Structures of Glycolipids Found in Trypanosomatids: Contribution to Parasite Functions

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Abstract: Neutral monohexosylceramides (CMHs) globosides (globotriasyl ceramides), other glycosphingolipids (GSLs) and more complex structures such as glycoinositol-phospholipids(GIPLs) and glycosyl phosphatidylinositol (GPI) anchors have been described in several members of the trypanosomatid family. These highly bioactive molecules are not only components of biological structures but also participants in host-parasite interactions such as macrophage invasion, antigenic presentation and signal transduction. Glycolipid structures have been studied using mass spectrometry (MS).This review describes a wide range of glycoconjugates with unique and complex structures that are present in several trypanosomatid species. Their structures are described in the context of their biological significance.

Keywords: Trypanosomatids, GSLs, GIPLs, GPI-anchor proteins, mass spectrometry.

INTRODUCTION

Glycoconjugates have been studied as components of several members of the trypanosomatidae family. These cell-surface molecules play important roles in parasite survival and infectivity. A wide range of glycoconjugates with different and complex structures are present in several species. Among them, we found lipid- containing carbohydrates (glycolipids) such as glycosphingolipids (GSLs), glycoinositol-phospholipids (GIPLs) and glycosylphosphatidyl inositol (GPI-APs) anchors [1].

Fig. (**1**) shows some of these glycoconjugates on a trypanosomatid plasma membrane.

Glycosphingolipid structures have been characterized from non-pathogenic trypanosomatids such as *Trypanosoma mega* and a bat trypanosomatid, as well as *Trypanosoma cruzi* [2-4].

The etiologic agents of tropical and subtropical diseases such as leishmaniasis (*Leishmania* spp.), African sleeping sickness (*Trypanosoma brucei*) and Chagas disease (*Trypanosoma cruzi*) are all members of the family trypanosomatidae and have been the target of extensive research. Many studies have focused on the unusually high levels of GPI-anchored molecules present in these organisms, which are thought to form a dense, homogeneous and protective coat on the parasite cell surface [5]. These molecules include the variant surface glycoprotein (VSG) in the bloodstream-form of *T. brucei* [6], metalloprotease Gp63 (or leishmanolysin) in *Leishmania* [7] and (GIPLs) in *Leishmania* [5]. Each parasite stage is already known to have different glycoconjugates [8, 9]. The aim of this mini-review is to describe the bioactive molecules found in trypanosomatids and correlate them with their biological significance.

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1. GLYCOSPHINGOLIPIDS (GSLs)

GSLs are membrane components of plant, animal and microbial cells. They are amphiphatic molecules containing mono- or oligosaccharide groups that are glycosidically attached to C-1 of an amino alcohol sphingosine. Complex sphingolipids have a fatty acid attached in an amide linkage (Fig. **2**). The fatty acids vary in chain length, degree of unsaturation (most are saturated), and presence or absence of a hydroxyl group.

GSLs have been implicated in many fundamental cellular processes including growth, differentiation and morphogenesis. GSLs modulate cell signaling by controlling the assembly and specific activities of plasma membrane proteins. They are highly bioactive and are involved in many aspects of cell signaling such as cell-cell interaction, cell-substratum interaction and cell-pathogen interaction. GSLs also are involved in the modulation of signal transduction, resulting in regulation of cell proliferation and differentiation [10, 11].

Using the carbohydrate moiety as a reference, GSLs can be divided into different classes including cerebrosides (GSLs containing mono- or oligosaccharide groups that are glycosidically attached to C-1 of the amino alcohol sphingosine), sulfatides (sulfate esters of some cerebrosides), globosides (GSLs containing two or more monosaccharide units) and gangliosides (similar to globosides but also containing sialic acid).

In eukaryotic organisms, there is a high diversity of GSL structures. Plants and fungi often contain glycosphingolipids with relatively simple carbohydrate structures, although clear differences in the structure of the ceramide backbone of these organisms are present [12]. Determination of GSL

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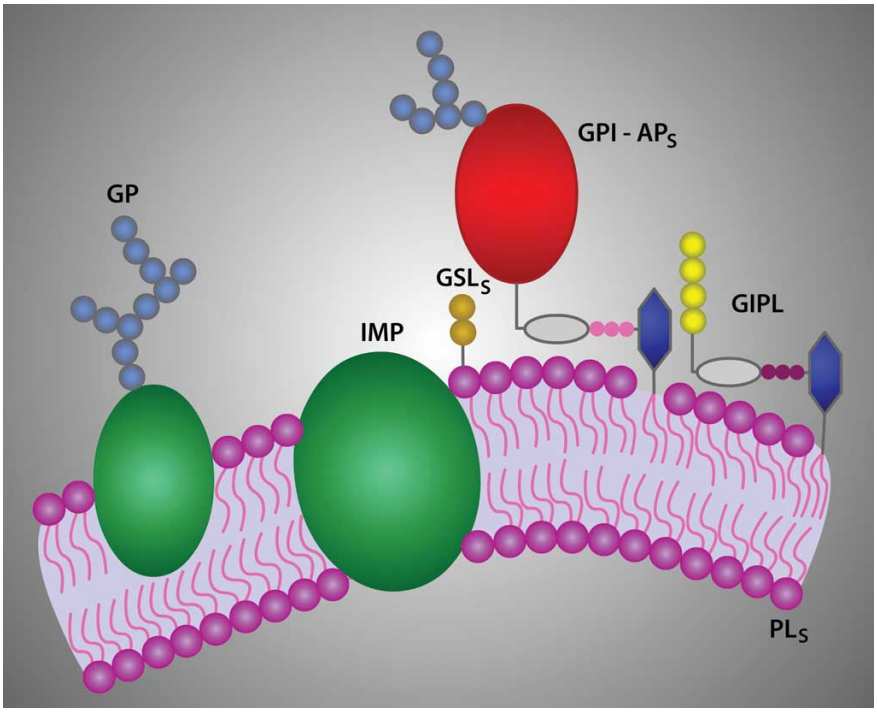

Fig. (1). Membrane glycoconjugates from trypanosomatids. GP, glycoprotein; IMP, integral membrane protein; GSLs, glycosphingolipids; GPI-APs, GPI-anchored proteins; GIPL, glycoinositolphospholipids; and PLs, phospholipids.

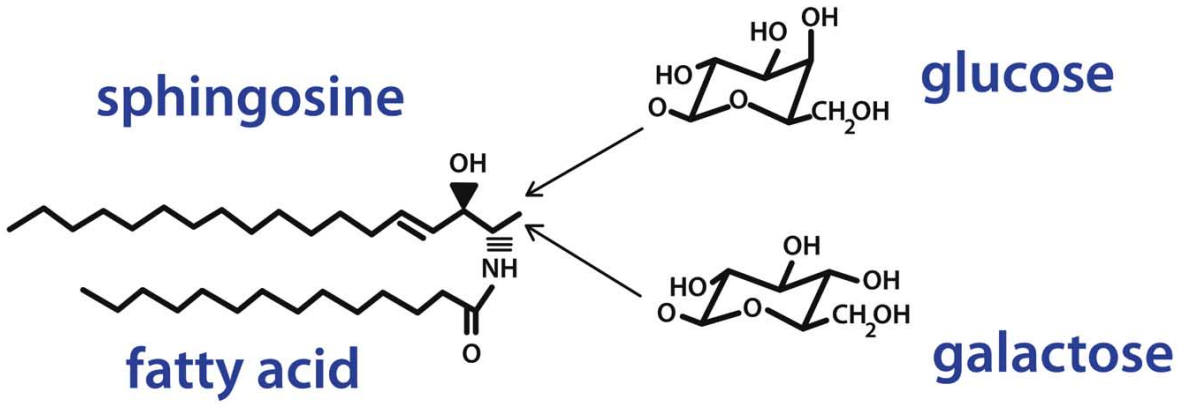

Fig. (2). Structure of a typical glycosphingolipid containing only one monosaccharide (monohexosylceramide) from mammalian cells. The glycolipids have a fatty acid attached in amide linkage to the sphingosine (d18:1) (forming ceramides). The monosaccharide (galactose or glucose) is attached glycosidically to C-1 of sphingosine.

structures is greatly dependent on the use of mass spectrometry (MS), such as fast atom bombardment MS (FAB-MS), electrospray ionization (ESI-MS) and dissociation MS (ESIMS/CID-MS); ^{1}H and ^{13}C-Nuclear Magnetic Resonance (NMR) have also been used successfully. A combination of these techniques is usually sufficient for complete structural elucidation [12].

Trypanosoma cruzi

The major neutral glycosphingolipids from the *T. cruzi* Y strain were identified as ceramide mono- and dihexosides (CMH and CDH), and their structures were elucidated using a combination of column chromatography, HPTLC, and gas-chromatography (GC) together with FAB-MS and 500 MHz^{-1} H-NMR spectroscopy [3]. The molecular species of CMH contain glucose or galactose, sphingosine (d18:1) and fatty acyl groups that are mainly C-24 saturated, monounsaturated or 2-hydroxy fatty acids (Table **1**). The different molecular species can be attributed to CMH molecules that differ only in the chain length of their hydroxy fatty acids. The ceramide dihexoside was identified as lactosylceramide with sphingosine (d18:1) as the long chain base and 16:0, 18:0, 24:0 and 24:1 fatty acids as the major components. No evidence of hydroxylated fatty acids was obtained by MS [3].

Recently, ceramide species containing C23:0 and C25:0 were also described among GIPLs from *T. cruzi* [13]. Glucosyl- and lactosylceramides were also isolated and their structures were characterized in another *T. cruzi* strain, Dm28c. [4] (Fig. **3**).

Table 1. Relative Distribution of Fatty Acid Chain Length Among Ceramide Monohexoside Fractions CMH-C_{OH} and CMH-C_n from *T. cruzi* (Y Strain) as Calculated from the $[M+H-60]^+$ Ions of the Per-O-acetylated Compounds. CMH-C_n, Ceramide Monohexosides from *T.cruzi* with -*n* Fatty Acids; CMH-C_{OH}, CMH with α-hydroxy Fatty Acids from *T.cruzi*

[M+H-60]+ (m/z)	Chain length of *n*-fatty acid	CMH-Cn (%)	Chain length of α-hydroxy acid	CMH-C_{OH} (%)
850	16:0	37	-	-
906	-	-	16:1	13
934	22:0	24	-	-
962	24:0	39	-	-
990	-	-	22:0	13
1004	-	-	23:0	15
1006	-	-	24:1	13
1020	-	-	24:0	33
1034	-	-	25:0	13

Based on the MS data, only sphingosine is present in the ceramide moiety.

Table 2. Relative Distribution of Fatty Acid Lengths of the Ceramide Dihexoside Fraction of CDH from *T. cruzi* (Y Strain) as Calculated from the $[M+H-60]^+$ Ions of Per-O-acetylated Species

[M+H-60]+ (m/z)	Fatty acid chain length	CDH (%)
1138	16:0	36
1166	18:0	35
1222	22:0	15
1250	24:0	14

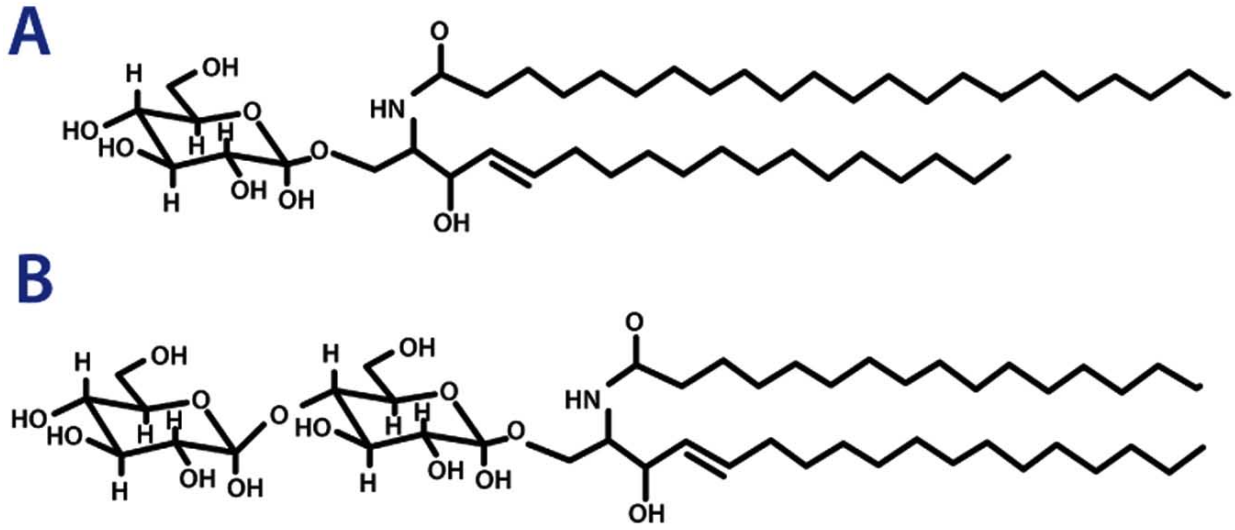

Fig. (3). Main structures of glycosylceramides from clone Dm28c of *T.cruzi (***A**) Glucosylceramide (CMH) and (**B**) Lactosylceramide (CDH).

The structure of the ceramide monohexoside was elucidated by GC-MS, FAB-MS and ^{1}H-NMR spectroscopy. Glucose was the only sugar present, and GC-MS analysis of the methanolyzed and trimethylsilylated CMH revealed three main peaks corresponding to C22:0, C23:0 and C24:0 non-hydroxylated fatty acids. Based on the FAB-MS spectrum and the analytical data for fatty acids, the major molecular species of long-chain base was sphingosine (d18:1), which was represented by an ion at mass to charge ratio (*m/z*) 264.

FAB-MS of the per-O-acetylated CDH fractions gave major molecular ions from $[M+H-60]^+$ at m/z 1223, 1237

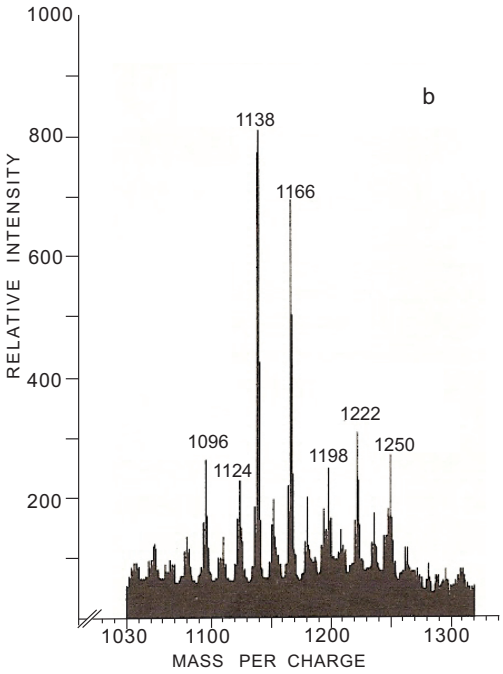

Fig. (4). FAB-MS analysis of the lactosylceramide (LacCer) of clone Dm28c of *T. cruzi.*

and 1251 (Table **2**). The ceramide moiety of CDH was represented by peaks at m/z 646.6 (d18:1/22:0 +Ac), 660.6 (d18:1/23:0 +Ac) and 674.6 (d18:1/24:0 + Ac). Ions at m/z 619, 331 and 289 derived from the hexose-hexose group were detected (Fig. **4**). The glycan sequence in CDH was confirmed by enzymatic degradation with β-galactosidase [4].

These data demonstrate a substantial diversity among *T. cruzi* glycosylceramides. Such differences may be associated with the different courses of Chagas disease observed with different *T. cruzi* clones [14].

GSLs are emerging as attractive targets for antimicrobial therapy [15]. GLS-binding antibodies with potent antimicrobial action have been recently described [12]. It is necessary to characterize the determinants of antigenicity of these molecules, aiming at the identification of antimicrobial antibodies with selective toxicity. Ceramide dihexoside isolated from clone Dm28c of *T. cruzi* was recognized by sera from *T. cruzi*-immunized rabbits to a much higher extent than the mono-glycosylated form. This result suggests that glycosylation is a determinant of antigenicity in *T.cruzi* GSL [4, 16]. Replacement of the ceramide moiety in this GSL by phosphatidylethanolamine resulted in a decrease of serological reactivity, indicating that intramolecular interactions between sugar and ceramide moieties are important for antigenicity. A previously characterized CMH from the Y clone of *T.cruzi* was strongly recognized by immunized sera. These results indicate that fatty acid hydroxylation and ceramide glycosylation influence the serological reactivity of *T.cruzi* GSL [4,16].

Few studies are currently found in literature concerning the biological role of these molecules in *T. cruzi*. Cossy-Isasi and collaborators [17] reported that parasite epimastigotes treated with gangliosides from bovine brain presented an altered lipid order that inhibited membrane enzymes and caused morphological alterations. Electron-lucent vacuoles opposite the cytostome, multilamellar bodies and dilated mitochondrion cristae, in addition to a disorganized kinetoplast and altered heterochromatin structure, were found in epimastigote forms [18, 19]. Trypomastigotes suffered a loss of cytoplasmic material and organelles when the ganglioside GM1 was present in the culture medium. Inoculation of murine models with the ganglioside GM1 has shown a strikingly nonlinear effect leading to a strong decrease in parasite load at low doses but reverting to a load increase at high doses. GM1-treated mice survived and recovered with normal frequency. Cardiomyocyte destruction concomitant with the disease was also significantly reduced by a moderate application of GM1 [18, 19].

1.2. *Trypanosoma mega*

A glycosphingolipid fraction from *Trypanosoma mega* was isolated and was further purified on a silicic acid column. Preparative thin-layer chromatography was used for final purification. The carbohydrate components of the glycolipid were fucose and galactose in approximately equimolar amounts. The neutral glycolipid of *T. mega* had a sphingosine base composition that consisted of sphingosine (d 18:1) and traces of dihydrosphingosine (d 18:0). Fatty acids forming amide groups with the sphingosine bases were analyzed by GC-MS and were a mixture of non-hydroxy and α-hydroxy fatty acids. Normal C16:0, C18:0 and 2-hydroxy C18:0 were the predominant fatty acids [2].

1.3. *Leishmania (L) amazonensis*

Glycosphingolipids were characterized in amastigote and promastigote forms of *Leishmania* (L*) amazonensis* [20].The structure of the main GSL present in the amastigote forms of this parasite was characterized as Gal*p*β (1→3) Gal*p*α (1→3) Gal*p*β (1→4) Glc*p*β (1→1) Cer and is referred to as a β-Gal-globotriasylceramide. The role of this glycolipid in macrophage infectivity was confirmed using Mabs directed to this molecule. A putative receptor/lectin of macrophages with a molecular mass of 30kDa for *L.amazonensis* GSL was suggested [21].

The specificity of the *L.amazonensis* interaction could be confirmed by the absence of binding of *L.chagasii* amastigotes, which do not express the β-Gal-globotriasylceramide glycoconjugate High concentrations of GSLs as well as sterols were detected in amastigote lipid rafts. Membrane domains were resistant to treatment with non-ionic detergents at 4 °C. Disruption of the membrane microdomains with methyl-β-cyclodextrin significantly reduced parasite infectivity suggesting a role of GSLs in macrophage invasion by species of *Leishmania* [19].

Other glycolipids such as IPC and GIPLs, along with sterols, were present in *L.amazonensis* promastigotes and preferentially distributed in membrane rafts [1].

2. GLYCOINOSITOLPHOSPHOLIPIDS (GIPLs) AND LIPOPHOSPHOGLYCANS (LPGs)

The cell surfaces of all trypanosomatids are rich in glycosylphosphatidylinositol (GPI)-anchored proteins and -glycans such as lipophosphoglycans (LPG) and other glycoconjugates, which are the free glycoinositol phospholipids

(GIPLs) that form protective surface coats and mediate essential host-parasite interactions [22-25].

GIPLs may be classified into three types: **i)** Type-1 GIPLs contain an α-Man residue linked (1→6) to the Man residue of the common motif (Manα1-4GlcNα1-6*myo*-inositol-1-HPO_4-) and are abundant in *T. cruzi* [26], *Leishmania donovani* and *Phytomonas* [28, 29], **ii)** Type-2 GIPLs are defined by the presence of an α-Man residue linked 1→3 to the Man residue of the common motif and have been described in *Leishmania* spp. [30, 31], **iii)** Type-3, the hybrid-type GIPLs, contain the branched structure Manα1→3(Manα1→6) Manα1→4GlcNα1-6*myo*-inositol-1-HPO_4-lipid and are found in some *Leishmania* species [28] and in *Herpetomonas samuelpessoai* [23]. The addition of oligosaccharide side chains and phosphorylated substituents, as well as distinct types of glycosidic linkages and lipid anchors, are responsible for the diversity of GIPL structures found in trypanosomatids [22,32].

2.1. *Trypanosoma cruzi*

The first free GIPL, called lipopeptideophosphoglycan, was that from the epimastigote form of *Trypanosoma cruzi*. In the parasite these GIPLs form a dense glycocalyx (approximately 10^7 GIPLs /cell) over the entire surface of the trypanosome [33].This was the first study to provide a precise quantitative analysis of GIPLs and mucins on the surface of both epimastigotes and trypomastigotes. Highly purified GIPLs from the *T. cruzi* Y strain were analyzed by nuclear magnetic resonance spectroscopy (NMR), mass spectrometry and chemical degradation [26, 27].

Variations in glycan structure and lipid composition were detected in *T. cruzi* GIPLs purified from different strains. The main GIPL species from the *T. cruzi* Y strain has the structure Gal*fβ*1→3Manα1→2(Gal*fβ*1→3)Manα1→2Manα1→6Manα1-4(2-AEP-6)GlcNα1→6*myo*-inositol-1-HPO_4-lignoceroylsphinganine [34]. However, GIPLs are mostly mixtures of beta-galactofuranose (β-Galf)-, ethanolamine phosphate (EtNP)- and 2-aminoethylphosphonate (AEP)-containing series 1 GIPLs (~structure Gal*f*β1→3Manα1→2(~AEP/EtNP-6)Manα1→2Manα1→ 6Manα1→4(AEP-6)GlcNα1→6-*myo*-inositol-*P*-ceramide) and series 2 GIPLs (~structure Gal*f*β1→3Manα1→2(~Gal*f*β1→3)Manα1→2Manα1-6Manα1→4(AEP-6)GlcNα1→6-*myo* -inositol-P-ceramide and Gal*fβ*1→3Manα1→2Manα1-6Manα1→4(AEP- 6)GlcNα1→6-*myo*-inositol-*P*-ceramide) [26, 27, 35, 36].

T. cruzi GIPLs are bioactive molecules and several biological effects have been described. First, GIPLs induce blockade of $CD4^+$ and $CD8^+$ T-cell activation *in vitro* by anti-CD3, superantigen or *T. cruzi* antigen. Furthermore, cell cycle blockade in T cells as well as reduced IL-2 secretion were observed. The suppressive effects of GIPLs on T cells are due to their ceramide moiety [34, 37]. Second, on the contrary, GIPLs were demonstrated to have a co-stimulatory effect on mouse T-cell hybridomas, enhancing IL-2 production induced by suboptimal doses of mitogenic stimuli [38]. Third, the purified GIPL ceramide moiety induced Ca^{2+} mobilization, [34, 38]. Fourth, GIPLs are involved in parasite attachment to the midgut of the insect vector. The administration of 0.5 μM of GIPLs inhibits up to 90% parasite infection in *Rhodnius prolixus* [39].

2.2. *Leishmania* spp.

In *Leishmania* , procyclic promastigotes, unlike amastigotes, express abundant quantities of a complex (protein-free) lipophosphoglycan (LPG) on their surface [22, 24] and the GPI anchored-metalloprotease (gp63) [40, 41]. Both glycoconjugates are thought to protect the promastigotes from hydrolytic enzymes in the sandfly gut, whereas LPG facilitates attachment to the insect gut epithelium. Transformation from non-infective dividing procyclics to infective non-dividing metacyclics can involve changes to the LPG structure [31, 42-44]. In addition, *Leishmania* species contain GIPLs that form protective surface coats that have also been implicated in virulence and have been shown to mediate essential host-parasite interactions. GIPLs are necessary for the viability of both the insect and mammalian (amastigote) stages of the life cycle [45].

2.2.a. LPG

The LPG is the most abundant macromolecule on the surface of *Leishmania* promastigotes (approximately $6x10^6$ copies per cell) during their development in the gut of the sandfly vector. Important roles have been described for the LPG coat including protection against the hydrolytic peptidase associated with bloodmeal digestion [46], binding of the parasite to the midgut wall [47], and, in some steps, it is required for the establishment of macrophage infections and for survival in the insect vector through complementation and oxidant resistance [24,25].

In all *Leishmania* species, the GPI anchor of LPG is composed of a 1-O-alkyl-2-lysophosphatidylinositol lipid anchor and a heptasaccharide core. A long phosphoglycan polymer composed of 15–30 [Galβ1,4Manα1-PO_4] repeating units (substituted with other sugars in some species) is attached to the heptasaccharide core and is terminated by a capping oligosaccharide [25, 28, 42, 48]. The chains of the phosphoglycan are assembled in the Golgi apparatus and are modified with monosaccharide or glycan side chains and terminal capping oligosaccharides [20, 49, 50]. The attachment of the *L. major* promastigote to the midgut of *P. papatasi* is mediated by the terminally exposed galactose residues of the LPG, and the microvillar-associated proteins act as ligands for the parasite LPG [51-53]. Alterations to the length of the phosphoglycan chain, as well as changes in the nature of the side chains, occur during promastigote development in the sandfly midgut. *In L. major* the transition from procyclic to metacyclic promastigotes is also associated with the capping of galactose side chains with arabinose residues [24, 44]. These changes result in an increase in the thickness of the surface coat and confer additional resistance to complement-mediated lysis, and they are also thought to be important in regulating the attachment of promastigotes to epithelial cells in the sandfly midgut [24, 43, 54].

2.2.b. GIPLs

In the *Leishmania* genus, GIPLs are necessary for the viability of both the insect and mammalian (amastigote) stages of the life cycle [32, 45]. Several types of structures have been found. For instance, in *Leishmania donovani*, type-1 GIPLs contain a αMan residue linked 1-6 to the Man residue of Manα1→4GlcNα1→-6*myo*-inositol-1-HPO_4-lipid [29]. This structure is present in *Leishmania donovani* [29].

The type-2 GIPLs are defined by the presence of a αMan residue linked 1→3 to the Man residue of the common motif and have been described in *L. major* [30], *L. mexicana* [31], *L. tropica* and *L.aethiopica* [55] and *L. adleri* [56]. The hybrid-type GIPLs contain the branched structure Manα1→ 3(Mana1 → 6) Manα →4GlcNa1→6myo - inositol-1- HPO_4-lipid and are found in *L. mexicana* and *L.donovani* [25,28], *L. tropica* and *L. aethiopica* [55].

2.3. *Trypanosoma dionisii*

Trypanosoma (Schizotrypanum) dionisii is a bat trypanosomatid that is non-pathogenic for humans. It originates from the Europe and Latin America and is related to *Trypanosoma cruzi*. Recently, studies with mammalian cells showed that *T. dionisii* is highly infective *in vitro*, particularly when the infection process occurs without serum. In this case, the invasion is affected by agents known to interfere with the *T. cruzi* invasion process [57].

The GIPLs of *T. dioinisii* were purified by reversed-phase and normal-phase liquid chromatography and analyzed by negative-ion mode electrospray-mass spectrometry (ESI-MS). The phosphatidylinositol moieties were released by nitrous acid deamination and identified as ceramide- and alkylacylglycerol-containing species. The GIPLs were based on the same Manα1-2Manα1-2Manα1-6Manα1-4(NH_2-CH_2CH_2-HPO_3-)GlcN-PI core with single terminal Gal*f* residue substitutions either on the terminal non-reducing Man or on the second αMan residue from the inositol and with either EtNP or AEP on the third αMan residue distant from the *myo*-inositol residue [58].

2.4. *Phytomonas* spp.

Phytomonas spp. are trypanosomatid parasites of plants. Their GIPLs were analyzed by chemical and enzymatic modifications, composition and methylation analyses, electrospray mass spectrometry and micro-sequencing after HNO_2 deamination and $NaBH_4$ reduction. The water-soluble head group of the second GIPL structure (see below) was also analyzed by ^{1}H NMR spectroscopy [29].

The GIPLs were analyzed in *Phytomonas* spp isolated from the rubber plant *Euphorbia characias* and they represent the first detailed characterization of surface molecules from this protozoa. Four GIPLs were detected with phosphatidylinositol moieties containing the fully saturated alkylacylglycerol lipids 1-*O*-hexadecyl-2-*O*-palmitoylglycerol and 1-*O*-hexadecyl- 2-01-*O*-hexadecyl-2-*O*-palmitoylglycerol and 1-*O*-hexadecyl--stearoylglycerol.These GIPLs are most similar to GIPL A of *T.cruzi* epimastigotes [35].

The structures of the GIPLs are: **i)** Manα1→2Manα1→ 6Manα1→4GlcNa1→-6PI, **ii**) Glcα1→2(NH_2- CH_2CH_2 - HPO_4) Manα1→2Manα1→ 6Manαc1 → 4GlcNα1 →-6PI, **iii)** Glcα1→2($NH_2CH_2CH_2$-HPO^4-)Manα1→2Manα1 →6 Manα1→-4(NH_2-CH_2CH_2-HPO_4-)GlcNα1→6PI and **iv)** Glcα1→2Glcα1→2(NH_2CH_2- CH_2-HPO_4-)Manα1→2Manα1 →6Manα1-4($NH_2CH_2CH_2$-HPO_4-)- GlcNα1→6PI. [29, 32].The presence of one and two α Glc residues are novel structural features for GIPLs. Unlike some *Leishmania* and *Endotrypanum* GIPLs [22] , *Phytomonas* GIPLs do not contain Gal residues.This finding may be significant since *Phytomonas* spp lives in the latex of *Euphorbia characias*, which contains a bivalent Gal-specific lectin , that might agglutinate the parasite [59].

The functions of these cell-surface GIPLs in trypanosomatid parasites remains obscure. However their abundance suggests that they may provide a protective role due to a dense negatively charged glycocalyx close to the surface of the plasma membrane, through which other macromolecules project [22].

3. GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)-ANCHORED PROTEINS

The glycosylphosphatidylinositol (GPI) anchor is a glycolipid structure that is added post-translationally to the C-terminus of many eukaryotic proteins. This modification anchors the attached protein in the outer leaflet of the cell membrane [32, 60-62]. The GPI anchor is a complex structure comprising a phosphoethanolamine linker, a glycan core and a phospholipid tail (Fig. **5**).

Proteins containing a GPI anchor are functionally diverse and play important roles in endocytosis, signal transduction, prion disease pathogenesis, complement regulation, antigenic presentation and the pathobiology of trypanosomal parasites [63-65]. In pathogenic protozoan parasites, the Tritryp group (e.g., *Trypanosoma cruzi, Trypanosoma brucei, Leishmania major*) molecules containing a GPI anchor may extensively coat the plasma membrane and are involved in host-parasite interaction processes, such as modulation and evasion of host immune responses.

3.1. *Trypanosoma cruzi*

T. cruzi GPI-anchored proteins are found in all evolutive forms and are encoded by thousands of members of multigene families, such as trans-sialidase (TS)/gp85 glycoprotein, mucin, mucin-associated surface proteins (MASP) and metalloproteinase gp63 [13]. Some of them, such as the TS/gp85 and mucins, have been shown to be very important for the infectivity of the parasite and for escaping the host immune response [8, 66-70]. Furthermore, GPI anchors from *T. cruzi* are pro-inflammatory molecules and are critical in modulation of the host immune response against the parasite [9, 71]. Taking into consideration these important functions, GPI- anchored proteins and GPI anchors themselves seem to be possible targets for new therapies against Chagas disease.

3.1.a. Mucins (TcMUC)

Mucins are hydrophilic glycoproteins that bear a dense array of *O*-linked oligosaccharides with side chains containing Gal and GlcNAc (about 60% carbohydrate by weight) and are anchored to the plasma membrane *via* a glycosylphosphatidylinositol (GPI) moiety. Metacyclic and cell-derived trypomastigote mucin-like molecules are sialylated by a parasite membrane-located *trans*-sialidase (TS) [72].

They were first described by Alves and Colli [73] as glycoproteins A, B, and C in non-infective epimastigotes. The core polypeptides of these glycoproteins are only 50–200 amino acids in length and their sequences are rich in Ser and Thr residues [67, 74-78]. The surface of *Trypanosoma cruzi* at different stages is covered by mucins. Acting at the interface between the parasite and both the vector and the infected host, these molecules provide protection against the

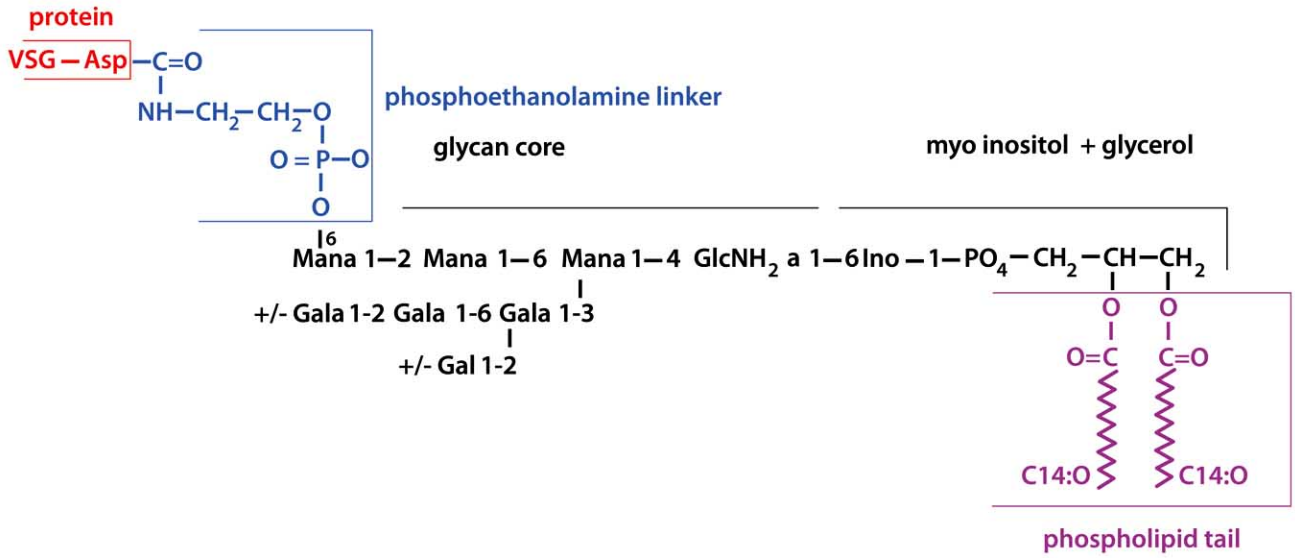

Fig. (5). Structure of the GPI anchor of *Trypanosoma brucei* bloodstream forms VSG. Based on Ferguson and collaborators [29].

vector and/or vertebrate-host-derived defense mechanisms and ensure the targeting and invasion of specific cells or tissues [8, 78].

T. cruzi mucins contain a complex family of mucin-like genes termed TcMUC. The groups of repetitive and non-repetitive genes were designated TcMUC I and TcMUC II, respectively. The majority of the mucin molecules present on the surface of the cell-derived trypomastigotes belong to the TcMUC II group [8]. In fact, amastigote mucins are probably from the TcMUC I family/group. A second mucin gene family was identified that, despite having similar flanking regions to those of the TcMUC gene products, encodes proteins with their own diverse and remarkably short central regions [79, 80]. This gene family was therefore termed TcSMUG, for *T. cruzi* small mucin-like gene family. TcSMUG comprises 70–80 genes that were originally divided in two groups (S for Small and L for Large) according to the size of their encoded mRNAs. Recently, the group S gene products have been identified as the major 35–50 kDa mucins expressed during the epimastigote stage [13].

Mucins can initially be divided into two major types: those present in the insect stages and those present in the mammalian stages. Mucins from both major insect-derived stages (epimastigotes and metacyclic trypomastigotes) run on SDS-PAGE as double or triple bands in the range of 35–50 kDa and have almost identical amino acid and carbohydrate compositions. The only structural difference is that, in the mucins isolated from epimastigotes, an alkylacylglycerol residue is found in the GPI anchor whereas in the metacyclic trypomastigote, it is replaced by a ceramide [8]. In the epimastigote and metacyclic mucins, the GPI glycan core is mainly composed of the linear structure Man1→2Man1→2Man1→6Man1→4GlcN [67, 81]. In the metacyclic trypomastigote forms, mucins may facilitate parasite development and growth in the insect vector by allowing trypomastigotes to survive the activities of digestive enzymes. In metacyclic trypomastigotes, which successfully initiate infection of the mammalian gastrointestinal tract, the peptidase-resistant mucin may confer the ability to survive at extremely low pH and protection from proteolytic enzymes present in gastric secretions [82].

Mucins from cell-derived trypomastigotes (tGPI-mucins) appeared on SDS-PAGE as a smear spanning a wide range of molecular masses (60–200 kDa). They shared the sialic acid-containing epitope Ssp-3, which is crucial for mammalian-cell attachment and invasion and which might be involved in diverting the complement cascade [74]. Mass spectrometric analysis of tGPI-mucins showed the presence of their GPI, oligosaccharide and peptide regions [83, 84]. The oligosaccharides were *O*-glycosidically linked mainly to Thr residues in the peptide backbone *via N*-acetylglucosaminyl units [77]. The attached lipid region is an alkylacylglycerol containing mainly unsaturated fatty acids at the *sn*-2 position of the glycerol moiety. The *O*-linked oligosaccharides are highly immunogenic to humans, resulting in the production of high-levels of trypanolytic anti-Gal antibodies [77]. In cell-derived trypomastigotes GPI glycan cores can be larger, containing a branch of Gal residues up to eight units in length [84], substituting a linear structure of Man1→2Man1→2Man1→6Man1→4GlcN [81]. The established and putative functions of the mucin components are as follows: **i)** variable region – immune evasion and adhesion; **ii)** core region – the main *O*-glycosylation scaffold and immunogenicity; **iii)** glycans – protection, adhesion and immunogenicity; **iv)** glycosylphosphatidylinositol (GPI) anchor – anchorage and immunomodulation (Fig. **6**; Table **3**).

The trypomastigote (strain Y obtained from $LLCMK_2$ cell culture) GPI structure was found to contain additional galactose residues and unsaturated acids in the *sn*-2 position of the alkylacyl-glycerolipid component. This feature is essential for the extreme efficiency of the trypomastigote GPI anchor in the induction of macrophage proinflammatory cytokines [84]. The TcMUC may also play an important protective role in the vertebrate forms and, in this case, an effective sialylation of the parasite seems to be critical. When the mucins are

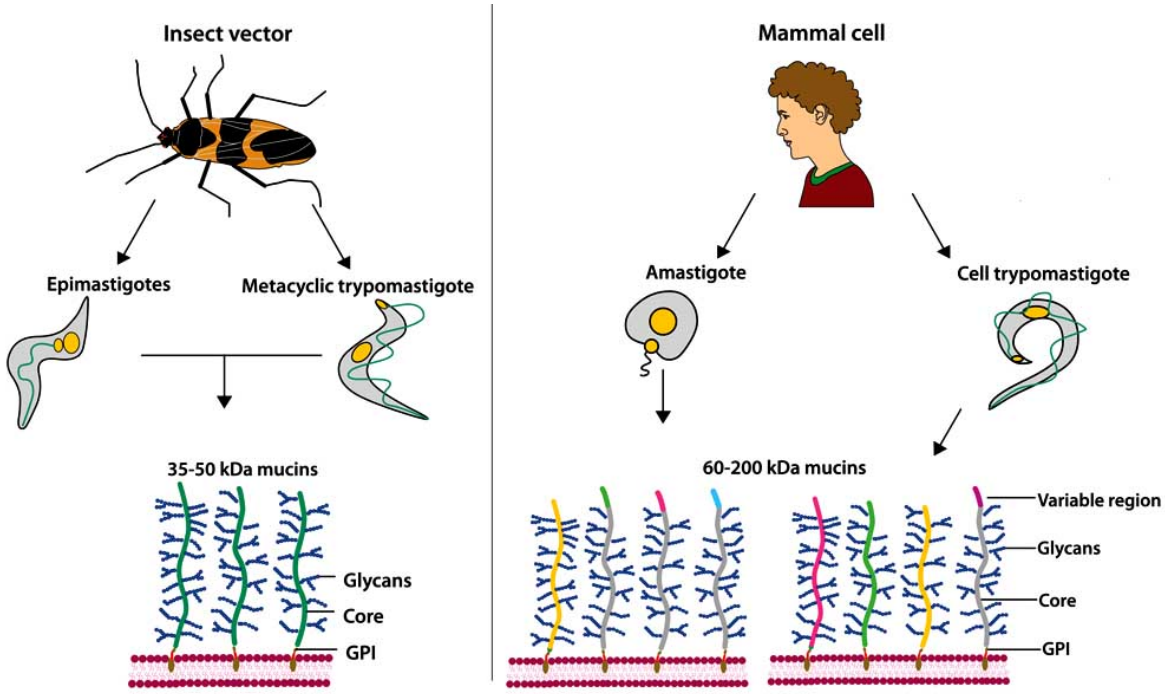

Fig. (6). Mucins in major *Trypanosoma cruzi* developmental forms in the insect vector and host cell (based on [8]).

Table 3. Some Functions of *Trypanosoma cruzi* Mucins

Stages / vector/ host	Functions	Ref.
Insect Epimastigote	Protection against peptidases	[67, 86]
Insect Metacyclic trypomastigote	Adhesion and invasion of mammalian host cells	[69, 87, 88]
	Ca^{2+} mobilization in the host cell	[89]
	Protection against peptidases	[82]
Mammal Blood trypomastigote	Cell attachment and invasion	[74]
	Induction of the synthesis of proinflammatory cytokines (TNF-α, IL-12) and nitric oxide (NO) by IFN-γ-primed murine macrophages	[67, 83, 84, 89, 90]
	Impair the B-cell Responses	[85, 91]
	Protect against complement-independent lysis	[67, 77]
Mammal Extracellular amastigotes	Impairment of B-cell responses	[85, 91-93]

sialylated, each parasite acquires about 1×10^7 sialic acid residues, resulting in a strong negative charge on the surface. This negatively charged coat is thought to provide protection against complement-independent lysis induced by human anti-galactosyl antibodies [67, 68]. The heterogeneity of the mucin core polypeptides expressed in mammal –dwelling stages of *T.cruzi* could have an additional protective effect against the host immune system. Antigenic cross-reactivity displayed by HV (hypervariable)–peptides might be one of the mechanism leading to the poor response directly towards them. A possible explanation could be the co-expression of multiple antigenically-related TcMUC I variants on the parasite surface impairing or delaying the maturation and/or leading to anergy of cross reactive B anf T lymphocytes [85].

3.1.b. Trans-Sialidase (TS)

Trans-sialidase (TS) is a glycoprotein that transfers sialic acid residues from host sialoglycoconjugates to parasite mucins but cannot use the CMP-sialic acid as a donor. TS activity has been postulated to enable *T. cruzi* to circumvent

its lack of *de novo* synthesis of sialic acid and is crucial for the viability and propagation of the parasite [72, 74, 94-96, 33, 101]. These molecules are present on the *T. cruzi* surface coat albeit in much lower numbers than mucins. Together with mucins they are essential for the infectivity of the parasite and its escape from the host-immune response [9, 71, 97-100]. *Endotrypanum* spp. (parasites of rain forest tree sloths) can also incorporate host-derived sialic acid into molecules of their own surface membrane [102].

The *ts* gene family comprises at least 1,400 members [103], which can be classified into three groups. [66] Two of these groups (TS and TS I) are expressed by trypomastigotes (non-replicative metacyclic forms in insect vectors and mammalian invasive bloodstream forms). Both of them are anchored by glycosylphosphatidylinositol (GPI) to the surface membrane. They have two main regions: an N-terminal catalytic region and a C-terminal extension with tandem repeats of 12 amino acids (SAPA repeats). Trypomastigotes derived from infected mammalian cells express and release 20 times more TS activity than axenic metacyclic trypomastigotes, which correspond to the infective forms derived from the insect vector [98]. After cleavage of its glycosylphosphatidylinositol (GPI) anchor by the action of a phosphatidylinositol-phospholipase C (PI-PLC), TSs are shed into the bloodstream to up-regulate the early infection in phagocytic and non-phagocytic cells and to exert other biological effects on several cell types [66, 104].

The lipid moiety of the glycoinositolphospholipid that anchors the trans-sialidase to the membrane was characterized and two different kinds of lipids are linked through a phosphate bridge to a glycoinositol structure: hexadecylglycerol (*Lyso*-1-O-hexadecylglycerol) and ceramide (N-palmitoyl-sphinganine) in a 1:3 ratio [105].

3.1.c. NETNES

MacRae *et al.* [106] described the occurrence of NETNES, a complex glycoprotein with only 13 amino acids with the sequence AQENETNESGSID, in *T. cruzi*. The glycoprotein (NETNES) is a 13-amino acid peptide with up to five post-translational modifications, including one or two N-linked glycans, two phosphate-linked mannose chains and a GPI anchor. The N-glycans are predominantly Manα1→6(Manα1→3) Manα1→6(Manα1→3)Manα1→4GlcNAcα1 →4GlcNAcα1-Asn; the phosphate-linked glycans are a mixture of (Manα1- 2)*O*-3Man1-P-Ser; and the GPI anchor has the structure Manα1-(ethanolamine phosphate)Manα1-2Manα1→ 6Manα1-4(2-aminoethylphosphonate-6)GlcNα1 →6-*myo*-inositol-1-P-3(*sn*-1-*O*-(C16:0) alkyl-2-*O*-(C16:0) acylglycerol). Four putative NETNES genes were found in the *T. cruzi* genome data base [106].

3.2. GPI-Anchored Proteins in Other Trypanosomatids

In trypanosomatids, other well characterized GPI-anchored molecules include the metallopeptidases, GP63 in *Leshmania* spp and VSG (variant surface glycoprotein) in *Trypanosome brucei*. Gp63 homologues have been found in all other trypanosomatids studied to date including heteroxenous members of *Trypanosoma cruzi* [107], *T. brucei*, [108-111], phytoparasitic *Phytomonas* spp. and numerous monoxenous species [112 , 113]. They very likely perform roles different from those in *Leishmania* spp. [114].

3.2.1. Trypanosoma brucei VSG

The African trypanosome *Trypanosoma brucei* is covered with a dense layer of variant surface glycoproteins (VSG), which protect it from lysis by host complement *via* the alternative pathway in the mammalian bloodstream [6, 109]. The parasite evades the immune system by periodically replacing the existing VSG coat with a different one. This phenomenon is known as antigenic variation, and it allows the trypanosome to maintain a chronic infection [115]. When bloodstream-form parasites are ingested by the tsetse fly, they differentiate into the procyclic form in the insect midgut and colonize it. Replacement of VSG with procyclin is a hallmark of the transformation of bloodstream stage trypanosomes into the procyclic form. The procyclic trypanosomes express a different cell surface coat that includes about 3 x 10^6 procyclin glycoproteins and about 1 x 10^6 poly-N-acetyllactosamine containing free GPIs [116-119]. Procyclins are glycosylphosphatidylinositol (GPI)-anchored proteins with either five or six pentapeptide repeats (GPEET procyclin) or up to 30 glutamic acid-proline dipeptide repeats (EP procyclin) that confer a rod-like structure to the protein [120-122]. Procyclin anchors are complex and are characterized by the presence of large poly disperse branched N-acetyllactosamine (Galβ1-4GlcNAc)- and lacto-N-biose (Galβ1-3GlcNAc)-containing side-chains that can be capped with α2-3-linked sialic acid residues [100]. The branched side-chains of the anchor form a dense glycocalyx that contributes to the protective function of the coat against digestive enzymes in the fly midgut [123]. The lipid moiety of gp63 is composed of alkylacylglycerol [22, 124].

GPEET and EP procyclins contain similar GPI membrane anchors. These are based on the ubiquitous ethanolamine-*P*-6Manα1-2Manα1- 6Manα1-4GlcNα1-6PI core: the PI lipid has a 2-*O*-acyl-myo-inositol-1-P-*sn*-2-l*yso*-1-*O*-acylglycerol structure [120-122].

3.2.2. Leishmania spp. *gp63*

Leishmania parasites are coated by a characteristic glycocalyx of molecular components that play a critical role in the initial contact between the parasite and its host environment. The gp63 from *Leishmania* spp, also referred to as a promastigote surface peptidase (PSP), leishmanolysin and major surface peptidase (MSP), is a metallopeptidase related protein associated with virulence and pathogenicity in this trypanosomatid [7, 125, 126].The enzyme corresponds to the most abundant surface glycoprotein in promastigotes and is anchored *via* a glycosylphosphatidylinositol (GPI) anchor [127]. Gp63 plays a crucial role in complement fixation and processing, which protect Leishmania in mammalian hosts [126 , 128]. Other studies have demonstrated that gp63 defends the parasite against antimicrobial peptides such as defensins and pexiganans [129]. The high catalytic activity of gp63 at mammalian body temperature favors the dissemination of the parasite as it digests constituents of the extracellular matrix of the host such as collagen type IV, fibronectin and laminin [130,131].

Several species of *Leishmania* spp. release proteolytically active gp63 into the extracellular medium presumably facilitating the propagation of the parasite [131,132].

In addition, fragments from gp63-processed fibronectin can protect parasites within macrophages, because they attenuate production of reactive oxygen intermediates and favor amastigote proliferation [133]. gp63 has also been suggested to maximize promastigote binding, to participate in internalization in macrophages and to promote complement-dependent adhesion [134]. Moreover coating polystyrene surfaces with gp63 enhances the *in vitro* spreading of fibroblasts [135].

The expression of specific gp63 genes in the intracellular amastigote form implies an intra-host cell function for this peptidase. Curiously, the identification of the myristoylated alanine-rich C kinase substrate related protein (MRP), a cytosolic protein associated with the actin network of macrophages, as a substrate of gp63 reinforces the potential of this enzyme to modulate host cell activities within the intracellular space [134].

Gp63 has been recently reported to cleave multiple intracellular proteins and to participate actively in p38 mitogen-activated protein kinase inactivation. A rearrangement of the actin cytoskeleton and marked modification of the profile of protein tyrosine phosphorylation in fibroblasts infected with *Leishmania major* has been described. Correspondingly, exposure to *L. major* resulted in degradation of the phosphorylated adaptor protein p130Cas and the protein-tyrosine phosphatase-PEST [134, 136, 137].

In addition, a recent study by Gomes and collaborators [138] reported that gp63 is the key *Leishmania*-virulence factor that modulates macrophage protein tyrosine phosphatases (PTPs) and revealed an essential role for PTP1B in the progression of cutaneous leishmaniasis in infected mice. The mechanism underlying protein tyrosine phosphatase (PTP) modulation involves the proteolytic activity of the *Leishmania* surface protease gp63. Furthermore, the authors reported a mechanism whereby *Leishmania* gp63 accesses the macrophage intracellular medium in part by a lipid raft-dependent mechanism, allowing a direct interaction with host protein substrates. The internalization of gp63, a key *Leishmania* virulence factor, into host macrophages is a strategy that the parasite uses to interact and survive within its host [138].

PERSPECTIVES

A huge number of glycosphingolipid structures (GSLs) have been identified in mammalian cells. These cell-surface molecules participate in cell physiology and play important roles in cell recognition and in the modulation of function of receptors, etc. In trypanosomatids, glycolipid analysis is undergoing rapid expansion. Glycosphingolipids (GSLs) have been characterized in *T. cruzi*, *Leishmania* spp. and some non-pathogenic trypanosomatids. Cell surface glycolipids/glycoproteins that form essential surface coats for survival of parasites in their various hosts were also identified. Many of these glycoconjugates are attached *via* glycosylphosphatidylinositol (GPI) anchors. However, the function of some of these molecules has remained largely unknown. A combination of isolation and separation technologies, as well as the use of mass spectrometry, for glycolipid structural characterization is required to unravel functional aspects of these cell surface molecules and to gain a better understanding of their role in infectious diseases. Considerable advances have recently been made in fields such as liquid-chromatography- Mass spectrometry (LC-MS) of glycolipids from mammalian cells [139], GPI-anchored proteomics of *Plamodium falciparum* [140], GPIomics of *T.cruzi* [13] and glycolipid arrays to study antitoxic malaria response [141]. The combination of these sensitive and powerful techniques has allowed us to increase our structural and functional knowkedge of a wide variety of glycoconjugates an other macromolecules expressed by different protozoa.

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