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Structure of the Glycosyl-phosphatidylinositol Membrane Anchor of the *Leishmania major* Promastigote Surface Protease*

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In common with many other plasma membrane glycoproteins of eukaryotic origin, the promastigote surface protease (PSP) of the protozoan parasite *Leishmania* contains a glycosyl-phosphatidylinositol (GPI) membrane anchor. The GPI anchor of *Leishmania major* PSP was purified following proteolysis of the PSP and anaylzed by two-dimensional ^1H-^1H NMR, compositional and methylation linkage analyses, chemical and enzymatic modifications, and amino acid sequencing. From these results, the structure of the GPI-containing peptide was found to be Asp-Gly-Gly-Asn-ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-1-PO_4-(1-alkyl-2-acyl-glycerol). The glycan structure is identical to the conserved glycan core regions of the GPI anchor of *Trypanosoma brucei* variant surface glycoprotein and rat brain Thy-1 antigen, supporting the notion that this portion of GPIs are highly conserved. The phosphatidylinositol moiety of the PSP anchor is unusual, containing a fully saturated, unbranched 1-*O*-alkyl chain (mainly C24:0) and a mixture of fully saturated unbranched 2-*O*-acyl chains (C12:0, C14:0, C16:0, and C18:0). This lipid composition differs significantly from those of the GPIs of *T. brucei* variant surface glycoprotein and mammalian erythrocyte acetylcholinesterase but is similar to that of a family of glycosylated phosphoinositides found uniquely in *Leishmania*.

The trypanosomatid protozoan parasites of the *Leishmania* family are the etiologic agents of the leishmaniases, a widespread group of diseases that affect man and other mammals in tropical and subtropical regions. The parasite is transmitted to the mammalian host as a flagellated promastigote by the bite of a sandfly (sub-family *Phlebotominae*). Parasites are internalized by cells of the mononuclear phagocyte system in which they subsequently differentiate and reproduce as amastigotes within the acidified phagolysosome of the infected cell (Behin and Louis, 1984).

A protein (M_r ~ 63,000) was described at the surface of promastigotes (Colomer-Gould *et al.*, 1985; Etges *et al.*, 1985) in amounts representing up to 1% of the total cellular protein depending on the species and strain of the parasite (Bouvier *et al.*, 1987). This protein, named gp63 or the promastigote surface protease (PSP),[1] is a membrane-bound zinc endopeptidase (Etges *et al.*, 1986b; Bouvier *et al.*, 1989; Chaudhuri *et al.*, 1989) which is active at the surface of fixed and live promastigotes (Etges *et al.* 1986b). Recently, a related protein was reported in the amastigote stage of the parasite (Medina-Acosta *et al.*, 1989; Frommel *et al.*, 1990). The protease is attached to the plasma membrane of the promastigote by a glycosyl-phosphatidylinositol (GPI) membrane anchor (for recent reviews on GPI anchors, see Ferguson and Williams, 1988; Low, 1989) which can be labeled with myristic acid (Etges *et al.*, 1986a). When the phospholipid moiety of the GPI is cleaved with a phosphatidylinositol-specific phospholipase C (PI-PLC), the protease becomes hydrophilic and exposes a new antigenic epitope, the cross-reacting determinant (CRD) (Bordier *et al.*, 1986). The CRD is common to soluble forms of other GPI-anchored proteins digested by PI-PLC (Ferguson and Williams, 1988; Zamze *et al.*, 1988).

The signal for GPI addition to protein lies in a COOH-terminal sequence, which typically consists of a relatively polar domain of about 7–12 polar amino acids followed by a stretch of about 12–20 hydrophobic amino acids. This sequence is cleaved and directly replaced by a prefabricated GPI anchor moiety (Ferguson and Williams, 1988; Caras *et al.*, 1989; Masterson *et al.*, 1989). The predicted amino acid sequence of PSP contains such a putative GPI addition sequence (Button and McMaster, 1988; Miller *et al.*, 1990).

Comparison of the two known GPI structures for *Trypanosoma brucei* variant surface glycoprotein (VSG) (Ferguson *et al.*, 1988) and rat brain Thy-1 antigen (Homans *et al.*, 1988) indicates that there is a common core structure that links the COOH-terminus of the mature protein to the lipid moiety *i.e.*, ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-1-PO_4. The partial structure of human erythrocyte acetylcholinesterase (EAChE) is also consistent with this common core region (Roberts *et al.*, 1988b). In addition to this common region, GPIs may also contain species-specific

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[1] The abbreviations used are: PSP, promastigote surface protease; GPI, glycosyl-phosphatidylinositol; VSG, variant surface glycoprotein; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-PLC, glycosyl-phosphatidylinositol-specific phospholipase C; CRD, cross-reacting determinant; EAChE, erythrocyte acetylcholinesterase; LPG, lipophosphoglycan; GIPL, glycoinositol phospholipid; BSA, bovine serum albumin; PI, phosphatidylinositol; TMS, trimethylsilyl; GC-MS, gas chromatography-mass spectrometry; sCt-gp, soluble COOH-terminal glycopeptide; 2,5-AHM, 2,5-anhydromannitol; HPLC, high performance liquid chromatography; COSY, correlated spectroscopy.

side chains of carbohydrate and/or ethanolamine phosphate moieties (Ferguson and Homans, 1989).

A greater heterogeneity is seen at the level of the lipid moiety. Dimyristyl glycerol is found in VSGs (Ferguson *et al.*, 1985a, 1985b), 1-alkyl-2-acyl glycerol in bovine and human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988a, 1987) and ceramide in contact site A glycoprotein of *Dictyostelium discoideum* (Stadler *et al.*, 1989).

In this study, the GPI anchor structure of *Leishmania* surface protease is described. This structure is compared with the anchor structure of VSG, Thy-1, and EAChE and with a series of glycosylated phosphoinositides found in the *Leishmania* parasites (Turco *et al.*, 1989; McConville and Bacic, 1989; McConville *et al.*, 1990).

EXPERIMENTAL PROCEDURES

Materials—Defatted-BSA, QEA-Sephadex A-25, and Sigma Sil-A were from Sigma (München, F.R.G.), octyl-Sepharose from Pharmacia (Uppsala, Sweden), jack bean α-mannosidase from Boehringer (Mannheim, F.R.G.), Pronase and *scyllo*-inositol from Calbiochem (La Jolla, CA), [9,10-^{3}H]myristic acid from Amersham Corp., D_2O (Gold grade) from Aldrich (Steinheim, F.R.G.), 6 N HCl from Pierce Chemical Co., NaB[^{3}H]$_4$ (15 Ci mmol^{-1}) from Du Pont-New England Nuclear (Dreieich, F.R.G.), AG3X4, AG50X12, and Chelex 100 from Bio-Rad (Glattbrug, Switzerland). PI-PLC from *Bacillus thuringiensis* and *Staphylococcus aureus* were generous gifts from Dr. M. G. Low (Columbia University, New York). Soluble-form variant surface glycoprotein variants MITat 1.5 and 1.4 (sVSG-MITat 1.5 and 1.4) were purified from *T. brucei* according to the method of Cross (1984). β-Glucose oligomer standards were prepared by partial hydrolysis of dextran. Glycoinositol phospholipids (GIPLs) were purified from *L. major* according to the method of McConville and Bacic (1989). PSP from *L. major* LEM 513 promastigotes was purified according to previously described protocols (Bouvier *et al.*, 1985; Bordier, 1988). Anti-CRD antibodies were as described in Zamze *et al.* (1988) and GPI-PLC, prepared as described in Stieger *et al.* (1986), was a gift from Dr. M.-L. Cardoso de Almeida (Escola Paulista de Medicina, São Paulo, Brazil). All other reagents were of the highest purity commercially available.

Biosynthetic Labeling with [^{3}H]Myristic Acid—Promastigotes in logarithmic phase of growth were washed twice with modified Schaefer's medium (Schaefer *et al.*, 1970; Etges *et al.*, 1986a) containing 1% defatted BSA, once in medium without BSA, and finally resuspended at 10^7 cells ml^{-1} in modified Schaefer's medium containing 1.5 μCi ml^{-1} of [^{3}H]myristic acid complexed with BSA (Hereld *et al.*, 1988).

After 20 h, the medium was supplemented to 5% HOSMEM II medium (Berens and Marr, 1978) and 2.5% fetal calf serum, and cells were grown for an additional 24 h. Promastigotes were harvested for PSP purification when they reached a density of 3×10^7 cells ml^{-1}.

Protein Quantification—The quantity of PSP was determined after electrophoresis and Coomassie Blue staining by densitometry using ovalbumin as a standard.

Mild Acid Treatment—Amphiphilic PSP was converted to its hydrophilic form using *T. brucei* GPI-PLC as described previously (Bordier *et al.*, 1986). Hydrophilic PSP and solube VSG were treated for 30 min at room temperature in 1 N HCl. After neutralization with NaOH, samples were heated and reduced for 3 min at 98 °C in the presence of 1% 2-mercaptoethanol.

Gel Electrophoresis and Immunoblotting—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, electroblotting on nitrocellulose paper, and visualization of the antigen-antibody complex with Protein-A-peroxidase were performed essentially as described previously (Bordier *et al.*, 1986) except that mini-gels (90 × 50 × 1 mm, 12% acrylamide) were used. Anti-MITat 1.2 antiserum at a dilution of 1:1000 (Zamze *et al.*, 1988) in 10 mM Tris, 140 mM NaCl, 1% gelatin at pH 7.5 was used to reveal CRD on blots.

Preparation of the GPI Peptide and Soluble COOH-terminal Glycopeptide—A scheme of the reactions used to analyze PSP GPI is shown in Fig. 1. Purified PSP (475 nmol, 31 mg) was combined with [^{3}H]myristic acid-labeled PSP (6.5×10^4 cpm). This material was reduced (in 3.3 ml of 300 mM Tris-HCl, pH 9, 1.8 M NaCl, 0.5% *N,N*-dimethyldodecylamineoxide, 50 mM dithiothreitol for 1 h at 37 °C) and *S*-carboxymethylated (by addition of 130 mM iodoacetamide, 14 h at 22 °C with stirring). The product was dialyzed against 0.1 M ammonium bicarbonate, 0.05% *n*-octyl glucoside, and digested with Pronase (2% w/w, 36 h, 37 °C) with a second addition of Pronase (0.2% w/w) after the first 24 h.

The detergent was removed by extraction with an equal volume of water-saturated 1-butanol. The aqueous phase, containing the GPI-peptide (compound 1), was acidified to pH 5 with acetic acid and loaded onto a column (0.5 × 18 cm) of octyl-Sepharose equilibrated in 0.1 M sodium acetate buffer, pH 5, containing 5% 1-propanol. The column was washed with 10 ml of the same buffer and eluted over 4 h with a 5–55% 1-propanol gradient at a flow rate of 4 ml h^{-1} (Fig. 2). Compound 1 was repurified on octyl-Sepharose using batch-elution with 37% 1-propanol in water.

Compound 1 was digested with 400 μl of PI-PLC from *B. thuringiensis* (7.5 μg ml^{-1} in 50 mM ammonium bicarbonate-CO_2 buffer, pH 7.5, for 30 h at 37 °C followed by a further 200 μl of PI-PLC for 16 h at 37 °C). Extraction of the digest with toluene indicated that only 48% of the [^{3}H]myristic acid label had been cleaved from compound 1. Due to the poor cleavage in the absence of detergent, the mixture containing compound 1 and compound 2 was freeze-dried and redigested with PI-PLC in the presence of detergent (60 μl of 13 μg ml^{-1} PI-PLC, 20 mM Tris acetate, pH 7.4, 0.1% Triton X-100, 8% glycerol, for 15 h at 37 °C). The digest was extracted with toluene (three times) to remove detergent and released lipid. The resulting COOH-terminal glycopeptide (compound 2) was desalted by descending paper chromatography for 16 h on Whatman 3MM paper in 1-butanol/ethanol/water (4:1:1, v/v), eluted from the origin with water and further purified by high performance liquid chromatography (HPLC) using a reverse-phase column (Hypersil 5 μm C_{18}, 4.6 × 250 mm) equilibrated with water. Compound 2 eluted in the void volume and was detected at 200 nm.

Alternatively, compound 1 (10 nmol) was digested (in 30 μl of 20 mM Tris acetate, pH 7.4, 1% glycerol, 37 °C for 20 h) with a mixture of *B. thuringiensis* (5 μg ml^{-1}) and *S. aureus* PI-PLCs (2 μg ml^{-1}). Water (100 μl) was added and the alkyl-acyl-glycerols were extracted twice with 250 μl of diethylether, then taken to dryness and converted to their trimethylsilyl derivatives before and after methanolysis (see below).

Dephosphorylation and N-Acetylation—Compound 2 was converted to compound 3 by aqueous HF dephosphorylation and *N*-acetylation as described under Generation of GPI Core Standard.

Nitrous Acid Deamination and NaBD$_4$ Reduction—Compound 2 was dissolved in 50 mM sodium acetate buffer, pH 4.0, containing 250 mM sodium nitrite (200 μl) and incubated at room temperature for 2.5 h. Boric acid (150 μl, 400 mM) was added, and the pH was adjusted to 11 with 1 M NaOH (50 μl). Products were reduced for 10 h with 2 M NaBD$_4$ in 1 M NaOH (50 μl), and excess reagent was destroyed with acetic acid. The deaminated and reduced glycopeptide (compound 4) was desalted by passage through 0.4 ml of AG50X12(H+) followed by evaporation twice with 5% acetic acid in methanol (250 μl) and twice with methanol (250 μl).

Preparation of Tritiated Neutral Glycan—Whole PSP (16 nmol, 1 mg) was dialyzed against water, dried, deacylated with 9 M ammonia (50 μl for 40 min at 45 °C), dried, and evaporated from water. PSP was then dephosphorylated with 50% aqueous HF as described under Generation of a GPI Core Standard. Defatted BSA (1 mg ml^{-1}) was added as a carrier and proteins were precipitated at 0 °C with 5% trichloroacetic acid. Anions were removed by passage through 0.6 ml of AG3X4(OH$^-$) over 0.2 ml of QAE-Sephadex A-25. The eluate was adjusted to pH 5 with 1 M HF and dried. Carbohydrates were extracted from the LiF residue with 70 μl of water, dried, and submitted to nitrous acid deamination as described above, except that 0.25 M acetate buffer was used. The sample was split in two equal parts and reduced at pH 10.5 with either 0.25 M NaBD$_4$ or 4 mM NaB[^{3}H]$_4$ (15 Ci mmol^{-1}) followed by 0.25 M NaBD$_4$ for 1.5 h. Labeled neutral glycan (compound 5) was separated from radioactive impurities by descending paper chromatography for 60 h as described above, followed by high voltage electrophoresis on Whatman 3MM paper in pyridine/acetic acid/water (3:1:387), pH 5.2, at 80 V cm^{-1} for 45 min. The labeled neutral glycan fraction was recovered from the origin by elution with water. Deuterated and tritiated neutral glycan were pooled, desalted by passage through a column of 0.2 ml each Chelex 100(Na$^+$) layered over AG50X12(H$^+$) over AG3X4(OH$^-$) over QAE-Sephadex A-25 equilibrated in water and filtered through a 0.2-μm Teflon membrane.

A standard of *T. brucei* VSG MITat 1.5 (2 nmol) was processed and labeled in parallel.

α-Mannosidase Treatment—Jack bean α-mannosidase was dialyzed against 0.1 M sodium acetate, pH 5. Compound 4 was treated with 2.5 units (100 μl) of enzyme for 18 h at 37 °C, prior to or after

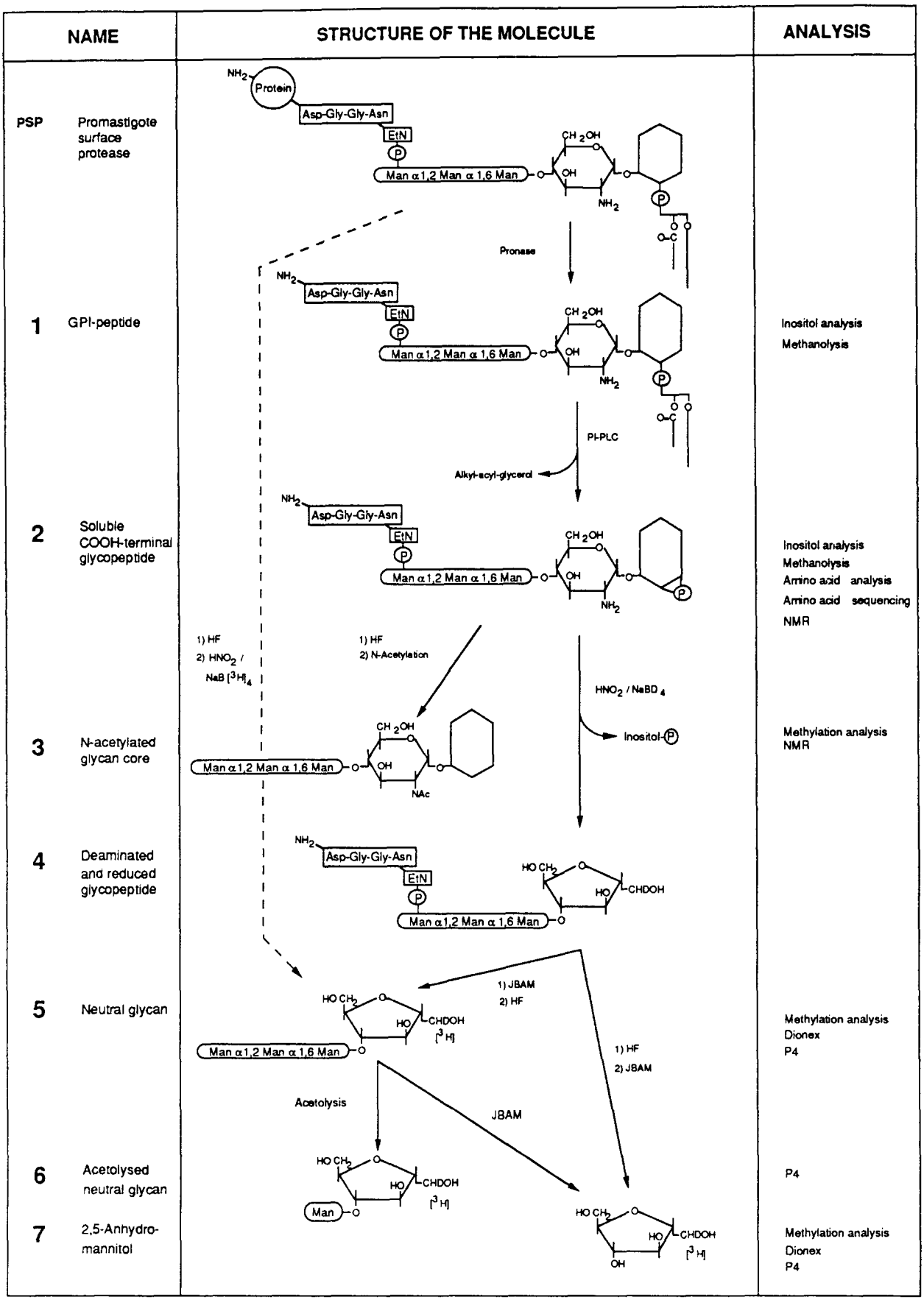


FIG. 1. **Scheme of reactions used to determine the structure of the membrane anchor of *Leishmania* promastigote surface protease.** The name and a reference number is given for each compound in the first column. The processing of the anchor and derived compounds is illustrated in the second column. The analyses performed on each compound are shown in the third column. Abbreviations: *EtN* = ethanolamine; *P* = phosphate; *Man* = mannose; *JBAM* = jack bean α-mannosidase.

HF dephosphorylation. ^{3}H-Labeled compound 5 was also submitted to α-mannosidase digestion. Digestions were stopped by heating for 2 min at 100 °C. Samples were desalted by passage through a column of 0.2 ml each AG50X12(H^+) over AG3X4(OH^-) and were filtered through a 0.2-μm membrane.

Acetolysis—^{3}H-Labeled compound 5 was peracetylated in acetic anhydride/pyridine (1:1) for 30 min at 100 °C and dried under vacuum. Acetolysis of the acetylated product was carried out in 30 μl of acetic anhydride/acetic acid/concentrated sulfuric acid (10:10:1) for 6 h at 37 °C. The reaction was stopped by the addition of 10 μl of pyridine and 500 μl of water. The acetylated products were extracted with 250 μl of chloroform, washed three times with 500 μl of water, then taken to dryness. Deacetylation was performed at 37 °C for 60 h in 200 μl of methanol and 35% NH_3 in water (1:1). The acetolyzed neutral glycan (compound 6), was dried and redissolved in water.

Liquid Chromatography—The ^{3}H-labeled glycan moieties were analyzed on a Bio-Gel P4 column (1.5 × 100 cm) held at 55 °C and eluted with water at 0.2 ml min^{-1}. Elution of the glycans and coinjected β-glucose oligomers (750 μg) were monitored with a radioactivity flow monitor (Ramona, Raytest) and a refractive index monitor (Erma Inc.), (Kobata, 1984). High performance liquid chromatography was performed using a Dionex model BioLC Carbohydrate Analyzer equipped with a pulsed amperometric detector, anion membrane suppressor, and radioactivity flow monitor. Radiolabeled glycans were injected with β-glucose oligomers (300 μg) onto a CarboPac column (4 × 250 mm) equilibrated in 0.15 M NaOH, 12.5 mM sodium acetate, and eluted with a linear gradient of sodium acetate to 50 mM over 50 min at 0.6 ml min^{-1}.

Gas Chromatography-Mass Spectrometry—All analyses were performed with a Hewlet-Packard 5890-MSD system, using a CPSil8CB column (30 m × 0.32 mm, Chrompack). Methylation analysis products were also analyzed using a SP2380 (25 m × 2 mm, Supelco) column.

Inositol Analysis—Inositol content was measured following acid hydrolysis (6 N HCl, 110 °C, 18 h) and trimethylsilyl (TMS) derivatization by selected ion monitoring GC-MS (Smith *et al.*, 1987) using *scyllo*-inositol as internal standard.

Neutral Sugar Analysis—Neutral sugars were measured by GC-MS following methanolysis and TMS derivatization as previously described (Ferguson *et al.*, 1988). For detection of phosphorylated sugars, TMS derivatives were dissolved in 20 μl of diethylether/methanol (9:1, v/v) for 5 min on ice and reacted for 10 min on ice with diazomethane-saturated diethylether. Samples were then dried and submitted again to TMS derivatization before analysis by GC-MS.

Lipid Analysis—Fatty acid and alkyl-glycerol content were measured simultaneously by GC-MS following methanolysis and TMS derivatization according to McConville and Bacic (1989). *L. major* GIPLs processed in the same way were used to generate alkyl-glycerol TMS standards to calibrate the system.

Methylation Analysis—Methylation analyses were performed using a modified procedure of Ciucanu and Kerek (1984). Samples were dissolved in dimethyl sulfoxide (50 μl) and methylated by sequential addition of a NaOH slurry in dimethyl sulfoxide (120 mg ml^{-1}, 50 μl, 20 min) and three additions of methyliodide (10, 10, and 20 μl) at 10-min intervals. Chloroform (0.5 ml) and 1 M sodium thiosulfate (1 ml) were added and the organic phase washed with water (three times 1 ml) and dried. Radiolabeled methylated sugars were redissolved in 100 μl of 25% acetonitrile in water (v/v) and eluted from a high performance liquid chromatography reverse-phase column (Hypersil 5 μm C_{18}, 4.6 × 250 mm) by an acetonitrile gradient (25–85% over 1.5 h). All methylated glycans were hydrolyzed in 0.25 M H_2SO_4, 93% acetic acid (100 μl, 80 °C, 2.5 h). The hydrolysate was neutralized by addition of 1 M NH_4OH (70 μl) and repeated evaporation with toluene. After reduction with $NaBD_4$ (150 μl, 13 mg ml^{-1}) in 0.33 M NH_4OH (3 h, 25 °C), the samples were acidifed and the boric acid removed by evaporation with 5% acetic acid in methanol (250 μl, twice) followed by methanol (250 μl, twice). Samples were acetylated with acetic anhydride (250 μl, 100 °C, 2.5 h), dried at reduced pressure, and the resulting partially methylated alditol acetates recovered by partitioning between water and dichloromethane and analyzed by GC-MS.

Generation of GPI Core Standard—The compound Manα1-2Manα1-6Manα1-4GlcNacα1-6*myo*-inositol was generated from *T. brucei* sVSG MITat 1.4. The structure of the GPI anchor of this VSG has been previously reported and the soluble form COOH-terminal glycopeptide (sCt-gp) fraction was purified from 80 mg of VSG as described (Ferguson *et al.*, 1988). The complex α-galactose side chain was quantitatively removed by digestion with coffee bean α-galactosidase (100 μl, 25 units ml^{-1} in 0.1 M sodium acetate, pH 5.0, 37 °C, 16 h). The digestion was terminated by heating at 100 °C for 5 mi and the products desalted by passage through a 0.2-ml column o AG50X12 (H^+), elution with water, and evaporation. The degalacto sylated sCt-gp was filtered (0.2-μm membrane) and purified fron released galactose by passage through a small column (1.5 × 20 cm of Bio-Gel P4 (400 mesh). Carbohydrate-containing fractions wer detected by spotting 2-μl aliquots onto silica thin layer chromatog raphy plates and staining with α-naphthol/H_2SO_4 (McConville an Bacic, 1989). The degalactosylated sCt-gp was dephosphorylated i 50 μl of 50% aqueous HF, 0 °C, 60 h. The digest was neutralized wit 280 μl of saturated LiOH and centrifuged. The supernatant and 10 μl of pellet-washings were combined and constituted the dephospho rylated fraction. The products were adjusted to 1 M $NaHCO_3$ by th addition of solid $NaHCO_3$ and *N*-acetylated at 0 °C by three addition of acetic anhydride (10, 10, and 20 μl) 10 min apart. The Manα1 2Manα1-6Manα1-4GlcNAcα1-6*myo*-inositol core was desalted b passage through a column of 0.2 ml of Chelex 100 over 0.8 ml o AG50X12 (H^+) over 2.5 ml of AG3X4 (OH^-) over 0.2 ml of QAE Sephadex A-25 and repurified by chromatography on Bio-Gel P4 i the absence of β-glucose standards. The GPI core was eluted at position equivalent to 5.2 glucose units on Bio-Gel P4. The core wa dried, exchanged into D_2O, and used as a standard for NMR spec troscopy.

Nuclear Magnetic Resonance—NMR spectra were recorded at 50 MHz in D_2O essentially as described previously (Ferguson *et al.* 1988).

Amino Acid Analysis—Samples containing a norleucine interna standard were dried, hydrolyzed (6 N HCl vapor, 16 h, 110 °C unde vacuum), dried, and evaporated from 30 μl water/ethanol/triethyla mine (2:2:1, v/v). Amino compounds were derivatized with 80 μl o water/ethanol/triethylamine/phenylisothiocyanate (1:7:1:1, v/v) fo 20 min in the dark, dried, and analyzed using the Pico Tag systen (Waters).

Ethanolamine hydrochloride and glucosamine hydrochlorid standards together with norleucine were analyzed with and withou HCl treatment.

Amino Acid Sequencing—Samples were sequenced by automate Edman degradation using an Applied Biosystem 470A Protein Se quenator attached to a 120A PTH Analyzer.

RESULTS

Preparation and Purification of the COOH-terminal Glyco peptide—PSP was reduced and *S*-carboxymethylated prior t degradation by Pronase. Detergent in the *S*-carboxymethyl ation mixture was removed by extraction with water-saturate butanol and the GPI-peptide purified by adsorption on octyl Sepharose and elution with 1-propanol (Fig. 2). The ratio o [^{3}H]myristic acid radioactivity to inositol content was con stant across the eluted peak, indicating that all the label wa associated with this inositol lipid. The overall yield fron whole PSP to compound 2 was 45%, with the majority of th

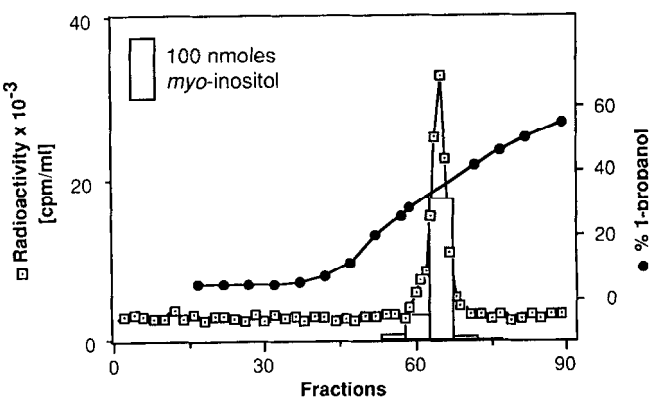


FIG. 2. **Octyl-Sepharose chromatography of the GPI-pep tide.** The [^{3}H]myristic acid-labeled GPI-peptide released by Pronas digestion of PSP was eluted from octyl-Sepharose by a gradient of 1 propanol (●). Fractions were analyzed for radioactivity (□) an pooled fractions for *myo*-inositol content (*bars*).

losses occurring during the dialysis step after reduction and *S*-carboxymethylation.

The Glycan Core—The compositional analysis of compounds 1 and 2 (Table I) show the presence of two Man residues and 1 GlcN residue/mol of *myo*-inositol. In addition Man 6-phosphate was detected, which is believed to originate from the Man 6-phosphate-ethanolamine bridge to the PSP polypeptide by analogy with results obtained for the GPI anchor of *T. brucei* VSG (Ferguson *et al.*, 1988). Methylation linkage analysis of compound 3 (Table II) indicates the presence of a linear glycan containing terminal-Man, 2-*O*-substituted Man, 6-*O*-substituted Man, 4-*O*-substituted GlcNAc, and a mono-substituted inositol. Methylation analysis of compound 5 is identical except that the GlcNAc derivative is replaced by a 4-*O*-substituted 2,5-anhydromannitol (2,5-AHM), due to deamination and reduction, and no inositol derivative was detected. The GlcNAc and inositol derivatives were observed in relatively low yield (Table II). However, this has been observed previously for other molecules containing the same Manα1-4GlcNα1-6*myo*-inositol sequence (Ferguson *et al.*, 1988; Turco *et al.*, 1989; McConville *et al.*, 1990). The reason for this low yield is still unclear. The difference between compound 3 and 5 implies a direct linkage between the GlcN residue and the *myo*-inositol. The mono-acetyl-penta-methyl inositol derivative observed in compound 3 coeluted on two different GC-columns with authentic 6-*O*-acetyl-1,2,3,4,5-penta-methyl inositol generated from methylation analysis of *L. donovani* lipophosphoglycan (LPG) (Turco *et al.*, 1989).

TABLE I
Compositions of compounds 1 and 2 relative to myo-inositol

	Molar ratio	
	Compound 1	Compound 2
myo-Inositol	1.0	1.0
Mannose	2.0	2.0
Galactose	0.2	0.3
Mannose 6-phosphate	+[a]	ND
1-*O*-Alkyl glycerols	1.0	0.0
Fatty acids	+	0.0
Glycine	ND[b]	1.8
Aspartic acid/asparagine	ND	1.7
Ethanolamine	ND	0.8
Glucosamine	ND	0.7
Inositol phosphate[c]	ND	+

[a] +, present but not quantified.
[b] ND, non-determined.
[c] Detected after deamination of compound 2.

The ^{3}H-labeled neutral glycan (compound 5) from PSP cochromatographed with an authentic Manα1-2Manα1-6Manα1-4(2,5-AHM) standard generated from sVSG MITat 1.5 on both Bio-Gel P4 and Dionex carbohydrate HPLC (Fig. 3). The digestion of both the PSP and the standard VSG-labeled glycan with jack bean α-mannosidase produced a single labeled component that cochromatographed with authentic 2,5-AHM.

Taken together, the methylation and α-mannosidase digestion data suggest that PSP anchor glycan is similar to that of the VSG standard (*i.e.* Manα1-2Manα1-6Manα1-4GlcN). However, it was possible that the order of the mannose linkages could be reversed in PSP (*i.e.* Manα1-6Manα1-2Manα1-4GlcN). To investigate this possibility, the glycan was subjected to partial acetolysis under conditions which preferentially cleave the Manα1-6Man glycosidic bonds (Kacourek and Ballou, 1969). The products (Fig. 3) include some uncleaved material (eluting at 4.2 glucose units) together with a fragment eluting at 2.3 glucose units on Bio-Gel P4 which correspond to a Man_1-2,5-AHM structure containing a single Man residue. This indicates that both the PSP and the VSG glycan structure are identical with the Manα1-2Manα1-6Manα1-4(2,5-AHM) structure. The alternative Manα1-6Manα1-2Manα1-4(2,5-AHM) structure would have produced a Man_2-2,5-AHM fragment of about 3.2 glucose units.

In order to determine the anomeric configuration of glucosamine and the linkage position to inositol, the soluble COOH-terminal glycopeptide (compound 2) was analyzed by two-dimensional ^{1}H-^{1}H-correlated spectroscopy (COSY) (Fig. 4*a*). Four anomeric protons of unit intensity and corresponding cross-peaks (correlating these H1 protons with their respective H2 protons) were observed in the low field region of the spectrum. The three cross-peaks at $\omega_1 = 5.04$ ppm, $\omega_2 = 4.08$ ppm, $J_{1,2} \sim 2$ Hz; $\omega_1 = 5.13$ ppm, $\omega_2 = 4.00$ ppm, $J_{1,2} \sim 2$ Hz and $\omega_1 = 5.21$ ppm, $\omega_2 = 4.07$ ppm, $J_{1,2} \sim 2$ Hz indicate the presence of 3 α-Man residues, which is consistent with the compositional and methylation analysis. The chemical shifts and the "reporter" resonances of glucosamine (H1, H2) and inositol (H6) did not correspond with those reported for other GPI structures containing the fragment GlcNα1-6*myo*-inositol (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Turco *et*

TABLE II
Methylation linkage analyses

Partially methylated alditol acetate	Origin	Compound 3	[^{3}H]Compound 5
2,5-Anhydromannitol (1,3,6-tri-*O*-methyl-4-*O*-acetyl)	4-*O*-Substituted 2,5-AHM	0.0	0.4[a]
Mannitol			
(2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl)	Terminal Man	0.9	0.9
(3,4,6-Tri-*O*-methyl-1,2,5-tri-*O*-acetyl)	2-*O*-Substituted Man	1.1	0.9
(2,3,4-Tri-*O*-methyl-1,5,6-tri-*O*-acetyl)	6-*O*-Substituted Man	1.0	1.0
(2,3,6-*Tri*-*O*-methyl-1,4,5-tri-*O*-acetyl)	4-*O*-Substituted Man	0.2	0.0
Galacitol (2,3,4,6-Tetra-*O*-methyl-1,5-di-*O*-acetyl)	Terminal Gal	0.2	0.0
2-*N*-Methylacetamido-2-deoxiglucitol (3,6-Di-*O*-methyl-1,4,5-tri-*O*-acetyl)	4-*O*-Substituted GlcNAc	0.3	0.0
Inositol Penta-*O*-methyl-mono-*O*-acetyl)	Mono-*O*-Substituted *myo*-inositol	0.5	0.0

[a] Low yield due to high volatility. 4-*O*-Substituted Glc was routinely detected, but also occurred in blank samples.

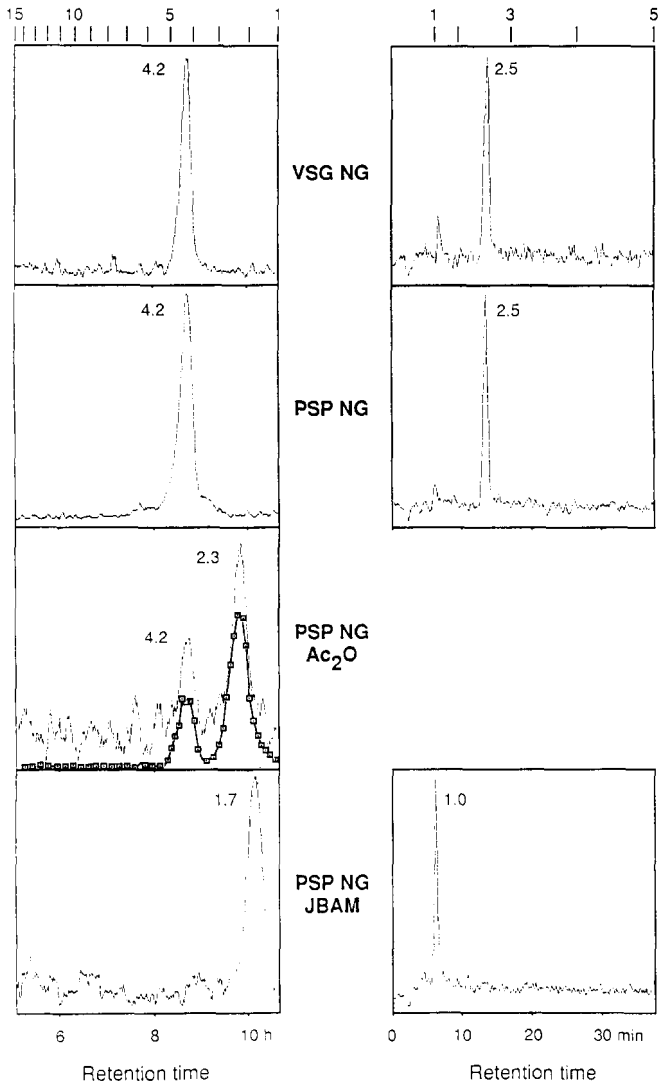


FIG. 3. **Liquid chromatography of labeled neutral glycans.** Labeled neutral glycans were chromatographed on either Bio-Gel P4 (*left panel*) or Dionex HPLC (*right panel*). The elution position of β-glucose oligomer internal standards are shown at the top of each panel. Neutral glycans were detected by on-line monitoring of radioactivity and also by counting fraction aliquots (*squares*) in one case. Figures adjacent to peaks indicate the elution positions relative to the β-glucose oligomer internal standards. For Bio-Gel P4 chromatography, these "glucose unit" values reflect the hydrodynamic volume of the neutral glycans. The equivalent value on Dionex HPLC has no specific meaning, but is characteristic of a given structure. Abbreviations are: *VSG NG,* neutral glycan from *T. brucei* MIT1.5 VSG GPI anchor (Manα1-2Manα1-6Manα1-4[2,5-AHM]; *PSP NG,* neutral glycan from PSP GPI anchor (compound 5, see Fig. 1); *PSP NG Ac_2O,* PSP NG that was submitted to partial acetolysis under conditions that preferentially cleave α1–6 linkages; *PSP JBAM,* PSP NG digested with jack bean α-mannosidase.

al., 1989; McConville *et al.,* 1990). In order to determine whether the sequence of the PSP glycan is related to that of all other GPI anchor structures examined to date (*i.e.* containing Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol), the COSY spectrum of compound 3 (Fig. 4*b*) was compared with that of an authentic GPI core standard, generated from sVSG MITat 1.4 (see "Experimental Procedures"). As shown in Table III, the chemical shifts and coupling constants of compound 3 and the GPI core standard are identical, thus strongly suggesting the equivalence of the two structures. The spectra of compound 2 and the analogous compound derived from sVSG are similar, but not identical (Table III). This shows that the H1 and H2 resonances of the monosaccharides are sensitive to changes in the attached peptide, from Asp-Gly-Gly-Asn in compound 2 to Asp in the sVSG-derived standard.

The location of the ethanolamine phosphate bridge was probed using α-mannosidase digestion of compound 4 before and after dephosphorylation with aqueous HF. The product of α-mannosidase digestion followed by HF dephosphorylation was deduced to be component 5 since methylation analysis showed the presence of terminal-Man, 2-*O*-substituted-Man, 6-*O*-substituted-Man, and 4-*O*-substituted-(2,5-AHM). By contrast, material digested with α-mannosidase after HF dephosphorylation contained mainly terminal-Man (arising from the released free mannose residues) together with only trace amounts of the other three derivatives. These results indicate that the ethanolamine phosphate was originally on the nonreducing terminal mannose residue. The same approach was previously used to establish the ethanolamine phosphate positions in both VSG (Ferguson *et al.,* 1988) and Thy-1 GPI anchors (Homans *et al.,* 1988).

Whole PSP was converted to its hydrophilic form by treatment with *T. brucei* GPI-PLC and subsequently treated with mild acid. The CRD present on GPI-PLC-cleaved PSP is entirely lost after mild acid treatment, in contrast to soluble VSG which is still significantly recognized by anti-CRD antiserum after mild acid treatment (Fig. 5). This result is in good agreement with the CRD epitope mapping studies of Zamze *et al.* (1988). The anti-CRD rabbit sera contain a population of antibodies which are dependent on the presence of an acid-labile *myo*-inositol-1,2-cyclic phosphate, generated by GPI-PLC action (Ferguson *et al.,* 1985a), and another population which recognize the α-galactose branch of *T. brucei* VSG GPI. For this reason, mild acid treatment only reduces sVSG anti-CRD reactivity. In contrast, the PSP anti-CRD reactivity is completely destroyed by mild acid, suggesting that the PSP anchor contains no α-galactose branch. This immunological prediction of the PSP GPI glycan structure is consistent with the complete chemical structure described in this study.

Lipid Structure—GC-MS analysis of compound 1, after acid methanolysis and trimethylsilylation, revealed the presence of acid stable monoalkylglycerols (Fig. 6). These were identified by their relative retention times and by the characteristic mass spectra of each ether derivative which contained the following diagnostic fragment ions: *m/z* $[M-15]^+$, $[M-90]^+$ and a base peak of *m/z* 205 in every case. The presence of *m/z* 205 $[CHOSi(CH_3)_3 - CH_2OSi(CH_3)_3]^+$ in all spectra, together with the absence of *m/z* 218 and 191 suggests that alkyl chains are found exclusively at the 1-position of the glycerol. The alkyl chain composition consisted of saturated, unbranched species having carbon chain length ranging from 18 to 26 (Fig. 6). The C24:0 alkyl chain was by far the most abundant species and accounted for more than 80% of the alkyl chains (Fig. 6).

Fatty acids methyl esters were also present in the methanolysate of compound 1. They consisted of laurate (C12:0), myristate (C14:0), palmitate (C16:0) and stearate (C18:0) in molar ratios indicated in Fig. 7. In order to investigate if lyso-alkyl glycerol was also present in the PSP anchor, TMS derivatives of the whole lipid released from compound 2 by PI-PLC were analyzed by GC-MS. The 1-*O*-alkyl-2,3-di-*O*-TMS-glycerol species could be detected in whole PSP lipids only after methanolysis of the sample. This result suggests that PSP anchor does not contain lyso-alkyl glycerols. For reasons which are unclear, we find that the 1-*O*-alkyl-2-*O*-

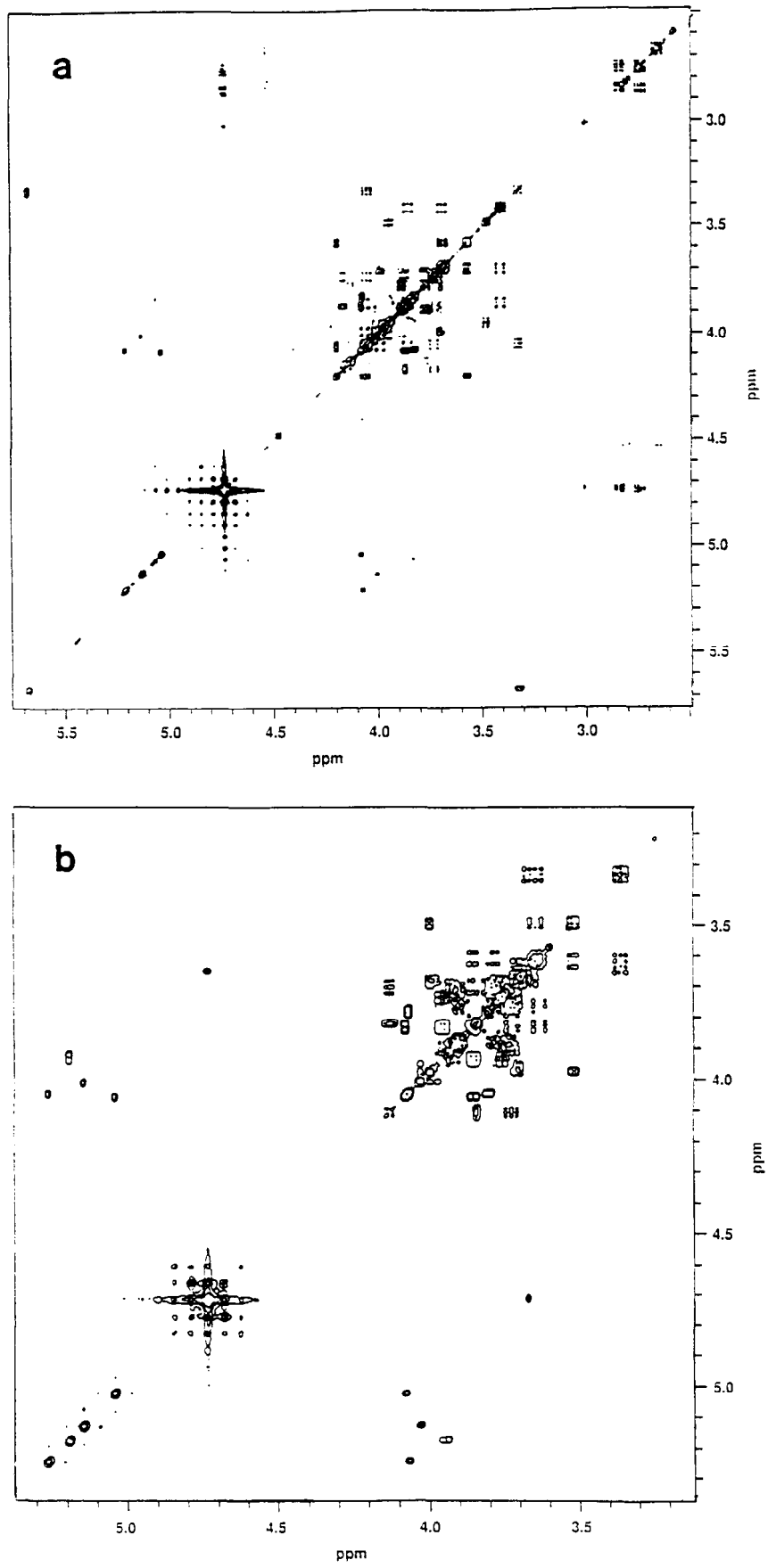


FIG. 4. **^{1}H-^{1}H NMR correlated spectroscopy.** ^{1}H-^{1}H correlated (COSY) spectra of (*a*) the soluble COOH-terminal glycopeptide and (*b*) the *N*-acetylated glycan core (compounds 2 and 3, respectively, in Fig. 1). Spectra were recorded as described previously (Ferguson *et al.*, 1988). Chemical shifts are reported relative to acetone (∂ = 2.225 ppm at 27 °C).

TABLE III

Chemical shifts and coupling constants of compound 2 and 3 in COSY experiments

	Compound 2				Compound 3		
	H1 (ppm)	H2 (ppm)	$J_{1,2}$ (Hz)		H1 (ppm)	H2 (ppm)	$J_{1,2}$ (Hz)
GlcN	5.68 (5.67)[a]	3.32 (3.35)	3.5 (3.5)	GlcNAc	5.20 (5.20)[b]	3.95 (3.95)	3.5 (3.5)
Man α1-6	5.21 (5.22)	4.07 (4.07)	2.0 (2.0)	Man α1-6	5.27 (5.27)	4.07 (4.07)	2.0 (2.0)
Man α1-2	5.13 (5.13)	4.00 (4.01)	2.0 (2.0)	Man α1-2	5.15 (5.15)	4.03 (4.03)	2.0 (2.0)
Man α1-4	5.04 (5.04)	4.08 (4.08)	2.0 (2.0)	Man α1-4	5.04 (5.04)	4.08 (4.08)	2.0 (2.0)
Inositol (H6)		3.85 (3.85)		Inositol (H6)		3.68 (3.68)	

[a] Numbers in brackets refer to resonances of standard Asp-ethanolamine-PO_4-6-Manα1-2Manα1-6Manα1-4GlcN-α1-6*myo*-inositol-1,2 cyclic-phosphate generated from sVSG MIT at 1.4 of *T. brucei*.

[b] Numbers in parentheses refer to resonances of standard Manα1-2Manα1-6Manα1-4 GlcNAcα1-6*myo*-inositol generated from sVSG MIT at 1.4 of *T. brucei*.

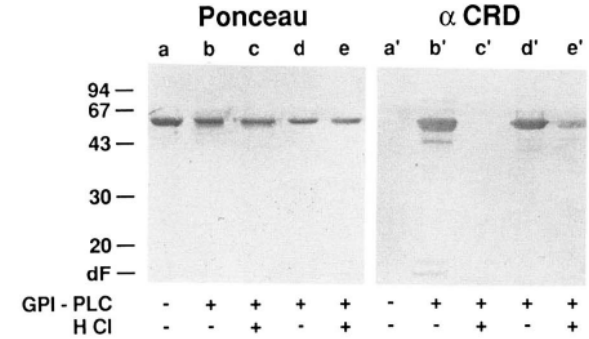


FIG. 5. **Cross-reacting determinant of PSP and VSG.** PSP (*lane a*), hydrophilic PSP generated by digestion with *T. brucei* GPI-PLC (*lane b*) and hydrophilic PSP treated with 1 M HCl (*lane c*), were analyzed by polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and stained with Ponceau red to visualize proteins. Soluble form VSG from *T. brucei* MITat1.4 was used as positive control prior to (*lane d*) or after (*lane e*) HCl treatment. The same blot was revealed with anti-CRD rabbit antiserum (*lanes a'–e'*). The CRD generated on PSP after GPI-PLC solubilization (compare *lanes a'* and *b'*) is totally acid sensitive (*lane c'*) whereas a weak but significant recognition is retained on VSG treated in the same way (compare *lanes d'* and *e'*). Migration position of molecular mass standards (in kDa) are shown on the left. *dF* = dye front.

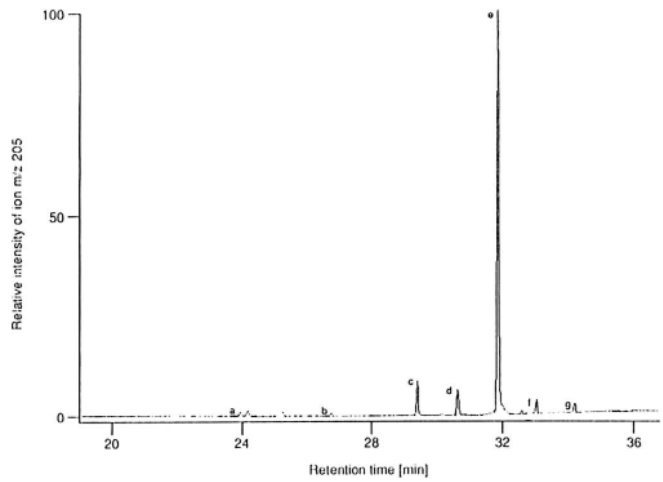


FIG. 6. **GC-MS of the 1-*O*-alkyl-glycerols of PSP.** Following methanolysis, the alkylglycerols of the GPI-peptide of PSP were analyzed by GC as their Me_3Si derivatives and identified by MS using selective ion monitoring for ions *m/z* 205, 398, 412, 426, 440, 454, 468, 473, 482, 487, 496, 501, 510, 515, 529, 543, 557, 571, and 585 which correspond to the base peak (*m/z* 205), and *m/z* $[M-15]^+$ and *m/z* $[M-90]^+$ of alkylglycerols with C18:0 (*a*), C19:0, C20:0 (*b*), C21:0, C22:0 (*c*), C23:0 (*d*), C24:0 (*e*), C25:0 (*f*), and C26:0 (*g*) alkyl chains. This analysis followed a similar one using linear scanning from *m/z* 40 to *m/z* 800 which showed the presence of only saturated 1-*O*-alkyl glycerol derivatives.

acyl-3-*O*-TMS-glycerol derivatives (of both PSP anchor and *L. major* GIPL standards) do not survive gas chromatography on CPSil8CB and other OV17-bonded phase columns. This is in contrast to saturated and unsaturated 1,2-di-*O*-acyl-3-*O*-TMS-glycerol derivatives which were easily detected under identical conditions.

COOH-terminal Peptide Structure—An amino acid analysis indicated that the Pronase had left more than one amino acid in the GPI peptide. Two mol of each Gly and Asx were detected (Table I) together with 1 mol of ethanolamine and glucosamine.

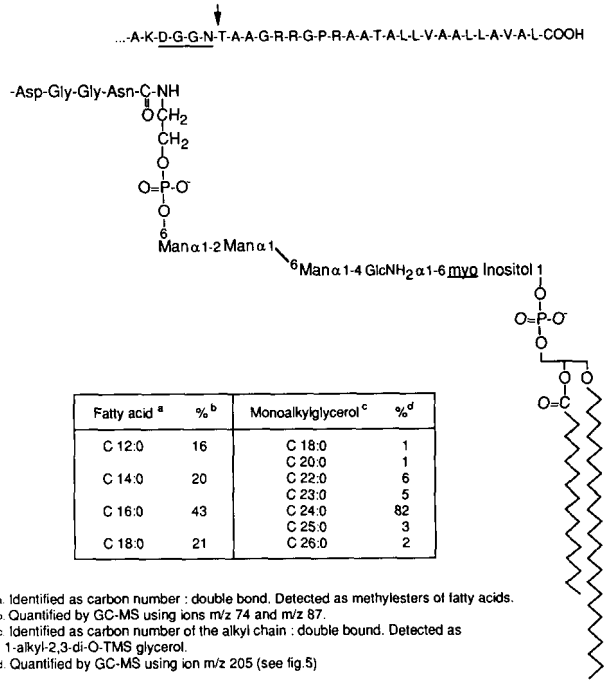
The amino acid sequence determined by automated Edman degradation was Asp-Gly-Gly-Asn (Fig. 7). This is a unique tetrapeptide in the PSP, corresponding to residues 405–408 in the amino acid sequence predicted from the gene (Button and McMaster, 1988; Miller *et al.*, 1990). The resonances of these amino acids were also observed in the NMR spectrum of compound 2 (Fig. 4*a*).

DISCUSSION

In the present work, the structure of the glycosyl-phosphatidylinositol anchor of the *L. major* promastigote surface protease was determined using NMR, compositional analyses, methylation linkage analysis, chemical modifications, exoglycosidase digestion, and amino acid sequencing. The isolated anchor (Fig. 7) consists of the COOH-terminal tetrapeptide of the mature protein (Asp-Gly-Gly-Asn) linked to a carbohydrate core by an ethanolamine phosphate bridge. No direct evidence was obtained to assign the ethanolamine position, but it seems reasonable to assume that it is in amide linkage to the carboxyl group of the COOH-terminal amino acid and in ester linkage to the phosphate of the non-reducing terminal Man 6-phosphate residue, by analogy with VSG, Thy-1 and human EAChE GPI structures (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Roberts *et al.*, 1988b). Three mannose residues and a non-acetylated glucosamine residue in a linear arrangement are linked to an inositol phospholipid which contains a 1-*O*-alkyl-2-*O*-acyl glycerol moiety. The fatty alcohol etherified on the *sn*-1 glycerol hydroxyl was found to be almost exclusively a 24-carbon-saturated alkyl chain, whereas smaller saturated fatty acids (C12:0, C14:0, C16:0, and C18:0) are esterified on the *sn*-2 hydroxyl of the glycerolipid.

We can rule out any contamination of the sample by the abundant glycolipids of *Leishmania* (LPG and/or GIPLs) as no significant amounts of terminal-Galf, 3-*O*-substituted-Galf, 3-*O*-substituted-Gal, 6-*O*-substituted-Gal, or 3-*O*-substituted-Man, which are characteristic of these molecules (Turco *et al.*, 1989; McConville *et al.*, 1990), were detected in the methylation analyses and NMR spectra.

Some galactose (0.2 residue/mol of *myo*-inositol) was de-



Fatty acid [a]	% [b]	Monoalkylglycerol [c]	% [d]
C 12:0	16	C 18:0	1
		C 20:0	1
C 14:0	20	C 22:0	6
		C 23:0	5
C 16:0	43	C 24:0	82
		C 25:0	3
C 18:0	21	C 26:0	2

a. Identified as carbon number : double bond. Detected as methylesters of fatty acids.
b. Quantified by GC-MS using ions m/z 74 and m/z 87.
c. Identified as carbon number of the alkyl chain : double bound. Detected as 1-alkyl-2,3-di-O-TMS glycerol.
d. Quantified by GC-MS using ion m/z 205 (see fig.5)

FIG. 7. **PSP GPI anchor structure.** The structure of the GPI anchor of PSP, as determined from the experiments described in this paper. The COOH-terminal amino acid sequence predicted from the gene of the PSP is shown at the top of the figure. The tetrapeptide found attached to the anchor is *underlined*. The *arrow* indicates the cleavage site for the removal of the GPI addition signaling peptide. The lipid is shown with the most abundant alkyl and acyl chains. The composition of the lipid components is given in the table.

tected in the sample by compositional analysis (Table I). From methylation analysis data (Table II), it was characterized as a terminal galactopyranose. A similar amount of 4-*O*-substituted Man was also present in the methylation analysis, suggesting that the Gal residue could be linked 1–4 to the Man residue at the non-reducing end of the glycan core. However, this galactose residue is not thought to be linked to the GPI anchor for two reasons. First, no heterogeneity could be seen in the radiolabeled compound 5 when run on Bio-Gel P4 or Dionex (Fig. 3). Second, this lack of heterogeneity in the labeled material was also observed in the reverse-phase HPLC analysis of the permethylated neutral glycan core (compound 5) (data not shown) and no terminal-Gal or 4-*O*-substituted-Man could be seen in the methylation analysis of this HPLC purified labeled product (Table II). Independent immunological evidence for the lack of *T. brucei* VSG type α-galactose side chains was obtained on whole PSP. It was shown (Zamze *et al.*, 1988) that a GPI-anchor lacking an α-galactose side chain is recognized by anti-CRD antibodies after PI-PLC digestion, but that mild acid treatment results in the complete loss of this epitope (due to inositol-1,2-cyclic-phosphate decyclization). If, however, a galactose chain is present, a significant recognition remains after mild acid treatment. In our case, the CRD present in GPI-PLC cleaved PSP is completely destroyed by mild acid (Fig. 5). This result is in agreement with the lack of any α-galactose side chain, analogous to that found on VSG, on the isolated PSP anchor.

The lack of heterogeneity of the carbohydrate core suggests that the transferases adding the α-galactose side chain in *T. brucei* VSG anchor may be absent in *Leishmania*. This would be in agreement with the hypothesis that these galactose side chains are specific to African trypanosomes and play a role in the architecture or function of the VSG coat (Homans *et al.*, 1989; Ferguson and Homans, 1988, 1989). In common with the *T. brucei* VSG GPIs, the PSP anchor contains only 1 ethanolamine/mol and lacks the extra ethanolamine phosphate side-chains found in all higher eukaryote anchors so far analyzed (reviewed by Ferguson and Williams, 1988; Low, 1989). The PSP anchor therefore seems to represent the minimum functional structure for a GPI anchor.

The conservation of the ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-1-PO_4 core region in *Leishmania* PSP is particularly relevant since the *Leishmania* are one of the very few groups of eukaryotes in which the dolichol-cycle of protein *N*-glycosylation diverge from the norm (Bosch *et al.*, 1988). Thus, the *Leishmania* might have been expected to differ also in the GPI glycosylation pathway.

The structural differences observed in *Leishmania* between GPI of protein (PSP) and glycolipids (LPG and GIPLs) (Turco *et al.*, 1989; McConville *et al.*, 1990) implies that distinct pathways are used for the biosynthesis of the two kinds of GPIs. Moreover, it excludes the hypothesis made by Rosen *et al.* (1989) that GIPLs might be precursors of protein GPI. However, common precursors might be used in the early biosynthetic steps as both GIPLs and PSP GPI contain a common carbohydrate portion, *i.e.* Manα1-4GlcNα1-6*myo*-inositol-1-PO_4 (McConville *et al.*, 1990).

In all eukaryote proteins so far investigated, the carbohydrate core of the GPI anchor appears to be conserved. In *Leishmania*, uniquely, a second type of GPI is found in the anchor of the GIPL glycolipids and the LPG, a molecule that plays a key role in *Leishmania* infectivity and survival (Turco, 1990), suggesting that these molecules may represent relatively recent evolutionary adaptations to a parasitic life style. This kind of anchor may have evolved from the conventional GPI anchor of proteins.

A striking feature of the PSP anchor phospholipid is the presence of an alkyl-acyl-glycerol. Although qualitatively distinct from the alkyl-acyl-glycerols of human EAChE (which has predominantly C18:0 alkyl and C22:4 acyl) (Roberts *et al.*, 1989a), the PSP lipid structure is more similar to this mammalian example than to that of the related kinetoplastid parasite *T. brucei* which uses exclusively myristic acid residues in a diacyl-glycerol (Ferguson and Cross, 1984; Ferguson *et al.*, 1985a, 1985b). In the *T. brucei* example, the phosphatidylinositol (PI) that is sequentially glycosylated to form a prefabricated GPI precursor (Masterson *et al.*, 1989) undergoes fatty acid-remodeling to dimyristoyl-GPI as a set of final reactions prior to the addition of the GPI to newly synthesized protein (reviewed in Doering *et al.*, 1990). This strict and specific lipid remodeling to dimyristoyl-GPI in *T. brucei* probably represents an unusual specialization unique to African trypanosomes. However, there is some evidence to suggest that some kind of lipid remodeling also occurs in the GIPL/LPG series in *L. major* (McConville *et al.*, 1990). Whether or not lipid remodeling occurs during the synthesis of the PSP anchor is unknown, but its alkyl content is almost identical to that of *L. major* LPG (McConville *et al.*, 1987). The total cellular PI pool of *Leishmania* includes inositol phosphosphingolipids (Kaneshiro *et al.*, 1986), dialkyl-PI (Singh *et al.*, 1988), diacyl-PI, and 1-*O*-alkyl-1′-enyl-2-acyl-PI (Wassef *et al.*, 1985) and *Leishmania* is able to incorporate 1-*O*-alkyl glycerol into alkyl-acyl-PI (Achterberg and Gercken, 1987). However, the amount and species of endogenous alkyl-acyl-PIs that could be used in GPI synthesis remains unclear.

The COOH terminus of the mature PSP was sequenced from compound 2 and found to be Asp-Gly-Gly-Asn. This sequence is unique in the amino acid sequence predicted from the gene (Button and McMaster, 1988; Miller *et al.*, 1990). The cleavage site occurs in a region devoid of large or strongly

hydrophobic amino acids, 25 residues before the predicted end of the protein and 9 residues before a run of hydrophobic amino acids that extend up to the end of the protein. These features are in agreement with the consensus structure observed by Ferguson and Williams (1988), Caras *et al.* (1989) and Berger *et al.* (1988) in the COOH-terminal sequences of other GPI-anchored proteins.

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