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Purification and Characterization of an Extracellular Phosphoglycan from *Leishmania donovani**

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An extracellular phosphoglycan (exPG), present in the culture medium of the promastigote form of *Leishmania donovani*, was purified and structurally characterized. The purification scheme included ethanol precipitation of the culture medium, anion exchange chromatography, hydrophobic chromatography on phenyl-Sepharose, and preparative polyacrylamide gel electrophoresis. Structural analysis by ^{1}H-^{1}H NMR, methylation linkage analysis, and glycosidase digestion revealed that the exPG consisted of the following structure: (CAP)→[PO$_4$-6Galpβ1–4Manpα1]$_{10-11}$-PO$_4$-6Galpβ1–4Man. The cap was found to be one of several small, neutral oligosaccharides, the most abundant of which was the trisaccharide Galpβ1–4(Manpα1–2)Man. The results indicated structural analogy to the cellular-derived lipophosphoglycan (LPG) from *L. donovani*. The important exceptions are a lack of the lipid anchor, the entire phosphosaccharide core, and several of the repeating disaccharide units. Although the function of exPG is presently unknown, it may play a protective role for the promastigote in the insect vector or during infection of a mammalian host.

The protozoan parasite *Leishmania donovani* is the causative agent of the human disease kala azar, or visceral leishmaniasis. These parasites have the remarkable ability to survive and proliferate in several very hydrolytic environments during their digenetic life cycle. As extracellular promastigotes, they multiply in the alimentary tract of the phlebotomine sandfly vector. During infection of host macrophages, they avoid the destructive effects of the oxidative burst, differentiate, and proliferate as amastigotes in the phagolysosomes. The ability to live in these hydrolytic environments may be due, at least in part, to the glycocalyx of lipophosphoglycan (LPG)[1] that covers the cell surface of the promastigote form (reviewed by Turco (1990)) and has recently been reported to be synthesized by *Leishmania major* amastigotes (Turco and Sacks, 1991; Glaser *et al.*, 1991), although it is not detected in *L. donovani* amastigotes (McConville and Blackwell, 1991). LPG has been implicated in the binding of the parasite to epithelial cells of the sandfly midgut (Davies *et al.*, 1990), in receptor-mediated phagocytosis of promastigotes (reviewed by Sacks (1989) and Talamas-Rohana *et al.* (1990)), in attenuation of the oxidative burst via inhibition of protein kinase C (McNeely and Turco, 1987; McNeely *et al.*, 1989), as well as in neutralization of toxic oxygen metabolites produced during the oxidative burst (Chan *et al.*, 1989).

Structurally, the LPG of *L. donovani* is composed of repeating phosphorylated disaccharide units of PO$_4$-6Galβ1–4Manα1 attached via a phosphosaccharide core to the phospholipid 1-*O*-alkyl-2-lyso-phosphatidylinositol (Orlandi and Turco, 1987, Turco *et al.*, 1987, 1989). Recently it has been demonstrated that the LPG of *L. donovani* contains nonreducing terminal oligosaccharide cap structures containing mannose and galactose, as well as a Glcpα1-PO$_4$ substitution of the core mannose distal to the phospholipid.[2]

In this study, we report the purification and structural characterization of an extracellular phosphoglycan (exPG) from *L. donovani*. We demonstrate that this exPG, like LPG, consists primarily of repeating phosphorylated disaccharides (PO$_4$-6Galβ1–4Manα1-) and nonreducing terminal oligosaccharide caps. Unlike LPG, we demonstrate that exPG lacks the lipid anchor, the phosphosaccharide core, and several of the repeating disaccharide units. The origin and function of exPG are presently unknown, but the structure presented here may provide insight into its biological significance.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION[3]

LPG-like substances, collectively termed excreted factor, have been previously reported to be present in conditioned

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[1] The abbreviations used are: LPG, lipophosphoglycan; exPG, extracellular phosphoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C; AαM, *Aspergillus phoenicus* α-mannosidase; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; PAS, periodic acid Schiff staining; TFA, trifluoroacetic acid.

[2] Thomas, J. R., McConville, M. J., Thomas-Oates, J. E., Homans, S. W., Ferguson, M. A. J., Gorin, P. A. J., Greis, K. D., and Turco, S. J. (1992) *J. Biol. Chem* **267**, in press.

[3] Portions of this paper (including "Experimental Procedures," "Results," Figs. 2–9, Tables I–III, and Footnote 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

A. exPG

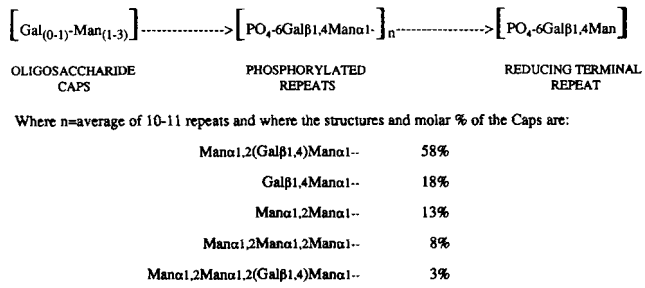
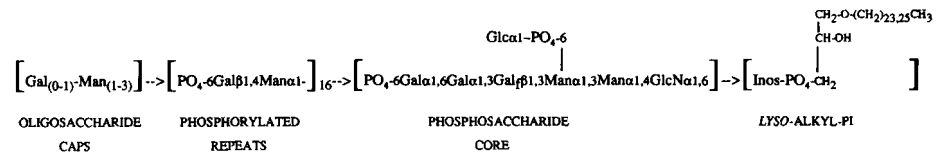


FIG. 1. **The proposed structure of the extracellular phosphoglycan from *L. donovani*.**

B. LPG



medium from *Leishmania* promastigotes (El-On *et al.*, 1979; Slutzky *et al.*, 1979; Handman and Goding, 1985; King *et al.*, 1987; Jaffe *et al.*, 1989, 1990). The components of the excreted factor can be organized into three categories. In one, LPG can form very tight complexes with albumin in the medium (King *et al.*, 1987). Analysis of this form indicates that the LPG in the medium is identical in all respects with the cell-associated LPG (King *et al.*, 1987). One probable interpretation is that the lipid portion of LPG interacts with the hydrophobic-binding pocket of albumin, facilitating its release from the surface of the promastigote. In a second category, the repeating phosphorylated saccharides, which are the prominent feature of LPG, have been shown to constitute a carbohydrate component of an acid phosphatase secreted by *L. donovani* (Bates *et al.*, 1990), *Leishmania tropica* (Jaffe *et al.*, 1990), and *Leishmania mexicana* (Ilg *et al.*, 1991). The third category of LPG-like substances found in conditioned medium is a hydrophilic form of LPG in which the lipid is absent from the glycoconjugate. It is the purification and structural characterization of this latter extracellular material that is reported here.

The proposed structure of the extracellular phosphoglycan (exPG) from *L. donovani* is shown in Fig. 1. The key features of the purified exPG that led to the proposed structure included the following. (i) It had a molecular weight somewhat smaller than that of delipidated LPG (about 8,300), as judged by TSK G2000SW gel filtration chromatography. (ii) The reducing terminal fragment of exPG was a disaccharide repeat of PO_4-6Galβ1–4Man, as determined by reduction studies with NaB^3H_4. (iii) Pretreatment of exPG with mild acid generated repeat units of PO_4-6Galβ1–4Man and neutral oligosaccharide caps in an 11:1 molar ratio, indicating the presence of about 11 repeat units/exPG molecule. (iv) Compositional analysis of exPG, reduced with NaB^2H_4, resulted in a 12:1 ratio of total mannose to total deuterated mannitol, again indicating approximately 10–11 repeat units/exPG. (v) The proton NMR spectrum of exPG was consistent with a polymer of repeating PO_4-6Galβ1–4Man units attached through mannosyl-α1-phosphate linkages. Taken together, these data show that exPG contains an average of 10–11 repeating phosphorylated disaccharides and one of several oligosaccharide caps, resulting in a calculated average molecular weight of 4,700–5,200.

The structure and relative abundance of the five neutral, oligosaccharide caps isolated from exPG are nearly identical with those found on the cell-associated LPG of *L. donovani*.[2] A sixth neutral fragment isolated by Dionex HPLC contained only free mannose (Fig. 6*a*). A time course of mild acid hydrolysis of exPG and quantitation of the released mannose by GC-MS showed a linear increase of mannose with increased hydrolysis time. It was therefore concluded that the mannose was produced as an artifact of the hydrolysis conditions due to minor hydrolysis of the PO_4-6Galβ1–4Man repeat units.

It was previously suggested that exPG originates by a phospholipase C cleavage of the membrane-bound LPG in *Leishmania* parasites (Handman and Goding, 1985; Jaffe *et al.*, 1989); however, no such endogenous enzyme activity has yet been demonstrated. The structure of exPG presented here argues against a single phospholipase cleavage due to the absence of any detectable phosphosaccharide core-like structure analogous to phosphatidylinositol-specific phospholipase C-treated LPG. Instead, this structure might suggest the possibility of a specific phosphodiesterase, although several attempts to demonstrate an *in vitro* enzymatic conversion of [^{3}H]LPG to [^{3}H]exPG have thus far been unsuccessful. It is possible that exPG may be secreted as an entity analogous to bacterial secretion of polysaccharides (Braatz and Heath, 1974). In either event, the similarity in the cap structures and repeat units strongly suggest that the biosynthetic machinery for the two molecules is likely to be the same. Metabolic labeling of variant promastigote lines that produce little or no LPG also produce no detectable amounts of exPG,[5] again suggesting a common biosynthetic pathway. Furthermore, to rule out the artifactual generation of exPG by chemical degradation of LPG during purification, a number of control experiments were performed. In these control studies, purified [^{3}H]LPG was subjected to the same purification conditions as those described for exPG. At each step, the [^{3}H]LPG was recovered with the lipid and phosphosaccharide core intact, thus precluding the artifactual generation of exPG due to degradation of LPG during purification.

The function of the exPG is unknown. Since the structure

[5] K. D. Greis, S. J. Turco, J. R. Thomas, M. J. McConville, S. W. Homans, and M. A. J. Ferguson, unpublished observations.

of exPG is nearly identical with the repeat units of LPG, it is reasonable to suggest that exPG might act in a similar functional capacity as LPG with respect to scavenging of oxygen metabolites (Chan *et al.*, 1989; El-On *et al.*, 1990; McNeely and Turco, 1990) and the chelating of cations that may be needed for host defense function (Eilam *et al.*, 1985). The amount of exPG produced during 3 days of culture (1–2 mg/liter) is approximately equal to the amount of LPG on the promastigotes' cell surface at that time.[5] ExPG is therefore likely to be present in the mouth parts of the sandfly vector in sufficient amounts to provide such protection. Furthermore, exPG may play a role in the development of metacyclic promastigotes during the insect vector stage. In one study, it appears that the promastigotes shed their surface LPG in the midgut of the vector, possibly to facilitate the anterior migration of the developing promastigotes (Davies *et al.*, 1990). However, it is presently unclear whether this released LPG-like material is in the form of exPG or LPG.

Although the possible role of exPG in the survival of leishmanial parasites remains to be resolved, the purification and structure presented here allows for experiments to be performed with a purified molecule. Therefore, the effector molecule(s) of the previously undefined excreted factor of leishmanial parasites may now be investigated.

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SUPPLEMENTAL MATERIAL
to
PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR PHOSPHOGLYCAN FROM *LEISHMANIA DONOVANI*
by
Kenneth D. Greis, Salvatore J. Turco, Jerry R. Thomas, Malcolm J. McConville, Steven W. Homans, and Michael A.J. Ferguson

EXPERIMENTAL PROCEDURES

Materials--*All materials were obtained as follows: Bacto-Brain Heart Infusion from Difco;* [2-^{3}H]mannose (20 Ci/mmol), [1-^{3}H]galactose (15 Ci/mmol), and NaB^3H_4 (120 mCi/mmol) from American Radiolabeled Chemicals; $NaBD_4$ from Fluka; *E. coli* alkaline phosphatase, Jack bean β-galactosidase and phenyl-Sepharose from Sigma; *Aspergillus phoenicus* α-mannosidase from Oxford Glycosystems; Jack bean α-mannosidase and bovine testicular β-galactosidase from Boehringer Mannheim; Sephadex G25, Sephadex G150, and TSK G2000SW HPLC column from Pharmacia/LKB; DE52-cellulose from Whatman; 3500 molecular weight cutoff dialysis membranes from Spectra/Por; and delipidated ^{3}H-mannose labeled LPG was prepared by PI-PLC treatment as described previously (Turco *et al.*, 1989). All other reagents were of the highest purity commercially available.

Parasites and Growth Conditions--*Leishmania donovani* (1S2D clone) were passaged in culture as previously described (King and Turco, 1988). The cells were grown at 25 °C in Dulbecco's Modified Eagle's Medium supplemented with 0.30 mg/ml bovine serum albumin, adenosine (0.05 mM), xanthine (0.05 mM), biotin (1 mg/L), Tween 80 (40 mg/L), hemin (5 mg/L), and triethanolamine (0.5 ml/L). For large-scale isolation of chemical quantities of the extracellular phosphoglycan (exPG), one liter cultures of Brain-Heart Infusion supplemented with triethanolamine (1 ml/L), hemin (5 mg/L) and adenosine (0.1 mM) were seeded with 50 ml of the starter culture. The cells were grown for 3 day at 25 °C with gentle shaking and harvested at a density of 3-5 x 10^7 cell/ml.

Carbohydrate Analyses--Phenol/sulfuric acid carbohydrate analysis as described by Dubois *et al.*(1956) was used to quantitate the amount of total galactose and mannose present during purification of exPG. Absorbance at 490 nm was monitored in comparison to a standard curve generated by using known quantities of a 1:1 mixture of galactose and mannose. The carbohydrate containing macromolecules separated by polyacrylamide gel electrophoresis were visualized by periodic acid/Schiff (PAS) staining as previously described (Zacharius *et al.*, 1969).

Anion Exchange Chromatography--ExPG was separated from neutral and other acidic carbohydrate components by successive passages over a 15 ml column of BioRad AG1-X2 (acetate form) anion exchange resin equilibrated in 10 mM NH_4OH. After applying the sample (suspended in 10 mM NH_4OH) to the column, neutral and acidic components were eluted batchwise with successive 50 ml washes with 0, 0.2, 0.4, 1.0, 2.0 M NaCl in 10 mM NH_4OH. A second passage on the column consisted of batch washes at 0 and 250 mM NaCl, followed by a gradient of NaCl (250 mM-1.0 M) in 10 mM NH_4OH.

Polyacrylamide Gel Electrophoresis (PAGE)--SDS-PAGE (Laemmli, 1970) was used to determine the purity of the exPG with respect to carbohydrate and protein using a Hoeffer Mighty Small minigel apparatus. The acrylamide concentration of the separating gel and the stacker were either 12.5% or 15% and 3.5%, respectively. Electrophoresis was carried out at 20 mA for 45-50 minutes. Preparative PAGE was performed according to the method of Laemmli (1970), but in the absence of sodium dodecyl sulfate (SDS). The separating gel was 12.5% acrylamide and the stacker was 3.5% acrylamide. Electrophoresis was carried out at 40 mA for 6.5-7 h.

Preparation of ^{3}H-labeled Extracellular Phosphoglycan (exPG)-- Promastigotes (10^6) harvested in exponential stage of growth (2 x 10^6-2 x 10^7 cell/ml) were centrifuged at 3000 x g for 7 min, washed once with 10 ml phosphate buffered saline (PBS; Hubbard and Robbins, 1979) and centrifuged again. The cells were resuspended in 8 ml of Bacto-Brain Heart Infusion supplemented with triethanolamine (1 ml/L), hemin (5 mg/L) and adenosine (0.1 mM) containing 500 μCi [2-^{3}H]mannose or [1-^{3}H]galactose. The cells were incubated with the isotope for 4 to 16 h at 25 °C. Subsequently, the cells were removed by centrifugation and an equal volume of 95% ethanol was added to the conditioned medium at 4 °C. The medium was dried under reduced pressure, resuspended in 1 ml of 40 mM NH_4OH and 1 mM EDTA and fractionated over a column of Sephadex G150 (2.5 x 55 cm) equilibrated in the same buffer. A broad peak of radioactivity that eluted shortly after the void volume was pooled, concentrated under reduced pressure and dialyzed (Mr cutoff=3.5 kDa) against 0.1 M acetic acid and 0.1 M NaCl. The dialysate was then fractionated by hydrophobic chromatography on phenyl-Sepharose as described below. The non-binding, hydrophilic radioactivity was termed ^{3}H-extracellular phosphoglycan (exPG). The ^{3}H-exPG isolated in this way was judged to be radioactively pure since all of the glycoconjugate was hydrolyzed into small fragments by mild acid (0.02 N HCl, 100 °C, 5 min) as analyzed by rechromatography on Sephadex G150 (data not shown). Furthermore, the major fragment released by mild acid hydrolysis was shown to be a phosphorylated disaccharide of galactose and mannose using methods described for the characterization of ^{3}H-mannose and ^{3}H-galactose labeled LPG (Turco *et al.*, 1984).

Preparation of NaB^3H_4-Reduced exPG--Approximately 20 nmoles of exPG was reduced with (50 μCi) NaB^3H_4 [120 mCi/mmol] in 100 μl of 0.05 N NaOH (3h, 37 °C) essentially as described by Takasaki and Kobata (1974). The reaction was stopped with 200 μl of 1.0 M acetic acid, twice evaporated to dryness with 1.0 M acetic acid under a stream of nitrogen, and desalted over a column of Sephadex G25 (1 x 5 cm). The ^{3}H-reduced exPG was repurified by anion exchange over AG1-X2 (acetate form) as above.

DE52-Cellulose Chromatography--Samples were applied to a column of DE52-cellulose (0.5 x 2 cm) equilibrated in 1.0 mM Tris, pH 8.0. After the fifth fraction was collected, a gradient of NaCl (0-0.1 M) in 1.0 mM Tris, pH 8.0 was applied to the column. Aliquots of each 0.6 ml fraction were assayed for radioactivity by liquid scintillation counting.

Phenyl-Sepharose Chromatography--^{3}H-labeled samples were suspended in 0.6 ml 0.1 M NaCl and

0.1 M acetic acid and applied to a column (1.0 ml) of phenyl-Sepharose equilibrated in the same buffer. The column was then sequentially washed with 3.0 ml of 0.1 M NaCl and 0.1 M acetic acid, 1.2 ml 0.1 M acetic acid, 0.6 ml H_2O, and 3.6 ml solvent E (H_2O/ethanol/diethyl ether/pyridine/NH_4OH; 15:15:5:1:0.017). For chemical purifications, the volume of the solvents and the column were scaled-up by a factor of 3.5.

$NaBD_4$ Reduction and Acetylation of exPG--About 2.0 nmoles of exPG containing 2.0 nmoles of *scyllo*-inositol as an internal standard were reduced with 200 μl 0.25 M $NaBD_4$ (3h, 25 °C). The reaction was terminated and the excess $NaBD_4$ was destroyed with 20 μl aliquots of 1.0 M acetic acid until effervescence was no longer visible. After desalting over AG50-X12 (H^+) and evaporation under vacuum with methanol, the sample was hydrolyzed with 2 N TFA (2h, 100 °C) in an evacuated capillary tube. Standard mixes of mannose/mannitol [(1:1),(5:1),(10:1),(15:1)] each containing 2.0 nmoles of *scyllo*-inositol were also hydrolyzed to determine the relative response factors of mannose and mannitol to the internal standard. After hydrolysis, the samples were dried once with H_2O and once with methanol to remove any residual TFA. The resulting hexoses and deuterated alditols were acetylated with 50 μl pyridine/acetic anhydride (1:2) (16 h, 25 °C or 1 h, 100 °C) in a sealed glass tube. The acetylated samples were dried under reduced pressure and suspended in fresh dichloromethane. The resulting acetylated hexoses and deuterated alditols acetates were quantitated by GC-MS and the mannose:deuterated mannitol ratio was calculated.

Monosaccharide Analysis--Samples containing *scyllo*-inositol as a internal standard were subjected to methanolysis and trimethylsilyl (TMS) derivatization prior to GC-MS analysis as described (McConville *et al.*, 1990). Analysis of phosphorylated sugars required methylation of the phosphate with ether saturated diazomethane at 0 °C (Ferguson *et al.*, 1988) followed by a second TMS-derivatization step prior to GC-MS analysis.

Methylation Analysis--Partially methylated alditol acetates (PMAA) were generated from neutral oligosaccharides using the method of Ciucanu and Kerek (1984) as described by Ferguson *et al.* (1988). The PMAAs were then analyzed by GC-MS.

Enzyme Digestion--Phosphorylated oligosaccharides were dephosphorylated with *E. coli* alkaline phosphatase (0.3-0.5 units) in 1 mM Tris/HCl, pH 8.0 (16 h, 37 °C). Neutral oligosaccharides were digested with either *Aspergillus phoenicus* α-mannosidase or jack bean α-mannosidase in 0.1 mM sodium acetate, pH 5.0 (16 h, 37 °C), or bovine testicular β-galactosidase or Jack Bean β-galactosidase in 50 mM phosphate-citrate buffer, pH 5.0 (16 or 40 h, 37 °C). The enzyme digests were deionized by passage through a column of AG50-X12 (H^+) over AG3-X4 (OH^-) followed by filtration through a 0.2 μM filter prior to HPLC injection.

High Performance Liquid Chromatography--HPLC was done on either a Dionex Model BioLC Carbohydrate Analyser equipped with a Pulsed Amperometric Detector (PAD) and a CarboPac PA1 anion exchange column (4 x 250 mm) or a Varian Model 5000 liquid chromatograph equipped with a TSK G2000SW gel filtration column (7.5 x 300 mm). Neutral and phosphorylated saccharides were separated by Dionex HPLC using programs A or B with a flow rate of 1.0 and 0.6 ml/min, respectively. Program A was an isocratic elution over 10 min with 20 mM NaOH. Program B started with an isocratic elution with 97% Buffer 1 (150 mM NaOH) and 3% Buffer 2 (150 mM NaOH, 500 mM sodium acetate) over 25 min. A linear gradient from 3% Buffer 2 to 100% Buffer 2 was applied over the next 15 min (25 to 40 min). Program B ended with an isocratic elution at 100% Buffer 2 over 20 min (40 to 60 min) followed by equilibration back to initial conditions over the next 20 min. Chemical quantities of intact exPG, delipidated LPG, or NaB^3H_4-reduced exPG were sized by TSK G2000SW gel filtration in 0.1 M ammonium acetate, pH 6.0 with a flow rate of 1.0 ml/min. The void and retention volumes of the column were determined with ^{3}H-Dextran and ^{14}C-glucose, respectively. The elution position of radiolabeled fractions was determined by liquid scintillation counting of an aliquot of each 0.25 min fraction, while chemical quantities of the glycoconjugates were determined by phenol/sulfuric acid carbohydrate analysis (Dubois *et al.*, 1956) of each fraction. Preparative samples isolated by HPLC were all deionized over a column of AG50-X12 (H^+) followed by evaporation under reduced pressure with toluene prior to further characterization.

Gas Liquid Chromatography-Mass Spectrometry--Combined GC-MS was performed on a Hewlett Packard 5890-5970 GC-MS system; electron ionization energy 70eV. Trimethylsilylated monosaccharides and partially methylated alditol acetates were analyzed on capillary columns (30 m x 0.25 mm) of SE-54 fused silica (Alltech Associates) and SP2380 fused silica (Supelco), respectively as described (McConville *et al.*, 1990). Acetylated hexoses and alditols were analyzed on a capillary column of SP2380 fused silica. The oven temperature was held at 80 °C for 1 min, increased to 200 °C at 30 °C/min, and then raised to 250 °C at 5 °C/min.

500-MHz ^{1}H NMR Spectroscopy--^{1}H NMR spectra were recorded at a probe temperature of 300K using a Bruker AM 500 Spectrometer, with a sweep width of 2,000 Hz and 8,000 real data points. Chemical shifts were referenced indirectly relative to acetone, δ=2.225 ppm at 300K. Samples were prepared for NMR studies by repeated dissolution in 99.96% D_2O [Aldrich] with intermediate flash evaporation. The samples were analyzed in 0.4 ml of D_2O.

RESULTS

Purification of the Extracellular Phosphoglycan--The medium from 3 day cultures of *L. donovani*, grown in 12 L of supplemented brain-heart infusion was used for large scale preparation of exPG as described in "Experimental Procedures". Purification was monitored by phenol/sulfuric acid carbohydrate analysis and by co-elution with 100,000 CPM of exogenous ^{3}H-mannose labeled exPG added to the preparation. Purification was initiated by ethanol precipitation of the exPG from the culture medium and extraction of the precipitate with dilute $MgCl_2$. The $MgCl_2$ was removed from the exPG by dialysis against 40 mM NH_4OH. The sample was applied to a column of AG1-X2 anion exchange resin and material was eluted with a gradient of NaCl. The first, batchwise, passage of the dialyzed extract over the anion exchange column eliminated > 90% of the carbohydrate positive material in the non-bound fraction, while the exPG was eluted with 1.0 M NaCl (data not shown). Rechromatography of the exPG pool over AG1-X2 ensured elimination of overlapping peaks of contaminating carbohydrate and resulted in coelution of the ^{3}H-mannose-labeled exPG and a single carbohydrate peak at 0.5 M NaCl (Fig. 2).

We had previously reported (King *et al.*, 1987) that substantial amounts of LPG from *L. donovani* are released intact into the culture medium. It was therefore necessary to remove any residual LPG from the exPG pool. This was accomplished by passage of the dialyzed exPG, obtained by chromatography on AG1-X2, over a column of phenyl-Sepharose. Under the conditions used, the hydrophilic exPG did not interact with the hydrophobic support whereas LPG or any other lipophilic material would be retained. Chromatography of exPG over phenyl-Sepharose removed about 15-20% of the carbohydrate (data not shown).

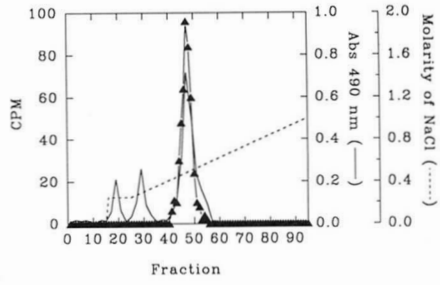

Figure 2. Anion exchange chromatography of the extracellular phosphoglycan. The 1.0 M NaCl eluant from the first, batchwise, passage of exPG over AG1-X2 was desalted, reapplied to an AG1-X2 column, and eluted with a gradient of NaCl (0.25-1.0 M) in 10 mM NH_4OH. The carbohydrate content was assayed by phenol/sulfuric acid analysis while the ^{3}H-exPG tracer was detected by liquid scintillation counting. Closed triangles, CPM; solid line, carbohydrate assay (Absorbance 490 nm); dashed line, NaCl gradient.

The purity of the exPG at this point was judged by PAS-staining of SDS-polyacrylamide minigels before and after pretreatment of the samples with mild HCl. From the characterization of ^{3}H-exPG (data not shown), it was determined that exPG, like LPG could be depolymerized with mild acid into small, phosphorylated disaccharide fragments. These small carbohydrate containing fragments could not be fixed into a polyacrylamide gel and consequently could not be visualized by PAS-staining of the gel. Therefore, the loss of PAS-stained carbohydrates after mild acid treatment was used as a criterion to establish the purity of exPG. An aliquot of the exPG preparation was subjected to electrophoresis and PAS-stained on a 12.5% SDS-polyacrylamide minigel as shown in Fig. 3A. For a comparison, an equal aliquot of phosphatidylinositol-specific phospholipase C (PI-PLC)-treated LPG was also electrophoresed. The delipidated LPG ran as a heterogeneous smear of carbohydrate near the gel front (lane 1). The exPG preparation (lane 3) contained an unknown high molecular weight (Mr) carbohydrate and a low Mr carbohydrate similar in size to the delipidated LPG. The structural similarity of the delipidated LPG and the low Mr carbohydrate from the exPG preparation was confirmed since both glycoconjugates were completely hydrolyzed by pretreatment with mild HCl (lanes 2 and 4, respectively). In addition, gel slices of an identical gel, when assayed for tritium, indicated that all of the ^{3}H-exPG eluted only in the fraction near the gel front (Figure 3B). The high Mr component from the exPG preparation was determined to be a contaminant, since it also appeared in mock purifications of Brain-Heart Infusion that had not been conditioned with promastigotes (data not shown).

Final purification of the exPG was achieved by removal of the undefined high Mr contaminant by preparative polyacrylamide gel electrophoresis in the absence of SDS. After electrophoresis, the gel was cut into a high Mr section and a low Mr section. The carbohydrate from each section of the gel was eluted by diffusion with 4 volumes of dilute NH_4OH over 16 h. Gel fragments and small contaminants that may have resulted from the preparative PAGE were removed by gel-filtration on G25 Sephadex and by dialysis. Subsequently, an aliquot of the exPG eluant was run on a 15% SDS-polyacrylamide minigel and stained for carbohydrate with periodic acid-Schiff staining (Figure 3C, lanes 6-10). Again for comparison, an aliquot of PI-PLC treated LPG was also electrophoresed (lane 10). Pretreatment of the PI-PLC treated LPG with dilute acid results in depolymerization and therefore loss of staining (lane 9). The purified low Mr carbohydrate from the exPG preparative gel (lane 8) migrated as a heterogeneous substance similar to the delipidated LPG (lane 10). Pretreatment of the purified exPG with mild acid resulted in complete hydrolysis as judged by the loss of PAS-staining (lane 7). These studies indicated that the low Mr component was an LPG-like molecule (exPG) with similar acid labile bonds.

When an identical minigel was stained for protein with silver stain (Figure 3C, lanes 1-5) as described by Wray *et al.* (1981), the lanes containing exPG or delipidated LPG exhibited no significant protein bands above background. Furthermore, the behavior of exPG on SDS-PAGE in the absence of mild acid pretreatment suggested that it had a similar, but not identical size to delipidated LPG (compare lanes 8 and 10). A representative purification scheme for exPG is summarized in Table I, indicating an overall purification of about 2,600-fold.

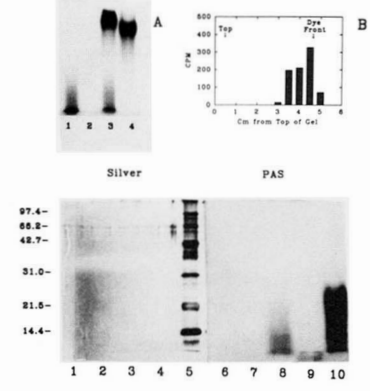

Figure 3. PAGE of exPG. ExPG and LPG samples were resolved by polyacrylamide gel electrophoresis as described in "Experimental Procedures." The carbohydrate and protein were visualized by PAS and silver staining of the gel, respectively. **Panel A:** A PAS-stained 12.5% SDS-polyacrylamide minigel of PI-PLC-treated LPG or exPG isolated by phenyl-Sepharose chromatography before and after pretreatment with mild HCl (0.02 N, 100 °C, 5 min). *lane 1*, PI-PLC-treated LPG; *lane 2*, PI-PLC-treated LPG after mild acid hydrolysis; *lane 3*, exPG isolated by phenyl-Sepharose; *lane 4*, exPG isolated by phenyl-Sepharose after mild acid hydrolysis. **Panel B:** Liquid scintillation counting of 0.5 cm gel slices from a duplicate run of Panel A, lane 3. **Panel C:** 15% SDS-PAGE of purified exPG or PI-PLC-treated LPG before and after pretreatment with mild HCl. *lanes 1 and 10*, PI-PLC-treated LPG; *lanes 2 and 9*, PI-PLC-treated LPG after mild acid hydrolysis; lanes *3 and 8*, purified exPG; lanes *4 and 7*, purified exPG after mild acid hydrolysis; *lanes 5 and 6*, protein standards. Lanes 1-5 were silver-stained; lanes 6-10 were PAS-stained. The protein standards include: phosphorylase b (97.4 kDa); BSA (66.2 kDa); ovalbumin (42.7 kDa); carbonic anhydrase (31 kDa); soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Table I

Summary of exPG Purification[a]

Procedure	Volume (ml)	CHO[b] (mg)	[³H]-exPG[c] (CPM)	Yield (%)	Specific Radioactivity (CPM/mg)	Purification (Fold)
Conditioned Medium	12,000	45,000	100,000	100	2.2	1
$MgCl_2$ Extract	100	3,800	76,300	76.3	20.1	9.1
AG1-X2 Anion Exchange I	18	15	47,800	47.8	3,190	1,450
AG1-X2 Anion Exchange II	18	11.1	38,000	38.0	3,420	1,560
Phenyl-Sepharose	8	8.8	34,300	34.3	3,900	1,770
Preparative PAGE	2.5	4.9	28,400	28.4	5,790	2,630

[a]12 liters of supplemented Brain-Heart Infusion was inoculated with promastigotes to a density of about 2 x 10^6 cells/ml and the cultures were incubated for 3 days at 25 °C with gentle shaking to a density of 3-5 x 10^7 cells/ml (see "Experimental Procedures"). The conditioned medium was separated from the cells by centrifugation and fractionated to isolate exPG.

[b]Total hexose as determined by phenol/sulfuric acid carbohydrate analysis (Dubois *et al.*, 1956).

[c]Purified [³H]-exPG was added to the conditioned medium after the removal of cells by centrifugation.

Structural Analysis of exPG.

Elucidation of the Reducing End of exPG--Initial structural studies of exPG focused on a comparison to the delipidated LPG of *L. donovani*. Compositional analysis by GC-MS (data not shown) indicated that exPG lacked both *myo*-inositol and a non-acetylated glucosamine, two known components of all glycosyl phosphatidylinositol (GPI) lipid anchors, including LPG. Determination of the reducing end of exPG was therefore an important first step in structural analysis. This was achieved by reduction of unlabeled, intact exPG with NaB^3H_4 (Takasaki and Kobata, 1974 &"Experimental Procedures"). To remove radiochemical contaminants, the ³H-reduced exPG was repurified by gel filtration on Sephadex G25 and anion exchange chromatography over BioRad AG1-X2 (data not shown). The repurified, reduced exPG was also examined by TSK G2000 HPLC and compared to the elution profiles of chemical quantities of non-reduced exPG and to delipidated LPG. The results indicated that NaB^3H_4 reduction, as expected, did not degrade exPG and that the size of exPG was smaller than PI-PLC-treated LPG (Fig. 4). To characterize the reducing end fragment, the reduced exPG was hydrolyzed with mild acid and the fragments were analyzed as by DE52-cellulose anion exchange chromatography (Fig. 5). A single ³H-labeled fragment (>98%) eluted from DE52-cellulose with a gradient of NaCl as a monophosphorylated molecule. Enzymatic digestion of this fragment with alkaline phosphatase released a neutral disaccharitol which comigrated on Dionex HPLC with a standard alditol of Galβ1-4Mannitol (Fig. 6). Furthermore, strong acid hydrolysis with 2 N TFA, 110 °C for 2 h or enzymatic digestion with Jack Bean β-galactosidase liberated free ³H-mannitol as judged by paper electrophoresis (not shown) and Dionex HPLC (Fig. 6). Taken together, these data indicated that the reducing end of exPG is PO_4-Gal(β)Man.

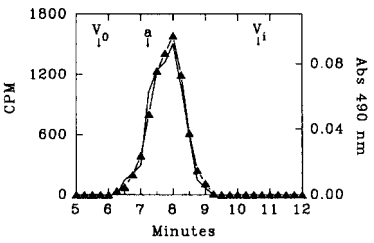

Figure 4. TSK G2000SW HPLC gel filtration chromatography of ³H-reduced exPG. HPLC conditions were as outlined in "Experimental Procedures." The ³H-reduced exPG was detected by liquid scintillation counting while the carbohydrate content of subsequent runs was assayed by phenol/sulfuric acid analysis. Closed triangles, CPM; solid line, carbohydrate profile (Absorbance 490 nm) of non-reduce exPG; a, peak elution position of chemical amounts of delipidated LPG.

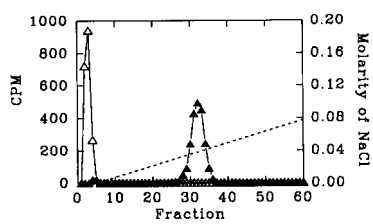

Figure 5. DE52 cellulose chromatography of the mild acid hydrolysis products of ³H-reduced exPG. The ³H-reduced exPG was hydrolyzed with 0.02 N HCl for 5 min at 100 °C, dried under reduced pressure, resuspended in 1 mM Tris-HCl, pH 8, and applied to a column of DE52 cellulose as described in "Experimental Procedures." Closed triangles, mild acid hydrolyzed ³H-exPG; open triangles, mild acid hydrolyzed and alkaline phosphatase treated ³H-exPG; dashed line, NaCl gradient. Standard monophosphorylated saccharides eluted in fractions 29-35.

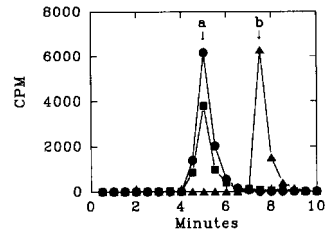

Figure 6. Dionex HPLC analysis of the dephosphorylated fragment produced by mild acid hydrolysis of ³H-reduced exPG. The single ³H-labeled, monophosphorylated fragment isolated by DE52-cellulose chromatography (Fig. 5) was dephosphorylated with alkaline phosphatase and analyzed by Dionex HPLC (program A) in the presence or absence of pretreatment with strong acid or Jack bean β-galactosidase as described in "Experimental Procedures." Closed triangles, the dephosphorylated fragment; closed squares, the dephosphorylated fragment after pretreatment with Jack Bean β-galactosidase; closed circles, the dephosphorylated fragment after pretreatment with 2 N trifluoroacetic acid for 2 h at 110 °C. Standards: a, mannitol; b, Galβ1-4Mannitol.

Separation of the Oligosaccharide Fragments Released from exPG by Mild Acid Hydrolysis--ExPG was depolymerized with 40 mM TFA, 100 °C, 8 min and the fragments were fractionated by Dionex HPLC. The neutral fractions were resolved by isocratic elution with 150 mM NaOH and 15 mM sodium acetate for 25 min (Fig. 7A). The sodium acetate concentration was then increased to 500 mM over the next 15 min followed by an additional 20 min of isocratic elution at 500 mM sodium acetate (Fig. 7B). These latter conditions are capable of eluting of mono- and diphosphorylated saccharides (McConville *et al.*, 1990). Under these conditions, one major monophosphorylated fragment and four neutral oligosaccharides were isolated. The neutral fragments were believed to be non-reducing terminal cap structures as seen for the LPG of *L. donovani*[2], *L. mexicana*[4], and *L. major* (McConville *et al.*, 1990) while the major phosphorylated fragment was found to be PO_4-6Galβ1-4Man (see below).

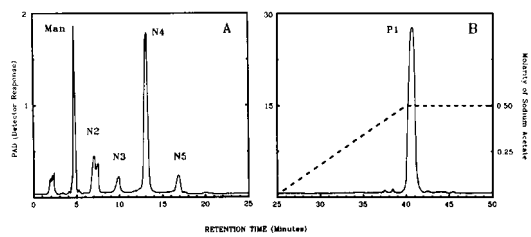

Figure 7. Dionex HPLC of the neutral and phosphorylated oligosaccharides generated from exPG by mild acid hydrolysis. ExPG was hydrolyzed with 40 mM trifluoroacetic acid for 8 min at 100 °C and fractionated by Dionex HPLC using program B. Each PAD response peak was collected and deionized as described in "Experimental Procedures" prior to further characterization. **Panel A**, the PAD response of the neutral oligosaccharides. Peaks N2, N3, N4, and N5 were 29%, 6%, 59% and 6% respectively of the total PAD response of the 4 peaks. The peak labeled "Man" was determined to be an artifact of hydrolysis (see "Discussion"). **Panel B**, the PAD response of the phosphorylated fragments. Only peak P1 contained any significant carbohydrate as judged by GC-MS compositional analysis (see "Results").

Characterization of the Neutral Cap Structures--The Dionex HPLC elution position and relative proportions of the exPG caps were nearly identical to those reported for *L. donovani* LPG[2]; therefore, it was likely that the exPG cap composition was the same as for LPG. To confirm this possibility, the structure of each exPG cap was examined. Compositional analysis of the major cap, N4 (Table II) as well as methylation linkage analysis in the presence or absence of pretreatment with Manα1-2Man specific *Aspergillus* α-mannosidase (Table III) indicated that N4 was the branched trisaccharide Gal$_p\beta$1-4(Man$_p\alpha$1-2)Man. The anomeric linkages were confirmed by exoglycosidase digestion followed by Dionex HPLC (Fig. 8A-C). Digestion of N4 with *Aspergillus* α-mannosidase (Fig. 8B) liberated mannose and a disaccharide of Gal1-4Man (7.5 min). The galactosyl residue in the disaccharide was confirmed to be in the β-configuration by digestion with bovine testicular β-galactosidase (Fig. 8C) which converted this disaccharide into the monosaccharides, galactose and mannose. These data confirmed that the N4 cap is the trisaccharide Gal$_p\beta$1-4(Man$_p\alpha$1-2)Man.

Table II

Monosaccharide compositional analysis of neutral and monophosphorylated oligosaccharides released from exPG by mild acid hydrolysis and separated by Dionex-HPLC

Dionex Fragment	Man	Gal	Gal-6-PO_4	nMoles[b] Hexose	nMoles[c] Oligosaccharide	Molar %[d]
	Molar Ratios[a]					
N2	1	0.4	0	29.8	14.9	31.8
N3	1	0	0	10.5	3.5	7.5
N4	1	0.6	0	80.8	26.9	57.5
N5	1	0.3	0	5.3	1.5	3.2
P1	1	0	1.1	1031*	515	--

[a]Oligosaccharides were prepared, analyzed and quantitated as described in "Experimental Procedures." The molar ratio were all normalized to mannose.

[b]Values were calculated by integration of total-ion chromatograms obtained by GC-MS using equal volumes of each Dionex-HPLC isolated oligosaccharide. *Includes both mannose and galactose-6-phosphate.

[c]Calculated from nMoles hexose divided by number of hexoses determined for each oligosaccharide.

[d]Molar % of each of the neutral oligosaccharides (Caps) compared to total moles of Cap fragments.

[4] Ilg, T., Etges, R., Overath, P., McConville, M. J., Thomas-Oates, J., Thomas, J., Homans, S. W., and Ferguson, M. A. J. (1992) *J. Biol. Chem.* **267**, in press.

N2 remained unresolved after HPLC, but the elution position of the two overlapping fragments as compared to standard disaccharides suggested that Galβ1-4Man was the earlier eluting fragment and Manα1-2Man as the latter. This was confirmed by methylation linkage analysis (Table III) and exoglycosidase digestion with Jack bean α-mannosidase (JBAM) followed by bovine testicular β-galactosidase (Fig. 8D-F). Treatment of N2 with JBAM (Fig. 8E) converted the latter fragment into hexoses, while β-galactosidase was required to hydrolyze the earlier fragment that remained unhydrolyzed by JBAM (Fig. 8F). These data established that N2 contains a mixture of the disaccharides $Gal_p\beta1$-4Man and $Man_p\alpha1$-2Man.

Table III

Methylation analysis of the oligosaccharide fractions released from exPG by mild-acid hydrolysis and separated by Dionex-HPLC

PMAA[a]	Origin	N2	N4 (Native)	N4 (AcαM)[b]	P1[d]
		Relative Moles[c]			
Galactitol					
2,3,4,6-Tetra-O-methyl	Terminal Gal_p	0.9	1	1	0.9
Mannitol					
2,3,4,6-Tetra-O-methyl	Terminal Man_p	1	1	--	--
3,4,6-Tri-O-methyl	2-Linked Man_p	0.3	--	--	--
2,3,6-Tri-O-methyl	4-Linked Man_p	0.7	--	0.8	1
3,6-Di-O-methyl	2,4-Linked Man_p	--	0.7	--	--

[a]Partially methylated alditol acetates (PMAAs) were prepared and analyzed as described in "Experimental Procedures."

[b]Disaccharide isolated by Dionex HPLC after pretreatment of N4 with *Aspergillus* α-mannosidase.

[c]Determined from integration of total-ion chromatograms obtained by GC-MS.

[d]P1 was dephosphorylated with alkaline phosphatase and reisolated prior to methylation analysis.

The elution position of N3 and N5 and their relative abundance compared to the caps of *L. donovani* LPG[2] suggested that N3 was the trisaccharide $Man_p\alpha1$-2$Man_p\alpha1$-2Man and N5 was the tetrasaccharide $Man_p\alpha1$-2$Man_p\alpha1$-2($Gal_p\beta1$-4)Man. The structure of N3 was confirmed as above by compositional analysis (Table II) and by digestion with *Aspergillus* α-mannosidase (data not shown). Compositional analysis of N5 (Table II) yielded a 3 to 1 molar ratio of mannose to galactose which is consistant with the above tetrasaccharide, but the limited amount of N5 precluded further analysis.

Characterization of the Monophosphorylated Fragment--Compositional analysis of the major fragment released by mild acid hydrolysis and isolated by Dionex HPLC (P1) showed that it contained mannose and galactose-6-phosphate in equal molar amounts (Table II). After dephosphorylation with alkaline phosphatase and isolation of the neutral disaccharide by Dionex HPLC (data not shown), methylation linkage analysis indicated that the dephosphorylated P1 was the disaccharide Galβ1-4Man (Table III). The β anomeric configuration of this galactosylmannose disaccharide was determined by hydrolysis of dephosphorylated P1 with bovine testicular β-galactosidase. The Galβ1-4Man disaccharide was completely converted into galactose and mannose as judged by Dionex HPLC (data not shown). These data suggested that the major carbohydrate component of exPG consist of PO_4-6$Gal_p\beta1$-4Man units polymerized via mild acid labile phosphodiester linkages.

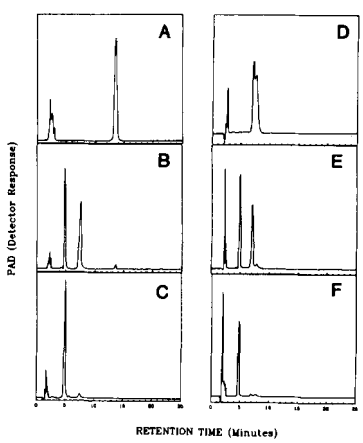

Figure 8. Dionex HPLC analysis of N4 and N2 after enzymatic hydrolysis with α-mannosidase and β-galactosidase. N4 and N2 were isolated by Dionex HPLC from mild acid treated exPG (Fig. 7A) and subjected to enzymatic digestion with specific exoglycosidases. The resulting fragments were separated by Dionex HPLC using program B. **Panel A**, N4 prior to enzymatic hydrolysis; **Panel B**, N4 after hydrolysis with *Aspergillus phoenicus* α-mannosidase; **Panel C**, the resulting disaccharide (panel B, 7.5 min) pretreated with bovine testicular β-galactosidase; **Panel D**, N2 prior to enzymatic hydrolysis; **Panel E**, N2 after hydrolysis with jack bean α-mannosidase; **Panel F**, the resulting disaccharide (panel E, 7.4 min) pretreated with bovine testicular β-galactosidase. The retention times of oligosaccharide standards were as follows: $Gal_p\beta1$-4($Man_p\alpha1$-2)Man, 13.5 min; $Man_p\alpha1$-2Man, 7.75 min; $Gal_p\beta1$-4Man, 7.4 min; Gal or Man, 4.9 min. The void volume of the column was about 1.3 ml corresponding to a retention time of about 2.2 min.

500-MHz NMR Spectroscopy of exPG--The anomeric configuration of the repeat units of exPG were determined by one dimensional ^1H NMR. As shown in Fig. 9, the ^1H NMR spectrum of exPG exhibits an intense doublet of doublets at 5.44 ppm, together with a intense doublet at 4.47 ppm. These are consistent with the presence of PO_4-6Galβ1-4Man units, and the anomeric configuration of the mannose can be defined as α from the magnitude of the spin coupling constant between the C1 and C2 protons, $^3J_{1,2}$ - 2 Hz. In addition, resonances characteristic of the phosphosaccharide core residues of LPG (Turco *et al.*, 1989) are absent which is consistent with the above chemical analysis. The lower intensity anomeric proton resonances observed in Fig. 9 are probably derived from the cap structures, and the small magnitude of the $^3J_{1,2}$ (~2 Hz) for these resonances suggests the presence of additional Man α residues in the cap. Finally, the doublet of doublets at 5.67 ppm is characteristic of Manα1-PO_4, which may also be tentatively assigned to be a cap structure.

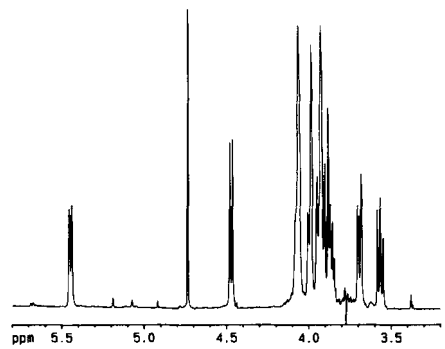

Figure 9. ^1H proton NMR spectrum of exPG. Chemical shifts are referenced relative to external acetone δ=2.225 ppm at 300K

Average Number of Repeat Units and Estimated Molecular Weight of exPG --Sizing chromatography by TSK G2000 HPLC suggested that exPG had a lower molecular weight than delipidated LPG (Fig. 4). To address this aspect in greater detail, exPG was reduced with excess $NaBD_4$, hydrolyzed with 2 N TFA, acetylated with pyridine/acetic anhydride (1:2), and then analyzed by GC-MS to determine the ratio of total mannose to deuterated mannitol at the reducing end of each exPG molecule ("Experimental Procedures"). The results indicated that there were 11-12 mannoses per deuterated mannitol of exPG. Taking into account an average of two mannose residues per cap structure and that the deuterated mannitol represents a Gal-Man repeat unit, it was concluded that there was an average of 10-11 repeating disaccharides per exPG molecule. This result was consistent with the molar ratio of total repeats (515 nmoles) to total caps (46.8 nmoles) as calculated from GC-MS monosaccharide compositional analysis of each fragment (Table II). Collectively, these data suggested that exPG contains about 10-11 repeat units per molecule with an average calculated molecular weight of about 4,700-5,200.