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Review Article

The Sphingolipid Biosynthetic Pathway Is a Potential Target for Chemotherapy against Chagas Disease

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The protozoan parasite *Trypanosoma cruzi* is the causative agent of human Chagas disease, for which there currently is no cure. The life cycle of *T. cruzi* is complex, including an extracellular phase in the triatomine insect vector and an obligatory intracellular stage inside the vertebrate host. These phases depend on a variety of surface glycosylphosphatidylinositol-(GPI-) anchored glycoconjugates that are synthesized by the parasite. Therefore, the surface expression of GPI-anchored components and the biosynthetic pathways of GPI anchors are attractive targets for new therapies for Chagas disease. We identified new drug targets for chemotherapy by taking the available genome sequence information and searching for differences in the sphingolipid biosynthetic pathways (SBPs) of mammals and *T. cruzi*. In this paper, we discuss the major steps of the SBP in mammals, yeast and *T. cruzi*, focusing on the IPC synthase and ceramide remodeling of *T. cruzi* as potential therapeutic targets for Chagas disease.

1. Introduction

Sphingolipids (SLs) belong to a diverse group of amphipathic lipids that have essential functions in eukaryotes. They are constituents of cellular membrane compartments and participate in a diverse array of signal transduction processes [1, 2]. The final products of the sphingolipid biosynthetic pathways (SBPs) are different in mammals, fungi, plants and protozoa. Thus, certain steps of this pathway are potential targets for chemotherapy against fungal [3] and protozoal infections [4–6]. Sphingomyelin (SM) is the primary phosphosphingolipid that is produced by mammalian cells, including in humans [7]. This molecule is formed by the transfer of the choline-phosphate head group from phosphatidylcholine (PC) to ceramide, a reaction catalyzed by SM synthase [8]. In contrast, plants, fungi, and some protozoa synthesize inositolphosphorylceramide (IPC) as their primary phosphosphingolipid [9]. In this pathway, the IPC synthase enzyme [10] catalyzes the transfer of inositol phosphate to ceramide. IPC makes up a relatively low proportion of fungal phospholipids. Nonetheless, it is essential, as IPC synthase-null mutants are not viable [11] and inhibitors of this enzyme kill fungal cells [12, 13].

Numerous proteins and glycolipids are attached to membranes by a glycosylphosphatidylinositol (GPI) anchor. This posttranslational modification is conserved among yeast, protozoa, plants and animals [14]. All of these groups except animals have GPI anchors containing IPC as an attached lipid. GPI biosynthesis is essential for mammalian embryonic development and the growth of yeasts and trypanosomes [15–17]. The biosynthesis and maturation of GPI anchors occurs during the ER-to-Golgi transit, beginning with the sequential addition of sugars and ethanolamine phosphates to phosphatidylinositol (PI). Subsequent structural remodeling reactions can happen during biosynthesis or after attachment to proteins. Most of these steps have been studied at the biochemical and molecular levels [18, 19]. Recently, it has been shown that GPI lipid remodeling reactions are important for maintaining the correct fate of GPI-anchored glycoconjugates and their proper association with microdomains in certain cellular processes [20, 21].

Several neglected tropical diseases worldwide are caused by a group of trypanosomatid protozoan parasites (also known as Tritryps), including the following: (i) African trypanosomes (*Trypanosoma brucei* subspecies), which cause sleeping sickness, (ii) multiple *Leishmania* species, which cause cutaneous and visceral forms of leishmaniases, and (iii) *Trypanosoma cruzi*, which causes Chagas disease [22–26]. Recent studies have shown that all of these parasites are capable of synthesizing IPC and that the expression of IPC is regulated during development.

The genome sequences of these pathogenic microrganisms have recently been published, allowing us to search for differences between the SL and GPI structures of mammals and Tritryps to identify novel drug targets. Here, we will discuss the major steps of the SBP in mammals, yeast and Tritryps. We will focus on the IPC synthase and ceramide remodeling of *T. cruzi* as potential therapeutic targets for Chagas disease.

2. Initial Steps in the *De Novo* Synthesis of a Sphingoid Long-Chain Base

In all eukaryotes, *de novo* SL biosynthesis starts with the condensation of L-serine and palmitoyl-CoA into 3-ketodi-hydrosphingosine (3-KDS), as shown in Figure 1 and Table 1. A pyridoxal 5′-phosphate-dependent enzyme called serine palmitoyltransferase (SPT) catalyzes this reaction. The SPT enzyme (Figure 1 and Table 1, Step 1) is a complex of two subunits, SPT1 and SPT2 [27]. In yeast, the small peptide TSC3 significantly enhances SPT activity [28]. Two open reading frames (ORFs) with homology to yeast *LCB1* and *LCB2* can be found in the Tritryp genome database. Although SPT1p and SPT2p function as a heterodimer, all experimental data indicate that the SPT2 subunit contains the catalytic site [28]. For this reason, most of the studies on Tritryps (mainly *L. major* and *T. brucei*) have focused on SPT2.

The expression of the *LmSPT2* gene (also called *LmLCB2*) is developmentally regulated. LmSPT2p is undetectable in the late stationary growth phase of promastigotes, as well as in metacyclic trypomastigotes and intramacrophage amastigotes [29, 30]. Deletion of the *SPT2* gene in *L. major* results in a complete loss of IPC and ceramide, whereas other alkyl/acyl and acyl/acyl phospholipids remain unchanged [29]. Although *spt2*$^-$ mutant promastigotes are viable and grow during log phase, they fail to efficiently differentiate into infective metacyclic trypomastigotes and die rapidly at this stage. This phenotype can be rescued either by the addition of sphingoid bases (3-ketodihydrosphinsosine or 3-KDS, dihydrosphingosine, sphingosine, and phytosphingosine), ethanolamine (EtN) or EtN-phosphate to the medium, or by complementation with the original *LmLCB2* gene [29–33]. However, neither ceramide nor SM can rescue the stationary phase defects or restore IPC synthesis [29]. Similar observations have previously been made in yeast and mammalian *SPT*-deficient mutants [34, 35]. Denny and Smith [30] showed that exocytic trafficking is compromised in *spt2*$^-$ mutants, but Zhang and colleagues [29, 32] observed little negative effect on vesicular trafficking. However, both groups found that *spt2*$^-$ parasites retain their ability to form membrane microdomains and lipid rafts [29, 32, 33]. It has been suggested that *Leishmania* can compensate for the loss of SLs by increasing its overall level of lipid synthesis, for example, by increasing ergosterol [31] or GIPL [33] production. These *spt2*$^-$ mutants are still able to establish infection in a mouse model, although with some delay [33], confirming that the first step in the *de novo* SBPs is unnecessary for either the survival of *Leishmania* within host macrophages or the resulting pathogenesis.

In contrast, the first step of the SBPs, which is catalyzed by SPT, is essential in *T. brucei*. This conclusion was based on pharmacological experiments with myriocin (Figure 1, Step 1), an inhibitor of SPT [36], and genetic experiments [37, 38]. These perturbations most profoundly affect viability, cellular proliferation and cytokinesis, with marginal effects on secretory trafficking and lipid raft formation. SL depletion can be rescued by the addition of 3-KDS, the immediate downstream intermediate in the SBPs, but not by the addition of ceramide or EtN [37, 38]. These results indicate that *T. brucei* absolutely requires *de novo* synthesis of SLs.

In the second step of SL biosynthesis (Figure 1 and Table 1, Step 2), the product 3-KDS is rapidly converted into dihydrosphingosine (DHS; sphinganine) in a NADPH-dependent manner by the 3-ketodihydrosphingosine reductase (KDSR) encoded by *TSC10* (temperaturesensitive suppressor of *cgs2*Δ) in *S. cerevisiae* [39] and by FVT-1 (follicular lymphoma variant translocation-1) in mammals [40]. Although KDSR activity has not been measured in parasites, *TSC10* homologues can be found in the genomes of Tritryps (Table 1). A predicted *TcKDRS* can be found in the GeneDB database in two genomic fragments (*Tc00.1047053510997.10* and *Tc00.1047053506959.64*) whose nucleotide and amino acid sequences are 98% identical. These probably correspond to the two haplotypes present in the hybrid CL-Brener strain [41].

3. Ceramide Synthase: The Central Axis of the SBPs

The next step in the SBPs, the synthesis of ceramide, is a key component of the pathway (Figure 1 and Table 1, Step 3). Ceramide is critical for cell growth and functions in several different cellular events, including apoptosis, growth arrest, endocytosis and stress responses [42–44]. Ceramide can be degraded by a ceramidase, or the sphingoid bases can be phosphorylated to produce DHS-1-P/SPH-1-P signaling molecules (Figure 1). Ceramide is synthesized mainly from the reaction of a fatty acyl-CoA with a sphingoid base catalyzed by an acyl-CoA:sphingosine *N*-acyltransferase or ceramide synthase (CerS) [45, 46]. An acyl-CoA-independent CerS activity has been described [47, 48] although it probably represents a reversal of ceramidase action. In yeasts, the long-chain base (LCB) DHS can be hydroxylated at C-4 by *SUR2/SYR2* [49] to form the sphingoid base phytosphingosine, which is later *N*-acylated by either of the two CerSs, encoded by *LAG1* (longevity assurance gene) or *LAC1* [50, 51], to yield phytoceramide.

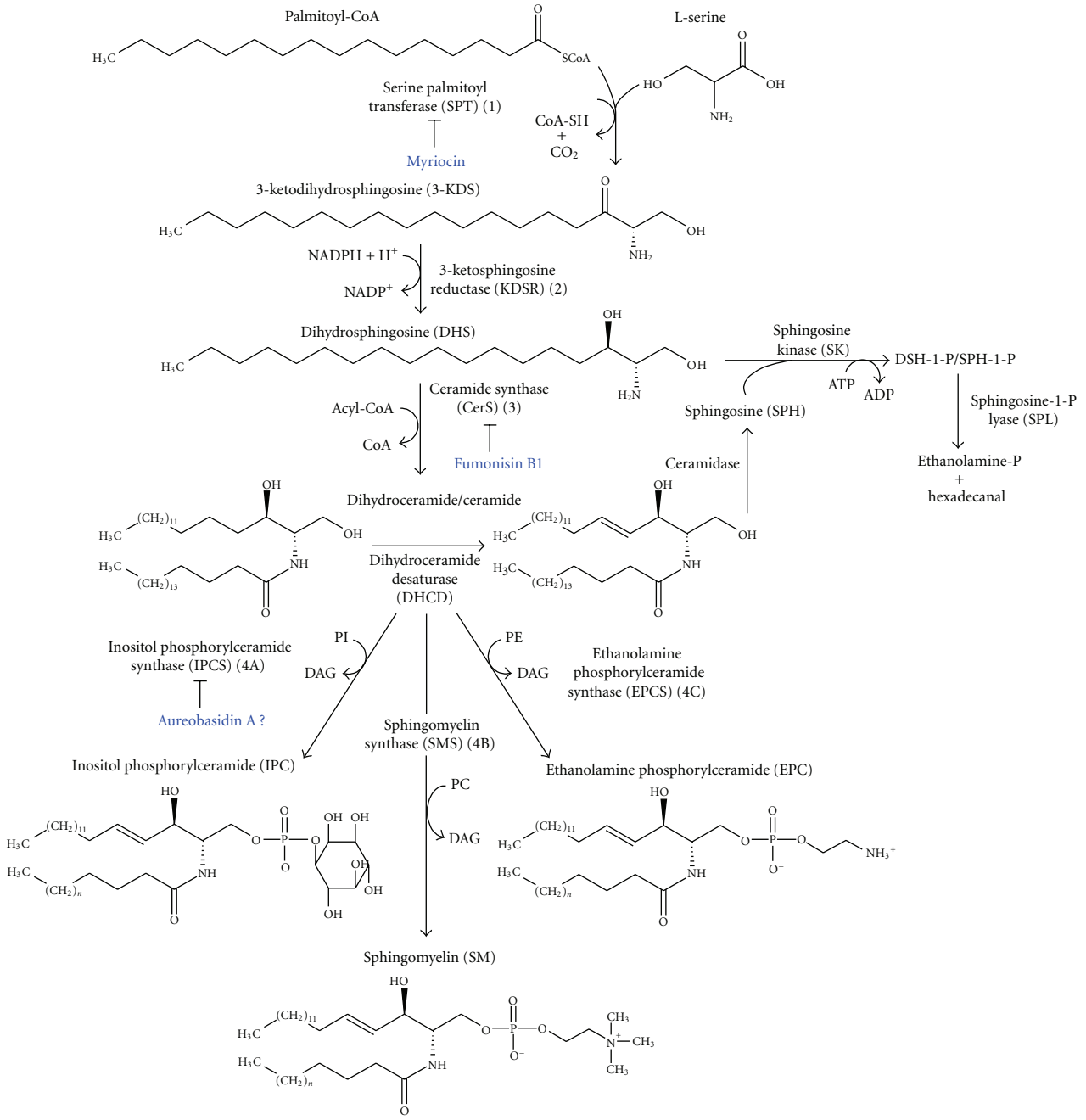

Figure 1: General scheme of the SBP in Tritryps. Substrates, products, enzymes and effective/potential (?) inhibitors of the four (1–4) initial SBP steps are indicated as described in the text and in Table 1.

In mammals, DHS can be directly *N*-acylated by a family of CerSs encoded by *CERS1-6* genes [52]. In addition, another essential component of the yeast CerS, known as Lip1p, was described recently. It forms a heteromeric complex with Lac1p and Lag1p and is required for CerS activity in yeast [53]. No orthologue of Lip1 has yet been found in nonfungal species.

In Trypanosomatids, ceramide can be found as a lipid component of phospholipids like SM, in *T. brucei,* and IPC, which is expressed in all Tritryps [54–62]. CerS activity has been identified [54] and characterized at the biochemical and molecular levels only in *T. cruzi* ([63], submitted). The TcCerS was initially identified by the incorporation of [^{3}H]palmitic acid into ceramides, which were chemically degraded to radiolabeled dihydrosphingosine and fatty acid [54]. More recently, TcCerS activity has been detected in a cell-free system using the microsomal fraction of epimastigote forms of *T. cruzi*. In this system, the enzyme was shown to employ both sphingoid long-chain bases (DHS and SPH) ([63], submitted). This activity requires acyl-CoAs,

Table 1: Genes required for sphingolipid biosynthesis and lipid remodeling steps in mammals, *Saccharomyces cerevisiae*, *Trypanosoma brucei*, *Leishmania major*, and *Trypanosoma cruzi*.

Step	Activity	Mammals	*S. cerevisiae*	*T. brucei*	*L. major*	*T. cruzi*
1	SPT	*LCB1* *LCB2*	*LCB1/LCB2* *TSC3*	*TbSPT1* *TbSPT2*	*LmSPT1* *LmSPT2*	*TcSPT1* *TcSPT2*
2	KDSR	*FVT-1*	*TSC10*	*Tb927.10.4040*[a]	*LmjF.35.0330*	*Tc00.1047053510997.10* *Tc00.1047053506959.64*[a]
3	CERS	*CERS1-6*	*LAG1/LAC1* *LIP1*	*Tb927.8.7730* *Tb927.4.4740*	*LmjF.31.1780*	*TcCERS1*
4	SLS	*SMS1* *SMS2*	*AUR1* *KEI1*	*TbSLS1-4*	*LmIPCS*	*TcIPCS1* *TcIPCS2*
5	ID	*PGAP1*	*BST1*	*GPIdeAc2*	—[b]	*Tc00.1047053508153.1040*[a]
6	GPIPLA2	*PGAP3*	*PER1*	—	—	—
7	LGPIAT-I	*PGAP2*	*GUP1*	*TbGUP1*	*LmjF.19.1000/LmjF.19.1320* *LmjF.19.1340/LmjF.19.1345* LmjF.19.1347[a]	*TcGUP1*[c]
8	CR	—	*CWH43*	—	*LmjF27.1770*[a]	*Tc00.1047053504153.120*[a]

[a] Corresponding homologues of each yeast gene were found by BLAST at NCBI and GeneDB (http://www.genedb.org/).
[b] Not found.
[c] *TcGUP* can be *Tc00.1047053508943.4*, *Tc00.1047053511355.59*, or *Tc00.1047053503809.90*.

with palmitoyl-CoA being preferred. In addition, Fumonisin B_1, a broadly active and well-known acyl-CoA-dependent CerS inhibitor (Figure 1, Step 3), blocks parasite CerS activity ([63], submitted). However, unlike what has been observed in fungi, the CerS inhibitors Australifungin [64] and Fumonisin B_1 [65] do not affect the proliferation of epimastigotes in culture ([63], submitted). Orthologues of the conserved Lag1-domain from yeast CerS, *LAG1*, were identified in a search of the Tritryp genome sequences (Table 1). The *T. cruzi* candidate gene (*TcCERS1*), which was hypothesized to encode the parasite's CerS orthologue, can functionally complement the lethality of a *lag1*Δ *lac1*Δ double-deletion yeast mutant that has no detectable acyl-CoA-dependent CerS ([63], submitted).

Glycoinositolphospholipids (GIPLs) are abundant surface glycoconjugates of *T. cruzi* and are involved in the pathogenesis of Chagas disease [66, 67]. GIPLs contain an IPC-lipid anchor that is formed by dihydroceramide *N*-acylated with palmitic or lignoceric acids [68–72]. TcCerS uses only palmitoyl-CoA as a substrate donor ([63], submitted); it is not known how the parasite incorporates C24:0 into ceramides. Recently, a novel fatty acid synthesis system was identified in the Tritryps [73]. In this system, synthesis is mediated by elongases that prime a butyryl-CoA molecule with malonyl-CoA units as the donor substrate and promote fatty acid extension to a length of 18 carbons or longer. Therefore, it is possible that this system elongates shorter fatty acids to C24:0 so that they can then be incorporated into ceramides. Alternatively, the substrate could be another fatty acid, like arachidonate (C20:4 from extracellular sources), which could be elongated and desaturated further to generate very-long-chain fatty acids [73]. Finally, IPC acyl-hydrolase and IPC acyl-transferase activities have been detected in membranes of *T. cruzi* [74, 75] and could be involved in the remodeling of the endogenous ceramide C16:0 fatty acids by an extracellular fatty acid (see below).

4. IPC Synthase Activity

The synthesis of IPC (Figure 1 and Table 1, Step 4A) occurs by the transfer of inositol phosphate from PI to the C-1 hydroxyl group of ceramide or phytoceramide. This reaction is catalyzed by IPC synthase, which is localized to the Golgi of yeasts. IPC synthase is encoded by *AUR1* (also called *IPC1*) [11]. As already mentioned, IPC represents a relatively low proportion of fungal phospholipids, but it is essential. IPC synthase-null mutants are not viable [11], and fungal cells are killed by the IPC synthase inhibitors Aureobasidin A (AbA) [12] and Rustmicin [13]. Recently, a critical protein interaction partner for yeast IPC synthase was identified and named Kei1p. It was shown that Kei1p is essential for both yeast IPC synthase activity and for its sensitivity to AbA [76]. As shown in Figure 1 (Step 4B), mammals cannot synthesize IPC; instead, they produce SM using two major SM synthases [8] encoded by *SM1* and *SM2* (Table 1, Step 4).

In yeasts, IPC is found as a lipid in complex SLs [9] or in mature GPI-anchored surface proteins. It is composed of a sphingoid-base with *N*-acylated C18:0–C26:0 fatty acids [18, 77, 78]. In *T. cruzi*, IPC is found in the majority of GIPLs (in epimastigotes) [67–70], the GPI anchors of Ssp4 antigen (in amastigotes) [54], *trans*-sialidase and Tc-85 glycoprotein (in trypomastigotes) [79, 80], mucins and 1G7-Ag (in metacyclic forms) [71, 81, 82]. In replacement of IPC, GPI-anchored proteins contain only 1-*O*-hexadecylglycerol-based PIs [71, 72, 81–84]. The lipid moiety of GIPLs also includes a small amount (2–8%) of 1-*O*-hexadecyl-2-acyl-PIs [72, 85]. Thus, there is a developmentally regulated expression [54] and distribution of ceramide in *T. cruzi* GPI-anchored components. In *T. brucei* bloodstream forms, GPI-protein anchors contain dimyristoylglycerol, whereas in *Leishmania,* these anchors are mainly composed of *sn*-1-alkyl-2-acyl-PI or *sn*-1-alkyl-2-*lyso*-PI [14]. In *Leishmania*, IPC is present together with other SLs and sterols in organized lipid rafts

[86] but it is never found attached to any GPI-anchored protein or GIPL [14]. In *T. brucei*, IPC has been found in insect-stage procyclic forms (PCFs), but its role remains unclear [38, 60, 87].

Another SL that is produced by *T. brucei* is SM. This lipid has been detected using a combination of methods, including metabolic labeling, enzyme treatments and high-resolution mass spectrometry, in both insect and mammalian stages of the parasite [61, 88]. The relative amount of SM in PCF cells is significantly lower than that in blood-stream forms (BSFs) [87], probably because the ceramide in BSFs is used in conjunction with PC to form SM, whereas in PCFs ceramide is also used to form IPC from PI [38, 61]. In BSF parasites of *T. brucei*, the unusual phosphosphingolipid ethanolamine phosphorylceramide (EPC, Figure 1, Step 4C) was detected for the first time by Sutterwala and colleagues [61]. Its presence was later confirmed by a lipidomic analysis [87].

The IPC synthases of *L. major* (*LmIPCS*) and *T. cruzi* (*TcIPC1* and *TcIPC2*) and the SL synthase (SLS) family of *T. brucei* (*TbSLS1-4*) were initially identified in the GeneDB database based on sequence similarity [4]. These are shown in Table 1 (Step 4). The *TbSLS* genes are organized in a unique linear tandem array. All Tritryp sequences are predicted to have six *trans*-membrane (TM) domains and two luminal motifs that likely constitute the catalytic domain. Each Tritryp sequence contains histidine and aspartate residues that mediate nucleophilic attack on the lipid phosphate ester bonds. This predicted topology more closely resembles that of mammalian SMSs, which also have the signature motifs (D1-4), than the fungal IPC synthase, which contains only the D3-4 motifs and is encoded by *AUR1/KEI1* genes [4, 8, 11, 61, 62].

All the *TbSLSs* genes are constitutively expressed in both stages of the life-cycle (PCF and BSF). Simultaneous knockdown of the four *TbSLS* genes using a pan-specific RNAi showed that the *TbSLS* gene products are required for cell viability [61]. The activity of each of the *TbSLS* gene products has been validated with genetic and biochemical analyses, as well as with a recently developed cell-free system for the synthesis of active polytopic membrane proteins. TbSLS1p is an IPC synthase and is expressed in PCFs, whereas TbSLS2p is an EPC synthase, and TbSLS3p and TbSLS4p are bifunctional SM/EPC synthases [61, 62]. Sequence alignments and site-specific mutagenesis indicate that the specific phospholipid head group donor depends on subtle differences in active site residues [62]. Taken together, the existing data support the ability of *T. brucei* to synthesize IPC (Figure 1, Step 4A), SM (Figure 1, Step 4B) and EPC (Figure 1, Step 4C).

The Lederkremer's group was the first to identify IPC in *T. cruzi* epimastigotes [57], trypomastigotes [58] and amastigotes [54, 75]. IPC synthase activity was initially found in the microsomal membranes of all life-cycle stages of *T. cruzi* [59]. The TcIPC synthase activity is consistent with the proposed reaction scheme for IPC synthase in fungi and plants, though there are differences in the optimal pH conditions, metal requirements and detergent preferences [59]. The classical inhibitors of fungal IPC synthase, rustmicin and AbA, do not inhibit *T. cruzi* IPC synthase *in vitro* (over the range of 0.9–7 μM) and do not affect the proliferation of epimastigotes in culture (>40 μM). However, AbA inhibits both the proliferation of amastigotes inside macrophages and the release of trypomastigotes from these cells in a dose-dependent manner [59]. The reduction in intracellular proliferation can be partially attributed to the effect of this drug on macrophage function, diminishing phagocytic capacity and nitric oxide production [59].

Similar results have been obtained with AbA in *T. brucei* SL synthesis [62], suggesting that the IPC synthase enzyme is not the main target of AbA in parasites. Nonetheless, TbSLSs remain potential chemotherapeutic targets, as *T. brucei* is critically dependent on *de novo* synthesis of sphingolipids to survive. Mass spectrometry of lipids extracted from AbA-treated *L. major* promastigotes has shown that there is no effect on IPC synthesis, unless very high concentrations of AbA are administered (>5.0 μM) [29].

5. The Lipid Remodeling Reactions

More than 20 genes involved in GPI biosynthesis and protein attachment have been identified. In most cases, these genes are conserved from yeast to mammalian cells [18, 89]. As in mammals, yeasts, *T. brucei* and *Leishmania*, the first steps of GPI anchor biosynthesis in *T. cruzi* do not include a ceramide precursor [90, 91]. Thus, ceramide is probably added during a later remodeling step in *T. cruzi* [75, 90, 91], as in yeasts [78, 92, 93]. Although there are differences in the ceramide composition, remodeling in yeast happens after the attachment of the GPI anchor to the proteins, whereas in *T. cruzi,* remodeling may occur on the GPI protein anchors and/or GIPLs.

Lipid remodeling of GPI-anchored proteins has been studied in fungi, mammals and *T. brucei* [20, 21]. The four most important enzymes and the genes involved in this process are listed in Table 1 (Steps 5–8); the lipid remodeling reactions are depicted in a simplified scheme in Figure 2.

In mammals, the first reaction is a deacylation to remove the fatty acid linked to position C-2 of the GPI anchor inositol ring (Table 1 and Figure 2, Step 5). This reaction is catalyzed by PGAP1p (postGPI attachment to protein 1) and occurs before the GPI-attached proteins leave the ER, as it is critical for efficient transit of GPI anchored proteins to the Golgi [94]. The second reaction is the removal of the unsaturated acyl chain from the *sn*-2 position of the alkyl-acyl-glycerolipid to form a *lyso*-GPI (Table 1 and Figure 2, Step 6). This reaction is catalyzed by PGAP3p [95]. The final reaction in mammals is the transfer of a saturated acyl chain (C18:0) to the *sn*-2 position of the *lyso*-GPI species (Table 1 and Figure 2, Step 7). PGAP2p is one protein involved in this process [96], but it is probably not an acyl-transferase because it has no homology to acyltransferases [21].

Mature GPI-anchored proteins in yeasts contain two different types of lipid moieties [77, 78, 92]. The first is a diacylglycerol with a C26:0 fatty acid at the *sn*-2 position. The second is a ceramide containing mainly C18:0 phytosphingosine and a C26:0 fatty acid [78]. In both cases, the C26:0 fatty acid may be 2-hydroxylated. As mentioned above

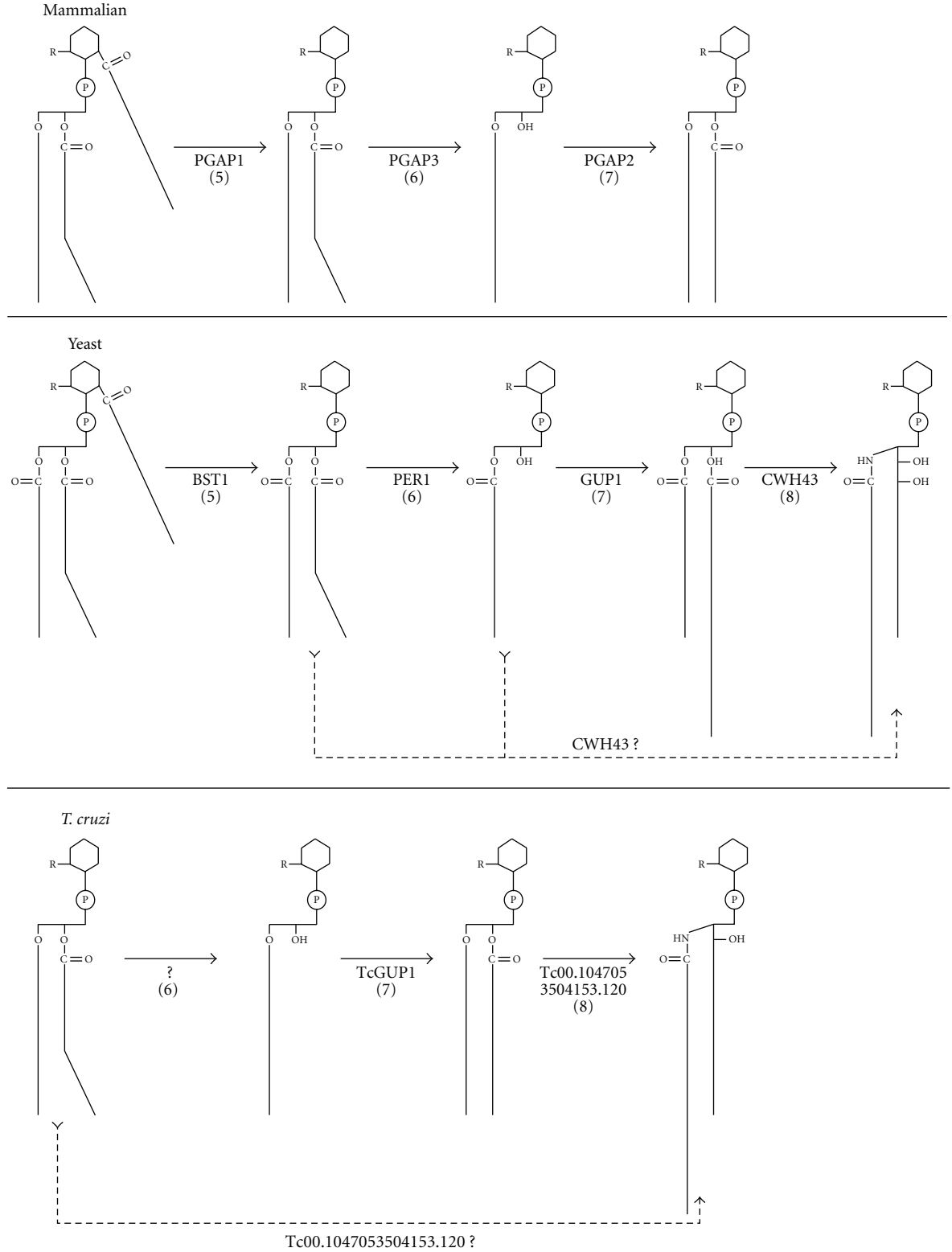

Figure 2: Lipid remodeling pathways in mammals, yeast and *T. cruzi*. "R" represents the entire glycan structure of each GPI anchor precursor linked to a protein. Although this assumption has been validated in mammals (top panel) and yeast (middle panel), no such experimental data are available for *T. cruzi* (bottom panel). Each step (in parentheses) has corresponding entries in Table 1.

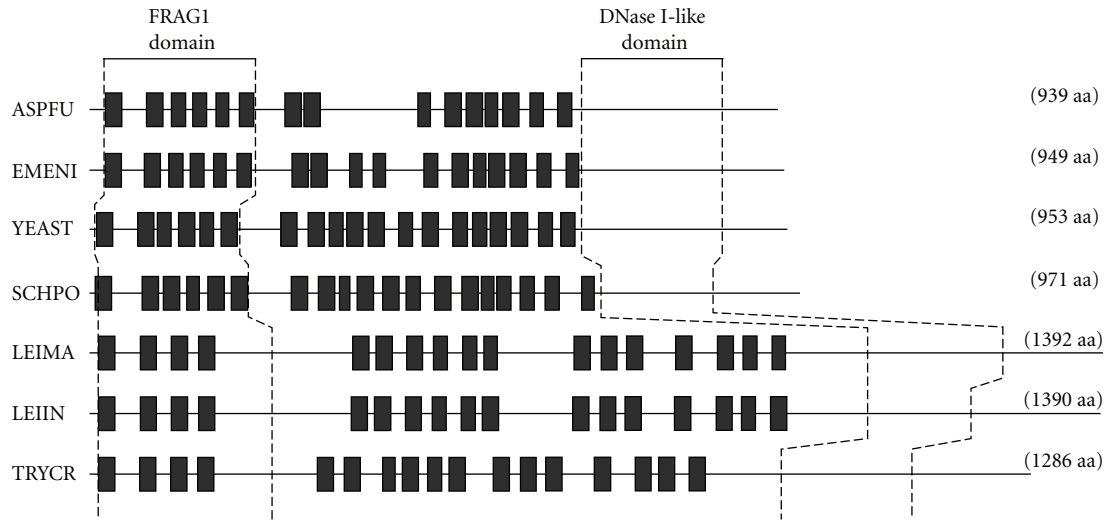

Figure 3: Comparison of the CWH43 proteins from fungi and Tritryps. The amino acid sequences of CWH43 proteins from *Aspergillus fumigatus* (ASPFU), *Emericella nidulans* (EMENI), *Saccharomyces cerevisiae* (YEAST) and *Schizosaccharomyces pombe* (SCHPO) were compared with the putative CWH43p candidates from *Leishmania major* (LEIMA), *Leishmania infantum* (LEIIN) and *Trypanosoma cruzi* (TRYCR). Thick horizontal bars indicate the relative positions of the membrane-spanning domains as predicted by the TMHMM Server. Dashed lines delineate the relative positions of the FRAG1 and DNase I-like domains, which are located at the N- and C-terminus, respectively.

for mammals, lipid remodeling of the yeast GPI-anchored proteins starts in the ER with the removal of the fatty acid linked to the C-2 position of the GPI anchor inositol ring (Table 1 and Figure 2, Step 5). This reaction is catalyzed by the PGAP1p orthologue BST1p [94]. Unlike mammals, which have a *sn*-1-alkyl-2-acyl-glycerolipid attached to the GPI, yeasts have a diacyl-glycerolipid (Figure 2). The next step is the removal of the C18:1 fatty acid at the *sn*-2 position of diacylglycerol to form a *lyso*-GPI (Figure 2, Step 6). This reaction is performed by GPI phospholipase A_2 (GPI-PLA_2), which requires PER1p (Table 1, Step 6) for its activity [94, 95]. Next, the free *sn*-2 position is filled with a C26:0 fatty acid (Figure 2, Step 7) by an acyltransferase called GUP1p [97]. Finally, the diacylglycerol lipid moiety is replaced by a ceramide (Figure 2, Step 8) with C18:0 phytosphingosine and a hydroxy-C26:0 fatty acid [78]. It has been reported recently that CWH43p (Table 1, Step 8) is responsible for this replacement [98, 99]. Indeed, the exchange reaction requires the C-terminus of CWH43p, and the association of the CWH43p-N with the CWH43p-C enhances the lipid remodeling reaction [99]. The alignment of CWH43p to its homologues in fission yeast (*Shizosaccharomyces pombe*), filamentous fungi, mice and humans has identified conserved residues that are important for the lipid remodeling function, including H802, D862 and R882, and protein stability (G57) [97, 99]. The N-terminal region of yeast CWH43 has a FRAG1 domain (Figure 3), which is also present in PGAP2p and is thought to act as a protein interaction motif that enhances stability under conditions of replicative stress [21]. The C-terminal region of CWH43 also has a DNase I-like motif (Figure 3) that is found in Isc1p, Inp51p, Inp52p, Inp53p and Inp54p. Isc1p is an inositol phosphosphingolipid phospholipase C [20], and the Inp51/52/53/54 proteins are phosphoinositol phosphatases [21]. This motif may be involved in the recognition of inositol phosphate, in which case the DNase I-like region in the C-terminal domain of CWH43p could be important for the recognition of PI on the GPI anchor.

The sequential remodeling reactions mentioned above comprise one of the three possible pathways for lipid remodeling in yeast (Figure 2, compare full with dashed lines). Another may be a divergent pathway, in which the *lyso*-GPI generated by PER1p is a direct substrate for the ceramide remodeling activity of CWH43p [99]. CWH43p could be involved in the direct exchange of glycerolipids containing an unsaturated fatty acid for the ceramide moiety. This third alternative is proposed to function as a backup if the first one, which is mediated by PER1p and GUP1p, is defective [94, 98, 99].

Acyl exchange also occurs in *T. brucei*, but in this organism, the remodeling happens before (during GPI anchor biosynthesis) and after the GPI anchor precursor is transferred onto the protein [100]. *T. brucei* contains two different GPI deacylation/reacylation pathways. One pathway (termed lipid remodeling) acts on lipid A′ (a GPI anchor biosynthetic precursor containing a *sn*-2-heterogenous fatty acid) to generate lipid A (containing *sn*-1, 2-dimyristoylglycerol), creating the intermediates lipid θ (*lyso*-GPI) and lipid A″ (*sn*-1-stearoyl-2-myristoylglycerol). The other pathway (termed lipid exchange) acts on both GPI proteins and lipid A and exchanges the original myristate for another myristate [100, 101]. The acyl transferase GUP1p has homologues encoded in the genomes of *T. brucei* and *T. cruzi* (Table 1, Step 7). In *T. brucei*, TbGUP1p is required for the acylation of lipid θ (*lyso*-PI) to generate lipid A″ (*sn*-1-stearoyl-2-myristoylglycerol) in the remodeling of GPI lipids [100, 101].

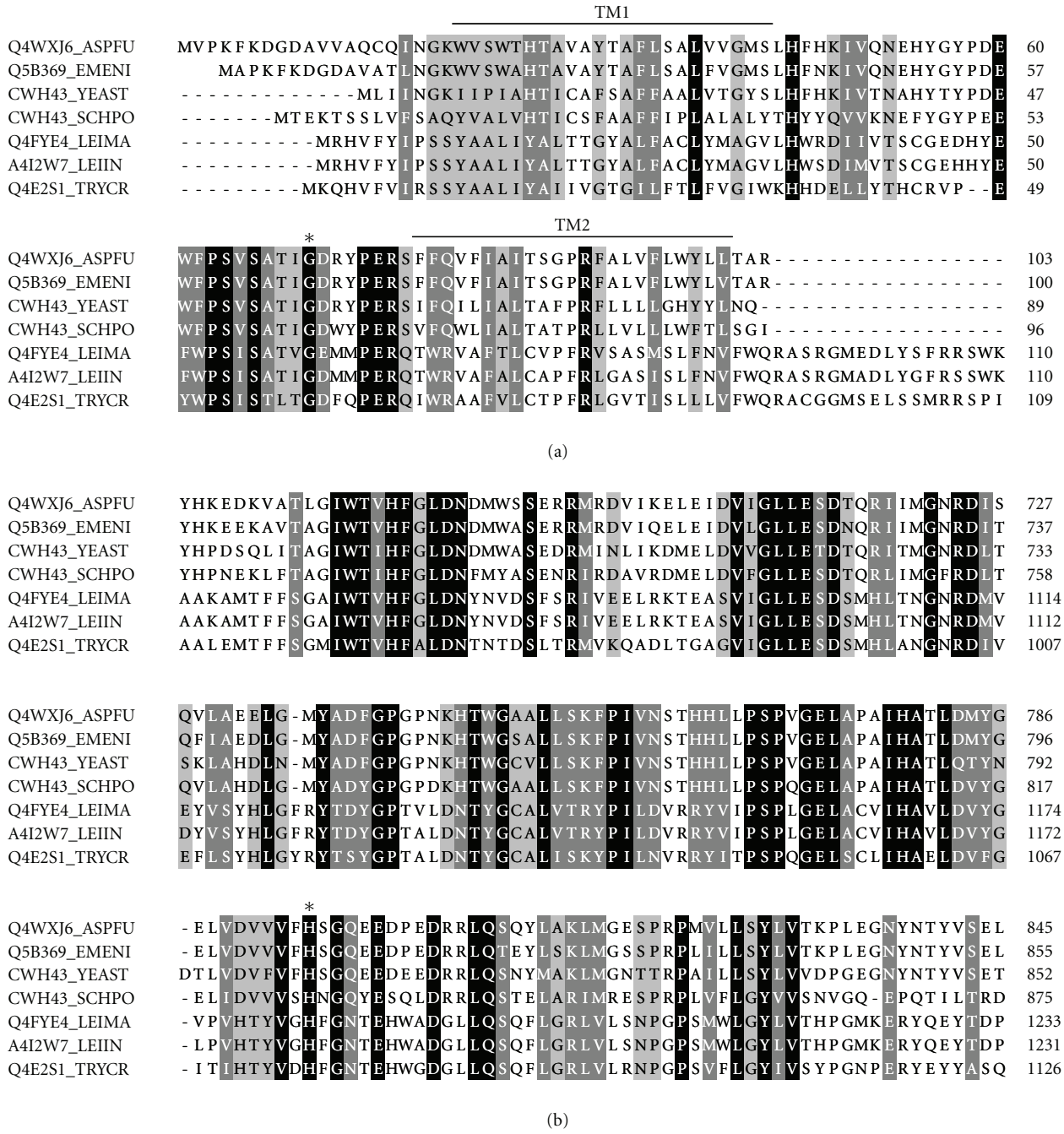

Figure 4: Alignment of the amino acid sequences of CWH43p homologues. Amino acid sequences of *Aspergillus fumigatus* (Q4WXJ6_ASPFU), *Emericella nidulans* (Q5B369_EMENI), *Saccharomyces cerevisiae* (CWH43_YEAST) and *Schizosaccharomyces pombe* (CWH43_SCHPO) proteins were aligned with the sequences of putative CWH43p candidates in *Leishmania major* (Q4FYE4_LEIMA), *Leishmania infantum* (A4I2W7_LEIIN) and *Trypanosoma cruzi* (Q4B2S1_TRYCR) using ClustalW. Identical amino acids are in reverse type, and conserved residues are shaded accordingly. The FRAG1 domain is shown in (a) the DNase I-like domain is shown in (b) Asterisks (∗) indicate the relative positions of amino acid residues that are essential for ceramide remodeling catalysis. TM, transmembrane stretches.

Although the remodeling of GPI anchors is important in several species to firmly anchor GPI proteins onto lipid bilayers and direct them to the correct cellular compartments and membrane domains [95], GPI lipid remodeling is not important for the stability and surface expression of the essential variant surface glycoprotein (VSG) [101]. The lack of GPI remodeling could be compensated *in vivo* by the myristate exchange pathway [100].

Like TbGUP1p, the *T. cruzi* GUP1p (Table 1, Step 7) can reacylate *lyso*-GPI anchors (Figure 2, Step 7), indicating that a similar pathway mediated by GUP1p is present in both yeasts and protozoa [97, 101]. Although several putative

GUP1 orthologues have been found in *Leishmania* (Table 1, Step 7), there is no experimental evidence for the existence of this kind of lipid remodeling in these parasites. Additionally, IPC has not been found to be linked to GPI anchors or GIPLs [14]. Therefore, targeted deletion studies in *Leishmania* could be used to determine the participation and function of these SBPs genes [29].

In *T. cruzi*, lipid remodeling occurs most likely as depicted in Figure 2. Unlike what has been described for mammals and yeasts, in this organism, the GPI anchor precursor molecule that is attached to the proteins is not acylated at position C-2 of the inositol ring [90]. Nonetheless, a putative TbGPIdeAc orthologue can be found in the *T. cruzi* genome database (Table 1, Step 5). In *T. cruzi*, intermediate GPI anchor precursors combine acylated and nonacylated inositol, as in *T. brucei* [90]. Thus, the putative orthologue (*Tc00.1047053508153.1040*) could be involved in deacylation during GPI biosynthesis. Although they are not acylated at the inositol ring, the GPI anchor precursors (either before or after attachment to proteins) contain a *sn*-1-alkyl-2-acyl-glycerolipid moiety [90] (Figure 2). However, Lederkremer's group has also detected a GPI-anchor precursor in *T. cruzi* that contains a mono-acyl-glycerol moiety [91]. There are no orthologues for the mammalian PGAP3p or yeast PER1p in the Tritryp genomes (Table 1, Step 6). It has been shown that *T. cruzi* lysates contain a PLA_2 activity that uses PI as a substrate [74], but it is not known whether this activity can also act on GPI-containing substrates. As mentioned above, TcGUP1p (Figure 2 and Table 1, Step 7) can reacylate *lyso*-GPI substrates [99, 101], but it is not clear which of the putative homologues encoded in the genome was used in those studies (Table 1, Step 7).

A putative orthologue of yeast CWH43p has been identified in the genomes of *T. cruzi* (*Tc00.1047053504153.120*), *L. major* (*LmjF27.1770*) (Table 1, Step 8) and *L. infantum* (*LinJ27_V3.1670*). This protein could have the ceramide remodeling activity that is supposed to exchange the *sn*-1-alkyl-2-acyl-glycerolipid of the GPI for a ceramide moiety in *T. cruzi* (Figure 2, Step 8 continuous or dashed lines). As already mentioned, no information is available on the function of these putative orthologues in *Leishmania* since ceramide is not found in their GPI anchors.

To learn more about the CWH43p orthologues in Tritryps, a multiple sequence alignment was prepared to compare these sequences with "bonafide" fungal CWH43p sequences. The results are presented in Figures 3 and 4. As shown in Figure 3, the Tritryp orthologues contain as many TM domains as the fungal CWH43p sequences. However, these domains have a greater distribution along the length of the sequences of *T. cruzi* and *Leishmania*. In addition, all the sequences have a highly conserved FRAG1 domain at the *N*-terminus and a DNase I-like domain at the C-terminus (Figure 3). A closer view of each of these conserved domains is shown in Figure 4. The FRAG1 domain has a high degree of identity across all sequences. The conserved residue G59 (G57 in yeast), which is essential for catalysis [99, 101], is located between the first and second TM domains (Figure 4(a), "∗"). A high degree of identity is also apparent in the DNase I-like domain at the C-terminus. The catalytically important residue H1076 (H802 in yeast) is conserved across all sequences (Figure 4(b), "∗"). Taken together, these data indicate that *T. cruzi* encodes a putative ceramide remodeling enzyme, which is essential in fungi and has no homologues in mammals.

6. Concluding Remarks

In *T. cruzi*, GPI-anchored glycoconjugates such as mucins, *trans*-sialidases, gp82/90 glycoproteins and GIPLs may extensively coat the plasma membrane of the parasite. These glycoconjugates are involved in many aspects of the host-parasite interaction, such as adhesion and invasion of host cells, modulation and evasion from the host immune response, and pathogenesis [66, 67, 83]. In addition, the GPI anchors, or certain parts of them, seem to act as strong proinflammatory molecules during the immune response against this parasite [66]. Therefore, mechanisms that interfere with the surface expression of GPI-anchored proteins and GIPLs or with the biosynthesis of GPI anchors are very attractive targets for new therapies against Chagas disease. Here, we discussed two novel targets in the SBPs of *T. cruzi*: the IPC synthase and ceramide remodeling. Because fungicidal inhibitors of IPC synthase activity do not affect the trypanosomal enzyme, the identification of novel inhibitors of this enzyme should be a goal of future research. This research direction could require the development of novel HTPS methods, such as the plate-based assay for screening *Leishmania* IPC synthase inhibitors that was recently developed by Mina and colleagues [102]. Unfortunately, recombinant parasite IPC synthase is prepared by overexpression in a fungal heterologous system, which has completely different optimal enzyme conditions and extra cofactors that would affect inhibition by novel candidates. Biochemical enzymatic assays have not been developed for ceramide remodeling, but recent advances have been made in monitoring the *in vitro* incorporation of ceramides into GPI-anchored proteins in *S. cerevisiae* [103]. These methods could be developed for use in *T. cruzi*.

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