-terminal half of GPI-PLCp could target GFPp to *T. brucei* membranes, and ii) deciphering a specific peptide segment in the COOH-terminal half that might mediate membrane binding. Towards this end, COOH-terminal fragments of GPI-PLCp were fused to the NH_2-terminus of GFPp (see Figure 1) and assessed for their ability to bind membranes (Figure 5). The full-length fusion protein binds to *T. brucei* membranes judging by the increase in the fraction of protein detected in the pellet (63%; up from 27%) upon addition of microsomes (Figure 5, lanes 1-4). Additionally, the COOH-terminal half of GPI-PLCp targeted GFPp to *T. brucei* membranes (Figure 5, lanes 5-8). After addition of membranes the distribution of protein between the supernatant and the pellet is reversed (from 25% to 60% protein in the pellet). Membrane targeting of GFPp was also observed with residues 238-358 of GPI-PLCp (Figure 5, compare lanes 9 and 10 to 11 and 12). Note the increase in the percentage of protein in the pellet from 36% to 60% upon the addition of membranes. A small increase in the fraction of protein found in

the pellet upon the addition of membranes was observed. However, membranes not did significantly effect the ratio of protein between the supernatant and the pellet, as happened with the other two COOH-terminal constructs. Therefore, the last sixty amino acids (298-358) do not contribute to the targeting of GFPp to *T. brucei* membranes (Figure 5, lanes 13-16).

In summary, these results indicate that the region between residues 238 and 298 are the adequate for binding GPI-PLCp to membranes.

DISCUSSION

Integral membrane proteins can be defined by two different sets of criterion. The first is based on biochemical properties, while the other hinges on predictions of the presence of membrane spanning regions using hydropathy plots. Biochemically, a membrane protein is considered integral if it is resistant to extraction with high salt (e.g., 1 M NaCl); concurrently it should be solubilized from membranes by reagents which disrupt the lipid bilayer (e.g. detergents). Hydropathy plots match the hydropathy index, which is the average hydrophobicity within a defined window [10]. The window used is usually 19 amino acids, chosen because that is the number of amino acids necessary to span the lipid bilayer in an α-helix [10].

Biochemically, GPI-PLCp is an integral membrane protein [2, 6, 8, 11, 12], but hydropathy analyses have failed to identify a trans-membrane region for the polypeptide [8]. It is possible that the biochemical data is correct, and that a palatable explanation exists for why computer analyses have failed to detect membrane-binding regions. Key to resolving the conundrum, is the ability to identify membrane binding regions in GPI-PLCp. Perhaps the biophysical properties of these membrane binding regions (MBR) will

reveal why computer analysis missed their identification. In the this work, we present two regions of GPI-PLCp, amino acids 60-120 and 238-298, which are sufficient to target a GFPp reporter to *T. brucei* membranes (Figure 4 and Figure 5).

Predicted properties of the membrane binding regions

Hydropathy plots [10] are classically used to predict potential trans-membrane regions of proteins within a window of 19 amino acids, which is the number of amino acids required to form a membrane spanning α-helix. On the Kyte-Doolittle scale, a value greater 1.25 is the lowest value recorded for a known trans-membrane protein [7], therefore this is the baseline to which other proteins might be compared. A peptide segment with a peak value above 1.25 is a possible membrane spanning domain [7].

An example of a hydropathy plot (19 amino acid window) using the Kyte-Doolittle scale is shown for *Saccharomysis cerevisiae* Sec61p (Figure 6A), a protein known to span the lipid bilayer 10 times [18] (Figure 6B). This plot predicts peptide hydropathy peaks ranging from 2 to 2.83, suggesting that Sec61p has trans-membrane regions. These membrane spanning domains have been proven experimentally [18], allowing for the production of the diagram of the protein (Figure 6B).

For GPI-PLCp, hydropathy analysis (19 residue window) does not predict any membranes-spanning regions (Figure 7A), suggesting that the polypeptide is not an trans-membrane protein. However, biochemical data indicates that the protein is bound integrally to membranes [2, 6, 8, 11, 12]. We hypothesize that GPI-PLCp binds to membranes by hydrophobic patches present in the protein's secondary structure.

To test the validity of this theory, we analyzed GPI-PLCp by hydropathy plot using a window of 8 amino acids (Fig. 7B). A series of alanines, the least aliphatic of the

non-polar amino acids, scores a 1.8 on the Kyte-Doolittle scale. Thus, a value of 1.8 (Kyte-Doolittle scale) was chosen to represent the minimum hydrophobicity needed to bind membranes. Using these parameters, five peaks of at least 1.8 were identified. Of these, one occurred in each of the two membrane binding regions (amino acids 60-120 (MBR-1) and 238-358 (MBR-2)) found in this study to direct membrane binding (Fig. 7B, asterisks). MBR-1 and MBR-2 were analyzed further using helical wheel analysis.

Amphipathic α-helices can be identified by helical wheel analysis. Such a survey of MBR-1 and MBR-2 is presented (Fig. 8A). Hydrophobic amino acids (valine, leucine, and phenylalanine) are found on one side of the proposed α-helix in MBR-1 (Figure 8A, black arch), forming a hydrophobic patch that could interact with a lipid bilayer. Similarly, MBR-2 shows the clustering of cysteines, valine, alanine, and phenylalanine on one side of a possible helix making another hydrophobic patch (black arch) that could contribute to membrane binding (Figure 8B). We propose that “hydrophobic patches” from MBR-1 and MBR-2 of GPI-PLCp interact with one leaflet of the lipid bilayer as shown in Fig. 9B. In support of this model, chaotropic agents which do not disrupt the lipid bilayer (e.g. 8 M urea), solubilize GPI-PLCp (R. Howard, unpublished data). However, high salt and high pH fail to remove GPI-PLCp from membranes (Al-Qahtani, unpublished).

The membrane binding mechanism proposed here operates in least two other proteins. Membrane binding of prostaglandin H_2 synthase-1 [14, 16] and mammalian PI-PLCδ [5] depend amphipathic α-helices which form “hydrophobic patches.” With GPI-

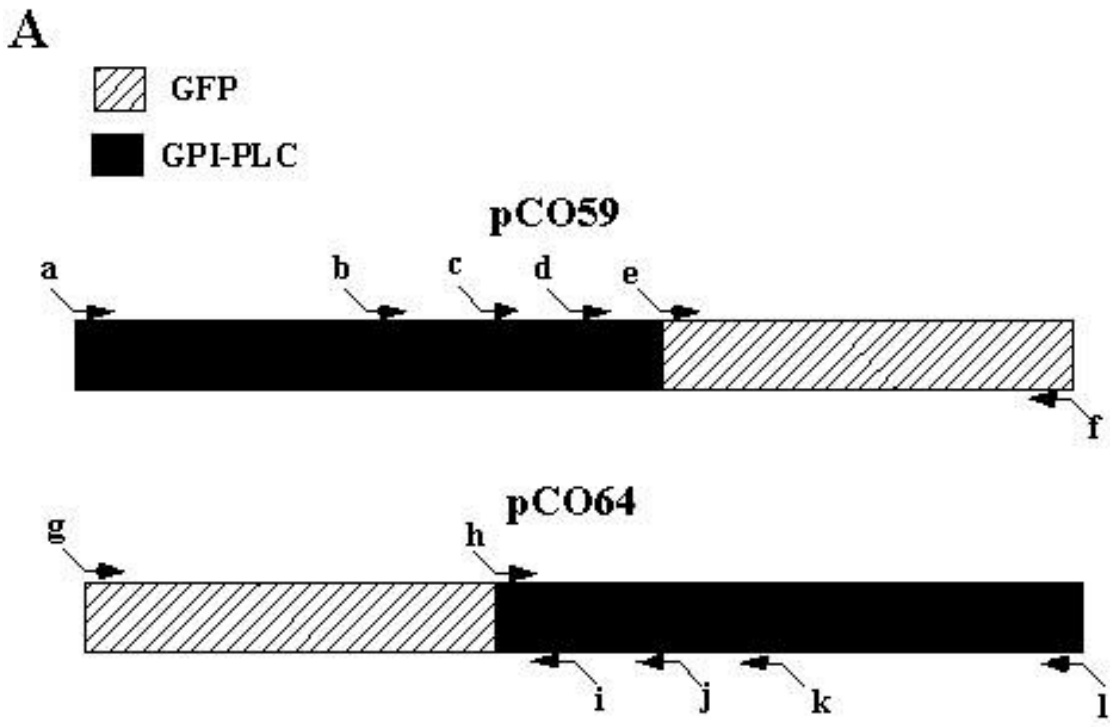
PLCp two distinct regions may be in operation (Figure 9A). Our model reconciles the observations on the biochemical properties of GPI-PLCp (i.e., tight binding to membranes) and computer assisted predictions of trans-membrane domains: The enzyme is an integral membrane protein but it does not span the phospholipid bilayer.

Table 1: Primers used in PCR to generate GPI-PLC fragments fused to GFP. Primers are listed in the first column according to their corresponding letter (see Figure 1). Sequences and primer features are given. For all primers the gene specific sequences are noted in lower case letters while other sequences are noted in capital letters. Underlined bases (solid line) show either the Hind III (for primers a-e) or BamH I (for primers f-j) restriction sites. All forward primers (a-e) have the T_7 promoter sequence (noted in bold) for transcription with T_7 RNA polymerase. A LACZ-ATC 5' UTR was introduced (dashed line) for enhanced translation in the rabbit reticulocyte lysate.

	Primer Name
a	$fT_7GPIPLC_{1-6}$
b	$fT_7GPIPLC_{179-184}$
c	$fT_7GPIPLC_{238-243}$
d	$fT_7GPIPLC_{298-303}$
e	$fT_7GFP1-6$
f	rGFP
g	$rGPIPLC_{55-60}$
h	$rGPIPLC_{115-120}$
i	$rGPIPLC_{174-179}$
j	rGPIPLC

Figure 1: A) Shows a schematic of the fused *GPI-PLC* (dark bar) and *GFP* (hatched bar) genes. The plasmid pCO59 harbors the *GPI-PLC* gene fused to the 5' end of *GFP*, and was used to make the C-terminal fusions of *GPI-PLC*, shown in panel B. pCO64 contains the *GPI-PLC* gene fused to the 3' end of *GFP*, and was used to make the N-terminal fusions of GPI-PLC, shown in panel B. Also noted are the PCR primers used to link the N- and C-terminal fragments. Descriptions of primer features can be found in Table 1. B) A list of the various *GPI-PLC* fragments. Constructs are named according to gene order and the *GPI-PLC* fragment is noted by a pair of numbers which represent the amino acids present in any given fusion protein.

Figure 1


A
GFP
GPI-PLC
pCO59
a
b
c
d
e
f
pCO64
g
h
i
j
k
l

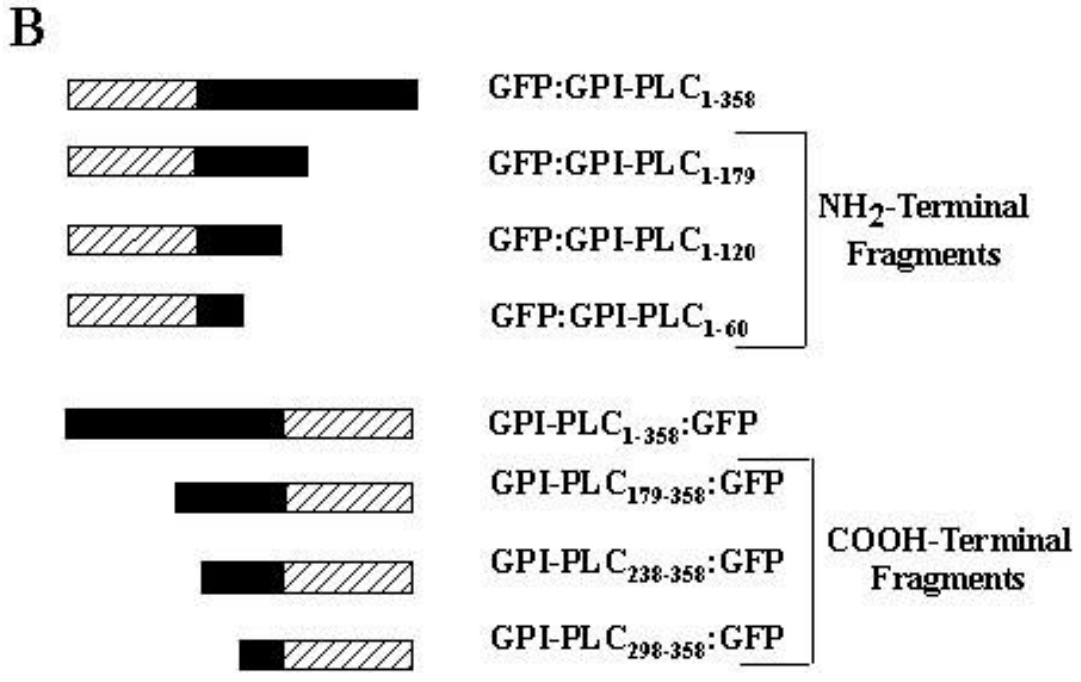

B
GFP:GPI-PLC1-358
GFP:GPI-PLC1-179
GFP:GPI-PLC1-120
GFP:GPI-PLC1-60
NH2-Terminal Fragments
GPI-PLC1-358:GFP
GPI-PLC179-358:GFP
GPI-PLC238-358:GFP
GPI-PLC298-358:GFP
COOH-Terminal Fragments

Figure 2: Translation of the GPI-PLCp:GFPp fusion proteins in a rabbit reticulocyte lysate. Purified (heat denatured) mRNA (2 μg) was translated *in vitro* in the presence of [^{35}S]methionine. Two microliters of the translation reaction was combined with 18 μl of 1X SDS-PAGE sample buffer. Samples were electrophoresed on a SDS-PAGE minigel (14%), and analyzed by phosphorimaging. GFPp and GPI-PLCp, lanes 1 and 2, are the negative and positive controls (respectively). The second panel (lanes 3-6) shows translation of the N-terminal portions of GPI-PLCp fused at the C-terminus of GFP. The third panel (lanes 7-10) shows C-terminal regions of GPI-PLCp fused with the N-terminus of GFPp. Molecular weight standards are indicated.

Figure 2

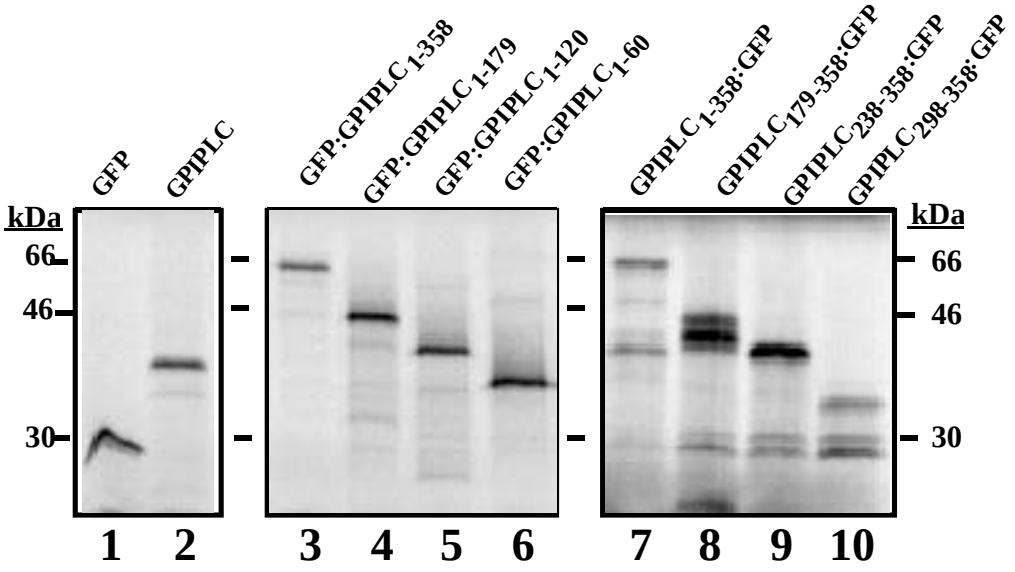
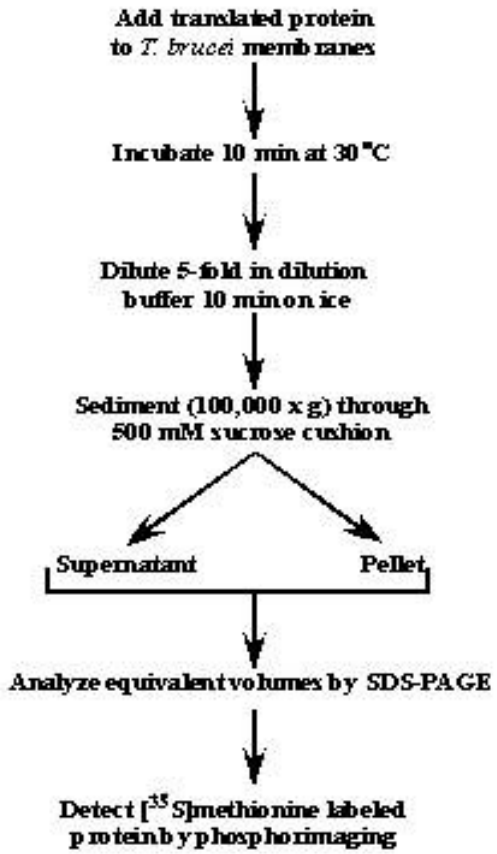

GFP
GPIPLC
GFP:GPIPLC1-358
GFP:GPIPLC1-179
GFP:GPIPLC1-120
GFP:GPIPLC1-60
GPIPLC1-358:GFP
GPIPLC179-358:GFP
GPIPLC238-358:GFP
GPIPLC298-358:GFP
kDa
66
46
30
kDa
66
46
30
1 2 3 4 5 6 7 8 9 10

Figure 3: Membrane binding assay. A) Translated protein was incubated at 30°C without (-) or with (+) *T. brucei* membranes (260 OD_{260} units). The mixture was diluted 3-fold and samples sedimented through a 500 mM sucrose cushion. A supernatant (S) and pellet (P) were collected. Equivalent volumes were analyzed by SDS-PAGE and phosphorimaging. B) GFPp is shown in lanes 1-4, and GPI-PLCp is depicted in lanes 5-6. Amount of radioactivity in each band was quantified using Quantity One software and graphed below the corresponding gel. A representative gel is presented: quantitated values shown are the average of 3 separate experiments. Background protein seen in pellet fraction in the absence of membranes was subtracted.

Figure 3

A


Add translated protein to T. brucei membranes
Incubate 10 min at 30 °C
Dilute 5-fold in dilution buffer 10 min on ice
Sediment (100,000 x g) through 500 mM sucrose cushion
Supernatant
Pellet
Analyze equivalent volumes by SDS-PAGE
Detect [35S]methionine labeled protein by phosphorimaging

B

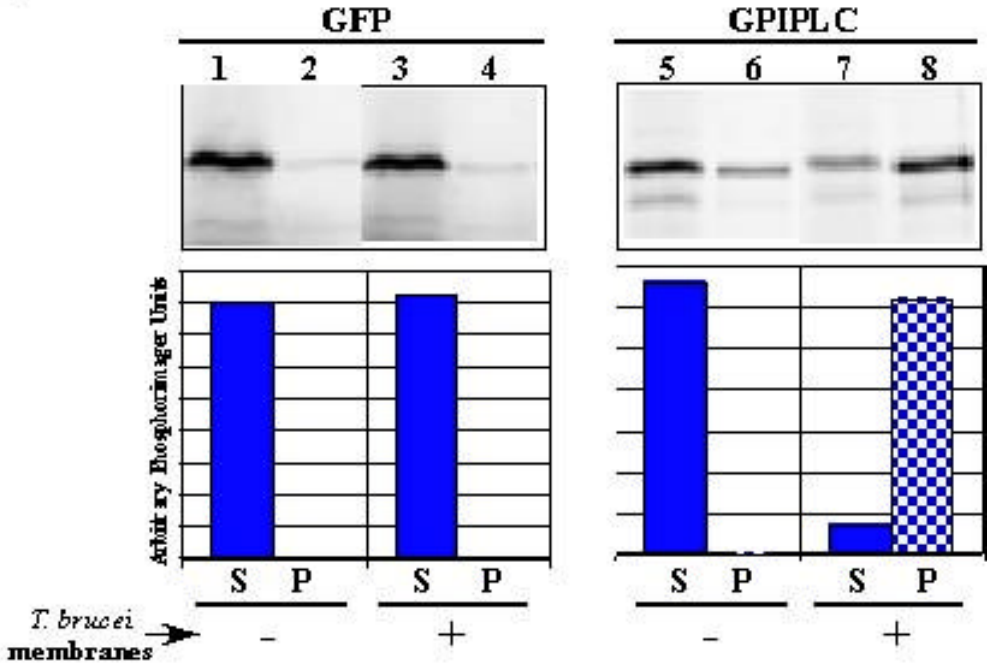

GFP
GPIPLC
1
2
3
4
5
6
7
8
Arbitrary Phosphorimager Units
S
P
S
P
S
P
S
P
T. brucei membranes
–
+
–
+

Figure 4: Effect of NH_2-terminal fragments of GPI-PLCp on GPI-PLCp membrane binding. Experiments were carried out as described in Figure 3. Fusion constructs are indicated. Graphs show an average of quantitation from samples in 3 different experiments. After phosphorimaging, data was quantified using Quantity One software (BioRad). Background protein seen in pellet fraction in the absence of membranes was subtracted.

Figure 4

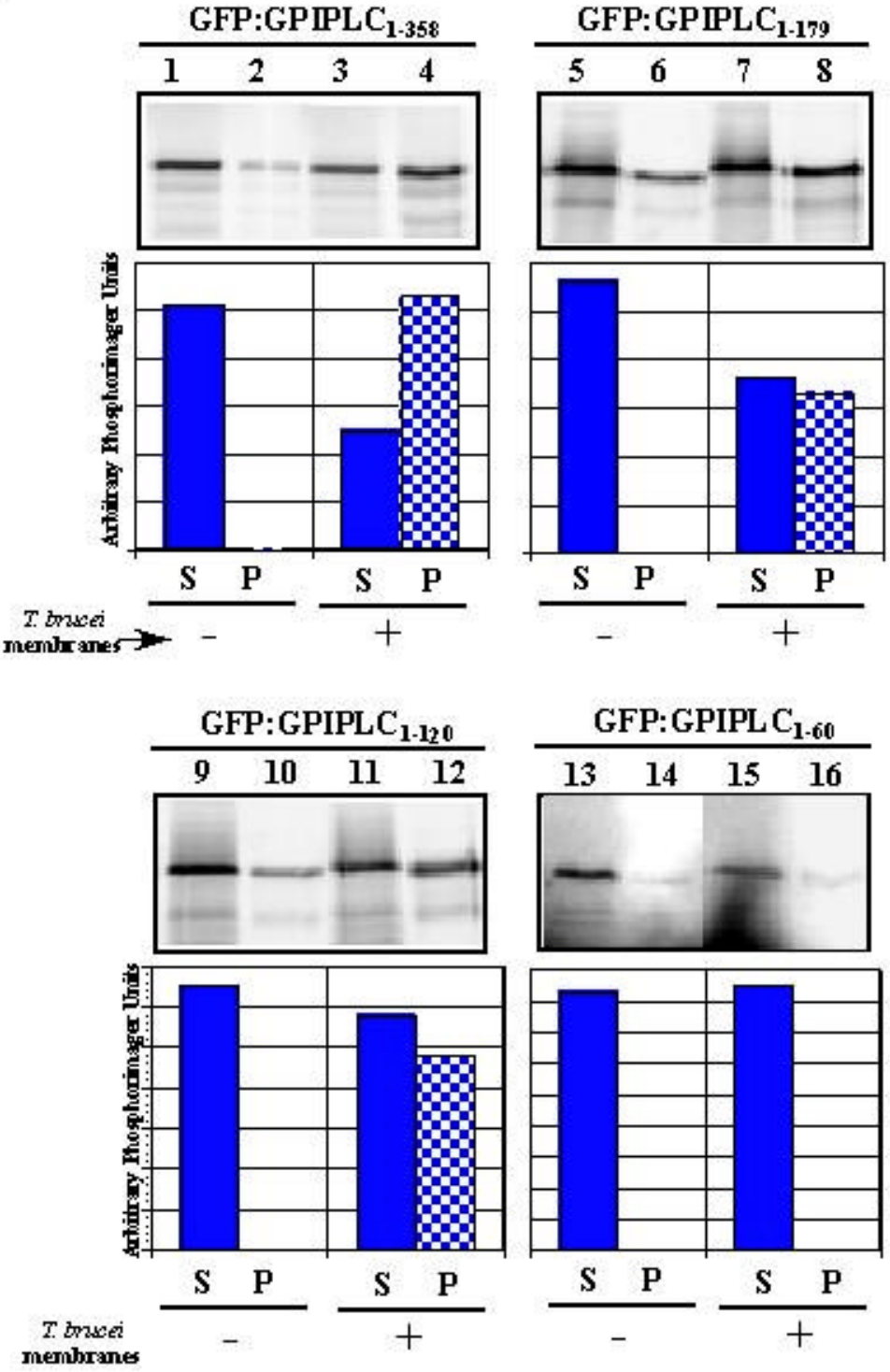

GFP:GPIPLC1-358
1 2 3 4
GFP:GPIPLC1-179
5 6 7 8
Arbitrary Phosphorimager Units
S P S P
S P S P
T. brucei membranes
- + - +
GFP:GPIPLC1-120
9 10 11 12
GFP:GPIPLC1-60
13 14 15 16
Arbitrary Phosphorimager Units
S P S P
S P S P
T. brucei membranes
- + - +

Figure 5: Membrane binding of C-terminal portions of GPI-PLCp. Experiments were carried out as diagramed in Fig. 3. Protein fusion constructs are indicated. Graphs show an average of quantitation of radioactivity from samples in 3 different experiments. Data was quantified using Quantity One software (BioRad). Background protein seen in pellet fraction in the absence of membranes was subtracted.

Figure 5

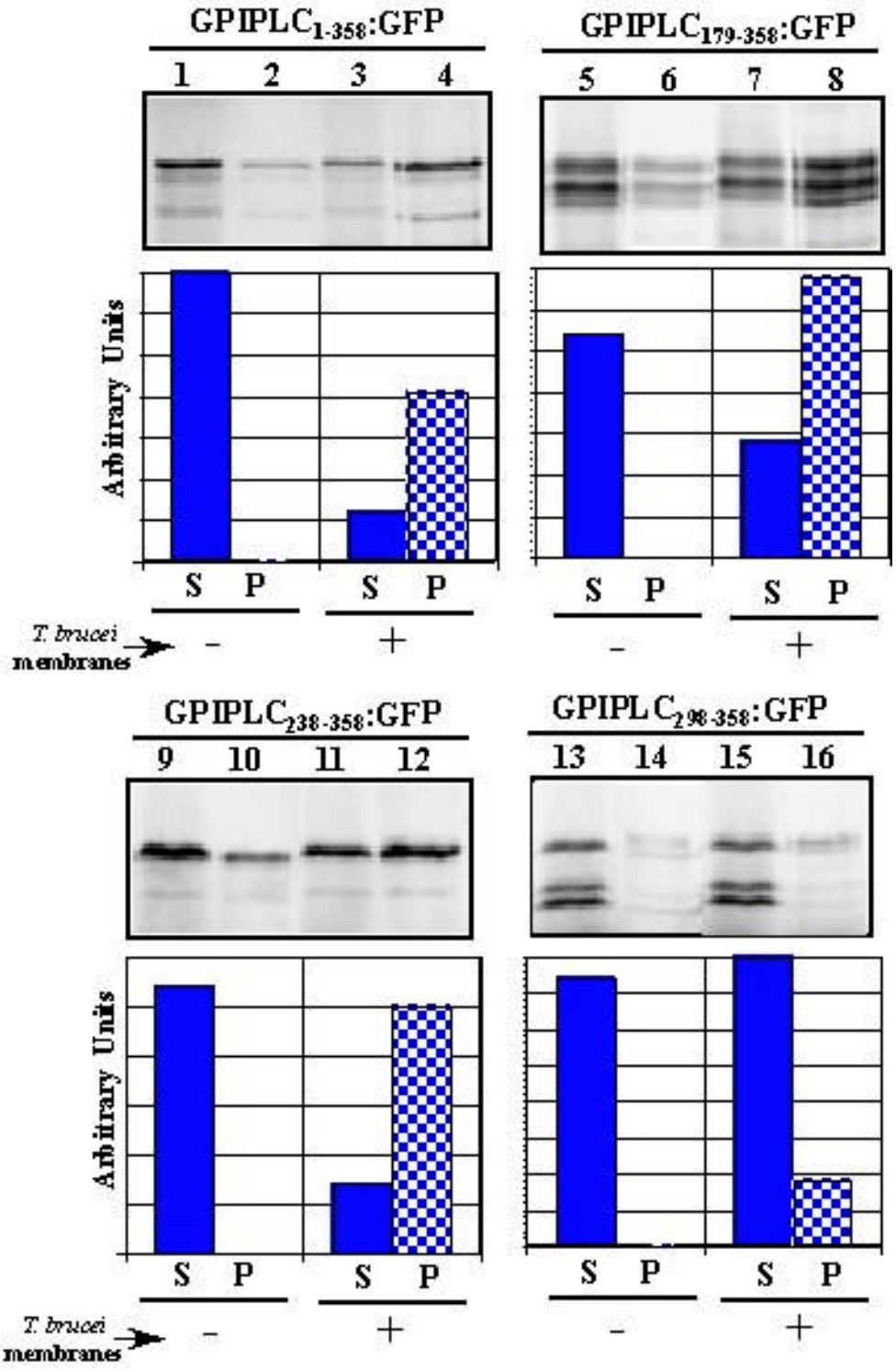
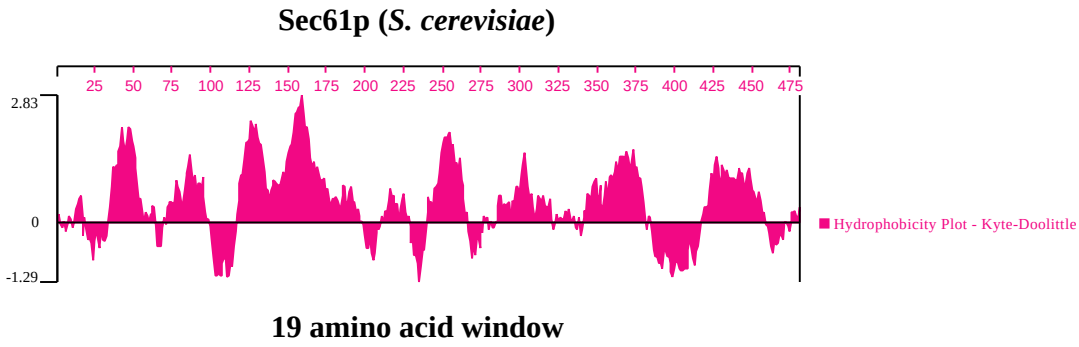

GPIPLC1-358:GFP
1 2 3 4
GPIPLC179-358:GFP
5 6 7 8
Arbitrary Units
S P S P
S P S P
T. brucei membranes
- +
- +
GPIPLC238-358:GFP
9 10 11 12
GPIPLC298-358:GFP
13 14 15 16
Arbitrary Units
S P S P
S P S P
T. brucei membranes
- +
- +

Figure 6: A) hydropathy analysis of Sec61p (from *S. cerevisiae*) using Lazergene software. The horizontal axis indicates the amino acid position while the vertical axis measures the peak hydrophobicity of certain amino acids using a Kyte-Doolittle scale within a 19 amino acid window. A value of 1.25, or higher, predicts a membrane spanning domain. B) Diagram of the 10 membrane spanning domains of Sec61p [18].

Figure 6

A


Sec61p (S. cerevisiae)
25 50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475
2.83
0
-1.29
Hydrophobicity Plot - Kyte-Doolittle
19 amino acid window

B

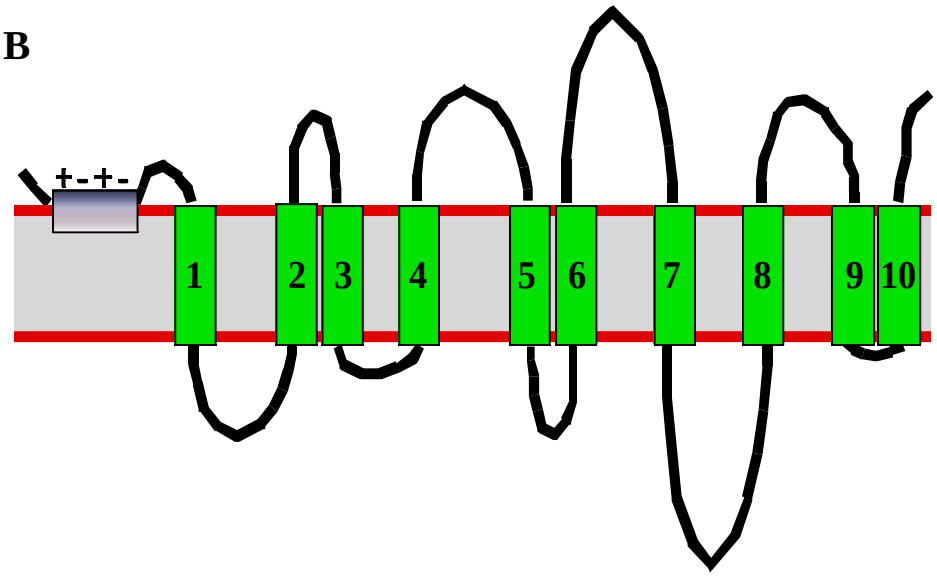
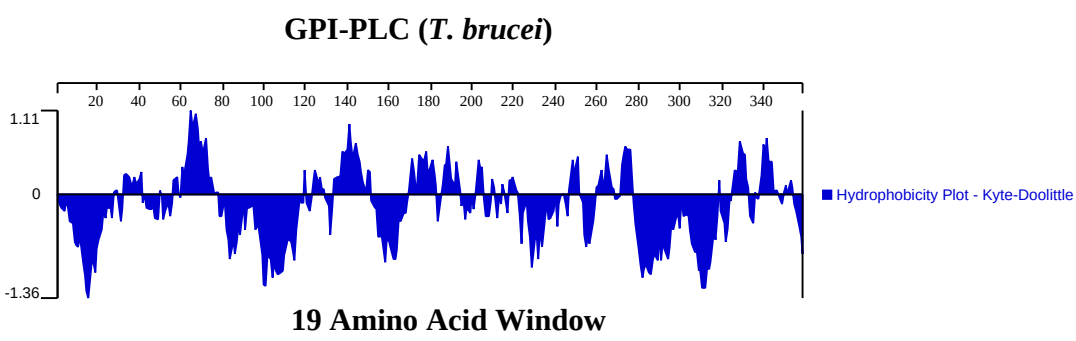

+-+-
1
2
3
4
5
6
7
8
9
10

Figure 7: A) Hydropathy analysis of GPI-PLC using a 19 amino acid window. B) Hydropathy analysis using a 8 amino acid window. Panels indicated by dotted lines outline GPI-PLC membrane binding regions (MBR 1 and 2) which are sufficient to direct membrane binding of protein. Peaks within this region greater than 1.8 using the Kyte-Doolittle scale are indicated (asterisks). Peaks indicated by arrows occur outside the regions known to direct protein binding to membranes.

Figure 7

A


GPI-PLC (*T. brucei*)
20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
1.11
0
-1.36
Hydrophobicity Plot - Kyte-Doolittle
19 Amino Acid Window

B

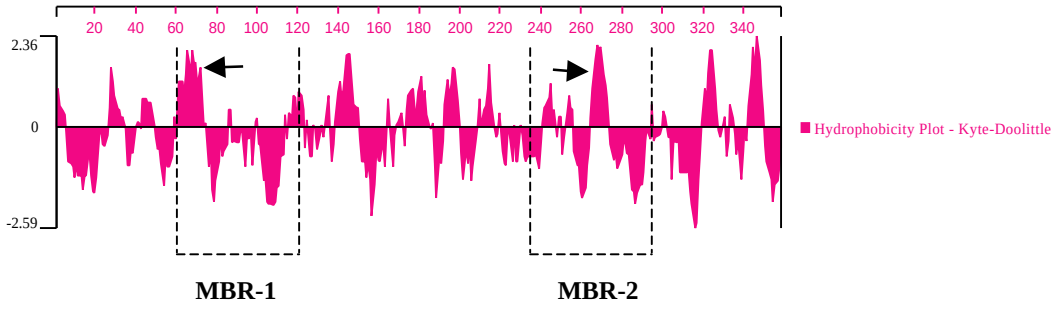
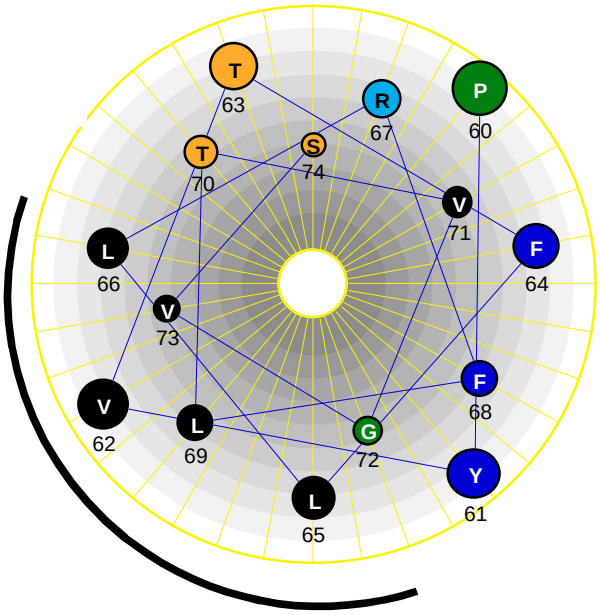

20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
2.36
0
-2.59
Hydrophobicity Plot - Kyte-Doolittle
MBR-1
MBR-2
8 Amino Acid Window

Figure 8: A) Helical wheel analysis of MBR 1 (Figure 7) (amino acids 60-74) predicts the orientation of a sequence of amino acids in the form of an α-helix. Amino acids are grouped according to properties and each group is indicated by a color, shown in the legend. Hydrophobic face is shown (black arch). B) Helical wheel analysis of amino acids 263-275 from MBR 2 show the presence of hydrophobic amino acids (solid arch).

Figure 8

A


T
63
R
67
P
60
T
70
S
74
V
71
L
66
F
64
V
73
F
68
V
62
L
69
G
72
Y
61
L
65

B

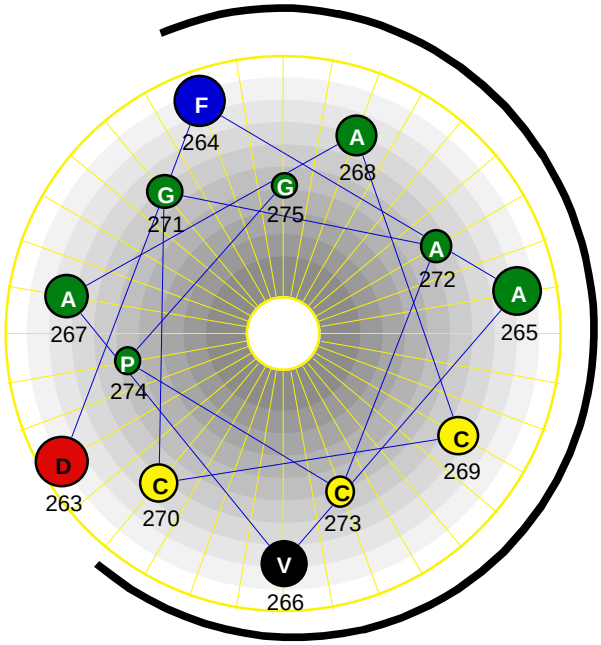
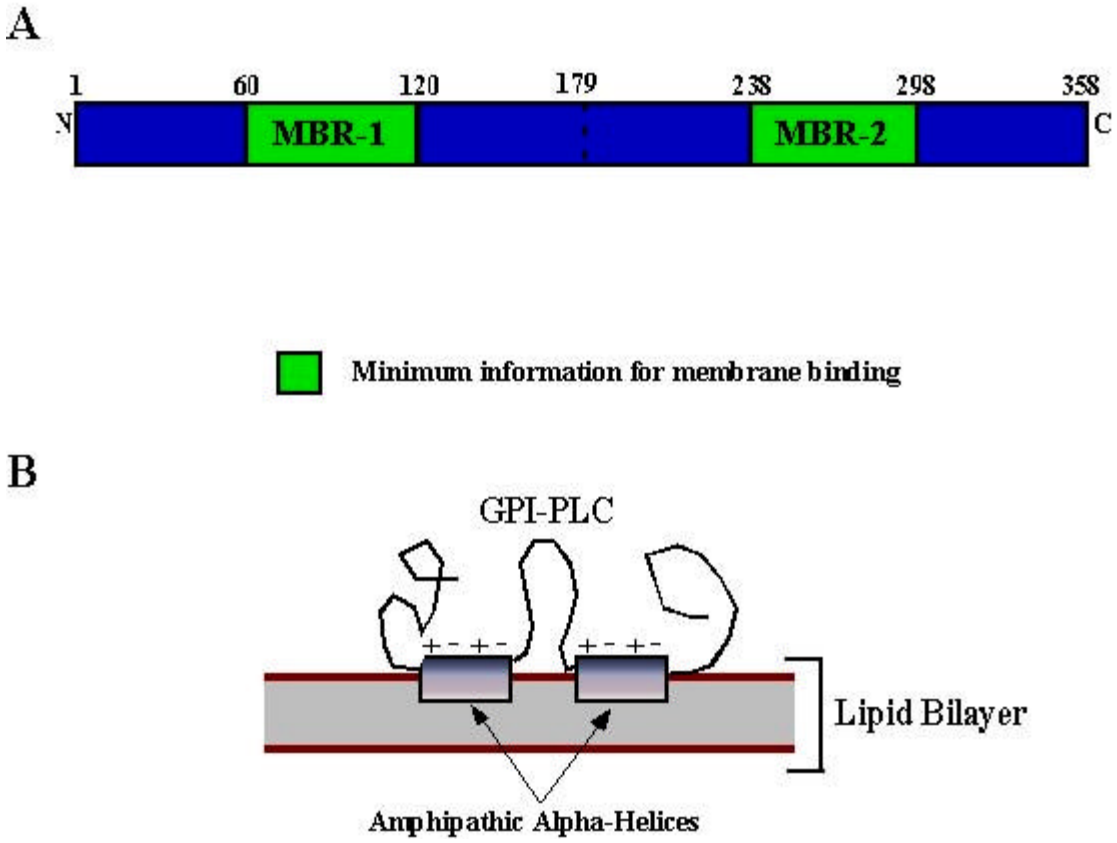

F
264
A
268
G
271
G
275
A
272
A
267
A
265
P
274
C
269
D
263
C
270
C
273
V
266

Figure 9: Mechanism of GPI-PLC membrane binding. A) Diagram of minimum requirement to direct membrane binding of GPI-PLCp. Regions between amino acids 60-120 and 238-298 (green) are sufficient to target proteins to membranes. B) Proposed models for GPI-PLCp membrane binding.

Figure 9


A
1
60
120
179
238
298
358
N
MBR-1
MBR-2
C
Minimum information for membrane binding
B
GPI-PLC
+ - + -
+ - + -
Lipid Bilayer
Amphipathic Alpha-Helices

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CHAPTER IV

DISCUSSION

The protozoan *Trypanosoma brucei* causes disease in both humans and cattle. Glycosylphosphatidylinositols (GPIs) are essential to these parasites. *T. brucei* express a GPI specific phospholipase C (GPI-PLCp). Heterologous expression of GPI-PLCp in *Leishmania* results in a GPI-negative phenotype. However, GPI-PLCp activity in *T. brucei* is suppressed, such that it does not cause a GPI deficiency. It is important to understand the mechanisms by which GPI-PLCp is regulated in *T. brucei*. GPI-PLCp is an integral membrane protein. Affinity of GPI-PLCp for membranes allows the protein to gain access to GPIs in the lipid bilayer.

In this work we found two regions from GPI-PLCp that are sufficient for membrane binding of a soluble reporter. These regions are between amino acids 60-120 and 238-298. Each stretch contains a smaller "patch" of hydrophobic amino acids which computer aided secondary structure programs predict could form amphipathic α-helices. We hypothesize that these α-helices could form "hydrophobic patches" that anchor the protein into the lipid bilayer.

In addition to directing membrane binding of GPI-PLCp, we report targeting of the protein to specific regions of the cell (i.e., the Golgi region of *Leishmania*). There is a direct correlation between intracellular location of GPI-PLCp and the ability of the enzyme to cleave GPIs in *Leishmania*. Targeting of GPI-PLCp to the Golgi region is dependent upon specific cysteines in the protein. We propose a "cysteine masking"

hypothesis, in which the presence of sulfhydryl groups on certain cysteines direct GPI-PLCp to the Golgi region where it cleaves GPIs. In *T. brucei* these cysteines would be "masked" by some factor (lipidation or protein-protein interactions may suffice), so that GPI-PLCp is directed away from GPIs. However, in *Leishmania* (where the enzyme activity is not suppressed), these exposed cysteines target GPI-PLCp to the Golgi region.

Constitutive activation of GPI-PLCp activity in *T. brucei* could potentially lead to the ability to produce GPI deficient parasites. Since GPIs are essential in these organisms, an understanding of how GPI-PLCp may be activated *in vivo* may provide leads for development of new drugs for treatment of trypanosomiasis.