Structural characterization of novel oligosaccharides of cell-surface glycoproteins of *Trypanosoma cruzi*

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Affinity-purified glycopeptides were prepared from *Trypanosoma cruzi* using the carbohydrate-specific monoclonal antibody WIC29.26. These glycopeptides contain rhamnose, fucose, xylose, and galactose, in the ratio 1:1:2:3. A series of oligosaccharides was released from the glycopeptides by mild acid hydrolysis, while, in contrast, no oligosaccharides were released by either peptide N-glycosidase F or conventional base-catalyzed β-elimination and reduction. This suggested the presence of a phosphodiester bond between the carbohydrate and peptide, which was further supported by the detection of phosphothreonine in the glycopeptides. The mild acid liberated (MAL) fraction was resolved into two major acidic oligosaccharides (MAL-P1 and MAL-P2), two minor neutral oligosaccharides (MAL-P1b and MAL-P2b) and a neutral fraction (MAL-N1) consisting of Gal and Xyl monosaccharides. The MAL-P1 and MAL-P2 oligosaccharides proved to be hexa- and heptasaccharides that shared a common xylose reducing terminus, but differed by one galactofuranose residue, and their negative charge was shown to be due to the presence of cyclic-phosphate attached to nonreducing terminal galactofuranose residues. The MAL-P1b and MAL-P2b oligosaccharides appeared to be nonphosphorylated versions of MAL-P1 and MAL-P2. Partial structures of MAL-P1 and MAL-P2 are suggested, based on compositional analyses, electrospray mass spectrometry, and tandem mass spectrometry before and after permethylation. The origin and significance of these unique trypanosomatid glycoconjugates is discussed.

Key words: glycoprotein/monoclonal antibody/oligosaccharide/structure/Trypanosoma cruzi

Introduction

*Trypanosoma cruzi* is the etiological agent of Chagas disease, which is endemic in Central and South America. The parasite undergoes distinct biochemical and morphological changes during a complex life cycle involving reduviid bugs and mammals. The nomenclature of each life cycle stage is centered on the point of emergence of the flagellum from the cell body. The parasite multiplies within the gut of its insect vector, the reduviid bug, as an epimastigote form. Differentiation occurs in the hind gut to produce metacyclic trypomastigotes, which are nondividing invasive forms transmitted to the mammalian host by contaminative infection via insect feces. The trypomastigotes invade different cell types in the mammalian host, including macrophages and muscle cells, and undergo division as amastigotes before release from the cells as trypomastigotes. This cycle of cell invasion continues indefinitely (Brener, 1973; Garcia and Azambuja, 1991). The biochemical changes that occur during differentiation between stages are important factors in establishing infection of insect vectors, invading mammalian cells, and evading the host immune system.

Several *T. cruzi* stage-specific surface antigens have been cloned and sequenced, examples of which include GP85 and other members of the trans-sialidase gene family from tissue culture trypomastigotes (Takle et al., 1989; Cross and Takle, 1993), GP82 from metacyclic trypomastigotes (Araya et al., 1994), and amastin from amastigotes (Teixeira et al., 1994). In epimastigotes, a 72 kDa glycoprotein was first identified on the cell surface using a carbohydrate-specific monoclonal antibody, WIC29.26 (Snary et al., 1981). The WIC29.26 epitope was subsequently found on the surface of epimastigotes of all *T. cruzi* strains examined, despite variations in posttranslational modification, which caused some masking of glycans epitopes (Kirchhoff et al., 1984; Schechter et al., 1986). The WIC 29.26 epitope is not found in the amastigote or tissue culture trypomastigote life-cycle stages (Snary et al., 1981). Recent experiments in our laboratory, however, have shown that GP72 protein appears to be expressed throughout the life-cycle (P.A.Haynes et al., unpublished observations), thus suggesting that developmental regulation of GP72 occurs at the level of posttranslational modification rather than protein expression.

The exact function of GP72 remains unclear, despite considerable study and a number of recent advances. It has been shown to be an acceptor for complement factor C3, which triggers the lysis of epimastigotes by mammalian sera via the alternative pathway (Joiner et al., 1985). It has also been demonstrated that metacyclogenesis can be inhibited by WIC29.26 in a model system, which indicates that GP72 may play a significant role in differentiation, possibly by interaction with lectins of the insect gut (Sher and Snary, 1982). All trypanosomes studied so far attach to organ surfaces within the insect vector, with the attachment occurring at the flagellum. As it has now been shown to be directly involved in flagellar adhesion to the cell body (Cooper et al., 1993; Ribeiro de Jesus et al., 1993), it seems likely that GP72 plays an important role in the parasite life cycle. The determination of the structure of the carbohydrates carried by GP72 and recognized by WIC29.26 may provide valuable insights into the nature of this host-parasite interaction.

Previous work in our laboratory has involved cloning and sequencing the gene encoding GP72 (Cooper et al., 1991) and preparing a GP72 null mutant by targeted gene replacement (Cooper et al., 1993). Deletion of GP72 caused an unusual phenotype, in which the flagellum was detached from the cell body after emerging from the flagellar pocket, and the overall
shape of the parasite was altered. The null mutant was unable to survive in the insect vector (Ribeiro de Jesus et al., 1993). The null mutants could be restored to normal morphology by complementation with episomal vectors expressing GP72, and glycosylation of the mature protein appeared to be the limiting step in the expression of functional GP72 (Nozaki and Cross, 1994).

Studies on the carbohydrate structure of GP72 have been limited by its very low abundance, which has been estimated at 0.04% of total cell protein (Ferguson et al., 1983). There is also considerable difficulty in purifying a single glycoprotein because several glycoproteins carry the same carbohydrate epitope (Cooper et al., 1991). Early studies indicated that the glycosylation recognized by WIC29.26 is highly unusual, as it contains high levels of phosphate and is rich in xylose, fucose, and rhamnose (Ferguson et al., 1983; Snary et al., 1987).

In this study, we have concentrated on carbohydrates that react with the WIC29.26 antibody, rather than purifying a single glycoprotein. We have obtained detailed structural information about several major component glycans purified by WIC29.26 affinity chromatography. These unique structures include phosphated hexa- and hepta-saccharides, which contain galactofuranose, rhamnose, xylose, fucose, and cyclic phosphate, and are most likely attached to peptide via a phosphodiester linkage.

Results

Characterization of WIC29.26 affinity-purified glycopeptides

Detergent lysates of T. cruzi epimastigotes were digested with Pronase and WIC29.26-binding glycopeptides were isolated by immunoprecipitation with WIC29.26-Sepharose beads. After extensive washing of the beads, the glycopeptides were eluted with a high pH buffer and repurified by gel filtration.

Amino acid analysis of the glycopeptide fraction (Table I) revealed the presence of significant amounts of Asx, Glx, Ser, Gly, Thr, Pro, Val, and Leu, together with lesser amounts of other amino acids and 2-aminoethylphosphonate (AEP). The AEP component has been found in other T. cruzi glycoconjugates, such as the glycoinositolphospholipid known as lipopetidophosphoglycan (Previtali et al., 1990; De Lederkremer et al., 1991), and in the glycosylphosphatidylinositol anchors of the cell surface mucins (Previtali et al., 1995; Serrano et al., 1995).

Monosaccharide analysis of the glycopeptide fraction (Table I) showed the presence of Rha, Fuc, Xyl, and Gal, together with lesser quantities of Man and Glc, a composition similar to that previously reported for intact GP72 purified from a clone of T. cruzi Y strain (Ferguson et al., 1983; Snary et al., 1987).

Phosphoamino acid analysis of metabolically radiolabeled cells revealed the presence of phosphoamino acids (Figure 1). The majority of the label was detected as phosphothreonine, which was present in an approximate ratio of 10:1 relative to phosphoserine. No phosphoarosine was detected, and no phosphoamino acids were detected in a control sample analyzed in parallel. The labeled compound detected close to the origin corresponds to the expected elution position of unhydrolyzed phosphate-containing peptides or proteins.

The glycopeptides contain galactofuranose

Reanalysis of the monosaccharide content, following mild periodate oxidation and reduction, a procedure that converts GalF residues with unsubstituted hydroxyl groups at C5 and C6 to arabinose (De Lederkremer et al., 1980), showed a reduction in the Gal content by 80% and a concomitant appearance of Ara. This result indicated that the majority of Gal present in the glycopeptide fraction was in the form of galactofuranose, a common component of T. cruzi glycoconjugates (De Lederkremer and Colli, 1995). The periodate-resistant fraction of galactose (approximately 20%) is probably also galactofuranose, but substituted at the C5 and C6 positions by cyclic phosphate (see below).

Release of oligosaccharides by mild acid treatment

Attempts to release oligosaccharides from the glycopeptide fraction using peptide N-glycosidase F and by β-elimination with concurrent reduction (Carlson, 1968) were unsuccessful. Similarly, attempts to generate radiolabeled oligosaccharitols, by β-elimination in the presence of NaN3H, were unsuccessful. On the other hand, mild acid hydrolysis released a series of oligosaccharides (Figure 2) that were separated by HPAEC. Five of these components were analysed further, and these are indicated in Figure 2. The two major negatively charged peaks were designated MAL-P1 and MAL-P2; the major neutral peak was designated MAL-N1; and two minor components (subsequently found to be related to MAL-P1 and MAL-P2) were designated MAL-P1b and MAL-P2b. Taken together with the presence of phosphothreonine, these data suggested the presence of phosphodiester linkages either between peptide and oligosaccharides, or between glycans, as this hydrolysis has been shown to quantitatively cleave hexose-1-Po4 linkages while leaving glycosidic linkages intact (McConville et al., 1990a).

Base-catalyzed β-elimination and reduction of oligosaccharides is inefficient when the linkage amino acid is at the amino-
Novel oligosaccharides of *Trypanosoma cruzi* glycoproteins

Front — P-Ser

— P-Thr

— P-Tyr

Fig. 2. HPAEC separation of MAL oligosaccharides released from WIC 29.26 affinity purified glycopeptides by mild acid hydrolysis. The oligosaccharides studied further are indicated.

Fig. 1. Cellulose thin-layer electrophoretogram of 32P-labeled phosphoamino acids purified from *T. cruzi* epimastigote cells by metabolic labeling and immunoprecipitation with WIC29.26 antibody. Lane 1, control immunoprecipitation without antibody; lane 2, sample immunoprecipitation.

The positions of the origin, solvent front and nonradioactive phosphoamino acid standards, visualized with ninhydrin, are as shown.

or carboxyl-terminus of glycopeptides. In order to ensure this was not the reason that no oligosaccharides were released from the glycopeptides by this treatment, the affinity purification was repeated on a smaller scale without pronase digestion, resulting in purification of glycoproteins rather than glycopeptides. The purified glycoproteins were also intractable to base-catalyzed β-elimination and reduction, but showed the same series of oligosaccharides released by mild acid treatment (data not shown).

The liberation of oligosaccharides, using mild acid hydrolysis conditions that cleave hexose-1-phosphate linkages while leaving most inter-residue glycosidic bonds intact (McConville *et al.*, 1990b), suggests that the MAL oligosaccharides were originally linked to each other and/or to the polypeptide via phosphodiester linkages. Carbohydrate attached to Ser or Thr residues via phosphodiester linkages requires stronger alkaline conditions for β-elimination than conventional O-glycosidic linkages (Ferguson *et al.*, 1983; Whitaker and Feeney, 1977), and this may explain the inability of the conventional β-elimination conditions used here to release the oligosaccharides.

When the mild acid hydrolysis procedure was repeated and followed by NaB₃H₄ reduction, a profile of radiolabeled oligosaccharitols was generated (Figure 3A) that was similar to the profile seen in Figure 2. The HPAEC retention times of the two major labeled oligosaccharitols corresponded exactly to those of the reduced forms of MAL-P1 and MAL-P2 (data not shown). The presence of a negative charge on the radiolabeled MAL-P1 and MAL-P2 oligosaccharitol peaks was confirmed by their binding to an anion exchange column. The additional peak (MAL-P3, Figure 2B) present in the radiolabeled oligosaccharitols was variable in yield and may represent dimers of MAL-P1 and/or MAL-P2.

**Characterization of radiolabeled MAL oligosaccharitols**

An aliquot of the radiolabeled MAL oligosaccharitols was treated with alkaline phosphatase. No change was observed in the size or retention time of the radiolabeled peaks (Figure 3B). Thus, the glycans do not contain any exposed phosphomonoester groups that are sensitive to alkaline phosphatase. Dephosphorylation of an aliquot of the labeled oligosaccharitols with 48% aqueous hydrofluoric acid resulted in the generation of small neutral labeled species (Figure 3C). However, since treatment with aqueous hydrofluoric acid will also cleave acid-labile glycosidic bonds (for example, Gal glycosidic bonds; Turco *et al.*, 1989) it was not clear whether the neutral species resulted from dephosphorylation or cleavage between the labeled reduced terminus and the charged residues, or both.

**Compositional analysis of mild acid liberated oligosaccharides**

Monosaccharide analysis of the MAL-N1 fraction (Table I) showed the presence of Gal and Xyl in a molar ratio of 3.5:1.0. Reanalysis of this fraction without acid methanolysis showed the same composition. Thus, the MAL-N1 fraction was composed of Gal and Xyl monosaccharides that appear to represent particularly acid-labile terminal residues.

Monosaccharide analysis of the MAL-P1 and MAL-P2 fractions (Table I) showed that these fractions were similar in composition, with MAL-P2 containing more Gal than MAL-P1. Moreover, the composition of these oligosaccharides is very similar to that of the starting glycopeptides, which indicates that the MAL oligosaccharides probably represent the majority of carbohydrate originally attached to the glycopeptides.

Positive-ion ES-MS analysis (Figure 4A,C) and negative-ion ES-MS analysis (Figure 4B,D) of MAL-P1 and MAL-P2 re-
MS data were: (Hex$_3$, dHex$_3$, Pent$_2$, cyclic phosphate) and (Hex$_3$, dHex$_3$, Pent$_2$, cyclic phosphate) for MAL-P1 and MAL-P2, respectively.

Positive-ion ES-MS spectra of fractions MAL-P1b and MAL-P2b (see Figure 2) revealed (M+NH$_4$)$_2^+$ pseudomolecular ions at m/z 916 and m/z 1078 (data not shown) that are consistent with components of mass 898 Da and 1060 Da. These masses suggest that MAL-P1b and MAL-P2b are nonphosphorylated versions of MAL-P1 and MAL-P2, respectively.

**Tandem mass spectrometry of MAL-P1 and MAL-P2**

The location of the cyclic phosphate group in MAL-P1 and MAL-P2 was suggested by the daughter ion spectra of the (M+NH$_4$)$_2^+$ parent ions of the two native compounds (Figure 5A,B). The daughter ions at m/z 225 and m/z 357, present in both spectra, are consistent with oxonium ions of a hexose
cyclic phosphate (P<Hex) and hexose cyclic phosphate linked to a pentose (P<Hex-Pent), respectively (see Figure 8). These daughter ion spectra were not changed by prior NaB<sub>3</sub>H<sub>4</sub> reduction of MAL-P1 and MAL-P2, indicating that the sequence (P<Hex-Pent) represents a nonreducing terminal sequence common to both structures. Taking into account the compositions of MAL-P1 and MAL-P2, the pentose must be Xyl while the hexose cyclic phosphate is most likely Gal-5,6-cyclic phosphate. The latter suggestion is based on the relative stability of the cyclic phosphate to acid treatment. Exposure of both structures to HCl, under conditions that decyclize inositol-1,2-cyclic phosphate (Ferguson et al., 1985), did not result in decyclization of the cyclic phosphate in MAL-P1 or MAL-P2, as monitored by ES-MS and HPAEC. Such stability is consistent with the presence of a cyclic phosphate on the exocyclic diol (C5 and C6) of Gal where the freedom of rotation about the C5-C6 bond should allow the five membered cyclic phosphate ring to adopt its most stable conformation.

The positive daughter ions at m/z 97, 109 and 115 (Figure 5A,B) can also be interpreted as phosphorous containing fragment ions arising from a cyclic phosphate group. The negative daughter ion spectrum of the (M-H)<sup>-</sup> parent ion of MAL-P2 contained fragment ions at m/z 79 and m/z 97, corresponding to (PO<sub>3</sub>)<sup>-</sup> and (H<sub>2</sub>PO<sub>4</sub>)<sup>-</sup> (Figure 5C). These ions are also generated by collision of the parent ions of sugar monophosphates. However, the collision energy necessary to generate these ions from MAL-P2 was considerably higher (150 V) than that required for a Man-6-P04 standard (40 V). The high collision energy needed for fragmentation of MAL-P2 is consistent with the relative stability of Gal-5,6-cyclic phosphate, which requires double cleavage events to generate the fragment ions. The absence of m/z 124 and m/z 140 ions, corresponding to (NH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-P0<sub>2</sub>)<sup>-</sup> and (NH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-OP0<sub>2</sub>)<sup>-</sup>, indicated that MAL-P2 did not contain AEP or ethanolamine phosphate substituents and, consistent with this, N-acetylation of MAL-P2 did not alter the mass of the main pseudomolecular ion in the negative-ion ES-MS spectra (Figure 6A).

The reducing terminal residue of MAL-P1 and MAL-P2 is xylose

The NaB<sub>3</sub>H<sub>4</sub> reduction of MAL-P1 and MAL-P2 resulted in the expected 3 Da increase in mass of each compound as...
judged by positive-ion ES-MS (data not shown). Monosaccharide analysis of the reduced compounds showed a reduction in the Xyl content with the concomitant appearance of (1-^2^H) Xylitol (data not shown). No other alditols were detected. These data define the reducing terminal residue of MAL-P1 and MAL-P2 as xylose.

**Permethyl and tandem mass spectrometry of MAL-P1 and MAL-P2**

Permethylation of NaB^3^H_4 reduced MAL-P1 and MAL-P2 produced fully methylated products, containing a single methyl group attached to the cyclic phosphate, as judged by negative-ion ES-MS: permethylated MAL-P1, (M-H)^- = m/z 1186, (M+Cl)^- = m/z 1222; permethylated MAL-P2, (M-H)^- = m/z 1390, (M+Cl)^- = m/z 1426. The negative-ion ES-MS spectrum of permethylated and NaB^3^H_4 reduced MAL-P1 is shown in Figure 6B. The daughter ion spectra of the (M+Na)^+ parent ions of the permethylated samples are shown in Figure 7, A and B. The fragment ions of reduced and permethylated MAL-P1 can be interpreted in terms of the sequential loss of a non-reducing terminal deoxy-hexose (dHex) residue, followed by an internal dHex residue, to yield a fragment of composition (P<Gal/ Xyl, Gal/) (see Figure 8A) and the presence of an (M-H)^- ion (m/z = 989) of a species that appears to have lost an unsubstituted Xyl residue from MAL-P1 during the process of N-acetylation and repurification (Figure 6A). These observations, together with the presence of free Xyl in fraction MAL-N1 (Table 1), are consistent with the presence of a mono-substituted reducing terminal Xyl residue which, in turn, suggests that the internal Xyl residue is a branch point with three substitutions (see Figure 8).

**Discussion**

Previous studies have shown that the carbohydrates recognized by WIC29.26 are highly unusual in composition (Ferguson et al., 1983) and appear to play an important role in the parasite life cycle (Sher and Snary, 1982). In this study, we have shown that the WIC29.26 carbohydrates are indeed unusual in a number of respects. A series of oligosaccharides was released from affinity purified glycopeptides by treatment with mild acid, and we have characterized these further, principally by mass spectrometry. The major charged species were phosphorylated hexa- and heptasaccharides, which contained rhamnose, xylose, fucose, and galactose. The galactose was present mostly in the furanos configuration, and a cyclic-phosphate was found at the nonreducing terminus of the major mild acid liberated (MAL) oligosaccharides. The deduced structural features, including those inferred from interpretation of minor fragments, are summarized in Figure 8. The fragmentation patterns indicated are those that would produce the observed molecular species.

The release of the MAL oligosaccharides does not appear to be a quantitative reaction, unlike, for example, release of N-linked oligosaccharides by peptide N-glycosidase F. Acid hydrolysis kinetic studies showed that the MAL oligosaccharides were at least partially degraded during the mild acid hydrolysis (data not shown), due to the considerable acid lability of pentose, deoxyhexose, and hexofuranose glycosidic bonds, when compared to those of hexopyranoses. For this reason, it cannot be assumed that the MAL oligosaccharides represent the entire spectrum of the native glycosylation. On the other hand, the composition of the MAL oligosaccharides is very similar to that of the starting glycopeptide fraction (Table 1), which indicates that the MAL oligosaccharides are representative of the
majority of the carbohydrate present in the glycopeptides. Notwithstanding the inherent difficulties, the study of the structure of these oligosaccharides provides revealing insights into glycoprotein biogenesis in T. cruzi.

The majority of the galactose in the MAL oligosaccharides is present in the furanose form. Galactofuranose is found mostly in bacteria, fungi, and protozoa, although it has been reported in several other organisms (Bock and Muller-Luennen, 1996). In particular, it has been found in the lipopolysaccharides and glycoinositolphospholipids of Leishmania and the lipopeptidophosphoglycan of T. cruzi. The metabolic pathways of both addition and removal of galactofuranose are not understood, but the absence of this pathway from mammalian hosts makes it a potential target for drug design (De Lederkremer and Colli, 1995). Galactofuranose is highly immunogenic in mammalian cells (Avila et al., 1989), and thus it seems likely that the presence of galactofuranose may be at least partially responsible for the considerable antigenicity of WIC29.26 carbohydrates.

The oligosaccharides of LPG structures are present as phosphodiester linked repeating units, which can be depolymerized using the same mild acid hydrolysis conditions as were used in this study to liberate MAL oligosaccharides from WIC29.26 affinity purified glycopeptides. In the case of LPG, mild acid hydrolysis produces oligosaccharides with nonreducing terminal hexose-6-phosphate residues, which are then susceptible to digestion with alkaline phosphatase to produce neutral species (McConville et al., 1990a; Ilg et al., 1992; Thomas et al., 1992). We found that the major MAL oligosaccharides were negatively charged, contained phosphate, but were insensitive to alkaline phosphatase. This was explained by positive-ion ES-MS and tandem ES-MS fragmentaion data, which revealed the presence of a hexose cyclic-phosphate at the nonreducing terminus of both MAL-P1 and MAL-P2. A structure such as this would be insensitive to alkaline phosphatase, and would also be consistent with the very high energy required in negative-ion tandem ES-MS-MS to produce phosphoanions. The unusually stable nature of the cyclic-phosphate group suggests it is probably a substituent of galactofuranose. A cyclic-phosphate attached to the exocyclic diol of galactofuranose (C5 and C6) would have a high degree of freedom of rotation, and would thus be able to adopt the most stable five membered ring conformation possible.

Fig. 8. Diagram of the deduced structure of MAL-P2, in both (A) native and (B) reduced and permethylated forms. The fragmentation points indicated produce ions detected in spectra shown in Figs. 4-7.

The cyclic-phosphate may be a part of the intrinsic oligosaccharide structure, as reported in Vibrio cholerae (Knirel et al., 1995), or, more likely, it may be an artifact produced by acid catalyzed cyclization of galactofuranose-6-phosphate during the mild acid liberation of the oligosaccharides. If the cyclic-phosphate is an artifact of mild acid hydrolysis, it could have originated from two different arrangements of the components we have identified. In the first possibility, the MAL-P1 and MAL-P2 may have been attached to peptide via phosphodiester linkage between the reducing terminal xylene and phosphothreonine, with the additional galactose and xylene found in MAL-N1 attached via phosphodiester to galactofuranose at the nonreducing terminus. In the second possibility, the Xyl reducing termini would be consistent with the presence of MAL-P1/MAL-P2 repeating units linked to each other by phosphodiester linkages. These would include phosphodiester linkage units of (Xyl1-PO4H5,6-Galf–) that may generate the cyclic phosphate during the hydrolysis procedure. The small proportion of the neutral MAL-P1b and MAL-P2b oligosaccharides seen in Figure 2 may therefore represent the terminal, or capping, structures of chains consisting of MAL-P1/MAL-P2 phosphosaccharide repeats. The ratio of MAL-P1b/MAL-P2b to MAL-P1/MAL-P2 (approximately 5:1) would then suggest that the average size of a glycopeptide sidechain is five MAL-P1/MAL-P2 phosphosaccharide repeats capped with one MAL-P1b/MAL-P2b unit. The putative phosphosaccharide repeat chains would be attached to peptide via a phosphodiester linkage between the reducing terminal Xyl residue of a MAL-P1/MAL-P2 repeat and phosphothreonine. The additional monosaccharides found in the MAL-N1 fraction could represent either alternative neutral capping structures, or particularly acid-labile terminal residues. It is likely that the native glycoprotein may contain both of these possible arrangements of glycan substituents, as most O-linked glycosylation is highly heterogeneous.

In either case, these are highly unusual glycoprotein oligosaccharides. The only other examples reported of oligosaccharides attached to proteins via phosphodiester linkage are the GlcNAc-1-phosphate attached to serine in lysosomal proteinases of Dictyostelium discoideum (Gustafson and Milner, 1980; Souza et al., 1995), the secreted acid phosphatase of Leishmania mexicana, which carries phosphodiester linked glycans that include many of the same oligosaccharide struc-
tured on LPG (Ig et al., 1994), and the partially characterized O-glycosylation of Dictyostelium discoideum spore specific antigen (Haynes et al., 1993).

The biosynthesis of novel oligosaccharide structures such as those we have described in this report is likely to involve unique glycosyltransferases and other related enzymes. A galactofuranosyltransferase has been cloned from Leishmania (Ryan et al., 1993) and a second gene (LP2G) has been identified (Descoteaux et al., 1995) that is responsible for the correct targeting and assembly of LP2G and related molecules implicated in parasite virulence. As with all biosynthetic pathways of parasites that are absent from the host cells, the identification of enzymes involved in the assembly of WIC29.26 oligosaccharides may provide attractive targets for chemotherapeutic disease intervention.

Materials and methods
WIC29.26 affinity purification of glycopeptides
T. cruzi epimastigote cells of strain Y-NIH were grown to late log phase in Liver Infusion Tryptose (Difco Laboratories) medium supplemented with 10% fetal calf serum (Sigma Chemical Co.) (Bone and Steiner, 1956), harvested by centrifugation, and lysed at 10^6 cells/ml in 1% sodium deoxycholate (Sigma Chemical Co.) with heating and sonication. Lysates were adjusted to 100 mM NH_4HCO_3 and 1% NP40 (Calbiochem), and digested with 0.5 mg/ml pronase (Boehringer Mannheim) for 48 h. The pronase digested lysates were immunoprecipitated batchwise with WIC29.26-Sepharose beads. The WIC29.26-Sepharose beads were washed five times with 0.1 M NH_4HCO_3, 0.5 M NaCl, 0.1% NP40, three times with 0.1 M NaHCO_3 and three times with water. Bound glycopeptides were eluted with 50 mM diethylamine (pH 11.5). Eluates were immediately frozen and lyophilized, and redissolved in water. The glycopeptides were then passed through a Bio-Gel P2 (Bio-Rad) column (50 x 1 cm) eluted with water, collected in the void volume, and concentrated under vacuum. A final desalting step was performed using an Oxford Glycosystems GlycoMap 1000 Carbohydrate analysis system, which was also used for final desalting of oligosaccharides prior to ES-MS.

Amino acid analysis
Amino acid analysis was performed using a Waters Pico Tag system. Samples were hydrolyzed in vapor phase with 6 M HCl containing 1% v/v phenol at 110°C for 16 h, dried from water/ethanol/triethylamine (2:2:1 by volume), derivatized with water/ethanol/triethylamine/phthalisobutyrate (7:1:1:1 by volume) for 20 min, and dried under vacuum before HPLC analysis.

Metabolic labeling and phosphoamino acid acid analysis
T. cruzi epimastigote cells of strain Y-NIH were grown for 16 h at 10^6 cells/ml in 4 ml phosphate-free Dulbecco's modified Eagle's Medium (Gibco BRL) supplemented with 10% fetal bovine serum (previously dialyzed against Hepes-buffered saline pH 7.4) and 2 mCi H_3^32P orthophosphate (New England Nuclear) Cells were lysed with 50 mM Tris (pH 8.2)/1% NP40, preclarified with Sepharose-glycine, and incubated for 16 h at 4°C with WIC29.26 antibody. Glycoprotein–antibody complexes were immunoprecipitated with protein-A Sepharose (Sigma), washed extensively, and separated on an SDS–PAGE gel. Following transfer to PVDF membrane (Millipore), the labeled glycoproteins were visualized by autoradiography and excised from the membrane. Bands were hydrolyzed in 6 M HCl for 2 h at 110°C, and the supernatant was dried under vacuum. Labeled phosphoamino acids eluted from the bands during hydrolysis were mixed with unlabeled phosphoamino acid standards and separated by thin layer electrophoresis on cellulose plates (Kodak). Unlabeled standards were visualized with ninhydrin and labeled standards were visualized by autoradiography.

Monosaccharide compositional analysis
Carbohydrate compositional analysis was performed by gas chromatography–mass spectrometry (GC-MS) of trimethylsilylated methylglycosides. Samples were mixed with scylllo-inositol internal standard, dried, and subjected to methanolysis in 50 µl 0.5 M HCl in dry methanol for 4 h at 85°C. Methanolyses were re-N-acetylated by the addition of 15 µl pyridine and 10 µl glutaric anhydride (30 min) prior to drying under vacuum and derivatizing with 15 µl trimethylsilyl reagent.

Aliquots of 1 µl were analyzed by GC-MS using an Ecombog 5SE-54 column (Alltech, 30 m x 0.25 mm) with He carrier gas at 0.5 ml/min. The temperature program used was 140°C for 2 min, increasing to 250°C at 5°C/min, then to 300°C at 15°C/min, and held at 300°C for 20 min. Electron-impact mass spectra were collected from linear scanning over m/z 40-650, and quantification was based on empirically determined response factors and integration of total ion current chromatograms.

Periodate oxidation and reduction
Samples were incubated in 50 mM NaOH in 50 mM sodium acetate, pH 4.5, for 30 min at room temperature in the dark. Excess periodate was destroyed by addition of ethylene glycol and reduction was achieved using 1 M NaBH_4 in 1 M ammonium hydroxide. The reaction was neutralized with 1 M acetic acid and passed through a column of 0.3 ml AG50-X12 (H^+). The eluate was dried under vacuum and redried twice from 250 µl methanol/5% acetic acid, twice from methanol, and flash evaporated twice with toluene.

High performance anion exchange chromatography
Oligosaccharides were identified and quantified using a Dionex high performance anion exchange chromatography (HPAEC) system fitted with a Carbowrap PA-1 column and a pulsed amperometric detector. Radiolabeled glycans were analyzed by scintillation counting of aliquots from collected fractions. Oligosaccharides were separated using a gradient of NaOAc in 150 mM NaOH. The gradient program was 10 mM initially, then linear gradients to 30 mM at 15 min, 200 mM at 40 min, 500 mM at 55 min, and held at 500 mM to 60 min. This program separates over a wide range of both size and charge, with monosaccharides eluting at approximately 7.5 min.

Glycan release and reduction
Mild acid hydrolysis was performed in 40 mM trifluoroacetic acid at 100°C for 8 min. Samples were cooled on ice and dried under vacuum. Reduction of oligosaccharides liberated by mild acid hydrolysis was achieved by redrying samples twice from 50 µl water, then adding 20 µl 100 mM NaOH and 36 mM NaBH_4 (10-15 Ci/mmol, New England Nuclear), and incubating for 2 h at room temperature. Reduction was completed by addition of 10 µl 0.5 M NaBH_4 and incubation for 2 h at room temperature. The reaction was neutralized with 1 M acetic acid and passed through a column of 0.3 ml AG50X-12 (H^+). The eluate was dried under vacuum and redried twice from 250 µl methanol/5% acetic acid, twice from methanol, and then flash evaporated twice with toluene.

Alkaline-catalyzed β-elimination and concurrent labeled reduction was performed by incubation in 100 mM NaOH at 37°C for 8 h. The reaction was completed and the reaction mixture neutralized as described above. Tritium-labeled glycans were dissolved in water, applied to Whatman 3 MM chromatography paper (40 cm x 3 cm) and subjected to descending chromatography for 60 h in 1-butanol/ethanol/water (4:1:6 v/v). The paper was dried and scanned for radioactivity with a Raytest RITA 3200 linear analyzer. Labeled glycans, running close to the origin, were eluted with water.

Aqueous HF dephosphorylation
Samples were dried and dephosphorylated with 50 µl 48% v/v HF at 0°C for 48 h. Excess HF was removed by repeated lyophilization.

Enzyme digestions
Calf intestinal alkaline phosphatase (Sigma) (5 U) was added to samples dissolved in 100 ml freshly prepared 0.1 M NH_4HCO_3, and digestions were incubated at 37°C for 16 h. Digests were lyophilized, rehydrated from water, and redissolved in 250 µl 0.5 M sodium phosphate, pH 7.5, containing 1% NP40 and 15 mM dithiothreitol, and digestions were incubated at 37°C for 24 h, with addition of an extra 20 U after 16 h.

Electrospray mass spectrometry
Samples were introduced into the electrospray source of a VG Quattro mass spectrometer in 20 µl aliquots at 5–10 µl/min in either 50% acetonitrile containing 10 mM ammonium acetate, for native oligosaccharides, or chloroform/
Abbreviations

AEP, 2-aminoethylphosphonate; CID, collision induced dissociation; dHex, deoxyhexose; ES-MS, Electrospray mass spectrometry; ES-MS-MS tandem electrospray mass spectrometry—the mass spectrometry–mass spectrometry; Galf, galactofuranose; GC-MS, gas chromatography–mass spectrometry; Hex, hexose; HPAC, high performance union exchange chromatography; MAL, mild acid liberated, P=Hex, hexose-cyclic-phosphate; Pent, pentose; PVDF, polyvinylidene difluoride, SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

References


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