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The Major Surface Antigens of *Entamoeba histolytica* Trophozoites are GPI-anchored Proteophosphoglycans

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Trophozoites of the parasitic protozoa, *Entamoeba histolytica*, synthesize a cell surface lipoglycoconjugate, termed lipophosphoglycan, which is thought to be an important virulence factor and potential vaccine candidate against invasive amebiasis. Here, we show that the *E. histolytica* lipophosphoglycans are in fact glycosylphosphatidylinositol (GPI)-anchored proteophosphoglycans (PPGs). These PPGs contain a highly acidic polypeptide component which is rich in Asp, Glu and phosphoserine residues. This polypeptide component is extensively modified with linear glycan chains having the general structure, $[Glc\alpha 1\text{-}6]_nGlc\beta 1\text{-}6Gal$ (where $n = 2\text{-}23$). These glycan chains can be released after mild-acid hydrolysis with trifluoroacetic or hydrofluoric acid and are probably attached to phosphoserine residues in the polypeptide backbone. The PPGs are further modified with a GPI anchor which differs from all other eukaryotic GPI anchors so far characterized in containing a glycan core with the structure, Gal_1Man_2GlcN-*myo*-inositol, and in being heterogeneously modified with chains of α-galactose. Trophozoites of the pathogenic HM-1:IMSS strain synthesize two distinct classes of PPG which have polydisperse molecular masses of 50-180 kDa (PPG-1) and 35-60 kDa (PPG-2) and are modified with glucan side-chains of different average lengths. In contrast, the non-pathogenic Rahman strain synthesizes one class of PPG which is only elaborated with short disaccharide side-chains (i.e. Glcβ1-6Gal). However, the PPGs are abundant in all strains (8×10^7 copies per cell) and are likely to form a protective surface coat.

Keywords: glycosylphosphatidylinositol; phosphoglycosylation; glycoprotein; lipophosphoglycan; trophozoite

Introduction

The protozoan parasite *Entamoeba histolytica* is the etiological agent of amoebic dysentry and amoebic liver abscess, infecting over 50 million people and causing 40-110 thousand deaths annually worldwide (Tannich, 1998). Infection is initiated by a resistant cyst stage which differentiates into amoebic trophozoites that colonize the large intestine. Trophozoites bind to the colonic mucosa and can either multiply asymptomatically or invade the intestinal epithelium and other tissues causing dysentery and sometimes lethal pathological conditions such as ulcerative colitis or abscesses of the liver. Symptomatic infections occur following contact-dependent cytolysis of epithelial and other host target cells by the trophozoite stage.

Molecules on the surface of the *E. histolytica* trophozoite are thought to play key roles in regulating host cell adhesion and cytolysis and may also be potential vaccine candidates. The best characterized of these molecules is a glycosylphosphatidylinositol (GPI) anchored GalNAc/Gal-inhibitable lectin which is required for trophozoite binding to host cell surface mucins (reviewed by Petri, 1996). Trophozoites also synthesize a second class of GPI-anchored macromolecules which are thought to contain a major phosphoglycan component and are

S.M-H and J.H.P. contributed equally to this study.

Abbreviations used: AHM, 2,5-anhydromannitol; GPI, glycosylphosphatidylinositol; HPTLC, high performance TLC; LPG, lipophosphoglycan; PPG, proteophosphoglycan; TFA, trifluoroacetic acid; HF, hydrofluoric acid; GU, glucose units.

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referred to as lipophosphoglycans (LPGs) (Stanley *et al*., 1992; Bhattacharya *et al*., 1992; Moody *et al*., 1997; Marinets *et al*., 1997). These molecules may be important virulence factors as antigenically distinct forms of LPGs are present on virulent and avirulent strains of *E. histolytica* (Srivastava *et al*., 1995; Moody *et al*., 1997; Marinets *et al*., 1997) and opsonic anti-LPG antibodies reduce the capacity of pathogenic *E. histolytica* trophozoites to kill mammalian cells (Stanley *et al*., 1992; Moody *et al*., 1998). Moreover, immunization with these antibodies prevents the development of liver abscess in animal models (Marinets *et al*., 1997). Initial studies provided no evidence for the presence of a polypeptide component in the LPGs and indicated that the phosphoglycan component was made up primarily of glucose and galactose-containing glycans that were attached to an undefined backbone by acid labile linkages (Stanley *et al*., 1992; Bhattacharya *et al*., 1992; Marinets *et al*., 1997). Recently, a combination of hydrophobic interaction and anion exchange chromatographies have been used to resolve two populations of LPGs which differ in charge and size from the pathogenic *E. histolytica* strain HM-1:IMSS (Moody *et al*., 1997). Protein staining suggested that one of these components (termed LPPG) contained an associated polypeptide component (Moody *et al*., 1997).

Here, we show that the trophozoite LPG/LPPGs from pathogenic and non-pathogenic strains of *E. histolytica* belong to a novel family of GPI-anchored proteophosphoglycans (PPGs) and that these molecules are likely to be the major macromolecules on the trophozoite surface. These PPGs are unusual in containing a highly acidic polypeptide backbone which is extensively modified with linear α1-6 linked glucan side-chains. Evidence is presented that these glycans are linked to the polypeptide *via* phosphodiester linkages. The GPI anchor of the PPGs is also unusual in containing a divergent glycan core, the first to be identified in any eukaryotic protein anchor, and in being modified with heterogeneous galactose side-chains. Based on the abundance of these molecules, and the finding that pathogenic and non-pathogenic *E. histolytica* synthesize structurally distinct PPGs, we propose that specific glycan epitopes in these molecules may be important determinants of amoebic virulence.

Results

Isolation and compositional analysis of PPG-1 and 2

Two distinct families of lipoglycoconjugates, previously referred to as LPPG and LPG, were extracted in 9 % (v/v) aqueous 1-butanol from delipidated *E. histolytica* HM-1:IMSS trophozoites and purified by octyl-Sepharose chromatography and anion exchange chromatography (Moody *et al*., 1997). Previous analyses have shown that these LPG and LPPG preparations migrate as polydisperse bands (100-190 kDa and 33-56 kDa, respectively) on SDS-polyacrylamide electrophoresis gels; that they both react with conconavalin A (but not galactose-binding lectins) and are sensitive to PI-specific phospholipase C digestion; and that they are free of other contaminating protein bands (Moody *et al*., 1997). While both purified fractions stained for carbohydrate with the periodate-silver reagent, only the high molecular weight LPPG fraction stained for protein (Moody *et al*., 1997). However, we present evidence here that both classes of molecules contain a phosphorylated polypeptide component and we now refer to LPPG and LPG as PPG-1 and PPG-2, respectively.

Compositional analyses suggested that PPG-1 and PPG-2 contained a polypeptide component which was highly enriched in Asp, Glu, Lys and Ser residues (Table 1). Approximately 60 % of the serine was present as phosphoserine which would further contribute to the overall acidic nature of the peptide component. The PPGs also contained a high content of Glc and Gal, which accounted for approximately 70 % and 60 % (w/w) of PPG-1 and 2, respectively. Low levels of ethanolamine, *myo*-inositol and mannose were also present in both fractions consistent with the presence of a GPI anchor (Stanley *et al*., 1992; Bhattacharya *et al*., 1992; Moody *et al*., 1997). When the amino acid composition was normalized to ethanolamine, PPG-1 was found to contain a larger polypeptide domain than PPG-2 (Table 1). Based on the total yield of these molecules from 10^9 cells (Table 1), we estimate that there are approximately 8×10^7 copies of PPG per trophozoite.

The PPGs are modified with α1-6 linked glucan side-chains

The monosaccharide analyses indicated that the PPGs lacked typical N- or O-linked glycans, consistent with the finding that none of the monosaccharides were released with *Flavobacterium meningosepticum* N-glycosidase F (data not shown). In contrast, approximately 80 % of the PPG-1 carbohydrate was released after mild-acid hydrolysis in 40 mM TFA, using conditions (ten minutes, 100 °C) that selectively hydrolyze hexose-1-PO_4-Ser linkages (Ilg *et al*., 1994). While these conditions released all the Glc, approximately 80 % of the galactose and all the mannose, *myo*-inositol and amino acid residues were quantitatively recovered in the octyl-Sepharose-bound fraction after this treatment (data not shown). The acid-released glycans were reduced with NaB^3H_4 to incorporate a radiolabel in the reducing terminus and analyzed by Bio Gel-P4 chromatography. As shown in Figure 1(a), the PPG-1 glycans comprised a series of oligosaccharides with a hydrodynamic volume equivalent to 4 to 25 glucose units (GU). A similar series of oligosaccharides was released from PPG-2, although the PPG-2 glycans were on average longer (10 to 20 GU) than the PPG-1 glycans (Figure 1(b)). Methylation linkage analysis of the

Table 1. Compositional analysis of the PPGs from virulent HM-1:IMSS and avirulent Rahman strains of *E. histolytica* trophozoites

	HM-1:IMSS		Rahman
	PPG-1	PPG-2	PPG
Amino acid residues[a]			
Asp	20	7	17
Ser (% P-Ser)[b]	17 (59)	7 (60)	13 (70)
Glu	11	6	8
Lys	10	3	8
Pro	7	2	2
Ala	7	2	2
Gly	6	2	1
Thr	2	3	1
Leu	1	2	1
Cys	1	1	tr
Val	tr[d]	1	tr
EtN	1	1	1
Other	tr	tr	tr
Monosacharides[c]			
Inositol	0.25	0.20	0.5
Man	2.0	2.0	2.0
Gal	21	8.1	5.9
Glc	128	25	3.7
Total monosaccharide (μg/10^8 trophozoites)	272	98	385

[a] The molar ratio of amino acid residues is given relative to ethanolamine (set at 1 mol/mol of protein).
[b] Percent phosphoserine was determined after hydrolysis of PPGs in 6 M HCl (three hours, 80 °C) and correction for partial dephosphorylation using authentic phosphoserine as a standard.
[c] The molar ratios of monosaccharides are given relative to mannose (set at 2 mol/mol of protein; see Results for justification).
[d] tr: trace refers to amino acid residues present at less than 0.4 mol/mol EtN.

acid-released glycan mixture revealed that the glucose was present as either terminal or 6-O-substituted residues, while all the galactose was 6-O-substituted (data not shown). Methylation analysis of the individual PPG-1 glycans (peaks 1-4 in Figure 1(a)) showed that all the Gal was present at the reducing terminus and confirmed that the Glc was only present as non-reducing terminal or 1-6 linked residues (Table 2). Treatment of the ^{3}H-labelled glycans with *Aspergillus niger* α-amyloglucosidase, an exoglucanase which degrades α1-4 and α1-6 linked glucans, generated a single oligosaccharide that eluted at 2.9 GU on Bio Gel-P4 (Figure 2(b)), suggesting that the 1-6 linked glucose residues were present in the α-anomeric configuration. The 2.9 GU oligosaccharide was resistant to digestion with *Saccharomyces cerevisiae* α-glucosidase (data not shown), but was reduced in size to 1.7 GU after treatment with sweet almond β-glucosidase (Figure 2(c)). Monosaccharide and methylation analysis of the Bio Gel-P4 purified 2.9 GU disaccharide revealed the presence of stoichiometric amounts of terminal-Glc and 6-O-substituted Gal (data not shown), indicating that all the acid-labile glycans contained the common core disaccharide, Glcβ1-6Gal. Collectively, these data indicated that the acid-labile chains of PPG-1 and 2 have the structure $[Glc\alpha1\text{-}6]_{2\text{-}23}$ Glcβ1-6Gal.

The quantitative release of all the glucose from the polypeptide component by very-mild-acid hydrolysis suggested that these glucan side-chains were linked to the polypeptide backbone *via* phosphodiester bonds. A direct linkage between the galactose and serine residues in the polypeptide was further indicated by the finding that approximately 60 % of the serine residues in the PPGs were phosphorylated (Table 1). Some of these glucans could also be linked *via* phosphodiester linkage to other peptide-linked glycans. In the latter case, we would expect to detect one or more phos-

Table 2. Methylation analysis of the acid-released glycans from HM-1:IMSS PPG-1

		P4 fraction (GU)			
PMAA	Origin	4	5	6	7
Glucitol					
2,3,4,6-tetra-O-methyl-	Terminal Glc	1.2	0.9	0.3	1.1
2,3,4-tri-O-methyl	6-substituted Glc	1.8	2.6	3.8	5.3
Galactitol[a]					
1,2,3,4,5-penta-O-methyl	6-substituted galactitol	0.6	0.8	0.5	0.4

[a] The recovery of all the galactose as 1,2,3,4,5-penta-O-methyl-6-O-acetylgalactitol confirmed that this monosaccharide was present at the reducing terminus of all the oligosaccharides.

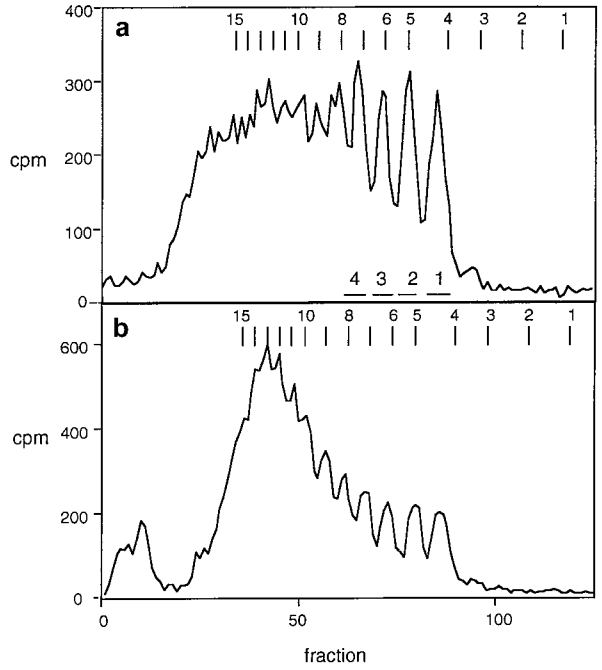

Figure 1. Bio Gel-P4 chromatography of acid-released glycans of PPG-1 and PPG-2. PPGs were purified from pathogenic HM-1:IMSS trophozoites and the glycans released after mild-acid hydrolysis (40 mM TFA, 100 °C, ten minutes) reductively labelled with NaB^3H_4. After removal of radiochemical impurities by descending paper chromatography and passage through mixed bed resins, the labelled glycans from (a) PPG-1 and (b) PPG-2 were analyzed by Bio Gel-P4 chromatography. The elution position of coinjected dextran oligosaccharides (degree of polymerization, 1-15) are indicated at the top of each profile. Oligosaccharides purified for methylation analysis (1-4) are indicated under each peak.

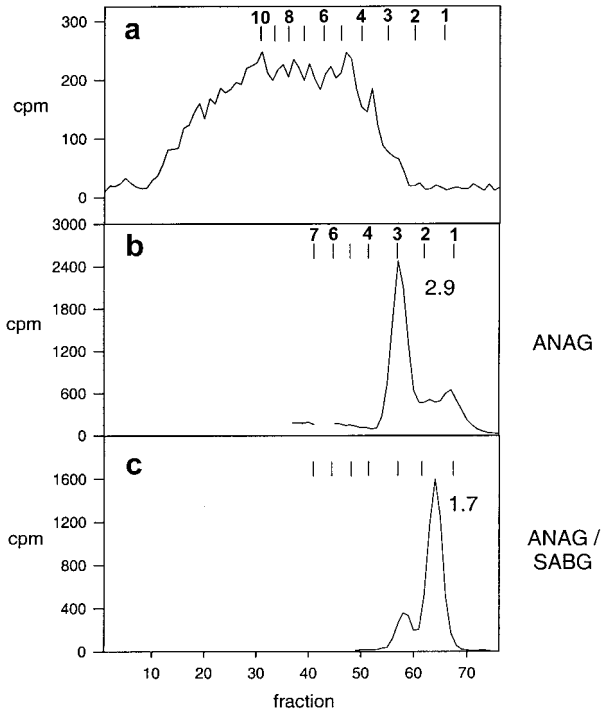

Figure 2. Exoglycosidase sequencing of peptide-linked PPG glycans. The acid-released ^3H-labelled glycans of HM-1:IMSS PPG-1 were analyzed by Bio Gel-P4 chromatography (a) before and (b) after *Aspergillus niger* α-amyloglucosidase (ANAG) treatment and (c) further digestion of the 2.9 GU peak in (b) with sweet almond β-glucosidase (SABG). The elution positions of coinjected dextran oligosaccharides are indicated at the top of each profile.

phomonosaccharides (the product of a cleavage across a hexose-PO_4-hexose bond) in the monosaccharide analyses. However, GC-MS analysis of the purified PPGs following methanolysis, diazomethane- and TMS-derivatization, failed to reveal the presence of any phosphomonosaccharides (data not shown), suggesting that all the glucans are linked directly to phosphoserine in the polypeptide component.

The PPGs contain a unique GPI anchor

As described above, only 20 % of the galactose was released from the PPGs by mild-acid hydrolysis, suggesting that these monosaccharides may be linked *via* a glycosidic linkage to either the polypeptide component or the GPI anchor. To test the latter possibility, the GPI anchors of the PPGs were radiolabelled by nitrous acid deamination and reduction with NaB^3H_4 . This procedure releases the inositol phospholipid moiety of the anchor and converts the core GlcN residue to [^{3}H]2,5 anhydromannitol (AHM). After removal of radiochemical contaminants on a G25 Sephadex column and acid hydrolysis, more than 90 % of the incorporated ^3H-label comigrated with authentic AHM on HPTLC (data not shown and Figure 3), suggesting that only the GPI moiety had been labelled.

The radiolabelled GPI glycans were released from the PPGs following HF dephosphorylation and analyzed by Bio Gel-P4 chromatography. Both PPG-1 and -2 contained similar GPI glycan moieties, which comprised a major 5.5 GU glycan species and an envelope of larger glycans that eluted between 6 and 25 GU (Figure 4(a) and (b)). These glycans were not digested with α-amyloglucosidase, the α- or β-glucosidases or jack bean α-mannosidase (data not shown), but collapsed to a major 3.0 GU oligosaccharide after treatment with coffee bean α-galactosidase (Figure 5(a) and (b)). The 3.0 GU oligosaccharide comigrated with Manα1-6Manα1-4AHM on HPTLC (Figure 6, lane 3) and was converted to AHM after jack bean α-mannosidase digestion (Figures 5(d) and 6, lane 5). Acetolysis of the HF-treated PPG-2 glycans, using conditions that preferentially cleave Manα1-6Man linkages (Ferguson, 1992) generated a major product that comigrated with Man_1AHM on HPTLC (Figure 6, lane 12), consistent with the pre-

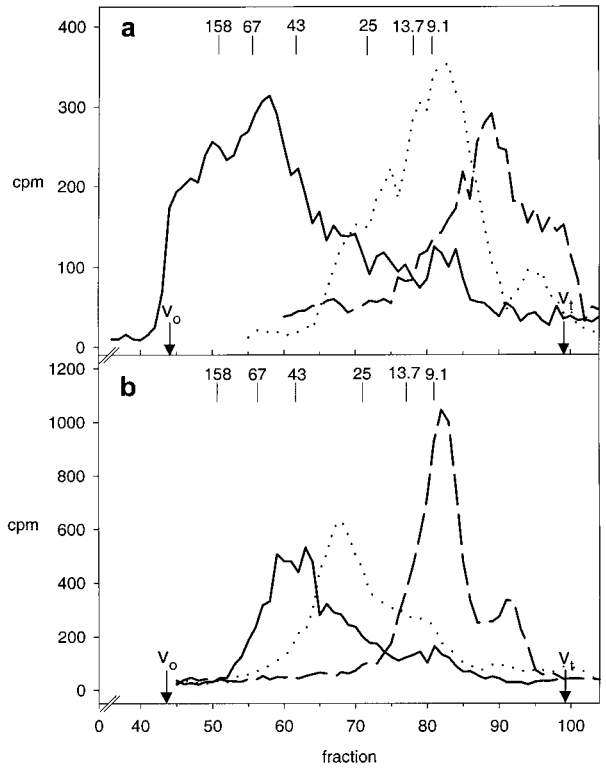

Figure 3. S-200 gel filtration of PPG-1 and PPG-2. The GPI glycan core of the purified HM-1:IMSS PPGs were deaminated and reduced with NaB^3H_4. The labelled glycopeptides were analyzed on a Sephacryl S-200 gel filtration column before and after Pronase treatment or mild acid hydrolysis (40 mM TFA, 100 °C, ten minutes). The elution profiles of untreated (continuous line), Pronase-treated (broken line), or mild acid-treated (dotted line) PPG-1 are shown in (a). The elution profiles of untreated (continuous line), Pronase-treated (broken line), or mild acid-treated (dotted line) PPG-2 are shown in (b). The elution positions of protein standards of defined molecular weights (given in kDa) are indicated.

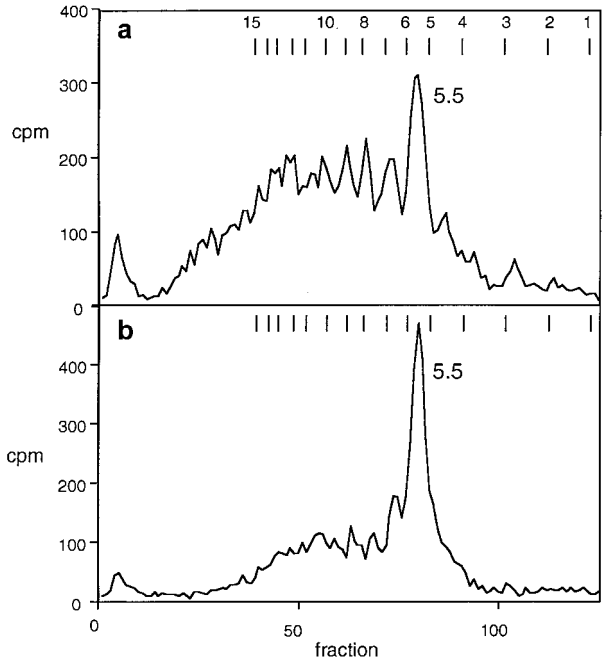

Figure 4. Bio Gel-P4 chromatography of the neutral GPI glycan moiety. Purified HM-1:IMSS PPG-1 and -2 were hydrolyzed in 40 mM TFA (ten minutes, 100 °C) to remove the peptide-linked glucans and the GPI-peptide recovered by octyl-Sepharose chromatography. The GPI glycan was radiolabelled by nitrous acid deamination and reduction with NaB^3H_4 and the neutral, HF-dephosphorylated glycans from (a) PPG-1 and (b) PPG-2 chromatographed on Bio Gel-P4. The elution positions of coinjected dextran oligosaccharides are indicated at the top of each profile.

sence of a Manα1-6Manα-GlcN core in the PPG anchor. To check that the coffee bean α-galactosidase preparation was free of contaminating α-mannosidase activity, a Man_4AHM standard (Manα1-2Manα1-2Manα1-6Manα1-4AHM generated from *Trypanosoma cruzi* LPPG) was exhaustively digested with both coffee bean α-galactosidase and jack bean α-mannosidase. As shown in Figure 6 (lanes 7-9) no digestion was observed with coffee bean α-galactosidase while digestion with jack bean α-mannosidase quantitatively converted this glycan to AHM as expected. Taken together, these data suggested that all the GPI anchors of the *E. histolytica* PPGs contain the novel sequence, Galα1-X Manα1-6Manα1-4AHM (where X signifies an unknown linkage), which is variably modified with 2 to 20 αGal residues.

In other eukaryotes, the carboxy terminus of a protein is linked to a GPI anchor *via* an ethanolamine-phosphate bridge (McConville & Ferguson, 1993; Ferguson *et al.*, 1999). The presence of a similar bridge in the PPGs was suggested by the presence of ethanolamine in the amino acid analyses (Table 1), and the finding that AHM-containing glycans were released after HF-treatment. To identify whether this putative phosphodiester bridge was linked to one of the galactose residues or one of the core mannose residues, the ^3H-labelled deaminated/reduced glycopeptide was treated with coffee bean α-galactosidase before HF dephosphorylation to remove any non-phosphorylated Gal residues. The neutral glycan which was subsequently released by HF treatment eluted at 3.9 GU on Bio Gel-P4 (Figure 5(c)) and with the glycan standard, Man_3AHM, on HPTLC (Figure 6, lane 2), but was resistant to jack bean α-mannosidase digestion (Figure 6, lane 4). These data showed that the α-galactosidase-treated glycopeptide had retained a single galactose residue on the GPI glycan core, and suggested that this galactose residue was modified with an HF-sensitive residue, possibly ethanolamine-phosphate. The HPTLC analyses revealed the presence of an additional minor band in all these digestions (Figure 6, lanes 2-5). This band was diminished by multiple treatments with α-galactosidase and had a faster

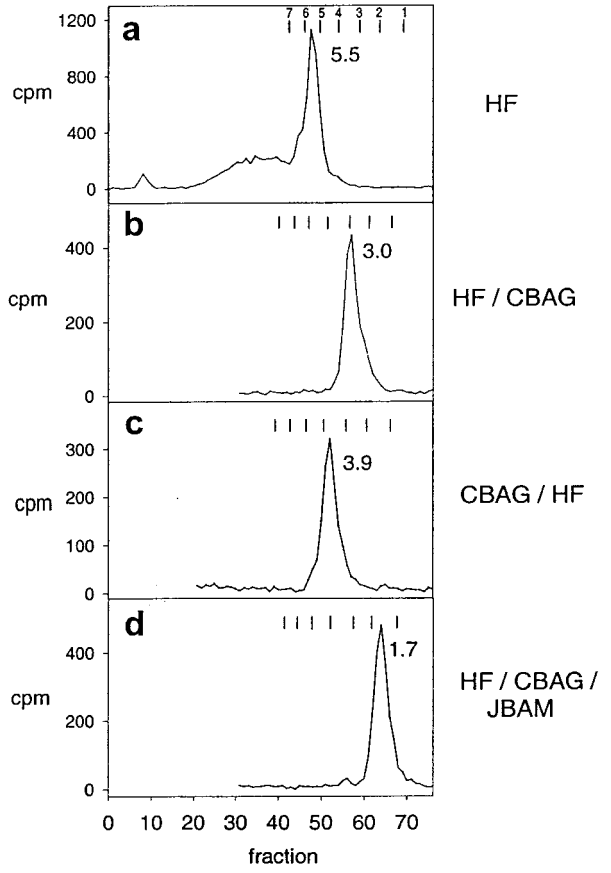

Figure 5. Bio Gel-P4 analysis of the PPG-1 GPI glycan. The deaminated/NaB^3H_4-reduced PPG-1 glycopeptide was subjected to the following treatments; (a) HF-dephosphorylation, (b) HF-dephosphorylation followed by coffee bean α-galactosidase digestion (CBAG), (c) CBAG digestion followed by HF-dephosphorylation, (d) HF-dephosphorylation followed by CBAG and jack bean α-mannosidase (JBAM). The neutral, ^3H-labelled glycans generated by these procedures were chromatographed on Bio Gel-P4. The elution positions of coinjected dextran oligosaccharides are indicated at the top of each profile.

HPTLC mobility after jack bean α-mannosidase treatment (Figure 6, compare lanes 3 and 5). The latter result indicated the presence of additional galactose residues (in a linkage that is largely resistant to α-galactosidase digestion) or another unidentified substituent on a proportion of the mannose residue proximal to the AHM.

These analyses suggested that the PPGs contained a GPI anchor with a novel glycan core. To confirm that this anchor was attached to the PPGs and not to a contaminating non-protein-linked GPI glycolipid, the deaminated/NaB^3H_4 reduced PPG-1 was chromatographed on a Sephacryl S-200 gel filtration column. The labelled PPG-1 eluted as a polydisperse, high molecular weight peak with an apparent molecular mass of 50-180 kDa (Figure 3(a), continuous line). This material was degraded by either Pronase or mild-acid hydrolysis. After Pronase digestion, all the label eluted near the V_t of the column (<8 kDa) (Figure 3(a), broken line), while mild-acid treatment generated a peak with a slightly higher apparent molecular weight (Figure 3(a), dotted line). These changes are consistent with the removal of the phosphoglycosylated polypeptide or the glucan chains alone, respectively. The deaminated/reduced PPG-2 glycopeptide also eluted as a polydisperse peak on Sephacryl S-200 but with a smaller apparent molecular mass than the labelled PPG-1 (35-60 kDa; Figure 3(b)). As with PPG-1, the labelled PPG-2 moiety was converted to lower molecular weight material following Pronase or mild-acid treatments (Figure 3(b)). Collectively, these data showed that both PPGs were modified with similar GPI anchors containing a novel galactosylated glycan core and additional galactose side-chains.

The acid-labile PPG side-chains from non-pathogenic strains of *E. histolytica* differ from those found in pathogenic strains

The avirulent Rahman strain of *E. histolytica*, synthesizes a single population of PPGs which have a similar SDS-polyacrylamide gel electrophoretic mobility to PPG-2 (Moody *et al.*, 1997). Compositional analysis showed that the purified Rahman PPG had a similar amino acid composition to HM-1:IMSS PPG-1, but a much lower level of glycosylation (Table 1). Significantly, the ratio of Glc/Gal was much lower in the Rahman PPG (Table 1), indicating a difference in the structures of the acid-labile side-chains. To investigate this possibility, the acid-released glycans of the Rahman PPG were radiolabelled with NaB^3H_4 and analyzed by Bio Gel-P4 chromatography. Remarkably, the Rahman PPG only contained a single acid-labile oligosaccharide species which coeluted with the core disaccharide, Glcβ1-6Gal, on both Bio Gel-P4 (2.7 Gu peak; Figure 7(a)) and HPTLC (Figure 7(b), compare lanes 3 and 5). As expected, this disaccharide was resistant to digestion with *A. niger* α-amyloglucosidase but comigrated with galactitol after digestion with β-glucosidase (Figure 7(b), lanes 6 and 7). These data suggested that the polypeptide backbone of the Rahman PPG was modified with the non-glucosylated core disaccharide, Glcβ1-6Gal.

Discussion

Here, we show that the previously identified LPGs and LPPGs of *E. histolytica* trophozoites comprise a novel class of GPI-anchored proteophosphoglycans which we have now termed PPG-1 and 2. These molecules are thought to be important parasite virulence factors and potential vaccine candidates against invasive amebiasis (Stanley *et al.*, 1992; Bhattacharya *et al.*, 1992; Moody *et al.*, 1997; Marinets *et al.*, 1997). The major structural features of the PPGs from pathogenic HM-1:IMSS trophozoites are summarized in Figure 8. Both PPGs con-

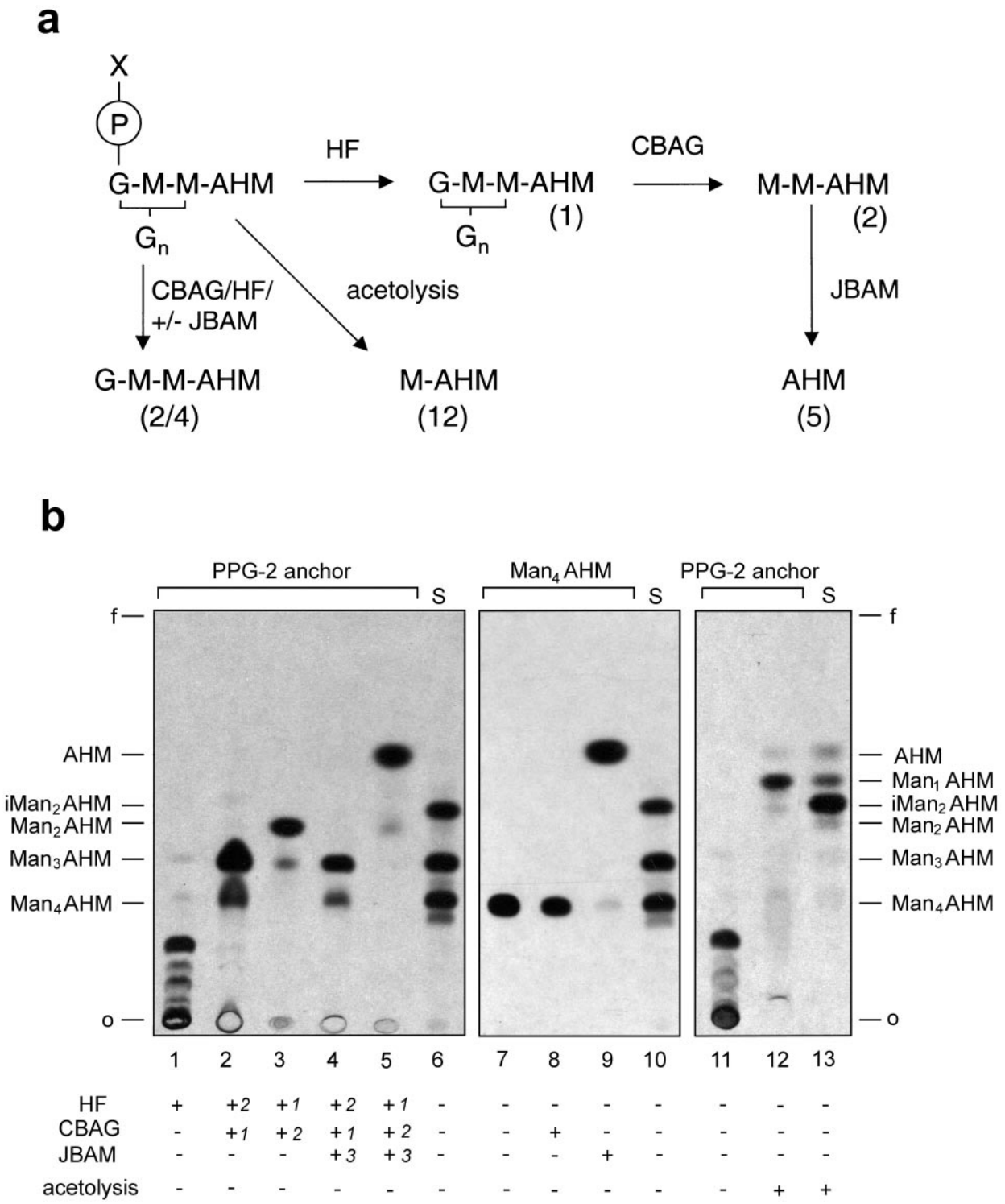

Figure 6. HPTLC analysis of the PPG-2 GPI glycans. The deaminated/NaB^3H_4-reduced glycopeptides of PPG-2 were treated with HF, coffee bean α-galactosidase (CBAG), jack bean α-mannosidase (JBAM) and partial acetolysis as indicated. (a) Schematic showing the proposed structures generated by each treatment. (b) HPTLC of the products of each treatment on PPG-2 anchor glycan (lanes 1-6 and 11-12) and a Man_4AHM standard generated from the *T. cruzi* lipopeptidoglycan (Manα1-2Manα1-2Manα1-6Manα1-4AHM) (lanes 7-9). A mixture of deaminated/NaB^3H_4 reduced glycans derived from *L. mexicana* GPIs were used as standards (S) in (a) and (b). These glycans (and fragments derived from them, lane 13) have the structures AHM, Manα1-4AHM (Man_1AHM), Manα1-3Manα1-4AHM ($iMan_2$AHM), Manα1-6Manα1-4AHM (Man_2AHM), Manα1-6[Manα1-3]Manα1-4AHM (Man_3AHM) and Manα1-2Manα1-6[Manα1-3]Manα1-4AHM (Man_4AHM). The HPTLCs were developed in solvent A. O = origin; f = solvent front.

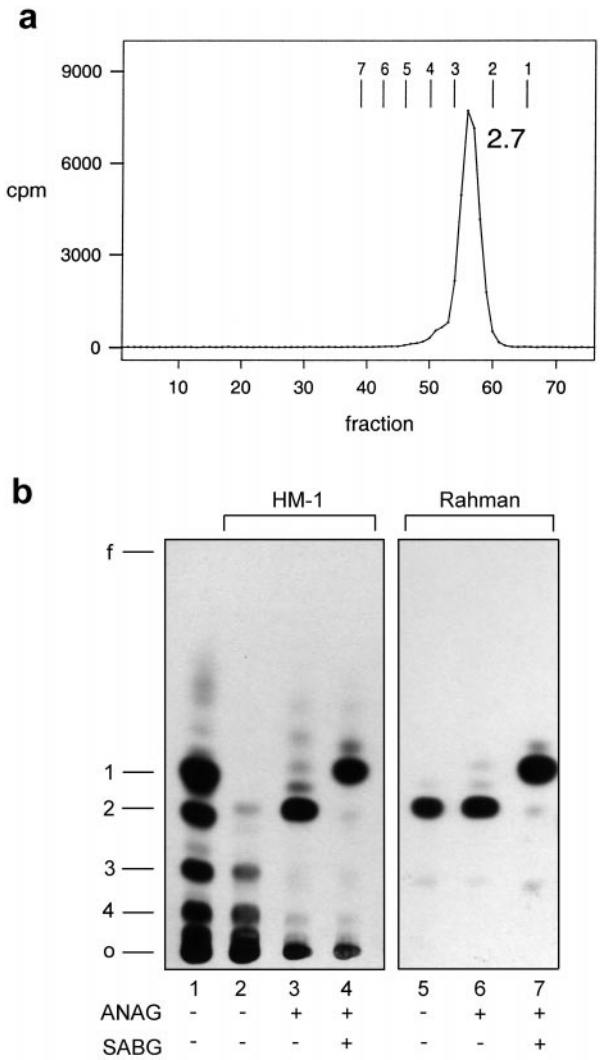

Figure 7. Rahman PPG is only modified with the disaccharide core. (a) Bio Gel-P4 chromatography of the single acid-released glycan from Rahman PPG. (b) HPTLC analysis of acid-released [^{3}H]glycans from HM-1:IMSS PPG-1 and Rahman PPG. The total [^{3}H]glycan mixtures were analyzed without treatment, or after digestion with α-amyloglucosidase (ANAG) or sweet almond β-glucosidase (SABG), as indicated. The migration positions of a mixture of reduced ^{3}H-labelled dextran oligosaccharides (lane 1) is indicated on the right-hand-side of the chromatogram. The HPTLCs were developed in solvent A. O = origin; f = solvent front.

tain a polypeptide backbone which is rich in acidic amino acid residues. This polypeptide is extensively modified with linear α1-6 linked glucan side-chains and a novel heterogeneously modified GPI anchor. The glucan side-chains range from 2 to 23 residues long and are assembled on the core disaccharide, Glcβ1-6Gal (Figure 8). While the linkage between the core disaccharide and the peptide backbone has not been unequivocally determined, it is likely to comprise the novel sequence Gal-1-PO_4-Ser, based on the fact that these oligosaccharides are quantitatively released after mild-acid hydrolysis in TFA or cold 48 % HF, and the identification of high levels of phosphoserine in the amino acid analyses. The possibility that some of these oligosaccharides may be attached *via* similar linkages to other oligosaccharides (instead of peptide) is unlikely given that no phosphomonosaccharides were detected in the monosaccharide analyses. Using both Sephacryl S-200 gel filtration and SDS-polyacrylamide gel electrophoresis (Moody *et al.*, 1997) we have shown that PPG-1 has a higher molecular weight than PPG-2. Our analyses suggest that this could reflect the presence of a longer polypeptide backbone in PPG-1 and/or a higher level of substitution with glucan side-chains. Interestingly, despite having a lower overall molecular weight, PPG-2 was modified with longer phosphoglucan side-chains (Figure 1). These differences could reflect peptide sequence specificity in the putative elongating glucosyltransferase or differences in the time that these molecules spend in organelles responsible for these modifications.

The attachment of glycans to polypeptides *via* a Gal-1-PO_4-Ser linkage has not been previously reported, although similar hexose/*N*-acetylhexosamine-1-PO_4-Ser linkages that have been found in a number of cell surface and secreted glycoconjugates from other lower eukaryotes. For example, the modification of phosphoserine with *N*-acetylglucosamine, mannose and xylose residues has been demonstrated, or proposed, to occur in *Dictyostelium discoideum* (Mehta *et al.*, 1996), *Leishmania* spp (Ilg *et al.*, 1994, 1996; Moss *et al.*, 1998) and *T. cruzi* (Haynes, 1998), respectively. In both *D. discoideum* and *Leishmania*, the phosphoglycosylation of serine residues is initiated by the transfer of a sugar-phosphate residue from a nucleotide sugar donor, rather than by the glycosylation of a serine-phosphate (Mehta *et al.*, 1996; Moss *et al.*, 1998). This type of modification is often restricted to specific cell surface or secreted glycoproteins within a particular cell suggesting that specific amino acid sequence motifs are recognized by the initiating enzymes (Mehta *et al.*, 1996; Moss *et al.*, 1998). Similarly, there appears to be a high degree of selectivity in the phosphoglycosylation of *E. histolytica* glycoproteins as there is no evidence that other serine-rich proteins (i.e. SREHP; serine-rich *E. histolytica* protein) are modified with PPG-like glucan side-chains (Stanley *et al.*, 1995).

The *E. histolytica* PPGs are also notable in containing a GPI anchor with a unique glycan backbone. All eukaryote GPI protein anchors characterized to date contain the backbone sequence, EtN-PO_4-Manα1-2Manα1-6Manα1-4GlcN-*myo*inositol-phospholipid, which may be variously modified with species-specific side-chains (reviewed by McConville & Ferguson, 1993; Ferguson *et al.*, 1999). The *E. histolytica* PPG anchors diverge from this conserved sequence in containing an anchor with the core structure, Gal_1Man_2GlcN-

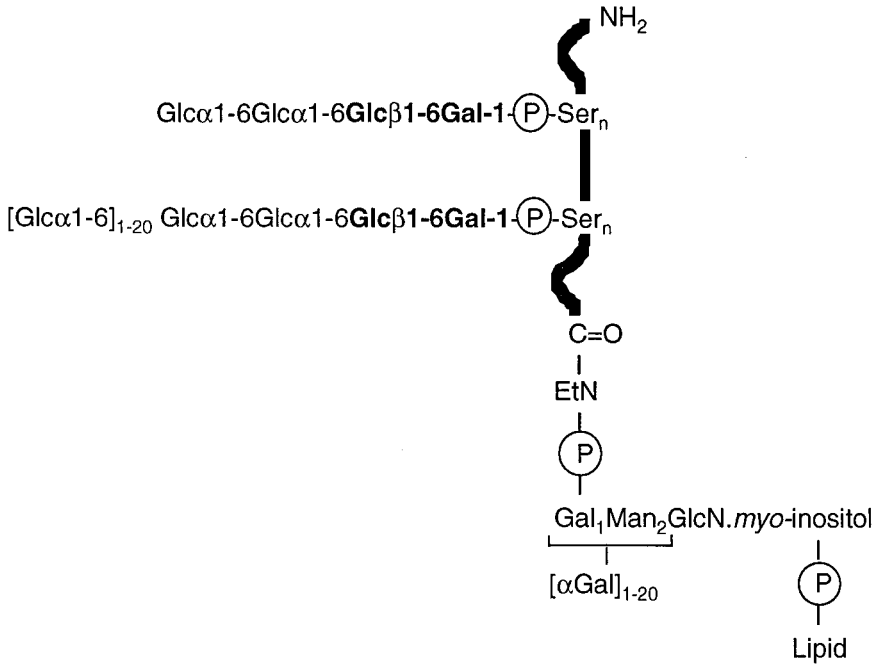

Figure 8. Schematic structure of *E. histolytica* PPG. The trophozoite PPGs of the pathogenic HM-1:IMSS strain contain three structural domains; (1) an acidic polypeptide backbone (heavy line), (2) a heterogeneous series of α1-6 linked glucans (4-25 residues long), possibly linked *via* phosphodiester bridges to multiple serine residues in the polypeptide backbone and (3) a novel GPI anchor that contains a divergent glycan core sequence and extensive side-chains of α-Gal. The structure of these side-chains and the nature of the GPI lipid moiety was not determined in this study. The acid labile glucan side-chains of the HM-1:IMSS PPGs contain the disaccharide core, Glcβ1-6Gal (shown in bold type). This disaccharide core is also added to the PPGs of the avirulent Rahman strain but is not elaborated with the α1-6 linked glucans.

*myo*inositol, where the terminal αGal residue replaces the α1-2 linked mannose residue of other eukaryotic protein anchors. It is extremely unlikely that this GPI moiety was derived from contaminating free (non-protein-linked) GPIs which can be abundant in some protozoa (McConville & Ferguson, 1993). Firstly, all of the labelled GPI glycan moieties were linked to Pronase and acid-sensitive components with the expected molecular weight of the PPG-1 and 2 components on Sephacryl S-200 (Figure 3). Second, essentially identical anchor structures were found in both PPG-1 and 2, so that the same GPI contaminant would have had to copurify with both families of molecules. Third, the PPG preparations were subjected to multiple extractions with organic solvents to remove any low molecular weight free GPIs. Finally, no evidence for the presence of conventional GPI sequence was found in several independent labelling experiments of different PPG preparations (data not shown), indicating the absence of any ''conventional'' anchors. This unusual GPI glycan core was further modified with a heterogeneous side-chain of 1-20 α-galactose residues. The sequencing data suggest that some or all of these residues (or other unidentified substituents) are linked to the Man residue proximal to the GlcN. The structures of these αGal-linked chains, their precise point of attachment to the anchor core and the nature of the phospholipid moiety in the PPG anchors are currently under investigation.

Based on the yields of purified PPG from both the virulent HM-1:IMSS and avirulent Rahman strains of *E. histolytica*, we estimate that there are approximately 80 million copies of PPG per trophozoite. These molecules have been localized to both the cell surface and intracellular vesicles (Marinets *et al*., 1997), and are likely to form a densely packed glycocalyx over the entire trophozoite surface, consistent with electron micrograph images of these cells (De Souza, 1995). Interestingly, structurally related glycoconjugates dominate the cell surfaces of other protozoa that live in hydrolytic environments. For example, the insect stages of *Trypanosoma brucei* , *T. cruzi* and *Leishmania* that proliferate within the digestive tract of their respective vectors are all coated by GPI-anchored glycoproteins or lipophosphoglycans. A hallmark of these molecules is the presence of a highly acidic protein or glycan backbone and extensive glycan side-chain modifications (that may also be charged) which are attached to either the polypeptide and/or the GPI anchor (McConville & Ferguson, 1993; Ferguson *et al*., 1993; Acosta Serrano *et al*., 1995; McConville *et al*., 1995; Ilg *et al*., 1996; Treumann *et al*., 1997). These studies support the notion that the expression of GPI-anchored acidic glycoconjugates confer a sur-

vival advantage on single celled eukaryotes that live in these hydrolytic environments.

At present, relatively little is known about the function of the *E. histolytica* PPGs. Opsonization of pathogenic trophozoites with anti-PPG antibodies inhibits trophozoite adhesion to host cells and contact-dependent cytolysis suggesting that they have an important role in regulating host-parasite interactions (Stanley *et al*., 1992; Marinets *et al*., 1997; Moody *et al*., 1998). In this respect, the PPGs could act as counter-ligands for unidentified host cell receptor(s). Alternatively, a densely packed PPG surface glycocalyx could modulate the activity of trophozoite surface receptors such as the GalNAc/Gal-inhibitable lectin (Petri, 1996; Renesto *et al*., 1997), either by sterically protecting them from proteolytic degradation or by regulating their ability to interact with host cell receptors. The former possibility is supported by the finding that the GalNAc/Gal-inhibitable lectin on cultured HM-1:ISS trophozoites is largely resistant to extracellular proteases, but becomes susceptible if trophozoites are coincubated with a cocktail of glycosidases derived from the intestinal bacterial flora (Variyam, 1996). The properties of the surface coat of PPGs is likely to be determined by both the length and number of glucan side-chains that are added to the polypeptide backbone. In this respect, it is of interest that the PPGs of the avirulent Rahman strain are only modified with short disaccharides. Other avirulent strains of *E. histolytica* also synthesize PPGs with truncated glucan side-chains (Patterson *et al*., unpublished data), raising the possibility that there may be a correlation between PPG glucosylation and trophozoite virulence. As molecular approaches are now available for genetically complementing *E. histolytica*, these natural glycosylation variants may prove to be useful in identifying genes involved in PPG phosphoglycosylation and in exploring the role of the PPG glucan side-chains in parasite virulence.

In summary, we have shown that a major class of molecules on the cell surface of *E. histolytica* trophozoites comprise a novel family of GPI-anchored PPGs. Changes in the structure of the PPG glycans are associated with loss of virulence consistent with other studies suggesting these molecules may be important virulence factors. The detection of these epitopes may thus be useful in diagnosing virulent strains. Finally, the identification of several novel glycan structures indicates the presence of new enzyme systems which could be targets for anti-amoebic agents.

Materials and Methods

Cell culture and reagents

Trophozoites of *E. histolytica* virulent strain HM-1:IMSS and avirulent strain Rahman (Bracha *et al*., 1995) were grown axenically in TYI-S-33 medium and harvested in the logarithmic phase of growth. Exoglycosidases were from Boehringer Mannheim, NaB^3H_4 (15 Ci/mmol) and EN^3HANCE spray were from New England Nuclear.

Isolation of PPGs

The PPGs were extracted from trophozoites by differential solvent extraction as described by Moody *et al*. (1997). Briefly, washed trophozoites were extracted twice in chloroform/methanol/water (1:2:0.8 by vol.) and the insoluble delipidated pellet further extracted twice in 9% (v/v) 1-butanol (4 °C, 12 hours) with constant mixing and sonication. After removal of insoluble material by centrifugation (4000 *g*, 20 minutes), the 1-butanol extract was lyophilized, resuspended in 0.1 M NH_4OAc containing 5% (v/v) 1-propanol and passed down a 10 ml column of octyl-Sepharose equilibrated in the same buffer. The column was washed with 0.1 M NH_4OAc containing 5% 1-propanol and the PPGs subsequently eluted with a linear gradient of increasing 1-propanol (5%-60%). Carbohydrate-containing fractions were detected by spotting aliquots of each fraction onto silica-gel 60 HPTLC sheets (Merck) and stained with orcinol-H_2SO_4 (McConville *et al*., 1990). Two PPGs populations were subsequently resolved by anion exchange chromatography on Fast Flow DEAE-Sepharose eluted in 30% 1-propanol and a linear gradient of NH_4OAc (Moody *et al*., 1997).

Compositional analyses

Amino acid residues were released by hydrolysis in 6 M HCl (110 °C, 15 hours), derivatized with *o*-phthalaldehyde and analyzed by reverse phase chromatography on a Hewlett-Packard Amino Quant system. For detection of phosphoamino acids, samples were hydrolyzed in 6 M HCl (six hours, 80 °C) before derivatization and quantified by HPLC as for the other amino acid residues (Ilg *et al*., 1994). Monosaccharides were released by solvolysis in anhydrous methanolic-0.5 M HCl and analyzed as their trimethylsilyl derivatives (McConville *et al*., 1990). For detection of phosphorylated monosaccharides, the trimethylsilyl derivatives were treated with diazomethane in diethylether (0 °C, ten minutes) and then resilylated before GC-MS (Ferguson, 1992). Total monosaccharide was quantitated by the phenol-sulphuric assay (Fox & Robyt, 1991).

Radiolabelling of peptide and GPI-linked glycans

Two experimental strategies were used to radiolabel the peptide- and GPI-linked glycans, respectively. In the first approach, the purified PPGs were subjected to mild-acid hydrolysis in 40 mM TFA (100 °C, ten minutes) which quantitatively released the peptide-linked glycans but not the GPI anchor (McConville *et al*., 1990). The acid-released glycans were separated from the GPI-anchored polypeptide by octyl-Sepharose chromatography as described above, except that a 1 ml column was used and bound material eluted with 1 ml aliquots of 30% and 40% 1-propanol instead of the linear 1-propanol gradient. Fractions containing the released glycans were pooled and labelled in 0.1 M NaOH containing 30 mM NaB^3H_4 (15 µl, 15 Ci/mmol) for two hours at 25 °C (McConville *et al*., 1990). Excess reductant was removed by addition of 1 M acetic acid and passage down a column of AG50 X12 (H^+), followed by repeated methanol washes. The labelled glycans were purified from radiochemical impurities by descending paper

chromatography in 1-butanol/ethanol/water (4:1:0.8 by vol.) for 48 hours. Glycans were eluted from the origin and desalted by passage down a column containing AG50 X12 (H^+) over AG3 X8 (OH^-) (McConville *et al.*, 1990).

In the second approach, the TFA-deglycosylated PPGs were deaminated in 0.5 M NaOAc (pH 4.0), containing 0.5 M HNO_2 (20 µl) for two hours at 37 °C (McConville *et al.*, 1990). The incubation was continued for a further two hours with addition of two aliquots of 0.5 M HNO_2 (15 µl) at one hour intervals. The mixture was extracted twice with water-saturated 1-butanol (200 µl) to remove the released phosphatidylinositol lipid moiety and the pH of the lower aqueous phase adjusted to 10 with 5 M NaOH. The 2,5-anhydromannose residue generated at the reducing terminus of the GPI glycan by nitrous acid deamination was then reduced to 2,5-anhydromannitol (AHM) with addition of NaB^3H_4 (15 µl, 30 mM, 15 Ci/mmol) and incubation for two hours at 25 °C (McConville *et al.*, 1990). The reduction was stopped by addition of acetic acid and radiochemical impurities removed by passage of the mixture down a Sephadex G25 column (1.3 cm × 43 cm) equilibrated and eluted in 0.1 M NH_4OAc. Material eluting in the void volume was lyophilized to remove volatile buffer salts.

Chemical and enzyme treatments of ^{3}H-labelled glycans

The deaminated/reduced glycopeptides were dephosphorylated in 48% aqueous HF (50 µl, 0 °C, 60 hours) and then neutralized with saturated LiOH (Ferguson, 1992) and desalted on a mixed bed column of AG50 X12 (H^+) over AG3 X8 (OH^-). Exoglycosidase digestions were performed in the following buffers; jack bean α-mannosidase in 0.1 M NaOAc, pH 5.0; coffee bean α-galactosidase in 0.1 M phosphate citrate buffer, pH 6.0; *A. niger* α-amyloglucosidase in 0.1 M sodium citrate (pH 4.5); sweet almond β-glucosidase and recombinant *S. cerevisiae* α-glucosidase in 0.1 M citrate buffer (pH 4.5). All digests were performed at 37 °C for 16 hours. In some cases, additional enzyme was added and the incubations extended for another 8 to 16 hours. Digests were stopped by boiling for three minutes and desalted on mixed bed resins containing AG50 X12 (H^+) over AG3 X8 (OH^-). Partial acetolysis was performed as described previously (Ferguson, 1992).

Gel filtration and HPTLC

The ^{3}H-labelled neutral PPG glycans were coinjected with a partial acid hydrolysate of dextran onto a column of Bio Gel-P4 (<45 µm mesh, 1.7 cm × 68 cm) held at 55 °C and eluted with distilled water at 0.2 ml/minute. Elution of radioactivity was monitored by scintillation counting of fractions (1 ml), while the elution of unlabelled dextran oligosaccharides was measured on-line with a refractive index monitor (Erma ERC-7512) (McConville *et al.*, 1990). The relative elution time of radiolabelled glycans to the dextran standards was expressed as GU (Ferguson, 1992). Neutral [^{3}H]glycans were also analyzed on aluminium-backed silica-gel 60 HPTLC sheets developed in 1-propanol/acetone/water (9:6:5 by vol., solvent A) (Schneider *et al.*, 1993). The HPTLC sheets were sprayed with EN^{3}HANCE spray and exposed against Biomax MR film (Kodak) at −70 °C. The ^{3}H-labelled glycopeptides were analyzed on a column of Sephacryl S-200 (1.3 cm × 48 cm) equilibrated and eluted in 0.1 M NH_4OAc at 0.75 ml/minute. Fractions (0.75 ml) were collected and the elution of radioactivity monitored by scintillation counting. The elution of a series of protein standards (9.1 kDa to 158 kDa, Sigma) were monitored using an on-line UV (A_{280}) monitor. Pronase (Boehringer Mannheim) digestions were performed in 10 mM $CaCl_2$ for 16 hours at 37 °C. The digestion was stopped by acidification with acetic acid, clarified by centrifugation and the reaction mixture loaded directly onto the column. Mild-acid hydrolysis was performed as described above and the neutralized hydrolysate loaded directly onto the column.

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