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Structure of the glycosylphosphatidylinositol membrane anchor of human placental alkaline phosphatase

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The glycosylphosphatidylinositol membrane anchor of human placental alkaline phosphatase was isolated by exhaustive proteolysis followed by hydrophobic interaction chromatography. The resulting glycosylphosphatidylinositol-peptide was subjected to compositional analysis and chemical and enzymic modifications. The neutral-glycan fraction, prepared by dephosphorylation followed by HNO_2 deamination and reduction, was sequenced using exoglycosidases and acetolysis. The phosphatidylinositol moiety was analysed by fast-atom bombardment mass spectrometry and gas chromatography–mass spectrometry. Taken together the data suggest the structure, Thr-Asp-ethanolamine-PO_4-Manα1-2Manα1-6Manα1-4GlcN-(*sn*-1-*O*-alkyl-2-*O*-acylglycerol-3-PO_4-1-*myo*-D-inositol), which contains an additional ethanolamine phosphate group at an unknown position.

INTRODUCTION

Glycosylphosphatidylinositol (GPI) membrane anchors serve to attach certain proteins to the outer leaflet of the plasma membrane or to the topologically equivalent lumenal face of secretory granules. GPI anchors appear to be ubiquitous among eukaryotes. They have been implicated in protective functions in the parasitic protozoa and, in mammalian cells, in intracellular protein targeting, potocytosis and signal transduction. Recent reviews on the structure, biosynthesis and function of GPI anchors can be found in Anderson et al. (1992), Brown (1992, 1993), Englund (1993) and McConville and Ferguson (1993).

Alkaline phosphatase was the first protein shown to be released from plasma membranes by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) (Ikezawa et al., 1976; Low and Finean, 1977). Human placental alkaline phosphatase (PLAP) is synthesized by a number of cell lines established from placenta and carcinoma. The involvement of GPI in the attachment of PLAP to the plasma membrane was established by metabolic labelling experiments with these cultured cells (Howard et al., 1987; Jemmerson and Low, 1987; Takami et al., 1988) and by chemical analysis of purified PLAP (Ogata et al., 1988; Micanovic et al., 1988).

In the present paper we describe the determination of the structure of the GPI anchor from human PLAP. This is the second detailed description of a human GPI structure, the other example being from human erythrocyte acetylcholinesterase (Roberts et al., 1988a,b; Deeg et al., 1992).

MATERIALS AND METHODS

Reagents

All solvents and general reagents were obtained from BDH Merck. Jack bean α-mannosidase was from Boehringer and *Aspergillus phoenicis* α-mannosidase was from Oxford Glycosystems.

Purification of human PLAP

The membrane form of PLAP was purified from human term placentas obtained within 2 h of delivery. Crude microsomes prepared from the placentas were suspended at about 10 mg/ml protein in 50 mM Tris/HCl, pH 8.5, mixed with 0.33 vol. of butan-1-ol and centrifuged. PLAP was purified from the aqueous phase (in the presence of 0.1 % Triton X-100) by concanavalin A–Sepharose affinity chromatography and Sephacryl S-300 gel-filtration and immunoaffinity chromatography on anti-PLAP–Sepharose, as previously described (Miki et al., 1986; Ogata et al., 1988). The resulting PLAP preparations contained a single 67 kDa polypeptide when analysed by SDS/PAGE.

Formation of the GPI-peptide

Pronase digestion of the alkaline phosphatase was performed at 37 °C for 16 h in 100 mM NH_4HCO_3, containing 0.1 % (v/v) Triton X-100 and 2 % (w/w) Pronase. Pronase was added from a freshly prepared 10 mg/ml solution in 10 mM calcium acetate. A further 0.2 % (w/w) portion of Pronase was added and the digestion was continued for a total of 24 h. The Pronase digest was extracted with an equal volume of water-saturated butan-1-ol, centrifuged and the butanol-saturated aqueous phase was subjected to octyl-Sepharose chromatography. The octyl-Sepharose 4B column (1 cm × 7 cm) was pre-equilibrated with 5 col. vol. of 5 % propan-1-ol in 100 mM ammonium acetate, pH 5.5. The sample was applied to the column at a flow rate of 2 ml/h and eluted with 10 ml of starting buffer. The flow rate was increased to 30 ml/h and the column was eluted with a linear gradient to 60 % propan-1-ol; 1 ml fractions were collected. To locate the elution position of the GPI-peptide, 2 μl samples of the fractions were spotted on to a silica Si60 h.p.t.l.c. plate (Merck), dried and stained for carbohydrate using an orcinol reagent [0.2 % (w/v) orcinol in water/ethanol/H_2SO_4 (1:15:2, by vol.), developed at 100 °C for 5 min]. The GPI-peptide was eluted in 37 % propan-1-ol, and the relevant fractions were pooled, dried

Abbreviations used: f.a.b.-m.s., fast-atom bombardment mass spectrometry; g.c.-m.s., gas chromatography–mass spectrometry; GPI, glycosylphosphatidylinositol; h.p.a.e.c., high-performance anion-exchange chromatography; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PLAP, placental alkaline phosphatase; TMS, trimethylsilyl; AHM, 2,5-anhydromannitol.

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and digested with 4 ml of papain (84 units/ml in 0.1 M sodium acetate, pH 6.0, 1 mM dithiothreitol, 1 mM EDTA and 0.05% *n*-octylglucopyranoside) for 24 h at 37 °C. The reaction was stopped by boiling (5 min), and the digest was extracted three times with equal volumes of toluene, followed by three times with equal volumes of butanol saturated with water. The aqueous phase was acidified to pH 5.0 with acetic acid and reapplied to the octyl-Sepharose column. The GPI-peptide-containing fractions were detected with orcinol, pooled and dried.

Composition analysis

The *myo*-inositol content of samples was measured after strong acid hydrolysis and trimethylsilyl (TMS) derivatization, using a Hewlett–Packard 5890-MSD gas chromatography–mass spectrometry (g.c.-m.s.) system and selected ion monitoring (Ferguson, 1992). Monosaccharide and lipid contents were measured by g.c.-m.s. after methanolysis and TMS derivatization (Ferguson, 1992). Amino acids, ethanolamine and glucosamine were analysed after strong acid hydrolysis and derivatization with phenylisothiocyanate, followed by separation using the Waters Pico-Tag system (Schneider et al., 1990; Ferguson, 1992).

Preparation of the radiolabelled neutral glycan

All the methods used are described in Ferguson (1992) unless otherwise stated. The neutral glycan was formed from the GPI-peptide by dephosphorylation using aq. 50% HF, followed by HNO_2 deamination and NaB^3H_4 reduction. The resulting radiolabelled neutral glycan was repurified by descending paper chromatography on Whatman 3 MM paper with butanol/ethanol/water (4:1:0.6, by vol.) as the mobile phase. The labelled neutral glycan was recovered from the origin by elution with water, dried and applied to a Bio-Gel P4 column, with a set of glucose oligomer internal standards, and eluted with water. The elution position (hydrodynamic volume) of the radiolabelled neutral glycan was expressed in glucose units by linear interpolation of its elution position between adjacent glucose oligomer peaks. The glycan was recovered and analysed by Dionex high-performance anion-exchange chromatography (h.p.a.e.c.), again with a set of glucose oligomer internal standards. The elution position of the radiolabelled neutral glycan was expressed in Dionex units by linear interpolation of its elution position between adjacent glucose oligomer peaks.

Microsequencing of the glycan core

The neutral-glycan fraction recovered from Dionex h.p.a.e.c. was split into three portions, dried and subjected to jack bean α-mannosidase digestion, *Aspergillus phoenicis* α-mannosidase digestion and acetolysis. The products were reanalysed by Dionex h.p.a.e.c. Jack bean α-mannosidase (30 μl at 25 units/ml) and *Aspergillus phoenicis* α-mannosidase (10 μl at 1 unit/ml) digestions were performed in 0.1 M sodium acetate buffer, pH 5.0, at 37 °C for 18 h. The products were passed over AG50 X12 (H^+), dried and residual acetic acid was removed by coevaporation with toluene. Acetolysis was performed as described by Ferguson (1992).

Location of the ethanolamine phosphate bridge

The GPI-peptide (2.5 nmol) was deaminated at 60 °C, by the addition of both 10 μl of 0.1 M sodium acetate (pH 4.0) and 10 μl of 0.5 M sodium nitrite (three times at 1 h intervals). The released phosphatidylinositol component was recovered by three extractions with 100 μl of butan-1-ol saturated with water and the aqueous phase was adjusted to pH 10.5 with 10 μl of 0.8 M boric acid and 2 M NaOH. Reduction was started immediately with 5 μl of 36 mM NaB^3H_4. After 1.5 h the reduction was completed by the addition of 50 μl of 1 M NaB^2H_4 and further incubation for 2 h. Excess reductant was destroyed by the addition of acetic acid. The products were desalted by passage through 0.5 ml of AG50 X12 (H^+), coevaporation with methanol (twice with 0.25 ml of 5% acetic acid in methanol and twice with 0.25 ml of methanol) and repurified by paper chromatography as described above. The deaminated/reduced GPI-peptide was treated with jack bean α-mannosidase, followed by dephosphorylation with aq. 50% HF. The product was analysed by Dionex h.p.a.e.c., recovered, desalted and subjected to further digestion with jack bean α-mannosidase. The product of this second digestion was reanalysed by Dionex h.p.a.e.c.

Fast-atom bombardment-mass spectrometry (F.a.b.-m.s.)

Negative-ion f.a.b.-m.s. of the phosphatidylinositol (PI) fraction was carried out using a VG Analytical 70-250SE mass spectrometer operated at an accelerating voltage of 7 kV. The ionizing beam was generated using an Ion Tech FAB gun, operated at 8 kV and 1 mA, with xenon as the bombarding gas. Scans (20 s/decade) were recorded on a VG 11-250 data system. The sample was resuspended in 10 μl of 40% propan-1-ol and 1 μl was applied to the probe using *m*-nitrobenzyl alcohol as the matrix.

RESULTS

The analytical strategy used is shown in Scheme 1. Membrane-form human PLAP was digested exhaustively with Pronase. Amino acid analysis of the GPI-peptide fragment, recovered by octyl-Sepharose chromatography, revealed the presence of a substantial peptide component (results not shown). This material was re-digested with papain and rechromatographed on octyl-Sepharose. Compositional analysis of this GPI-peptide preparation indicated that it contained only the C-terminal dipeptide (Thr-Asp), together with the GPI-anchor components *myo*-inositol, ethanolamine, mannose, glucosamine and mono-alkylglycerol (Table 1).

The GPI-peptide was dephosphorylated with aq. 50% HF, deaminated and reduced with NaB^3H_4. The resulting radiolabelled neutral-glycan fraction was analysed by Bio-Gel P4 gel filtration and Dionex h.p.a.e.c. A single glycan species was observed at 4.2 glucose units and 2.4 Dionex units respectively. These chromatographic properties are identical with those described for an authentic standard of Manα1-2Manα1-6Manα1-4AHM (where AHM is 2,5-anhydromannitol) (Ferguson, 1992). This sequence was confirmed for the alkaline phosphatase GPI neutral glycan by exoglycosidase and acetolysis digestions and reanalysis by Dionex h.p.a.e.c. (Table 2 and Scheme 1).

A further portion of the GPI-peptide was deaminated and reduced with NaB^3H_4. This deaminated/reduced GPI-peptide was digested with jack bean α-mannosidase and then dephosphorylated with aq. 50% HF. The product on Dionex h.p.a.e.c. was shown to be the 2.4 Dionex unit Manα1-2Manα1-6Manα1-4AHM species. This dephosphorylated material could be fully digested with jack bean α-mannosidase (Table 2). These results indicate that the non-reducing terminal α-mannose residue was originally resistant to the jack bean α-mannosidase as a result of substitution with a phosphoryl moiety (Scheme 1).

After methanolysis and TMS derivatization, the lipid components 1-*O*-($C_{16:0}$)alkyl-glycerol-TMS_2 and 1-*O*-($C_{18:0}$)alkyl-

Scheme 1 Reactions used to determine the PLAP GPI anchor structure

JBAM indicates digestion with the broad-specificity jack bean α-mannosidase. APAM indicates digestion with the *Aspergillus phoenicis* Manα1-2Man-specific α-mannosidase. Ac_2O indicates cleavage by Manα1-6Man-selective partial acetolysis. AHM* is 2,5-anhydro[1-^{3}H]mannitol.

Table 1 Composition of PLAP relative to *myo*-inositol

+, Detected but not quantified.

Compound	Molar ratio
myo-Inositol	1.0
Mannose	1.1
Ethanolamine	2.4
Glucosamine	1.5
Asx	1.2
Thr	1.2
C_{16}-1-*O*-monoalkylglycerol	+
C_{18}-1-*O*-monoalkylglycerol	+
$C_{18:0}$ fatty acid	+
$C_{18:1}$ fatty acid	+

Table 2 Summary of treatments performed on the glycan core

HF, aq. HF dephosphorylation; APAM, *Aspergillus phoenicis* α-mannosidase digestion; Ac_2O, partial acetolysis; JBAM, jack bean α-mannosidase digestion.

Order of treatment							
HF	APAM	Ac_2O	JBAM	HF	JBAM	Product	Dionex value (Dionex units)
+	−	−	−	−	−	Man_3-AHM	2.4
+	+	−	−	−	−	Man_2-AHM	2.1
+	−	+	−	−	−	Man_1-AHM	1.1
+	−	−	+	−	−	AHM	1.0
−	−	−	+	+	−	Man_3-AHM	2.4
−	−	−	+	+	+	AHM	1.0

glycerol-TMS_2 were observed by g.c.-m.s. [characteristic ions at m/z 205 and $(M-15)$ and $(M-90)$ were recorded]. The PI moiety released by HNO_2 deamination was recovered by butan-1-ol extraction and analysed using negative-ion f.a.b.-m.s. (Figure 1). Taking into account the g.c.-m.s. data, the two major $(M-H)^-$ pseudo-molecular ions at m/z 823 and 849 are consistent with PI species of 1-*O*-($C_{16:0}$)-alkyl-2-*O*-$C_{18:0}$)-acylglycero-3-phosphoinositol and 1-*O*-($C_{18:0}$)-alkyl-2-*O*-($C_{18:1}$)-acylglycero-3-phosphoinositol respectively.

DISCUSSION

The membrane-form PLAP preparation was found to be relatively stable to Pronase digestion, but it could be digested with papain to yield a GPI-dipeptide fragment. However, from 117 nmol of PLAP, only 27 nmol of GPI-dipeptide were re-

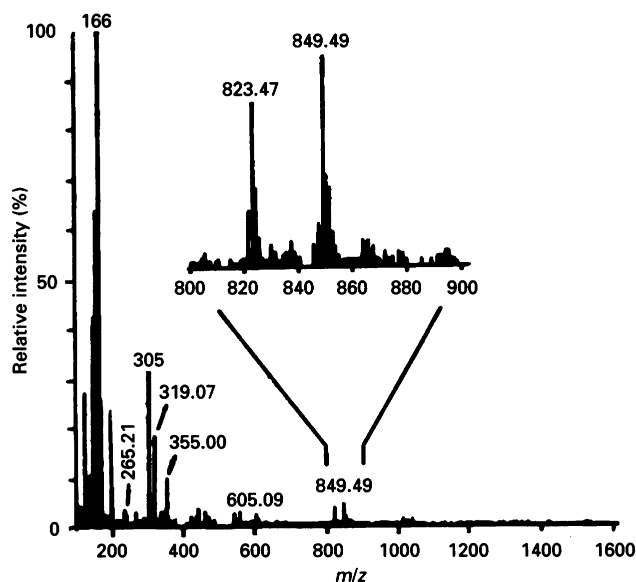

Figure 1 Negative-ion f.a.b.-m.s. spectrum of the PI moiety released on HNO_2 deamination of the PLAP GPI-peptide

The inset shows the *m/z* 800 to *m/z* 900 range in greater detail. All of the ions, except *m/z* 823 and *m/z* 849, were observed in the negative ion spectrum of a blank control sample taken through the deamination and extraction procedure.

covered from the octyl-Sepharose column. The remainder was found (by inositol analysis) to be associated with a precipitate, suggesting that the low yield was due to the poor solubility of the membrane-form PLAP.

The data described here are in good agreement with those obtained by preliminary compositional analysis (Ogata et al., 1988) and suggest that the GPI anchor of PLAP has the structure shown in Figure 1. The g.c.-m.s. compositional data, on the GPI-peptide, and f.a.b.-m.s. data, on the isolated PI moiety, suggest that the anchor contains a 1-*O*-alkyl-2-*O*-acylglycerol moiety. The stereochemistry of the glycerol and *myo*-inositol components may be inferred from the sensitivity of the alkaline phosphatase GPI anchor to *Bacillus thuringiensis* PI-PLC (Low et al., 1991). Thus the phospholipid component may be described as an *sn*-1-alkyl-2-acyl-3-PO_4-1-D-*myo*-inositol. Alkylacyl-PI species are quite common in GPI anchors and they have been previously identified in bovine and human erythrocyte acetylcholinesterase (Roberts et al., 1988a), human erythrocyte decay-accelerating factor (Walter et al., 1992), human folate-binding protein (Luhrs and Slomiany, 1989; Lee et al., 1992) and *Leishmania major* promastigote surface protease (Schneider et al., 1990).

The release of PI by HNO_2 deamination and the concomitant generation of 2,5-anhydromannose (reduced to 2,5-anhydromannitol) is consistent with the existence of a glycosidic linkage between the glucosamine residue and the *myo*-inositol ring. The neutral glycan obtained after dephosphorylation, deamination and reduction was defined as Manα1-2Manα1-6Manα1-4AHM. Taken together these data define the GPI glycan structure as Manα1-2Manα1-6Manα1-4GlcN-*myo*-inositol. This structure is identical with the minimum conserved GPI-core structure found in all GPI anchors to date (McConville and Ferguson, 1993). The linkage between the GlcN and the inositol ring has not been deduced in this study but, by analogy with other examples, it is likely to be GlcNα1-6-*myo*-inositol.

In common with all other higher-eukaryote GPI structures, the alkaline phosphatase anchor contains more than one ethanolamine residue (Homans et al., 1988; Walter et al., 1990; Deeg et al., 1992; Kamitani et al., 1992; Puoti and Conzelmann, 1992). The compositional data suggest that alkaline phosphatase contains two ethanolamine residues. One of these will constitute the ethanolamine phosphate bridge to the polypeptide (located on the non-reducing terminal α-mannose residue). The location of the second residue is unknown, but it is most likely present as ethanolamine phosphate linked to the 2-position of the reducing terminal α-mannose residue, as has been observed for the rat brain Thy-1 GPI anchor (Homans et al., 1988).

In summary, the PLAP GPI anchor is relatively simple in structure and almost identical with that described for human erythrocyte acetylcholinesterase (Deeg et al., 1992). These structures lack the sialic acid and/or galactose and/or *N*-acetylgalactosamine carbohydrate side chains found in the GPI anchors of other mammalian proteins from rat brain (Homans et al., 1988), hamster brain (Stahl et al., 1992) and pig and human kidney (Brewis et al., 1993).

Note added in proof (received 21 July 1994)

The GPI structure of another human glycoprotein, CD59, has been published recently (Nakano et al., 1994).

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