Relevant Glycoconjugates on the Surface of *Trypanosoma cruzi*

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Although Chagas disease has been discovered 90 years ago, only after 1970 substantial progress has been made in the knowledge of molecular aspects of the host-parasite interaction. The amount of information gathered in the past 30 years was considerable, making it virtually impossible to cover all aspects of the biochemistry and molecular biology of the *Trypanosoma cruzi*, the etiologic agent of the disease. *T. cruzi* colonizes different habitats, from the intestinal tract of an insect to the cytoplasm of a mammalian cell, with a brief passage by the blood of the vertebrate host. An adaptation of the parasite to these different environments should occur and, consequently, differences in the composition of the plasma membrane should be expected among the parasite stages. In fact, in each parasite stage, molecules belonging to the surface membrane are the ones most probably involved in interaction with the hosts. In the vertebrate side of the infective cycle, the parasite has to leave the blood by crossing the capillary walls, the basal laminae and the extracellular matrix before encountering receptors located on the host cell plasma membrane. In the invertebrate phase of the cycle, epimastigotes must attach to the perimicrovillar membrane of the reduviid intestine in order to get prepared to differentiate to metacyclic trypomastigotes.

This review will cover mainly the evolution of knowledge which occurred with the parasite macromolecular structures residing in the plasma membrane and originally described by our laboratory.

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In fact, by extracting epimastigote forms with phenol it was possible to separate in polyacrylamide gels four bands developed by the Schiff reagent that were called bands A, B, C, and D (Alves & Colli 1975). Band D was baptized as lipopeptido phosphoglycan (LPPG) (Lederkremer et al. 1976), and more recently *T. cruzi* Type-1 GIPL (Ferguson 1997), and bands A, B, C have been shown to be compounds which are the main sialic acid acceptors on *T. cruzi* membranes, including the now well studied mucins (Acosta Serrano et al. 1995). In addition, trypomastigotes have on their surface a family of glycoproteins (Abuin et al. 1989) probably involved in parasite interiorization (Alves et al. 1986) and adhesion (Giordano et al. 1994, 1999, Alves 1996) which have been collectively called Tc-85 (Katzin & Colli 1983).

Excellent reviews on related issues were recently published on surface receptors and transporters of trypanosomatids, including the glucose transporter (Tetaud et al. 1997, Borst & Fairlamb 1998) and trans-sialidase (Cross & Takle 1993, Colli 1993, Schenkman et al. 1994).

LPPG AND GALACTOFURANOSE

Phenolic extraction of epimastigote forms led to the identification of four glycoconjugates stained by the Schiff reagent and called bands A, B, C, and D (Alves & Colli 1975). Extraction of that complex mixture with chloroform:methanol:water (10:10:3) allowed the purification of band D (Lederkremer et al. 1976). The latter contained mannose, galactose, glucosamine, phosphorus, fatty acids, inositol and long chain alcoholic bases. Due this complex composition the substance was baptized as LPPG. The term peptide was introduced because a faint reaction with dyes specific for proteins was observed. Later it was demonstrated that single aminoacids or even dipeptides may be bound to the sugar residues by ester linkages (Lederkremer et al. 1985a).

The main fatty acids are palmitic and lignoceric acids with the latter bound as amide to sphingosine bases forming ceramides (Lederkremer et al. 1977).

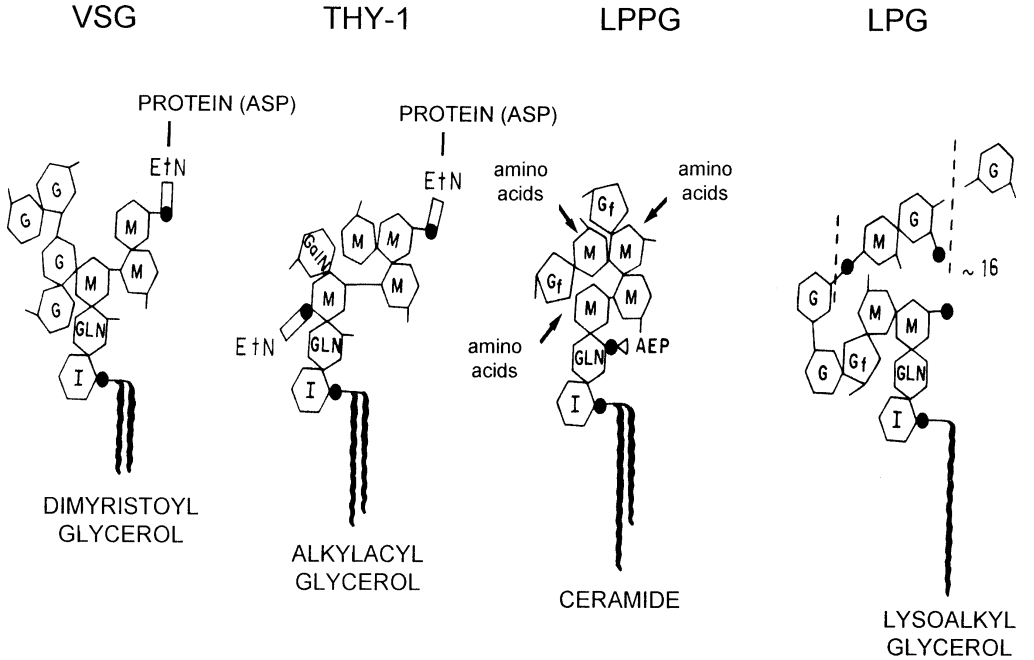
The binding of an inositol-phosphate group to the ceramide was demonstrated in our laboratory (Lederkremer et al. 1978). By 1978, the basic structural feature of a glucosamine-inositol-phosphate linked to a ceramide in LPPG was known and it was also demonstrated that LPPG was the major component of the epimastigote form plasma membrane (Alves et al. 1979).

Our laboratory also demonstrated that galactose is quantitatively liberated from LPPG by diluted trifluoroacetic acid under conditions that only liberate furanoic rings (Lederkremer et al. 1980). That sugar is easily attacked by metaperiodate eliminating carbon 6 and oxidizing carbon 5. If that treatment is followed by compound reduction with tritiated sodium borohydride the final product will be a labeled L-arabinose. These experiments have been made and the existence of terminal galactofuranose residues in a protozoan glycoconjugate was demonstrated for the first time in the literature (Lederkremer et al. 1980). Results pointing to the same direction were also found by Gorin et al. (1981) who described the existence of furanoic galactose bound to the three position of [1-2] bound mannose residues (Mendonça-Previato et al. 1983, Lederkremer et al. 1985b). It took 10 years to elucidate the structure of the oligosaccharide from LPPG. Previous studies had shown the presence of mannose [1-2] and [1-6] bound to the ceramide fraction (Lederkremer et al. 1985b) and, finally, the complete structure was published by two independent groups (Previato et al. 1990, Lederkremer et al. 1990, 1991).

More recently the glycoinositolphospholipids (GIPLs) received great attention since it was demonstrated that they act as anchors of proteins on the eukaryotic cell membrane and, for instance, of a phosphosaccharide in Leishmania (Turco & Descoteaux 1992).

Figure shows the structure of LPPG as compared to glycophosphatidilinositol anchors (Cross 1990) and the lipophosphoglycan (LPG) from *Leishmania donovani* (Turco et al. 1989), the latter with galactofuranose internal to the oligosaccharide chain. In anchors, a residue of aspartic acid from the protein is linked to an ethanolamine phosphate residue (Cross 1990). LPPG does not contain ethanolamine phosphate but the phosphonic (carbon-phosphorus bond) analogs 2-aminoethylphosphonic acid (Ferguson et al. 1982, Lederkremer et al. 1985a, Lederkremer et al. 1991) and 2-amino-3-phosphopropionic acid (Lederkremer et al. 1985a).

Other GIPLs found in Leishmania, as well as LPG, contain a glycerolipid instead of a ceramide in the apolar portion (McConville et al. 1990, 1993). These GIPLs, as well as others described in *Leptomonas samueli* (Previato et al. 1992) and



Structure of four glycoinositolphospholipids. VSG and THY-1 are, respectively, anchors from VSG (*Trypanosoma brucei*) and THY-1 (rat brain). LPPG is the lipopeptidophosphoglycan (*T. cruzi*) and LPG is lipophosphoglycan (*Leishmania*). G: galactose; M: mannose; I: inositol; GLN: glucosamine, GalN: galactosamine; EtN: ethanolamine; AEP: aminoethylphosphonic acid; G_f: galactofuranose; ASP: aspartic acid; (•) phosphate.

TABLE I

Galactofuranose-containing structures in some trypanosomatids

Organism	Structures	Ref.
GPI-simile structures		
Trypanosoma cruzi (epimastigotes)	Lipopeptidophosphoglycan (LPPG)	a-d
Trypanosoma cruzi (epimastigotes)	GIPL-A and GIPL-B	e
Leishmania donovani and *Leishmania* sp.	Lipophosphoglycan (LPG)	f,g
Leishmania major	GIPL-1, GIPL-2, GIPL-3, GIPL-A, LPGp	h
Leishmania mexicana	GIPL-2, GIPL-3, LPGp	i
Leptomonas samueli	GIPL	j
Endotrypanum schaudinni	GIPL	k
Leishmania adleri	GIPL	l
Glycoproteins		
Trypanosoma cruzi (trypomastigotes)	Surface glycoproteins (80-90 kDa)	m
Trypanosoma cruzi	Mucins	n, o, p
Leptomonas samueli	N-linked glycoproteins	q
Herpetomonas samuelpessoai	N-linked glycoproteins	q
Crithidia fasciculata and *C. harmosa*	N-linked glycoproteins	r
Endotrypanum schaudinni	N-linked glycoproteins	s

a: Lederkremer et al. 1976; b: Lederkremer et al. 1980; c: Lederkremer et al. 1991; d: Previato et al. 1990; e: Lederkremer et al. 1993; f: Turco et al. 1989; g: Turco & Descoteaux 1992; h: McConville et al. 1990; i: McConville & Ferguson 1993; j: Previato et al. 1992; k: Previato et al. 1993; l: Previato et al. 1997; m: Arruda et al. 1989; n: Previato et al. 1994; o: Previato et al. 1995; p: Acosta-Serrano et al.1995; q: Moraes et al. 1988; r: Mendelzon & Parodi 1986; s: Merello et al. 1995.

Endotrypanum schaudinni (Previato et al. 1993), the latter with ceramide in the apolar portion, contain terminal galactofuranose (Table I).

LPPG is routinely isolated from parasites in the stationary phase of growth (4-5 days). However, two GIPLs can be isolated from cells in exponential growth. These lipids differ in the apolar portion since one contains hexadecylpalmitoyl glycerol bound to a galactofuranose-containing glycan, whereas the other contains ceramide. Notwithstanding, in these cases, 20% of the galactose residues are in the pyranoic configuration (Lederkremer et al. 1993).

These findings pose several unresolved problems of crucial importance: (1) would the synthesis of these compounds be made from a common precursor, with posterior remodelling of the lipid portion or, in other words, could a glycerolipid be substituted for by a ceramide in one single reaction step, or vice-versa? (2) or would the synthesis of GIPLs be made entirely *de novo* accompanying stages transitions? (3) could pyranoic galactose rings be transformed in furanoic galactose residues after incorporation inside an oligosaccharide chain? or (4) alternatively, would the latter be incorporated into a macromolecule via a UDP-Gal_f precursor? Nassau et al. (1996), have been able to obtain clones of UDP-galactopyranose mutase from *E. coli* K-12 capable to convert reversibly UDP-gal_p to UDP-gal_f with the equilibrium favoring the formation of the pyranoic compound. More recently, mutases have been cloned in *Klebsiella pneumoniae* (Koplin et al. 1997) and *Mycobacteria* (Weston et al. 1998). The search of that enzyme in *T. cruzi* is imperative since the design of inhibitors to block its activity may render a very specific chemoterapeutic agent since mammalians do not seem to possess galactofuranose (cf. Lederkremer & Colli 1995).

It is interesting to note that in all GIPLs, furanoic galactose, when present, is bound to an α-D-mannose by a $\beta 1 \rightarrow 3$ bond, as a non reducing terminal sugar – as in LPPG (Lederkremer et al. 1985b, 1991, Previato et al. 1990, Carreira et al. 1996) – or as an internal unit inside an oligosaccharide chain – as in Leishmania LPG (Turco & Descoteaux 1992). That specificity suggests the existence of a β(1-3) galactofuranosyltransferase for biosynthesis and a β(1-3) galactofuranosyl hydrolase for degradation. Evidently, the elucidation of the metabolic pathways involving this sugar is very important for the development of chemotherapeutic agents without affecting the host.

Would the function of LPPG be merely structural? One should not forget that this molecule also exists in cell cultured trypomastigotes, although 10-fold less represented (Golgher et al. 1993). In Leishmania, LPG is important in parasite adhesion to the insect intestinal cells during parasite differentiation (Pimenta et al. 1994, Butcher et al. 1996).

In collaboration with several groups one of us (WC) is trying to show the active participation of LPPG in the binding of epimastigotes to the perimicrovillar membrane of the reduviid insect.

LPPG was found to block *in vitro* CD4(+) and CD8(+) T cell induced mitogenesis and to reduce secretion of IL-2 among others. These and other features seem to be due to the ceramide moiety rather than the phosphoinositol oligosaccharides (Gomes et al. 1996). Using macrophages it was possible to show that the ceramide moiety of LPPG is able to induce *in vitro* fluid phase endocytosis as well as apoptosis in the presence of IFN-γ and independently of nitric oxide secretion. Release of viable parasites from infected macrophages was augmented in the presence of ceramide and IFN-γ. These observations suggest a role for the ceramide portion of LPPG in spite of the fact that similar results can be obtained with synthetic ceramides (Freire-de-Lima et al. 1998).

A serum elicited in rabbits against LPPG has shown, by immunoelectronmicroscopy, a homogeneous distribution on the surface of epimastigotes allowing to determine the existence of 1-1.5 x 10^7 molecules of LPPG on the epimastigote surface (Golgher et al. 1993), a measurement which is in accordance with estimates made by other methods (Lederkremer et al. 1991). An average of 10^6 molecules was found in trypomastigotes but labeling was heterogeneous and only 15% of the trypomastigote forms were found labeled. There was an intense labeling of vesicles in the cytoplasm from both parasite stages (Golgher et al. 1993). It has been found that sera from chronic chagasic patients contain antibodies to LPPG mainly directed against galactofuranose (Golgher et al. 1993) but they did not protect against a parasite challenge (Travassos & Almeida 1993).

Terminal furanoic galactose was also found in mannose-rich oligosaccharides in the lower trypanosomatids *Crithidia fasciculata* e *C. harmosa* (Mendelzon & Parodi 1986), *L. samueli* e *H. samuelpessoai* (Moraes et al. 1988). This residue can be liberated by a β-galactofuranosidase from *Penicillium fellutanum*, but the bond position has not been determined. Galactofuranosyl residues have also been described in N-linked chains of 80-90 kDa glycoproteins from the trypomastigote forms (Arruda et al. 1989). Most probably, the latter were the mucin-like glycoproteins that were extensively studies in the past seven years (see below).

One would expect that galactofuranose, being absent from mammalian glycoconjugates, should be highly antigenic and, in fact, terminal β-D-galactofuranose epitopes are recognized by antibodies elicited by the 80-90 kDa glycoproteins of *T. cruzi* trypomastigote forms. These antibodies partially inhibit parasite entry into the host cell (Schnaidman et al. 1986, Arruda et al. 1989). A serum against epimastigotes reacts with LPPG (Mendonça-Previato et al. 1983) and immunoprecipitation is abolished when galactofuranose units are oxidized by diluted periodate. Recently, anti-gal_f monoclonal antibodies to a lipid antigen from *Paracoccidioides brasiliensis* reacted with *T. cruzi* epimastigotes and *Leishmania major* promastigotes, confirming the antigenicity of the sugar and previous data showing the existence of gal_f in these parasites (Suzuki et al. 1997).

MUCINS

Very abundant molecules, presently mainly on the surface of epimastigotes and metacyclic forms, mucins are members of a family of GPI-anchored, heavily O-glycosylated mucin-like glycoproteins (Schenkman et al. 1993, Previato et al. 1994, 1995, Acosta-Serrano et al. 1995). Mucins isolated from epimastigotes and metacyclic trypomastigotes migrate as 35-43 kDa and 35-50 kDa bands in SDS-PAGE, respectively (Previato et al. 1985, Yoshida et al. 1989) and as a broad smear (60-200 kDa) when isolated from trypomastigotes (Almeida et al. 1993, 1994). It has been estimated that 3 x 10^6 mucin molecules are present on the surface of epimastigotes and 1.5 x 10^6 on metacyclic trypomastigotes. Mucins, are the major substrates of the trans-sialidase, an enzyme secreted into the medium which has been related to the invasion of host cells by the parasite. Heavy glycosylation occurs on serine and threonine through N-acetylglucosamine rather than the common N-acetylgalactosamine. The enzyme involved in biosynthesis of N-acetylglucosamine-linked glycans was recently characterized as a uridine diphospho-N-acetylglucosamine:polypeptide-N-acetylglucosaminyltransferase (α-1-O-threonine) present in microsomal membranes (Previato et al. 1998). Structural studies showed that 20% of the molecules have no other sugar besides the hexosamine, but the majority is biantennary with galactose (1-4, 1-6 or 1-3, 1-6)-linked to the hexosamine in each ramification. The structures of the oligosaccharide alditols isolated after β-elimination of the mucins isolated from epimastigotes of the G strain (38/43 kDa) were established by NMR spectroscopy and methylation analysis as in Table II (Previato et al. 1994, 1995, Acosta Serrano et al. 1995).

Sialic acid is added to the mucins by the trans-sialidase, linking sialic acid (α 2-3) to terminal βGal_p of the oligosaccharides. Interestingly, no Gal_f residues were detected in mucins isolated from epimastigotes of the Y strain. Variability in the

TABLE II

Oligosaccharides from mucins

Oligosaccharitols	epi (%)	meta (%)
GlcNac-ol	21	22
Gal_f β1-4 GlcNac-ol	8	8
Gal_f β1-4 (Gal_p β1-6) GlcNAc-ol	15	13
Gal_p β1-3 Gal_p β1-6 (Gal_f β1-4) GlcNAc-ol	7	8
[(Gal_p β1-3)(Gal_p β 1-2) Gal_p β1-6] (Gal_f β1-4) GlcNAc-ol	17	16
[(Gal_p β1-3)(Gal_p β1-2) Gal_p β1-6] (Gal_p β1-2 Gal_f β1-4) GlcNAc-ol	10	12

Schenkman et al. 1993, Previato et al. 1994, 1995, Acosta-Serrano et al. 1995.

structure of the O-linked oligosaccharides was found among mucins isolated from different strains of epimastigotes, but not when they were isolated from the same strain (Previato et al. 1994, 1995, Acosta-Serrano et al. 1995). The trypomastigotes also express abundant mucin-like glycoproteins, although apparently larger than the mucins from epimastigotes or metacyclic trypomastigotes (Yoshida et al. 1989, Almeida et al. 1994). The non reducing end of part of the O-linked oligosaccharides have terminal α-galactose, a non-substrate for the trans-sialidase. These residues are recognized by antibodies anti-gal present in the serum of chagasic patients a feature that has been used to propose a diagnostic method for Chagas' disease (Almeida et al. 1993, 1994).

Differences in GPI-anchor composition of mucins isolated from epimastigotes, metacyclic trypomastigotes or cell-cultured trypomastigotes were found. Epimastigotes contain only sn-1-alkyl-2-acylglycerol, the same lipid found in trypomastigotes, whereas ceramide lipids were found in 70% of the GPI-mucins isolated from metacyclic forms (Almeida et al. 1994, Acosta-Serrano et al. 1995, Heise et al. 1995). Fatty acids of the GPI anchor from trypomastigotes are mostly unsaturated (C18:1, C18:2), in contrast to the saturated fatty acids found in the other stages (C18:0 sphinganine long chain base and mainly C24:0 and C16:0 fatty acids). These differences have been related to the synthesis of cytokines by macrophages induced by mucins isolated from trypomastigotes, but not by mucins isolated from epimastigotes (van Voorhis 1992, Silva et al. 1995, Aliberti et al. 1996, Camargo et al. 1997). Unusually the glucosamine was 6-O-substituted with 2-aminoethylphosphonate, and 2-aminoethylphosphonate was also present on the third mannose residue distal to glucosamine in the GPI-anchor of the mucin isolated from epimastigotes from the Y strain (Previato et al. 1995).

Interestingly, mucin genes belong to a multigene family, with a minimal number of 484 members per haploid genome (DiNoia et al. 1995, 1998, Salazar et al. 1996, de Freitas et al. 1998). The deduced molecular mass of the core proteins is small (from 17-21 kDa) (DiNoia et al. 1995). The regions encoding the N and C termini that contain, respectively, the leader sequence and the membrane anchoring signal are highly conserved, with the central regions which encode the target sites for O-glycosylation showing great variability. The region putatively coding for the N-terminal domain of the mature protein is hypervariable, being different in most of the sequenced transcripts. Gene-specific probes show that the relative abundance of different mRNAs varies within the same parasite clone (DiNoia et al. 1998, Freitas-Junior et al. 1998) Two distinct groups of mucin-like genes were proposed: one group containing KP(1-2)T(6-8) repeats, a motif found in mammalian mucins and expressed preferentially in the trypomastigote forms, and the other – expressed in all stages – having no repeats with highly variable sequences in the central portion and rich in threonine, proline and serine.

THE Tc-85 GLYCOPROTEIN FAMILY

Properties of Tc-85

The existence of at least one important trypomastigote specific surface glycoprotein was inferred when it was observed that anti-trypomastigote serum, exhaustively immunoadsorbed with epimastigote forms, was able to immunoprecipitate a protein with a molecular mass of 85 kDa (Zingales et al. 1982). Through systematic studies of lectin binding to the surface of several stages of *T. cruzi*, Katzin and Colli (1983) found in the trypomastigote form a glycoprotein band of 85 kDa which could be eluted from a WGA-Sepharose column with 0.1 M N-acetil-D-glucosamine. This glycoprotein was named Tc-85 (Katzin & Colli 1983).

Three monoclonal antibodies that recognized Tc-85 were raised (Alves et al. 1986). These antibodies immunoprecipitated an 85 kDa band which,

in fact, contained more than one protein, all with the same molecular mass but with different isolectric pHs (pH 5.5-9.0) as it could be seen in bidimensional polyacrylamide gels (cf. Andrews et al. 1984). When present in the culture medium these antibodies partially inhibited the internalization of trypomastigote forms in LLC-MK_2 cells (Alves et al. 1986, 1987, Abuin et al. 1989).

Using the monoclonal antibodies it was possible to define some properties of the Tc-85 protein family (Gonçalves et al. 1991, Abuin et al. 1996a): (1) the molecules are synthesized with a molecular mass of 94 kDa; (2) precursor processing is abolished by cysteine-proteases but not by serine-proteases; (3) the half-life of Tc-85 is 3.5-4 h (Abuin et al. 1996a); (4) the protein (s) expressed on the surface is continuously shed to the culture medium in membrane vesicles, although the phenomenon does not appear to be specific for Tc-85 since other antigens are also shed through membrane vesiculation (Gonçalves et al. 1991).

The following observations point to the fact that Tc-85 is, in fact, a pool of highly related molecules belonging to the same protein family: (1) the monoclonal antibodies H1A10, H1H8 e 6A2 that recognize only one band in unidimensional gels of tissue culture trypomastigotes of the Y strain are able to immunoprecipitate three bands of 82, 78 and 74 kDa from metacyclic forms of the Y strain or the CL-14 clone obtained from axenic media (Alves et al. 1987, Abuin et al. 1989); (2) monoclonal antibody 6A2 which recognizes Tc-85 in culture trypomastigotes of the Y strain is unable to immunoprecipitate any protein in the YuYu strain, in spite of the fact that this strain contains 85 kDa polypeptides which are recognized by the other two monoclonal antibodies – H1A10 and H1H8. This result can only be explained by a microheterogeneity inside this protein family so that the epitope recognized by the monoclonal antibody 6A2 is absent from the 85 kDa glycoproteins of the YuYu strain (Alves et al. 1987, Abuin et al. 1989); (3) when the parasites are treated with tunicamycin, monoclonal antibody H1A10 still recognizes a 75 kDa band in unidimensional gels. If the immunoprecipitates are subjected to bidimensional electrophoresis the usual pattern of several proteins with the same molecular mass but distinct pIs appears. These patterns, however, are different when the Y and YuYu strains are compared, the latter possessing relatively more acidic proteins than those from the Y strain (Abuin et al. 1989).

Taken together, these results strongly suggest the existence of an 85 kDa glycoprotein family whose members should have similar sequences but differing enough depending, at least, on the strain and origin of the trypomastigotes.

The trans-sialidase superfamily

The existence of sialoglycoconjugates on the surface of *T. cruzi* was established more than 15 years ago. Treatment of the parasites with neuraminidase induced several changes in parasite properties (references in Colli 1993). These and other findings led Previato et al. (1985) to search for alternative pathways of sialic acid incorporation in *T. cruzi* epimastigote forms. These authors have shown that epimastigotes grown in media containing fetal calf serum contained sialic acid as opposed to parasites grown in the absence of serum. Neuraminidase-treated epimastigotes lost the ability to agglutinate with WGA, known to bind to sialic acid, and acquired the capacity to agglutinate with PNA, a lectin that recognizes galactose (Gal). The incubation of the desyalylated parasites with fetuin (which contains sialic acid) or sialyllactose – but not free sialic acid – restored the WGA receptors (sialic acid) and concomitantly masked the PNA receptors (Gal). The authors proposed that sialylation occurred by trans-glycosylation reactions different from the route in which CMP-sialic acid is the intermediate.

In 1987, Zingales et al. demonstrated for the first time the sialic acid trans-glycosylase activity in trypomastigote forms of *T. cruzi*. When trypomastigotes were incubated with radioactive fetuin, the label was transferred to sialoglycolipids. Sonicated lysates of trypomastigotes catalyzed labeling of endogenous and exogenous glycoconjugates, the latter belonging to the classes G_T, G_D and G_M. The activity in the trypomastigote forms was 10-20 fold higher than that found in epimastigotes. These experiments unequivocally demonstrated that sialic acid transfer from fetuin to the glycolipid was not mediated by a pool of free sialic acid but occurred directly from one molecule to the other. The enzyme described in epimastigotes and trypomastigotes was not detected in intracellular amastigotes (Briones et al. 1995).

Schenkman et al. (1991) showed that the specific epitope Ssp-3 from the trypomastigote stage, originally defined by the monoclonal antibody 3C9 (Andrews et al. 1987) was sialylated. These acceptors of sialic acid correspond to proteins with a high degree of glycosylation having a broad range of molecular masses, now called mucins which were discussed above.

Pereira's group, responsible for the discovery of neuraminidase activity in *T. cruzi* (Pereira 1983), isolated and sequenced the neuraminidase gene (TCNA) of *T. cruzi* (Pereira et al. 1991). Parodi et al. (1992) demonstrated that the activities of neuraminidase and trans-sialidase co-precipitated with the same antibody, and Schenkman et al. (1992) unequivocally showed that both activities

belonged to the same enzyme. Finally, when the deduced amino acid sequence from TCNA was compared with the sequence of SAPA there was 84% homology corresponding to 93% of sequence identity in the nucleotide sequence. SAPA (Pollevick et al. 1991) is the "shed acute phase antigen", an immunodominant parasite antigen generating an early humoral response in human acute and congenital infections. That similarity showed that SAPA, neuraminidase and trans-sialidase are associated to the same enzyme protein (cf. Cross & Takle 1993, Colli 1993).

The gene of that enzyme displays important characteristics: (1) at least two perfect copies of the consensus sequence (Asp box) found in bacterial neuraminidases are present (Roggentin et al. 1988): Ser-X-Asp-X-Gly-X-Thr-Trp. Bacteria have four consensus sequences; (2) the carboxi-terminal sequence is made of 44 repeats of 12 amino acids (TCNA) or 14-47 repeats of the same 12 amino acids (SAPA): -Asp-Ser-Ser-Ala-His-[Ser/Gly]-Thr-Pro-Ser-Thr-Pro-[Ala/Val]-; (3) at the carboxi-terminal region, upstream of the repeats there is a sequence common to all members of the family: -Val-Thr-Val-X-Asn-Val-Phe-Leu-Tyr-Asn-Arg- (see below). This sequence written with the one letter symbol: VTVXNV FLY NR is commonly referred to in our laboratory as the FLY sequence; (4) the carboxi-terminal portion contains a sequence of hydrophobic amino acids typical of GPI membrane-anchored proteins with the predicted anchor binding site residing 9-12 amino acids upstream the hydrophobic segment.

The Gp-85 glycoprotein family

The discovery of Tc-85 stimulated several groups to search for the coding genes of that family. Immunoadorbing exhaustively anti-trypomastigote serum with epimastigote forms, an approach described by Zingales et al. (1982), was the method employed by these groups to select clones expressing genes coding for proteins compatible with a molecular mass of 85 kDa. Some clones have been fully or partially sequenced: TSA-1 (Fouts et al. 1991); Tt34c1 (Takle & Cross 1991); SA85-1.1, 1.2, 1.3 (Kahn et al. 1991) and pTt21 (Takle et al. 1992). After these first disclosures, other groups mentioned in Table III found genes having high degree of homology with the previously cloned sequences. These sequences show 60-70% sequence identity with TCNA or SAPA and all have the FLY consensus sequence and two non-degenerated Asp boxes. In addition, all have putative glycosylation sites located 9-12 amino acids upstream the hydrophobic sequence at the carboxy terminal end. The members of the Gp-85 family do not show the repeats *in tandem* characteristic of TCNA or SAPA and the proteins do not have neuraminidase activity.

To close the circle it would be necessary to show whether the Tc-85 family, defined to com-

TABLE lII

Trypomastigote proteins implicated in adhesion or internalization of the parasite into non-phagocytic cells

Source	Antigen kDa	Characteristics	Ref.
TCT, MT	85	Precursor of 95 kDa; O, N – glycosylation; GPI-anchor; trans-sialidase/gp 85 super-family; binds to laminin	1 - 7
TCT	82-85	Glycoprotein, binds to fibronectin and collagen	8
TCT	60	Penetrin, binds heparan sulfate, heparin, collagen	9,10
TCT	83	Glycoprotein, binds to receptors of 74 kDa	11,12
TCT, MT	80-220[a]	Trans-sialidase (neuraminidase) activity; GPI anchor; trans-sialidase/gp 85 super-family	13 - 15
TCT	?	Binds to polypeptides of 32-34 kDa	16
TCT	?	Binds to β-adrenergic and muscarinic receptors	17
TCT	?	Binds to TGF-β receptors	18
TCT, MT	35-50[a]	Mucins, GPI anchor	19 - 21
MT	90	Glycoprotein, GPI anchor, trans-sialidase/gp 85 super-family	22 - 25
MT	82	Glycoprotein, GPI anchor, trans-sialidase/gp 85 super-family	26, 27
MT	68-70[a]	Carbohydrate binding protein (Gal, Man) to receptors of 58-65 kDa	28

TCT: tissue culture trypomastigotes; MT: metacyclic trypomastigotes; *a*: exist also in epimastigote forms; 1: Abuin et al. 1989; 2: Alves et al. 1986; 3: Couto et al. 1990; 4: Couto et al. 1993; 5: Giordano et al. 1994; 6: Giordano et al. 1999; 7: Katzin & Colli 1983; 8: Ouaissi 1988; 9: Herrera et al. 1994; 10: Ortega-Barria & Pereira 1991; 11: Lima & Villalta 1988; 12: Villalta et al. 1993; 13: Colli 1993; 14: Cross & Takle 1993; 15: Schenkman et al. 1994; 16: Davis & Kuhn 1990; 17: von Kreuter & Santos-Buch 1989; 18: Ming et al. 1995; 19: Ruiz et al. 1993; 20: Schenkman et al. 1993; 21: Yoshida et al. 1989; 22: Franco et al. 1993; 23: Güther et al. 1992; 24: Schenkman et al. 1988; 25: Yoshida et al. 1990; 26: Araya et al. 1994; 27: Ramirez et al. 1993; 28: Bonay & Fresno 1995.

prise proteins that bind to WGA and are recognized by monoclonal antibody H1A10, belong or not to the multigenic family described above. In fact, two DNA inserts, a genomic DNA fragment and a full-length cDNA encoding the H1A10 epitope were cloned and characterized. The gene was sequenced with an open reading frame of 2.361 base pairs coding for a protein with an expected molecular mass of 84.549 daltons and pI = 5.00. Results showed that both inserts have high sequence identity with all reported members of the Gp85/trans-sialidase gene superfamily. The epitope has been mapped by competition of antibody binding to a Tc-85 recombinant protein with peptides having sequences predicted by the Tc-85 DNA sequence, which contains also putative N-glycosylation sites and carboxy-terminal GPI anchor insertion sites (Giordano et al. 1999). This gene has 82% and 62% sequence identity with SA85-1 (Kahn et al. 1991) and TSA-1 (Fouts et al. 1991), respectively, and contains two non degenerated Asp boxes and the FLY sequence.

The Tc-85 GPI anchor

Several parasite surface proteins are inserted in the membrane by glycosylphosphatidylinositol showing the motif Man(α1-4)GlcN(α1-6)PI containing a non-acetylated glucosamine bound to phosphatidylinositol (cf. McConville & Ferguson 1993). The presence of a GPI anchor in Tc-85 was suggested initially by labeling with radioactive palmitic acid. After hydrolysis of Tc-85 with phospholipase C, the lipidic portion was identified as 1-O-hexadecylglycerol and the sugar structure was determined as Man(α1-2)Man(α1-6)Man(α1-4) anhydromannitol (Couto et al. 1993). The fact that Tc-85 labeled with palmitic acid is found in the culture medium suggests that shedding of the glycoprotein occurs without GPI cleavage, contrary to other molecules where anchor cleavage was directly implicated in molecule liberation from the membrane. Shed Tc-85 could not be hydrolysed by phospholipase C, suggesting a modification at the inositol ring which has been proved to be an esterification by palmitic acid (Abuin et al. 1996b).

The carbohydrates of Tc-85

The carbohydrates were determined using the fraction of Tc-85 which binds to WGA columns and, thus, containing sialic acid. Alkaline treatment under conditions that liberate N- and O-glycosydic linkages allowed the separation of two chains. Possibly, there are two O-linked chains made of a mannose disaccharide. The N-glycosidic chains are complex and made of sialic acid, L-fucose, galactose, mannose and N-acetyl glucosamine. The presence of sialic acid was confirmed by hydrolysis with neuraminidase, acid treatment and treatment with sodium periodate and reduction with tritiated borohydride followed by chromatographic detection. The partial structure of the N-glycosydic chain was determined in the oligosacchride which has been liberated from Tc-85 with endo-β-N-acetylglucosaminidase F. The sequential treatment of this oligosaccharide with different glycosidases and analysis of the liberated residues allowed to propose the structure of the oligosaccharide bound to the protein as Man(β1-4)GlcNAc(β1-4) GlcNAc(β-Asn). Two mannose residues are bound α (1-6) and a (1-3) to the terminal mannose, forming the anthena and the oligosaccharide Gal(α1-3) Gal(β1-(4)?) GlcNAcβ(1-2)Man is bound to one of these mannoses (Couto et al. 1987, 1990). The binding of sialic acid and fucose to that structure has not been determined. The complete determination of the Tc-85 oligosaccharide structure is important to check whether it is or not common to all members (100-1000) of the Gp-85 family. Most probably each protein molecule has more than one oligosaccharide anthena differing in the last sugar residue: some would have Gal(α1-3) Gal(β1-(4)?) and others sialic acid (α2-3) Gal(β1-(4)?).

The function of Tc-85

The trypomastigote form present in the vertebrate host circulation necessarily establishes contacts with structures as basal laminae, extracellular matrices, and cell membranes before invading a vertebrate cell to initiate the parasite intracellular cycle. To understand the invasive process, several laboratories try to identify surface molecules from the trypomastigote forms which could explain adhesiveness and invasiveness. One popular approach uses monoclonal and polyclonal antibodies which may inhibit *in vitro* cultured cells invasion, followed by molecule identification by immunochemical techniques. Another approach uses affinity chromatography to bind parasite molecules to known host molecules, previously coupled to the affinity resins.

Laminin, a trimer glycoprotein, is an important component of the basal membrane. Different combinations of the α, β and γ chains allow the construction of the 11 isoforms as yet described. These isoforms have distinct expression patterns even in the same adult individual and very little is known on the specific role of each isoform. As they are difficult to obtain, the majority of the studies are made with laminin from the murine tumor called Engelbreath-Holm-Swarm (EHS), easy to purify in reasonable amounts.

An 85 kDa glycoprotein with pI ranging from 5.6-6.7 (LBG-laminin binding glycoprotein), present in trypomastigotes (and absent in epimastigotes), binds to columns of laminin-

Sepharose (Giordano et al. 1994). The binding is independent of the carbohydrates from both molecules and is located in the laminin fragment E8, obtained by treatment of laminin with elastase and described in the literature as a locus for the binding of different cell lineages to laminin. In addition, we have shown that LBG is recognized by monoclonal antibody H1A10, strongly suggesting that at least part of the Tc-85 family members – from which LBG is one the most acidic – could be involved in parasite adhesion to the host structures. Radioimmunoassays allowed to determine a number of LBG molecules on the trypomastigote surface seven times less than the total number of molecules recognized by the monoclonal antibody (Giordano et al. 1994). Finally, the protein encoded by the full-lenght cDNA insert that belongs to the Tc-85 family and has a predicted pI = 5.00 (LBG) binds to cells and *in vitro* to laminin, but not to gelatin or fibronectin, in a saturable manner (Giordano et al. 1999). These results pose several immediate questions: would other molecules belonging to the Tc-85 family be involved in adhesion processes to other matrix or membrane elements? What would be the number of family members? Are all members expressed concomitantly on the trypomastigote surface? Does each member possesses specificity of binding to host molecules or are they polyvalent, as other adhesion molecules? It is interesting to remind that the surface of trypomastigotes contains an 85 kDa glycoprotein that binds to fibronectin – a component of the extracellular matrix – a binding dependent on the RGD sequence (Ouaissi 1988). The RGD sequence is present in laminin but is absent from the fragment E8, where LBG binds. Would the fibronectin-binding glycoprotein be a member of the Tc-85 family?

Other proteins have been related with adhesion to or invasion of host cells by the parasite indicating that most probably, due to the complexity of the system and the variety of environments, the parasite utilizes several molecules to infect the host (Table III). The differential invasive properties among strains (cf. McCabe et al. 1984) and differentiation forms (cf. Hoft 1996), as well as the respective antigens will not be discussed for lack of space but they are listed in Table III.

In order to check the possible host cell membrane receptors for the Tc-85 family members, anti-idiotypic antibodies (anti-ID) to purified Fab fragments from monoclonal antibody H1A10 have been prepared. The idea was that part of the anti-ID antibodies thus obtained would mimic the morphology of the Tc-85 antigen and, thus, help to recognize the receptor in the host cell membrane (Pan et al. 1995). The anti-ID antibodies reacted with with different tissue slices from mice and different cultured cells recognizing three polypeptides (130-150, 73 and 43 kDa) on the surface of cultured cells. The inhibition of *T. cruzi* invasion in cultured cells by the anti-ID antibodies strongly suggest that at least one of these polypeptides could be the receptor for *T. cruzi* in the host cell (cf. Alves 1996). Which members of the Tc-85 family react with these polypeptides?

In order to answer these and other questions involving the adhesion of *T. cruzi* to host elements, cloning and expression of different Tc-85 family members and their possible receptors on the host cell membrane are under way.

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