Structural characterization of a novel glycosyl-phosphatidylinositol from the protozoan *Tetrahymena mimbres*

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A glycolipid metabolically labelled with [¹⁴C]GlcN was isolated from the free-living protozoan *Tetrahymena mimbres*. The glycolipid was sensitive to a bacterial phosphatidylinositol-specific phospholipase C, and the headgroup was shown to contain a phosphorylated Man α 1-2Man α 1-3Man α 1-4GlcN glycan. The *Tetrahymena* glycolipid is structurally unique among the glycosylphosphatidylinositols that have so far been characterized, including those from several protozoan parasites of humans.

INTRODUCTION

Glycosyl-phosphatidylinositol (GPI) anchors have been identified as alternatives to hydrophobic transmembrane amino acid sequences for membrane attachment of a variety of eukaryotic cell-surface proteins (Ferguson & Williams, 1988). In higher eukaryotes only a minority of cell-surface proteins are anchored by a GPI anchor, including hydrolytic enzymes, antigens and cell-adhesion molecules (Ferguson & Williams, 1988; Low, 1989; Cross, 1990; Thomas *et al.*, 1990). In parasitic protozoa, however, GPI anchors can be the predominant form of membrane protein attachment, e.g. in *Trypanosoma brucei* with its variant surface glycoprotein (VSG) coat (see Ferguson *et al.*, 1988). Protozoa that parasitize humans also produce cell-surface GPIs as free glycolipids with no covalently attached protein. These include the glycoinositol phospholipids (GIPLs), lipophosphoglycan (LPG) and so-called lipopeptidophosphoglycan (LPPG). The primary structures of several protozoan GPIs have been determined. These include LPG from *Leishmania donovani* (Orlandi & Turco, 1987; Turco *et al.*, 1987, 1989) and *Leishmania major* (McConville *et al.*, 1990b), GIPLs from *L. major* (McConville *et al.*, 1990a), LPPG from *Trypanosoma cruzi* (Prevati *et al.*, 1990) and the membrane protein anchors of *T. brucei* VSG (Ferguson *et al.*, 1988) and the *L. major* promastigote surface protease (gp63) (Schneider *et al.*, 1990). Comparison of these structures with those of non-protozoan GPIs [e.g. rat brain Thy-1 (Homans *et al.*, 1988)] suggests that they all contain a common Man α 1-4GlcN α 1-6Ins-lipid structural motif, but beyond this the glycans of the protein-linked and free GPIs diverge in structure. No GPI from a protozoan that does not parasitize humans has been structurally characterized, particularly with regard to the glycan headgroup.

Tetrahymena is a ciliated protozoan with a world-wide distribution. Most species have been found in fresh water as free-living organisms, but some have been reported to live as symbionts in slugs, clams or worms, or as parasites in the larvae of midges and mosquitos (see Hill, 1972). Membranes of *Tetrahymena mimbres* contain GPI and GPI-linked proteins (Pak *et*

al., 1991; Ryals *et al.*, 1991), as well as acylated proteins (Ryals & Thompson, 1988). GPI-anchored proteins have also been found in the ciliated protozoan *Paramecium* (Capdeville *et al.*, 1987). We have isolated a glycolipid from *T. mimbres* and determined the structure of its glycan headgroup. The *Tetrahymena* glycolipid has structural features common to both the GPIs that anchor membrane proteins and those from parasitic protozoa that have no covalently attached protein.

EXPERIMENTAL

Materials

Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was kindly provided by Dr. M. G. Low (Columbia University, New York). Octyl-Sepharose CL 4B was obtained from Pharmacia, Bio-Gel P-4 from Bio-Rad, *Aspergillus phoenicis* α -D-mannosidase (APAM) from Oxford Glycosystems, jack-bean α -D-mannosidase (JBAM) from Boehringer Mannheim, and D-[1-¹⁴C]glucosamine from Amersham.

Isolation of the glycolipid

Tetrahymena mimbres cultures were grown in the presence of D-[¹⁴C]GlcN and glycolipids were extracted as previously described (Pak *et al.*, 1991; Ryals *et al.*, 1991). Labelled cells were rapidly cooled and centrifuged for 5 min at 400 g. The cell pellet was extracted with 5 vol. of cold acetone, centrifuged at 10 550 g for 5 min and re-extracted with 5 vol. of chloroform/methanol/water (10:10:3, by vol.). After centrifugation and drying under a nitrogen stream, the extract was vortex-mixed with chloroform/methanol/water (10:10:6, by vol.) and centrifuged at 5000 g for 15 min. The aqueous phase was removed, dried and re-dissolved in 1 ml of 0.1 M-ammonium acetate/5% propan-1-ol, pH 7.2. The sample was loaded at a flow rate of 4 ml/h on to a column (1 cm $\times$ 16 cm) of octyl-Sepharose equilibrated in the same buffer. The column was washed with loading buffer (50 ml) and glycolipids were eluted with a 60 ml linear gradient of propan-1-ol in water (5–60%, v/v) at a flow rate of 20 ml/h (McConville *et al.*, 1987). Fractions (1 ml) were collected

Abbreviations used: AHM, 2,5-anhydromannitol; APAM, *Aspergillus phoenicis* α -mannosidase; Du, Dionex unit; GIPL, glycoinositol phospholipid; GPI, glycosyl-phosphatidylinositol; Gu, glucose unit; Hex, hexose; JBAM, jack-bean α -mannosidase; LPG, lipophosphoglycan; LPPG, lipopeptidophosphoglycan; PAD, pulsed amperometric detector; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein.

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Table 1. Chromatographic properties of the *T. mimbres* GPI glycan

The [^{14}C]GlcN-labelled glycan was released from the octyl-Sepharose-purified GPI by treatment with PI-PLC, then deaminated with nitrous acid, reduced with NaBH_4 and dephosphorylated with aqueous HF. Details of the enzyme digestions, Bio-Gel P-4 chromatography and high-pH anion-exchange chromatography (Dionex h.p.l.c.) are given in the Experimental section.

Enzyme treatment	Product	Elution position	
		Bio-Gel P-4 (Gu)	Dionex h.p.l.c. (Du)
—	$\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}3\text{Man}\alpha 1\text{-}4[^{14}\text{C}]\text{AHM}$	4.2	2.25
APAM	$\text{Man}\alpha 1\text{-}3\text{Man}\alpha 1\text{-}4[^{14}\text{C}]\text{AHM}$	3.2	2.01
JBAM	$[^{14}\text{C}]\text{AHM}$	1.7	—*

* The ^{14}C -labelled JBAM product co-eluted with authentic AHM under conditions that resolve monosaccharides (see Fig. 1).

and radioactivity was determined by liquid scintillation counting of 10 μl aliquots. Concentrations of propan-1-ol were determined by measuring the refractive index.

Preparation of the glycan headgroup

Purified glycolipid was resuspended in 50 μl of 20 mM-Tris/acetate/0.1 % Triton X-100, pH 7.4, containing 0.4 units of PI-PLC. After incubation for 16 h at 37 °C, detergent and glycolipids were removed by partitioning four times into 150 μl of toluene. Material remaining in the aqueous phase was loaded on to a 1 cm $\times$ 10 cm column of octyl-Sepharose as described above. Under these conditions, lipid-free species do not bind to the column and elute in the buffer wash (McConville *et al.*, 1987). Elution of the [^{14}C]GlcN-containing compounds was monitored by liquid scintillation counting.

Deamination with nitrous acid and reduction with sodium borohydride were performed as described by Ferguson *et al.* (1988). The unbound fraction from the second octyl-Sepharose column was freeze-dried and redissolved in 200 μl of 0.1 M-sodium acetate/acetic acid, pH 4.0, and 200 μl of freshly prepared 0.5 M-sodium nitrite was added. After 3 h at room temperature, 200 μl of 400 mM-boric acid and 200 μl of 1.0 M-NaOH in 1.0 M- NaBH_4 was added. After 16 h the sample was acidified with 1 M-acetic acid and desalted by passage through Dowex AG50-X12 (H^+ form). Borate was removed by evaporation four times with 5 % acetic acid in methanol, and residual acetic acid was removed by evaporation with methanol.

Dephosphorylation in 48 % aqueous HF was performed as previously described (Ferguson *et al.*, 1988).

Exoglycosidase digestion

Digestion with APAM and JBAM was performed with 10 μunits and 0.25 units respectively in 10 μl of 0.1 M-sodium acetate, pH 5.0, for 16 h at 37 °C. Reactions were stopped by heating at 100 °C for 5 min.

Bio-Gel P-4 and high-pH anion-exchange chromatography

The Bio-Gel P-4 column (1.6 cm $\times$ 93 cm, 400 mesh) was equilibrated in water and held at 55 °C. Samples were introduced using a Rheodyne low-pressure injector and eluted with water at a flow rate of 12 ml/h. A Dionex BioLC Carbohydrate Analyzer with a CarboPac PA-1 column (4 cm $\times$ 250 mm) and pulsed amperometric detector (PAD) was used for high-pH anion-exchange chromatography. The column was equilibrated in 150 mM-NaOH/12.5 mM-sodium acetate and eluted with a linear gradient from 12.5 to 125 mM-sodium acetate in 30 min at a flow rate of 0.6 ml/min for separation of neutral oligosaccharides (McConville *et al.*, 1990b). Elution volumes of radioactive oligosaccharides are reported relative to those of a co-injected series of dextran oligosaccharides (Yamashita *et al.*, 1982), which

were detected using a refractive index monitor (Erma) or PAD, and expressed in either glucose units (Gu) or 'Dionex units' (Du) for P-4 and high-pH anion-exchange chromatography respectively. Radiolabelled oligosaccharides were detected by using an on-line radioactivity monitor (Raytest Ramona) and/or liquid scintillation counting of fractions. Refractive index, Ramona and PAD signals were collected and analysed using Raytest software. Monosaccharides were separated by high-pH anion-exchange chromatography (Hardy *et al.*, 1988) using the PA-1 column eluted with 15 mM-NaOH at a flow rate of 0.6 ml/min.

RESULTS AND DISCUSSION

Glycolipids extracted from [^{14}C]GlcN-labelled cells in chloroform/methanol/water were fractionated by octyl-Sepharose chromatography (McConville *et al.*, 1987), which yielded a single peak of radioactivity eluting at 20–25 % propan-1-ol. [Further extraction of the pellet remaining after chloroform/methanol/water extraction with with butan-1-ol-saturated water (McConville *et al.*, 1990b) produced no radiolabelled material that was retained on octyl-Sepharose, indicating that no large glycolipids such as the LPGs of *Leishmania* were present.] The [^{14}C]GlcN-labelled glycolipid purified by octyl-Sepharose chromatography was shown to be sensitive to PI-PLC. After PI-PLC treatment, the radiolabel (84 %) no longer bound to octyl-Sepharose, indicating that the glycan headgroup containing the glucosamine had been released from a lipid moiety. The substrate specificity of *Bacillus thuringiensis* PI-PLC (see Low, 1989) suggests that the radiolabelled glycolipid is a derivative of phosphatidylinositol, with a D-myo-inositol residue linked at O-1 via a phosphodiester to a lipid moiety.

The glycan headgroup was deaminated with nitrous acid and reduced with NaBH_4 . This reaction simultaneously converts non-N-acetylated glucosamine residues, which are diagnostic of GPIs (Ferguson & Williams, 1988), to 2,5-anhydromannitol (AHM) and cleaves the glycosidic linkage between the glucosamine and myo-inositol of GPIs, releasing the inositol from the rest of the glycan headgroup. Bio-Gel P-4 chromatography of the PI-PLC-released, deaminated and reduced glucan resulted in elution of all of the radiolabel in the void volume (results not shown), indicating that the [^{14}C]AHM-containing oligosaccharide was negatively charged. (The net negative charge of the Bio-Gel P-4 matrix results in the exclusion of negatively charged molecules when water is used as the eluent.) However, after treatment with cold aqueous HF, which removes phosphate groups from oligosaccharides without cleavage of glycosidic linkages, the radiolabelled glycan eluted from Bio-Gel P-4 at about 4.2 Gu (Table 1). This elution position from Bio-Gel P-4 is consistent with a neutral Hex₃AHM oligosaccharide (Schneider *et al.*, 1990), and the change in elution behaviour indicates that

Fig. 1. High-pH anion-exchange chromatography of the neutral headgroup of the ^{14}C -labelled glycolipid

The neutral ^{14}C AHM-containing glycan that eluted from Bio-Gel P-4 at 4.2 Gu (Table 1) was chromatographed (a) using a Dionex Carbo-Pac PA-1 column equilibrated in 150 mM-NaOH/12.5 mM-sodium acetate and eluted with a linear gradient of sodium acetate (12.5–125 mM). Elution positions of a co-injected series of dextran oligosaccharides are indicated (a and b), and elution positions of peaks of radioactivity are reported in Du (at top of Figure). Radioactivity was measured using an on-line monitor (a) or liquid scintillation counting (b and c). The radiolabelled glycan eluting at 2.25 Du (a) was treated with APAM and rechromatographed (b). The precise elution position was determined to be 2.01 Du by comparing the PAD (dextran) and Ramona (radioactivity) traces (results not shown). The radiolabelled glycan eluting at 2.01 Du was treated with JBAM and rechromatographed in 15 mM-NaOH to resolve monosaccharides; elution positions of AHM and glucose (Glc) standards are indicated (c).

the glycan contained at least one phosphate residue prior to HF treatment. The radiolabel after HF treatment eluted from Dionex h.p.l.c. at 2.25 Du (Fig. 1a). Since $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$ elutes from Dionex h.p.l.c. at 2.5 Du (Schneider *et al.*, 1990), it can be concluded that the *T. mimbres* glycolipid does not have the same conserved glycan backbone that has been found in all GPI protein anchors characterized so far (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Schneider *et al.*, 1990).

The HF-treated glycan was digested with APAM, an exoglycosidase which is specific for $\text{Man}\alpha 1\text{-}2$ residues (where $p =$

pyranose) (Yamashita *et al.*, 1980; Amano & Kobata, 1986). The consequent change in the elution positions of the radiolabel from Bio-Gel P-4 (Table 1) and Dionex h.p.l.c. (Fig. 1b) indicated the loss of a single mannosyl residue. Furthermore, elution from Dionex h.p.l.c. at 2.01 Du indicates that the Hex_2AHM product of APAM digestion is not $\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{AHM}$, which elutes at 1.9 Du (M. A. J. Ferguson, unpublished work), but is identical to the $\text{Man}\alpha 1\text{-}3\text{Man}\alpha 1\text{-}4\text{AHM}$ derived from *Leishmania* LPGs and GIPLs (McConville & Blackwell, 1991). Further digestion of the HF-treated glycan with the pan-specific α -mannosidase from jack bean (Li, 1967) removed two α -linked mannopyranosyl residues. The radiolabelled JBAM product eluted from Bio-Gel P-4 at 1.7 Gu (Table 1), which is consistent with it being ^{14}C AHM. Analysis by Dionex h.p.l.c. under conditions that separate monosaccharides (Fig. 1c) confirmed the ^{14}C -labelled product of JBAM digestion to be AHM.

Taken together, the results suggest the following minimum structure for the *Tetrahymena* glycolipid:

It should be noted that the total number of phosphate residues, their location within the oligosaccharide, and whether there are additional moieties attached to them was not determined. In addition, it is possible that the neutral oligosaccharide product of APAM digestion was $\text{Man}\alpha 1\text{-}4\text{Man}\alpha 1\text{-}(3 \text{ or } 6)\text{AHM}$ and not $\text{Man}\alpha 1\text{-}3\text{Man}\alpha 1\text{-}4\text{AHM}$, as suggested by its co-elution with the latter. However, it is unlikely that the alternative structures, $\text{Man}\alpha 1\text{-}4\text{Man}\alpha 1\text{-}(3 \text{ or } 6)\text{AHM}$, would co-elute with $\text{Man}\alpha 1\text{-}3\text{Man}\alpha 1\text{-}4\text{AHM}$ from Dionex h.p.l.c., and neither of the two alternative structures are consistent with the $\text{Man}\alpha 1\text{-}4\text{GlcN}$ motif found to date in all GPIs. Finally, it is not known whether the glucosamine residue is in $\alpha(1\text{-}6)$ linkage to the *myo*-inositol, since this information is lost upon cleavage by nitrous acid deamination. Future studies using larger amounts of material and analytical techniques such as g.l.c.-m.s. and n.m.r. spectroscopy will be necessary to resolve these details of the primary structure.

The structure of the glycan headgroup of the *Tetrahymena* GPI described herein is unique compared with the GPI structures that have so far been determined (Fig. 2). By having a mannosyl residue that is 3- rather than 6-substituted, it differs from the minimum $\text{Man}_3\text{GlcN-Ins}$ backbone found in protein-linked GPIs (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Schneider *et al.*, 1990), in the candidate precursor glycolipid isolated from *T. brucei* (Mayor *et al.*, 1990) and in the LPPG of *T. cruzi* (Prevato *et al.*, 1990). This 3-substituted mannosyl residue is, however, common to the LPGs and GIPLs isolated from *Leishmania* spp. The terminal $\text{Man}\alpha 1\text{-}2\text{Man}$ sequence, on the other hand, is common to the protein-linked GPIs, the *Trypanosoma* precursor glycolipid and the LPPG of *T. cruzi*. The glycan of the *Tetrahymena* GPI thus has features characteristic of both classes of GPI.

GPIs are likely to be added to newly synthesized proteins as a pre-formed glycolipid precursor (see Doering *et al.*, 1990). A candidate precursor glycolipid has been isolated from *T. brucei* that has the structural features consistent with such a role (Krakow *et al.*, 1989; Mayor *et al.*, 1990). In particular, it has a free amino group as part of an ethanolamine-phosphate moiety (see Fig. 2), which could participate in the formation of an amide bond between the GPI anchor and the C-terminal amino acid of the protein. A glycolipid has been detected in *Tetrahymena* that (1) displays a similar t.l.c. behaviour as the *T. brucei* glycolipid, (2) can be biosynthetically labelled with ^{14}C GlcN and ^3H -ethanolamine, and (3) appears to be incorporated into GPI-

Fig. 2. Comparison of the *Tetrahymena mimbres* glycolipid with other GPI structures

The *Tetrahymena* glycolipid has structural features in common with the other GPIs that have been characterized. The GIPLs and LPG of *Leishmania* spp. (Turco *et al.*, 1989; McConville *et al.*, 1990b), LPPG from *Trypanosoma cruzi* (Prevato *et al.*, 1990), protein-linked GPIs from rat brain Thy-1 (Homans *et al.*, 1988), *Trypanosoma brucei* VSG (Ferguson *et al.*, 1988) and *Leishmania major* PSP (Schneider *et al.*, 1990), and putative precursor glycolipid from *Trypanosoma brucei* (Mayor *et al.*, 1990) all share a common structural motif (large box). The *Tetrahymena* and *Leishmania* glycolipids have a mannosyl residue linked $\alpha(1-3)$ to the common core (small box), while the next mannosyl residue in protein-linked GPIs and LPPG is linked $\alpha(1-6)$ to the core (medium-sized box). The Man α 1-2Man sequence is shared by the *Tetrahymena* glycolipid LPPG and the protein-linked GPIs (medium-sized box). Note that the number, position and substitution of the phosphate residues on the *Tetrahymena* glycolipid have not been determined. The branching galactofuranosyl residue of LPPG is located on the penultimate mannosyl residue as shown (R. M. de Lederkremer, C. Lima, M. I. Ramirez, M. A. J. Ferguson, S. W. Homans & J. E. Thomas, unpublished work). Abbreviation: 2-AEP, 2-aminoethylphosphonic acid.

anchored proteins during pulse-chase experiments (Pak *et al.*, 1991; Ryals *et al.*, 1991). It is not clear whether this putative precursor glycolipid is the same as the GPI described herein, because they were not prepared in the same manner. Nevertheless, if the novel GPI described herein is related to the putative precursor described by Ryals *et al.* (1991), then the protein-linked GPIs of *T. mimbres* are structurally unlike any others that have been so far characterized.

Grant support from the Wellcome Trust to M.A.J.F. and the Welch Foundation (F-350) and the National Science Foundation (DCB-8802838) to G.A.T. is gratefully acknowledged. We thank M. G. Low for the gift of PI-PLC and M. J. McConville and W. J. Masterson for helpful discussions.

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