Structures of the Glycosyl-phosphatidylinositol Anchors of Porcine and Human Renal Membrane Dipeptidase

COMPREHENSIVE STRUCTURAL STUDIES ON THE PORCINE ANCHOR AND INTERSPECIES COMPARISON OF THE GLYCAN CORE STRUCTURES

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The glycan core structures of the glycosyl-phosphatidylinositol (GPI) anchors on porcine and human renal membrane dipeptidase (EC 3.4.13.19) were determined following deamination and reduction by a combination of liquid chromatography, exoglycosidase digestions, and methylation analysis. The glycan core was found to exhibit microheterogeneity with three structures observed for the porcine GPI anchor: Man\(_a\)-1\(\rightarrow\)2Man\(_a\)-1\(\rightarrow\)6Man\(_a\)-1\(\rightarrow\)4GlcN (29\% of the total population, Man\(_a\)-1\(\rightarrow\)2Man\(_a\)-1\(\rightarrow\)6(GalNAC\(_b\)-1\(\rightarrow\)4)Man\(_a\)-1\(\rightarrow\)4GlcN (33\%), and Man\(_a\)-1\(\rightarrow\)2Man\(_a\)-1\(\rightarrow\)6(Gal\(_\beta\)-1\(\rightarrow\)3GalNAC\(_b\)-1\(\rightarrow\)4)Man\(_a\)-1\(\rightarrow\)4GlcN (38\%). The same glycan core structures were also found in the human anchor but in slightly different proportions (25, 52, and 17\% respectively). Additionally, a small amount (6\%) of the second structure with an extra mannose \(\alpha\)-linked to the non-reducing terminal mannose was also observed in the human membrane dipeptidase GPI anchor. A small proportion (maximally 9\%) of the porcine GPI anchor structures was found to contain sialic acid, probably linked to the GalNAC residue. The porcine GPI anchor was found to contain 2.5 mol of ethanolamine/mol of anchor. Negative-ion electrospray-mass spectrometry revealed the presence of exclusively diacyl-phosphatidylinositol (predominantly distearoyl-phosphatidylinositol with a minor amount of stearyl-palmitoyl-phosphatidylinositol) in the porcine membrane dipeptidase anchor. Porcine membrane dipeptidase was digested with trypsin and the C-terminal peptide attached to the GPI anchor isolated by removal of the other tryptic peptides on anhydrotrypsin-Sepharose. The sequence of this peptide was determined as Thr-Asn-Tyr-Gly-Tyr-Ser, thereby identifying the site of attachment of the GPI anchor as Ser\(^{368}\). This work represents a comprehensive study of the GPI anchor structure of porcine membrane dipeptidase and the first interspecies comparison of mammalian GPI anchor structures on the same protein.

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Glycosyl-phosphatidylinositol (GPI)\(^1\) membrane anchors are present in organisms at most stages of eukaryotic evolution, including protozoa, yeast, slime molds, invertebrates, and vertebrates, and are found on a diverse range of proteins. They are primarily responsible for the anchoring of cell-surface proteins in the plasma membrane and may be considered as an alternative to the hydrophobic transmembrane polypeptide anchor of integral membrane proteins. Many other functions have been proposed for GPI anchors, including roles in intracellular sorting, transmembrane signaling, and the novel endocytic process of potocytosis. A GPI anchor might also allow the protein to associate in membrane microdomains or be selectively released from the cell-surface by phospholipases. These functions, as well as the structure, biosynthesis, and distribution of GPI anchors have been extensively reviewed (Ferguson and Williams, 1988; Cross, 1990; Thomas et al., 1990; Ferguson, 1991; Ferguson 1992a; Englund, 1993; McConville and Ferguson, 1993).

Although over 100 examples of GPI-anchored proteins have been described very few GPI anchor structures have been characterized in detail. To date partial or complete structures have been described in a variety of protozoal proteins, including Trypanosoma brucei variant surface glycoprotein (VSG) (Ferguson et al., 1988) and procyclic acidic repetitive protein (PARP) (Field et al., 1991; Ferguson et al., 1993). Leishmania major promastigote surface protease (Schneider et al., 1990), Trypanosoma cruzi 1G7 antigen (Güther et al., 1992), and Tc51 glycoprotein (Coutu et al. 1993). They have also been characterized in yeast glycoproteins (Fankhauser et al., 1993), Dictyostelium discoideum prespore-specific antigen (Haynes et al., 1993), and Torpedo electric organ acetylcholinesterase (Mehler et al., 1993). To date eight GPI structures on mammalian proteins have been characterized: rat brain Thy-1 antigen (Homans et al., 1988), human erythrocyte acetylcholinesterase (Roberts et al., 1988a; Deeg et al., 1992), hamster brain scrapie prion protein (Stahl et al., 1992), bovine liver 5'-nucleotidase (Taguchi et al., 1994), human placental alkaline phosphatase (Redman et al., 1994), human urine CD59 (Nakano et al., 1994).

\(^1\) The abbreviations used are: GPI, glycosyl-phosphatidylinositol; AHM, 2,5-anhydromannitol; AHT, anhydrotrypsin; Du, Dionex units; ES-MS, electrospray-mass spectrometry; ETNP, ethanamine phosphate; GC-MS, gas chromatography-mass spectrometry; Gu, glucose units; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; HVE, high voltage electrophoresis; MDP, membrane dipeptidase; NANA, N-acetyleneuraminic acid; NCAM, neural cell adhesion molecule; PARP, procyclic acidic repetitive protein; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PMAA, partially methylated alditol acetate; TMS, trimethylsilyl; TPCK, L-1-tosylamido-2-phenylethylchloromethyl ketone; VSG, variant surface glycoprotein; cpm, counts/minute.
mouse skeletal muscle neural cell adhesion molecule (NCAM) (Mukasa et al., 1995), and human spleen CD52 (Treumann et al., 1995) and these are detailed in Fig. 1. From these studies it is apparent that the GPI anchor consistsof a highly conserved core structure of ethanolamine-P(O)\_4-
\(6\text{Man}_1-2\text{Man}_1-6\text{GlcNH}_2\alpha1-6\text{myo-inositol}-1\)-P(O)\_4-lipid. Often attached to this conserved core are variable side chains which may be protein-, tissue-, and/or species-specific (McConville and Ferguson, 1993). Examples include an \(\alpha\)-galactose branch on some \(T.\) brucei VSG molecules and additional \(\alpha\)-mannose residue(s) on a number of structures (including yeast, slime mold and mammalian glycoproteins, and \(T.\) cruzi 1G7 antigen). A single sialic acid (\(N\)-acetylneuraminic acid, NANA) residue is present on a proportion (30%) of the scrapie prion protein anchors (Stahl et al., 1992) while an average of five sialic acid residues are found on the \(T.\) brucei PARP anchor (Ferguson et al., 1993). In contrast to lower eukaryotic GPI anchor structures, all metazoan GPI structures studied to date contain at least one additional ethanolamine phosphate residue, although the exact position(s) and linkages have only been determined in rat brain Thy-1 and human erythrocyte acetylcholinesterase (Homans et al., 1988; Roberts et al., 1988a; Deeg et al., 1992). The function, if any, of side chain modifications in general remains obscure, although the \(\alpha\)-galactose branch in VSG has been suggested to be involved in the dense packing of the protective surface coat of the trypanosome (Homans et al., 1989).

From the lipid moieties that have so far been determined it appears that this part of the anchor structure can be quite variable. They range from ceramide in most yeast glycoproteins (Fankhauser et al., 1993) and slime mold pre-spore antigen (Haynes et al., 1993), to sn-1-alkyl-2-acylglycerols in \(T.\) cruzi 1G7 antigen (Heise et al., 1995), human erythrocyte acetylcholinesterase (Roberts et al., 1988a, 1988b), human placental alkaline phosphatase (Redman et al., 1994), and several other mammalian GPI-anchored proteins (reviewed in McConville and Ferguson, 1993), sn-1,2-diacylglycerols in \(T.\) brucei VSG (Ferguson et al., 1985), Torpedo acetylcholinesterase (Butikofer et al., 1990) and human CD52 (Treumann et al., 1995), and an unusual sn-1-acyl-2-lyso-glycerol in \(T.\) brucei PARP (Field et al., 1991). In addition, several GPI anchors contain an addi-

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**Fig. 1. Structures of known mammalian GPI anchors.** The conserved core region contains an extra ethanolamine phosphate (EtNP) in all mammalian structures. Various side chain modifications of carbohydrate, additional EtNP, and/or palmitate (\(R_1-R_4\)) are found as indicated (see text for references except human and porcine membrane dipeptidase; this study). In some proteins certain residues may only be present in a proportion of GPI anchors, and this is indicated by ±. OH indicates that no modification is thought to be present. \(R_5\) = lipid moiety present. n.d. = not determined.
tional fatty acid (palmitate) in ester linkage to the myo-inositol ring. Examples include human erythrocyte acetylcholinesterase (Roberts et al., 1988b), T. brucei PARP (Field et al., 1991; Ferguson, 1992c) and one form of human CD52 (Treumann et al., 1995). The latter study showed that the modification is at the 2-position of the myo-inositol ring.

The mRNAs of known GPI-anchored proteins encode an N-terminal signal sequence, to direct the protein to the endoplasmic reticulum, and a C-terminal GPI attachment signal sequence. This sequence is believed to hold the nascent protein in the membrane prior to host anchorage and is cleaved post-translationally with the concomitant addition of the GPI anchor. The anchor attachment site (ω) may be one of six amino acid residues, all of which have small side chains (Ala, Asn, Asp, Cys, Gly, or Ser) (Moran et al., 1991; Gerber et al., 1992). In addition, the residue at the ω + 2 position is restricted to Ala, Gly, or Ser (Gerber et al., 1992).

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Membrane dipeptidase (MDP, dehydropeptidase-I, renal dipeptidase, microsomal dipeptidase, EC 3.4.13.19) is a zinc metalloenzyme found predominantly in the brush-border membrane of the kidney and also in the lung (for review see Keynan et al., 1995). The enzyme is capable of hydrolyzing a wide variety of dipeptides (Campbell, 1970; Armstrong et al., 1974) may have a role in the metabolism of glutathione and leukotriene D4 (Kozak and Tate, 1982; Campbell et al., 1995). The enzyme is capable of hydrolyzing a wide variety of dipeptides (Campbell, 1970; Armstrong et al., 1974) may have a role in the metabolism of glutathione and leukotriene D4 (Kozak and Tate, 1982; Campbell et al., 1995).

In the present study we have continued our molecular characterization of the GPI anchors on porcine and human MDP. The complete structure of the porcine MDP GPI anchor has been determined, including the nature of the lipid species present, the extent of sialylation, and the site of GPI anchor attachment in the polypeptide chain. In addition, we have compared the GPI anchor glycan core structures of porcine and human MDP.

EXPERIMENTAL PROCEDURES

Materials—Pig kidneys were obtained from ASDA Farm Stores, Loft-housegate, West Yorkshire, U.K., and post-mortem human kidneys were from Leeds General Infirmary or St. James’ University Hospital, Leeds, U.K. Citrullin was from Merck, Sharp and Dohme Research Laboratories, Rahway, N.J.; Soylo-inositol was from Calbiochem-Novabiochem Ltd., La Jolla, CA. Bovine serum albumin, diithiothreitol, iodoacetic acid, myo-inositol, 3- and 6-sulphate, glycerol, NaOD, and methyl 22948
of fractions for carbohydrate composition.

Carbohydrate Compositional Analysis—Compositional analysis was performed as described in Ferguson (1992b) using methanolation followed by re-N-acetylation and trimethylsilyl (TMS) derivatization. The resulting neutral sugar, hexosamine, and sialic acid derivatives were analyzed by gas chromatography-mass spectrometry (GC-MS), and silylated inositol (1 nmol) was used as an internal standard.

Methylation Analysis—Methylation analysis was carried out on the porcine neutral glycan fraction (9.5 nmol), prior to fractionation by liquid chromatography, and on the N-acetylated glycan core fraction (150 nmol). Methylation analysis was performed using a modification of the method of Ciucanu and Kerek (1984) as described in Ferguson (1992b). Analysis of the resulting partially methylated alditol acetates (PMAAs) was by GC-MS. GC-MS analyses were performed using both SE-54 and SP2380-bonded phase columns to allow detection of hexosamine PMAAs and the resolution of mannose and glucose PMAAs, respectively.

Isolation of Sialylated Glycans from the GPI Anchor of Porcine Membrane Dipeptidase—Hyphophilic porcine MDP was subjected to deamination, reduction, dephosphorylation, and desalting essentially as described in Schneider and Ferguson (1995). Briefly, MDP (8.2 mg, 175 nmol) was dissolved in 0.3 M sodium acetate, pH 4.0 (1 ml) and deaminated and reduced as described, except that only 5% of the sample (8.5 nmol) was reduced with NaBD₄ and the remainder was reduced with NaBD₃ (166.5 nmol). Following dialysis against water, both deaminated and dephosphorylated fractions were freeze-dried and the neutral glycan fraction obtained.

To measure the extent of desialylation due to the mildly acidic conditions of the aqueous HF dephosphorylation step, NaB₃H₄-reduced 3'-sialyl-lactitol (NANA₂-3Galβ1-4[L-3H]glucitol) and 6'-sialyl-lactitol (NANA₂-6Galβ1-4[L-3H]glucitol) standards (10 nmol of each) were subjected to aqueous HF treatment and N-acetylation as described above. The extent of desialylation was measured by separating the neutral desialylated product (Galβ1–3Galβ1–4[AHM and Man₁β1–2Man₁β1–2Man₁β1–4AHM) from the starting material by HVE. The neutral and acidic glycans were quantitatively eluted from the electrophoretogram and taken for scintillation counting.

Analysis of the Phosphatidylinositol Moieties of the Membrane Form of Porcine Membrane Dipeptidase—The amphiphilic membrane form of porcine MDP (2 nmol) was dissolved in 50 μl of water and extracted three times with 50 μl of butan-1-ol saturated with water, to remove any contaminating phospholipids. The sample was subsequently freeze-dried and redissolved in 15 μl of 0.3 M sodium acetate buffer, pH 4.0. Deamination was performed by adding sodium nitrite, 7.5 μl of a fresh 1 M solution, and allowing for 1 h at room temperature, followed by further deamination at 56°C with a further 15 μl of buffer and 7.5 μl of sodium nitrite solution. The released PI moieties were recovered by extraction once with 100 μl, and twice with 50 μl, of butan-1-ol saturated with water. The combined butan-1-ol phases were washed twice with 200 μl of water saturated with butan-1-ol and dried in a stream of N₂.

The samples were resolved in 100 μl chloroform/methanol (2:1) containing 1 mM NaOH and introduced in 20 μl aliquots into the electrospray source of a VG-Biotec Quattro triple-quadrupole mass spectrometer (Fisons Instruments, U.K.) at 10 μl/min via a Michrom UMA microblock HPLC system (Michrom Associates, CA). Negative ion mass spectra were recorded after optimizing the source conditions for maximal PI pseudomolecular ion response with standards of soybean PI and bovine PI. Typical conditions were: capillary voltage, 3.0 kV; high voltage lens, 0.3 kV; focus lens, 40 V; skimmer lens, 45 V. Experiments employing collision-induced dissociation tandem mass spectrometry (parent ion and daughter ion spectra) used argon in the collision cell (cell pressure 2.5 × 10⁻³ mbar). Ions were accelerated into the collision cell through a potential difference of 60 V. All data were processed using MassLynx software (Fisons Instruments, U.K.).

Distribution of the site of GPI Anchor Attachment to Porcine Membrane Dipeptidase—Porcine hydrophilic MDP (0.94 mg, 20 nmol) was redissolved with 20 μl dithiothreitol (1 h at 37°C) and carboxymethylated with 50 μl iodoacetic acid (1 h at 37°C in the dark) and trypsin-digested for 16 h at 37°C using a protein/TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin ratio of 50:1 (w/w) as described in Adachi et al. (1990b). The sample was carefully adjusted to pH 5.0 with 5% (v/v) acetic acid, and an aliquot (equivalent to 1.0 nmol) was freeze-dried and redissolved in 50 μl sodium acetate/1.2 M CaCl₂, pH 5.0 (equilibrium buffer, 0.1 ml final volume). This was applied to an anhydrotrypsin (AHT)-Sepharose column prepared as described in Ishii et al. (1983) (10 mg AHT, 3.5 ml bed volume, 1-ml void volume and pre-equilibrated in equilibrium buffer (100 ml)). The column was sealed and mixed overnight, the AHT-Sepharose was allowed to settle in the column, and the first 15 ml of the run-through fraction was collected while equilibration buffer was applied at 10 ml/h. The run-through fraction was freeze-dried and redissolved in 0.1 ml of water. This sample was subjected to reverse-phase HPLC using a μBondapak C₁₈ steel column (3.9 × 300 mm) with a UV (214 nm) detector at a flow rate of 2.0 ml/min and a 30-min gradient of 0–70% (v/v) acetonitrile in 0.08% H₃PO₄, at pH 2.5 followed by 5 min elution at the start of the gradient correspond to the major peaks were pooled, freeze-dried, and subjected to solid-phase peptide sequencing as described in Findlay et al. (1989) and Hooper et al. (1990).

**RESULTS**

Purification of Membrane Dipeptidase—The hydrophilic forms of both porcine and human MDP were purified from kidney cortex following PI-PLC solubilization to apparent homogeneity as assessed by SDS-polyacrylamide gel electrophoresis (data not shown). The apparent molecular masses were 47 and 59 kDa for the porcine and human forms, respectively, in agreement with Littlewood et al. (1987) and Hooper et al. (1990). The amphiphilic form of porcine MDP was purified following solubilization with n-octyl-β-D-glucopyranoside to apparent homogeneity with a molecular mass of 45 kDa, in agreement with Hooper and Turner (1989).

Sequencing of the GPI Glycerol Core—Neutral glycans terminating with AHM were isolated from both porcine and human MDP. An aliquot of each of these samples (1 × 10⁶ cpm, 4–5% of total radioactivity) was subjected to Dionex HPLC and each resolved peak then further chromatographed using Bio-Gel P₄ gel-filtration. The results obtained for the porcine neutral glycan fraction are illustrated and show that two peaks were resolved on Dionex HPLC (Fig. 2a, 37°C). These peaks chromatographed as a single species on Bio-Gel P₄, whereas the other was further separated into two distinct species (Fig. 2b, c). The elution positions of the three resolved neutral glycan structures (B, C, and D) on both Dionex HPLC and Bio-Gel P₄ gel-filtration, and their relative abundance, are summarized in Table I. Two of the structures (C and D) were assigned the provisional structures Man₁α1–2Man₁α1–6GalNα1–3Galβ1–4Man₁α1–4AHM and Man₁α1–2Man₁α1–6Man₁α1–4AHM based on the known chromatographic properties of other GPI neutral glycans (Ferguson, 1992b) and in the knowledge that, to date, no two neutral glycans have been found to co-elute on both Dionex HPLC and Bio-Gel P₄ gel-filtration. It was not possible to assign a provisional structure to neutral glycan structure B as a GPI glycan with these chromatographic properties has not been previously reported. The results of the human neutral glycan liquid chromatography (data not shown) were similar to those for the porcine neutral glycans, except that an additional neutral glycan structure (A), that eluted at 3.5 Du on Dionex HPLC and 6.5 Du on Bio-Gel P₄ gel-filtration, was resolved. Based on these elution positions and comparison with previously determined GPI neutral glycans this structure was provisionally assigned as Man₁α1–2Man₁α1–2Man₁α1–6-
structure B appears to differ from structure C by the presence of 1–4 linked α-Man residue and the subterminal Man residue of neutral glycan structure A were similarly defined for the human structures B, C, and D. The exoglycosidase digestions of human structure A presented in Fig. 3a were also assessed by Dionex HPLC and Bio-Gel P4 gel-filtration chromatography. The jack bean α-mannosidase digestion product eluted at 1.0 Du and 1.7 Gu. These chromatographic properties are consistent with Manα1–6Manα1–4AHM and AHM, respectively (Ferguson, 1992b). All of the exoglycosidase digestions performed in Fig. 3b on the porcine structures B and C, to the point where the structure was the same as porcine structure D, were also assessed by Dionex HPLC and Bio-Gel P4 gel-filtration chromatography, and these results were also consistent with the intermediate structures presented. Identical results were also obtained for the human structures B, C, and D. The exoglycosidase digestions of human structure A presented in Fig. 3c were also assessed by Dionex HPLC and Bio-Gel P4 gel-filtration chromatography. The jack bean α-hexosaminidase digestion product eluted at 3.0 Du and 5.1 Gu, the A. saitoi α-mannosidase digestion product eluted at 2.1 Du and 3.2 Gu, and the jack bean α-mannosidase digestion product eluted at 1.0 Du and 1.7 Gu. These results are consistent with Manα1–2Manα1–2Manα1–6Manα1–4AHM, Manα1–6Manα1–4AHM, and AHM, respectively (Ferguson, 1992b).

Methylation analysis of the total porcine neutral glycan fraction (Table II) is consistent with the exoglycosidase results described above. The presence of 4,6-di-O-substituted Man defines the linkage position of the GalNAc residue to the 4-position of the α-Man residue adjacent to the AHM. The presence of terminal-GalNAc is consistent with the presence of structure C and the presence of terminal-Gal and 3-O-substituted GalNAc confirms the presence of the Galβ1–3GalNAc branch proposed for structure B. Methylation analysis of the N-acetylated glycan core sample (Table II) showed, in addition, the presence of 4-O-substituted GlcNAc (in place of the 4-O-substituted AHM) and 6-O-substituted myo-inositol. These results further define the original GPI structures as containing GlcN1–6-myoinositol termini.

Isolation of Sialylated Glycans from the GPI Anchor of Porcine Membrane Dipeptidase—GPI glycan was prepared from porcine MDP by deamination, reduction, dephosphorylation, and N-acetylation, as described under “Experimental Procedures.” Following radiochemical decontamination the glycans (containing a radioactive tracer, \(2 \times 10^3 \text{ cpmp} \)) were applied to a QAE-Sephadex A25 (OH\(^-\)) column and eluted with a gradient of ammonium acetate. Three radioactive peaks were resolved.
(Fig. 4), freeze-dried, and each fraction was analyzed for monosaccharide composition by GC-MS. Peak 1, eluting in the column void volume, contained Man, GalNAc, and Gal (in a molar ratio of 3.0:0.9:0.4) but no sialic acid. This monosaccharide ratio is consistent with the mixture of neutral glycans B, C, and D indicated in Table I. Peak 2 was considered to be irrelevant residual radiochemical contamination since it contained only a trace of Man. Peak 3 contained Man, GalNAc, Gal, and sialic acid (in a molar ratio of 3.0:1.7:0:4:1.7). The equimolar GalNAc and sialic acid contents, and the low Gal content, suggest that the major sialylated glycan is Man\(\text{a1-2Man}\text{a1-2Man}\) (i.e. glycan C in Table II). The complete absence of fucose, a component of the complex sialylated N-linked oligosaccharides of MDP,\(^2\) suggested that peaks 1 and 3 are composed solely of GPI glycan material. The total radioactivity recovered in peaks 1-3 was 1.92 \(\times\) 10\(^5\) cpm which represented a recovery of 96% of the total radioactivity applied to the QAE-Sephadex column. The Man contents of peaks 1 and 3 were 22.6 and 10.5 nmol, respectively. From this the sialylated glycan (peak 3) were calculated to represent 4.5% of the recovered glycans. The extent of desialylation due to aqueous HF treatment was calculated to be 31 and 52% for m/z 815 and 837, and m/z 865 (Fig. 5a). The low resolution parent ion scan (for parents of the PI-specific daughter ion at m/z 241, corresponding to the inositol-1,2-cyclic phosphate fragment ion (Sherman et al., 1985) revealed that the m/z 865 parent ion was due to a PI species, whereas the m/z 815 ion was not (Fig. 5b). The nature of the m/z 837 ion was equivocal by this analysis. The mass of the major ion at m/z 865 is consistent with that predicted for the [M-H]\(^+\) pseudomolecular ion of distearoyl-PI (or any other isometric PI species). The daughter ion spectrum of m/z 865 (Fig. 5c) shows the presence of the PI-specific m/z 241 fragment ion (i.e. [inositol-1,2-cyclic phosphate]\(^-\)), together with another intense daughter ion at m/z 283. The latter ion is the carboxylate ion of stearic acid (i.e. [CH\(_3\)\(\text{CH}_2\)\(_9\)COO\(^-\)])). The absence of any other significant daughter ions shows that the parent ion contains only this fatty acid. Thus, the major PI species released from porcine MDP by nitrous acid deamination must be distearoyl-PI. From this unequivocal identification of the m/z 865 ion, the minor ion at m/z 837 (28 mass units lower) is likely to be due to a stearoyl-palmitoyl-PI species. Significantly, no m/z 885 ion, corresponding to the [M-1]\(^+\) ion of the major PI species (1-stearoyl-2-arachidonoyl-PI) of mammalian cells (Michell, 1975; Kerwin et al., 1994) was observed. This latter finding strongly suggests that there was no significant PI-phospholipid contamination of the sample.

**Identification of the PI Moieties of the Membrane Form of Porcine MDP—High resolution negative ion ES-MS analysis of the PI-containing fraction, isolated by nitrous acid deamination and butan-1-ol extraction, revealed major pseudomolecular ions at m/z 815, m/z 837, and m/z 865 (Fig. 5a). The low resolution parent ion scan (for parents of the PI-specific daughter ion at m/z 241, corresponding to the inositol-1,2-cyclic phosphate fragment ion (Sherman et al., 1985) revealed that the m/z 865 parent ion was due to a PI species, whereas the m/z 815 ion was not (Fig. 5b). The nature of the m/z 837 ion was equivocal by this analysis. The mass of the major ion at m/z 865 is consistent with that predicted for the [M-H]\(^+\) pseudomolecular ion of distearoyl-PI (or any other isometric PI species). The daughter ion spectrum of m/z 865 (Fig. 5c) shows the presence of the PI-specific m/z 241 fragment ion (i.e. [inositol-1,2-cyclic phosphate]\(^-\)), together with another intense daughter ion at m/z 283. The latter ion is the carboxylate ion of stearic acid (i.e. [CH\(_3\)\(\text{CH}_2\)\(_9\)COO\(^-\)])). The absence of any other significant daughter ions shows that the parent ion contains only this fatty acid. Thus, the major PI species released from porcine MDP by nitrous acid deamination must be distearoyl-PI. From this unequivocal identification of the m/z 865 ion, the minor ion at m/z 837 (28 mass units lower) is likely to be due to a stearoyl-palmitoyl-PI species. Significantly, no m/z 885 ion, corresponding to the [M-1]\(^+\) ion of the major PI species (1-stearoyl-2-arachidonoyl-PI) of mammalian cells (Michell, 1975; Kerwin et al., 1994) was observed. This latter finding strongly suggests that there was no significant PI-phospholipid contamination of the sample.

**Determination of the Site of GPI Anchor Attachment to Porcine Membrane Dipectidase—Porcine hydrophilic MDP was reduced, S-carboxymethylated, and trypsin-digested. SDS-polyacrylamide gel electrophoresis performed under non-reducing conditions on aliquots after each treatment confirmed that the enzyme had been successfully reduced and trypsin-digested (results not shown). Trypsin digestion selectively cleaves proteins on the C-terminal side of the basic amino acid residues Arg and Lys and the trypsin digesion of MDP therefore contained 28 peptides (determined using the cDNA-derived amino acid sequence (Rached et al., 1990)). All these peptides, except for the C-terminal peptide, have a C-terminal Arg or Lys, and this fact was exploited to isolate the C-terminal peptide by affinity chromatography using AHT-Sephadex. AHT is a catalytically inert derivative of trypsin but still retains a strong
binding affinity toward the products of trypsin digestion. The AHT-Sepharose should therefore bind all the peptides except for the C-terminal peptide (Ishii et al., 1983). Following AHT-Sepharose chromatography, the run-through fraction was subjected to reverse-phase HPLC to assess the previous step, and this revealed that the AHT-Sepharose chromatography appeared to have bound all but three of the peptides in the trypsin-digested sample as three significant peaks were pres-
Methylation linkage analyses of the neutral glycan and N-acetylated glycan core fractions of the porcine membrane dipeptidase GPI anchor

| PMAAs Origin | Peak area* |
|--------------|-----------|
| **Neutral glycan** | **N-Acetylated glycan** |
| | |
| 1,3,6-Tri-O-methyl-4-O-acetyl-2,5-anhydromannitol | 4-O-Substituted AHM | 0.4 | 0.0 |
| 2,3,4,6-Tetra-O-methyl-1,5-di-O-acetyl-mannitol | Terminal Man | 1.0 | 1.0 |
| 3,4,6-Tri-O-methyl-1,2,5-tri-O-acetyl-mannitol | 2-O-Substituted Man | 1.0 | 0.7 |
| 2,3,4,6-Tetra-O-methyl-1,5,6-tri-O-acetyl-mannitol | 6-O-Substituted Man | 0.2 | 0.4 |
| 2,3,4,6,7-Tetra-O-acetyl-1,5,6,7-tetra-O-acetyl-mannitol | 4,6-Di-substituted Man | 1.0 | 0.3 |
| 1,5-Di-acetyl-3,4,6,tri methyl 2-N-methylacetamido-2-deoxy-galactitol | Terminal Gal | 1.0 | 0.6 |
| 1,3,5-Tri-acetyl-4,6-di methyl-2-N-methylacetamido 2-deoxy galactitol | 3-O-Substituted GalNAc | 0.2 | 0.2 |
| 3,4,6-Tri-O-methyl-1,5-di-O-acetyl-2-N-acetyl amido 2-deoxy galactitol | Terminal GalNAc | 0.2 | 0.4 |
| 4,6-Di-O-methyl-1,3,5-tri-O-acetyl-2-N-methyl acetamido 2-deoxy galactitol | 3-O-Substituted GalNAc | 0.2 | 0.2 |
| 4,6-Di-O-methyl-1,3,5-tri-O-acetyl-2-N-methyl acetamido 2-deoxy galactitol | 3-O-Substituted GalNAc | 0.2 | 0.2 |
| 3,6-Di-O-methyl-1,3,5-tri-O-acetyl-2-N-methyl acetamido 2-deoxy glucitol | 4-O-Substituted GalNAc | 0.4 | 0.0 |
| 1,2,3,4,5-Penta-O-methyl-6-O-acetyl myo-inositol | 6-O-Substituted myo-inositol | 0.0 | 0.6 |

* The relative ratios of the total ion-current peak areas of each of the PMAAs are shown compared to the PMAA of terminal Man (assigned an arbitrary unit of 1.0).
The GPI anchor of Hex-HexNAc and NANA-Hex-HexNAc side chains (Stahl et al., 1992). The Galβ1–3GalNAc motif is also common in glycoprotein and mucin O-linked oligosaccharides (Carlstedt et al., 1985; Van Halbeek et al., 1988; Hokke et al., 1994), and it is possible that the same β1–3 galactosyltransferase is used to modify both O-linked GalNAc residues and GPI anchor GalNAc residues.

The possibility that some of the GPI glycans were originally sialylated was investigated by releasing the GPI anchor of porcine MDP by aqueous HF dephosphorylation under conditions that retain half to two-thirds of the sialic acid residues, depending on the linkage. The low yield of sialylated GPI glycans obtained after anion-exchange chromatography suggested that a maximum of 9% of the original GPI anchors contained sialic acid (presuming β2–6 linkage and no polysialylation). The low abundance of sialylated species, and their inherent lability to the aqueous HF conditions necessary to prepare them, prevented a detailed structural characterization. However, the composition of the sialylated fraction suggests that the presence of GalNAc is a prerequisite for sialylation whereas only a small fraction of the sialylated species contained Gal. This leads us to tentatively suggest that in the case of the porcine MDP GPI anchor most, if not all, of the sialic acid is attached to the side chain GalNAc residue. The presence of sialic acid in GPI anchors has been reported only twice. In one case, that of T. brucei PARP, an average of 5 sialic acid residues/anchor were found linked β2–3 to the terminal βGal residues of branched polylactosamine side chain structures (Ferguson et al., 1993). In the other example, that of hamster brain scrapie prion protein, about 30% of the anchors were found to be monosiallylated with the sialic acid attached to a Hex-HexNAc side chain, predominantly in the form NANA-
The porcine MDP was shown to contain 2.5 mol of ethanolamine/mol of GPI anchor indicating that, in addition to the ethanolamine phosphate bridge, there is an extra ethanolamine moiety in the GPI anchor and, probably in half the structures, a third ethanolamine phosphate residue is present. The presence of at least one extra ethanolamine phosphate in the GPI anchors of bovine liver 5′-nucleotidase (only 3.5% of total population) (Taguchi et al., 1994), Torpedo acetylcholinesterase in 10–20% of the structures. Two additional ethanolamine phosphates are also thought to occur in a proportion of the GPI anchors of bovine liver 5′-nucleotidase (up to 30% by compositional analysis) (Mehlert et al., 1993), human placental alkaline phosphatase (up to 40% by compositional analysis) (Redman et al., 1994), and human CD52 (40% of the total population) (Treumann et al., 1995). The presence of two additional ethanolamine phosphate residues was not found in rat brain Thy-1 nor hamster brain scrapie prion protein suggesting that this is not a ubiquitous feature of all metazoan GPI structures (Homans et al., 1988; Stahl et al., 1992). The presence of this third ethanolamine phosphate in about half of the porcine MDP anchors would cause microheterogeneity of charge, in addition to the microheterogeneity of the carbohydrate residues.

In general, the GPI PI moieties are substantially different from the cellular pool of PI phospholipids (McConville and Ferguson, 1993). For example, several of the mammalian GPI anchors contain exclusively alkylacyl-PIs (Roberts et al., 1988b; Walter et al., 1990; Redman et al., 1994) (see Fig. 1) as opposed to sn-1-stearoyl-2-arachidonoyl-PI that is the predominant cellular PI species in these organisms (Michell, 1975; Kerwin et al., 1994). In the case of porcine MDP, the predominant PI moiety is a diaclyl-PI (diacyl-PI). Although diacyl-PI moieties are quite common in non-mammalian GPI anchors (e.g. in Torpedo acetylcholinesterase (Bültkof er et al., 1990), T. brucei VSG (Ferguson et al., 1985), Saccharomyces cerevisiae gp125 (Fankhauser et al., 1993), and human CD52 (Truemann et al., 1995)), the presence of predominantly diacyl-PI has only been reported previously for one of the two forms of human CD52 (Truemann et al., 1995). The only other example of a porcine GPI anchor diacyl-PI that contains predominantly one type of acyl chain is that of T. brucei VSG. The dimyristoyl-PI moiety of the VSG anchor is produced by a process of fatty acid remodeling (Masterson et al., 1990), whereby the original heterogeneity in the PI moiety (Doering et al., 1994) is removed by sequentially replacing the sn-2 and sn-1 fatty acids with myristate. While most mammalian GPI intermediates and precursors contain alkylacyl-PIs, some contain diacyl-PIs (Puoti and Conzelmann, 1993). Thus, it is possible that some kind of analogous fatty acid remodeling may occur on the diacyl-PI containing GPI intermediates in the cells producing CD52 and porcine MDP. Alternatively, these cells may simply select for diacyl-PI as the precursor for the GPI intermediates that will be transferred to these proteins.

The results of the trypsin digestion of porcine MDP and isolation of the C-terminal peptide on AHT-Sepharose identified the site of GPI anchor attachment as Ser368. Thus the mature protein lacks the predominantly hydrophobic C-terminal sequence of 25 residues predicted from the cDNA (Rached et al., 1990). The Ser anchor attachment site (ω) has a pair of Ala residues immediately C-terminal to it. This is consistent with the proposals of Gerber et al. (1992), who have found that the N-terminal residue C-terminal to the site of anchor attachment (ω + 2 position) is generally restricted just to Ala, Gly, or Ser. Also displayed in Fig. 6 is the cDNA-derived protein sequence for human MDP and the previously determined GPI anchor attachment site of Ser369 (Adachi et al., 1990b). The human and porcine proteins are 80% homologous and, from sequence alignment studies, it appears that in the porcine MDP either Ser368 or Ser369 has been mutationally deleted. However, it is impossible to predict from the cDNA codon information which Ser is absent. Whatever the case both the enzymes utilize Ser in the same part of the protein sequence for GPI anchor attachment and have Ala at the ω + 2 position.

In conclusion, we have determined the glycine core structures of the GPI anchors on porcine and human MDP, representing the first interspecies comparison of mammalian GPI anchor structures on the same protein. The glycine core structures were remarkably similar, with three major structures observed in approximately equal amounts: Man1–2Man1–6Man1–4GlcN, Man1–2Man1–6GALNAc1–4Mann–1–4GlcN, and Man1–2Man1–6Galβ1–3GalNAc1–4Man1–4GlcN. Also the GPI anchor on porcine MDP was found to be anchored to the protein at Ser368 to contain 2.5 mol of ethanolamine/mole of anchor, and almost exclusively distearoyl-PI. In addition, a small proportion of the porcine GPI structures were shown to contain sialic acid residues, probably attached to GaINAc.
Structures of the Glycosyl-phosphatidylinositol Anchors of Porcine and Human Renal Membrane Dipeptidase: COMPREHENSIVE STRUCTURAL STUDIES ON THE PORCINE ANCHOR AND INTERSPECIES COMPARISON OF THE GLYCAN CORE STRUCTURES
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