A β-N-Acetylg glucosaminyl Phosphate Diester Residue Is Attached to the Glycosylphosphatidylinositol Anchor of Human Placental Alkaline Phosphatase

A TARGET OF THE CHANNEL-FORMING TOXIN AEROLYSIN*

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Glycosylphosphatidylinositol (GPI)-anchored proteins are ubiquitous in eukaryotes. The minimum conserved GPI core structure of all GPI-anchored glycopeptides has been determined as EtN-PO₄-6Man₁–4GlcN-myo-inositol-PO₄-H. Human placental alcaline phosphatase (AP) has been reported to be a GPI-anchored membrane protein. AP carries one N-glycan, (NeuAcα₂–3)GalαβGlcNAc-M₃Man₃GlcNAc(±Fuc)GlcNAc, and a GPI anchor, which contains an ethanolamine phosphate diester group, as a side chain. However, we found that both sialidase-treated soluble AP (sAP) and its GPI-anchored glycan bound to a PVL-Sepharose column, which binds β-GlcNAc residues. PVL binding of asialo-sAP and its GPI-anchored glycan was diminished by digestion with diplococcal β-N-acetylg glucosaminidase or by mild acid treatment. After sequential digestion of asialo-sAP with β-N-acetylg glucosaminidase and acid phosphatase, the elution patterns on chromatofocusing gels were changed in accordance with the negative charges of phosphate residues. Trypsin-digested sAP was analyzed by liquid chromatography/electrospray ionization mass spectrometry, and the structures of two glycopeptides with GPI-anchored glycan were confirmed as peptide-EtN-PO₄-6Man₁–2(GlcNAcβ₁–PO₄-6)Man₁–6(±EtN-PO₄)–Man₁–4GlcN, which may be produced by endo-α-glucosaminidase. In addition to AP, GPI-anchored carcinoembryonic antigen, cholisterase, and Tamm-Horsfall glycoprotein also bound to a PVL-Sepharose column, suggesting that the β-N-acetylg glucosaminyl phosphate diester residue is widely distributed in human GPI-anchored glycosylphosphatidylinositol. Furthermore, we found that the β-N-acetylg glucosaminyl phosphate diester residue is important for GPI anchor recognition of aerolysin, a channel-forming toxin derived from Aeromonas hydrophila.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) is a glycoprotein that is widely distributed in the plasma membrane. The isozymes derived from various mammalian organs show different enzymatic and immunoenzymechemical properties (1). Human placental alkaline phosphatase (AP) was the first protein that was identified as being released from the plasma membrane by bacterial phosphatidylinositol-specific phospholipase C (2, 3). The structures of the N-linked glycan and GPI anchor of AP have been already reported. Although the amino acid sequence indicates that AP has two potential N-glycosylation sites (4–6), only Asn(240) was suggested to be actually glycosylated (4). Endo et al. (7) reported that the structure of the N-linked glycan of AP is NeuAcα₂–3Galβ₁–4GlcNAcβ₁–2Manα₁–3(SiaNeuAcα₂–3Galβ₁–4GlcNAcβ₁–2Manα₁–6Manα₁–4GlcNAcβ₁–4(Fucα₁–6)GlcNAc, as derived by a combination of sequential exoglycosidase digestion and methylation analysis. On the contrary, the structure of the GPI-anchored glycan of AP was investigated by Redman et al. (8) and reported to be Thr-Asp-EtN-PO₄-6Man₁–2Manα₁–6Manα₁–4GlcN(s-n-1-O-alkyl-2-O-acetylglycerol-3-PO₄-1-myo-d-inositol), with an additional ethanolamine phosphate group. However, the ethanolamine phosphate had been analyzed quantitatively by amino acid analysis and metabolic labeling method (19). In this study using a combination of β-GlcNAc-specific PVL-Sepharose column chromatography, LC/ESI-MS analysis, chromatofocusing, nitrous acid deamination treatment, and periodate oxidation, we found that not only ethanolamine phosphate, but also a β-N-acetylg glucosaminyl phosphate diester (GlcNAc-P) residue is present in AP and is positioned as side chains of its GPI-anchored glycan. GlcNAc-P has not been reported previously in GPI-anchored glycans, probably because it is easily hydrolyzed by mild acid treatment.

We also investigated whether GlcNAc-P residues were asso
ciated with GPI-anchored glycans of human carcinomaembryonic antigen (CEA), cholinesterase, and Tamm-Horsfall (T-H) glycoprotein using PVL-Sepharose column chromatography. All of these glycoproteins carry both N-glycans (9–12) and GPI-anchored glycans, and most of them effectively bound to a PVL-Sepharose column. Because nonreducing terminal βGlcNAc residues were not present in their N-glycans, the GlcNAc-P residue appeared to be linked to GPI-anchored glycans as a side chain. These results suggest that the GlcNAc-P residue is widely distributed on GPI-anchored glycoproteins of the human cell membrane.

Aerolysin is a toxin released by Aeromonas hydrophila (13). Binding of aerolysin to receptors on target cells promotes receptor oligomerization, and this phenomenon is followed by membrane insertion and channel formation. Although GPI-ceptor oligomerization, and this phenomenon is followed by binding of aerolysin to receptors on target cells promotes receptor oligomerization, and this phenomenon is followed by membrane insertion and channel formation.

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EXPERIMENTAL PROCEDURES

Materials—Pronase K, trypsin, wheat germ acid phosphatase, and NaN3 were purchased from Sigma. NaB3H4 (490 mCi/mmol) was purchased from PerkinElmer Life Sciences. Diplococcal β-N-acetylhexosaminidase was purified from a culture of Diplococcus pneumoniae according to the method of Glasgow et al. (18). N-Acetylglucosamine and Arthrobacter ureafaciens N-acetylhexosaminidase (10 milliunits), or wheat germ acid phosphatase (10 μg) at 37 °C for 2 h.

Preparation of Soluble Form of AP and Its Derivatives—Non-treated and treated sAPs were separated on a Mono P HR5/20 chromatofocusing column (0.5 cm inner diameter × 9.6 cm long) at 4 °C. After the column was equilibrated with 0.025 M histidine HCl, pH 6.2, 1 μg of sAP was injected onto the column. Elution was carried out isocratically with 10 × diluted polybuffer 74 (Amersham Biosciences), pH 4.0, at a flow rate of 1 ml/min. The effluent was monitored using a spectrophotometer and enzyme-linked immunosorbent assays (ELISAs).

PVL-Sepharose Column Chromatography—PVL was purified from the fruiting bodies of Phanerochaete chrysosporium (5). To purify PVL-Sepharose, Sepheryl S-300, and hydroxylapatite columns. Mild acid-treated sAP was prepared as follows. 10 μg of sAP was incubated in 50 μl of 0.01 M HCl at 100 °C for 30 min, to hydrolyze GlcNAc-phosphodiester linkages (20), and the pH was adjusted to 7. AP treated with phosphatase-immobilized beads (Sigma) after adjusting the pH to 8. Following the application of 20 μl of 0.2 M boric acid was added, and the column was washed with 50 ml of PBS at 37 °C for 2 h. The glycoconjugates were purified by Dowex AG-50 column chromatography. In direct labeling with NaB3H4 reduction, 100 μl of NaB3H4 was added to the mixture, and this was incubated at room temperature for 2 h. Subsequently 0.2 ml of 0.2 M boric acid was added, and the mixture was reduced with NaB3[H]. [3H]-Labeled GPI-anchored glycans were purified by Dowex AG-50 column chromatography. In direct labeling with NaB3H4 reduction, 100 μl of NaB3H4 was added to the mixture, and this was incubated at room temperature for 2 h. Subsequently 0.2 ml of 0.2 M boric acid was added, and the mixture was reduced with NaB3[H]. [3H]-Labeled GPI-anchored glycans were purified by Dowex AG-50 column chromatography.

Preparation of 3H-labeled GPI-anchored Glycan from sAP by Nitrous Acid Deamination or Direct Labeling with NaB3H4—Reduction—One mg of sAP was digested with 0.1 mg of Pronase K in 100 μl of Tris-HCl buffer, pH 8.0, at 37 °C for 2 h. The glycoconjugates were purified by Dowex AG-50 column chromatography. In direct labeling with NaB3H4 reduction, 100 μl of sAP was treated with 0.1 mg of Pronase K in 50 μl of Tris-HCl buffer, pH 8.0, at 37 °C for 2 h. [3H]-Labeled GPI-anchored glycans were purified by Dowex AG-50 column chromatography.

LC/ESI-MS of Trypsin-digested sAP—One mg of sAP was digested with 0.1 mg of trypsin (sequence grade, Sigma) in 100 μl of Tris-HCl buffer, pH 8.0, at 37 °C for 2 h. LC/ESI-MS was carried out using a CapLC system (Waters Corp.) with an Xterra MS-18 column (1.0 × 50 mm; 5 μm; Waters Corp.). The eluent was 0.1% trifluoroacetic acid in acetonitrile (A pump). The flow rate was 20 μl/min, and the effluent was monitored at 210–400 nm. The gradient conditions were 5% of B for 5 min, 5–25% of B at 1%/min, and 25–60% of B at 0.5%/min. The ESI voltage was set at 3.5 kV, the desolvation gas (nitrogen) temperature was 150 °C, and the ion source block temperature was 80 °C. Two scan functions were recorded simultaneously to detect molecular masses of sAP tryptic fragments and fragment ions from sugars. The first scan function was from 70 to 2,500 atomic mass units in 2 s with the low cone voltage at 25 eV, and the second scan function was from 70 to 1,200 atomic mass units in 0.5 s with a high cone voltage of 70 eV.

Solid Phase Binding Assay—Proaerolysin was purchased from Proter Biotech (Victoria, Canada) and was biotinylated by EZ-link Sulfo-NHS-LC-biotin (Pierce) according to the manufacturer’s instructions, which was then dia lyzed against PBS. The binding of proaerolysin to AP was measured using a solid phase binding assay. ELISA plates were coated with AP at 4 μg/ml in 200 μl of PBS at pH 9.6, at 4 °C for 18 h. The plates were washed with 0.05% Tween 20 in PBS, pH 7.3, blocked with PBS containing 0.05% Tween 20 and 1% bovine serum albumin, and then treated with sample. After washing with 0.05% Tween 20 in PBS, 5 μg/ml goat anti-CEA polyclonal antibody was added and incubated for 1 h at 37 °C. After further washing, AP-conjugated rabbit anti-goat IgG antibody was added and incubated for 1 h at 37 °C. After washing, 6.7 μmol p-nitrophenylphosphoric acid disodium salt in 0.1 M carbonate buffer, pH 9.6, was added, and absorbance at 405 nm was measured with a spectrophotometer.

PVL Column Chromatography of T-H Glycoprotein—T-H glycoprotein was purified from 1 liter of pooled urine obtained from normal adults as follows (24). 0.58 mol of NaCl was added to the urine, and it was stirred at 4 °C for 18 h. The precipitate formed was collected by centrifugation, solubilized with distilled water, and dialyzed against distilled water. After centrifugation, the supernatant was freeze dried and redissolved in PBS. An aliquot of T-H glycoprotein was added to SDS-PAGE using 10% polyacrylamide gels to check the purity. It showed a single band of 80 kDa corresponding to the molecular mass of T-H glycoprotein. One mg of the purified T-H glycoprotein was applied to a PVL column, and each fractionated T-H glycoprotein was measured using a protein assay (Bio-Rad).

Chromatofocusing of sAP and Its Derivatives—Non-treated and treated sAPs were separated on a Mono P HR5/20 chromatofocusing column (0.5 cm inner diameter × 9.6 cm long) at 4 °C. After the column was equilibrated with 0.025 M histidine HCl, pH 6.2, 1 μg of sAP was injected onto the column. Elution was carried out isocratically with 10 × diluted polybuffer 74 (Amersham Biosciences), pH 4.0, at a flow rate of 1 ml/min. The effluent was monitored using a spectrophotometer and enzyme-linked immunosorbent assays (ELISAs).
Dowex AG-50 (H+)—After adding acetic acid, the reduced sample flowed through a column of buffer, pH 9.5, was added, and the mixture was left at 30 °C for 1 h, 10 mg of NaBH₄ in 500 μl of 0.1 M sodium borate buffer, pH 9.5, was added, and the mixture was left at 30 °C for 3 h. After adding acetic acid, the reduced sample flowed through a column of Dowex AG-50 (H⁺ form) (2-ml bed volume). The eluates were evaporated, and the sample was freed from boric acid by repeated evaporation with methanol. The sample was then hydrolyzed in 1 ml of 2 N HCl at 100 °C for 2 h and thoroughly dried up. The sample was radiolaabeled with NaB₃H₄ to monitor the recovery of the following steps. sAP was digested thoroughly with 1 mg of Pronase K in 100 μl of PBS at 37 °C for overnight. The glycopeptides were dried and dissolved in 100 μl of 0.05 M acetic buffer, pH 4.5, containing 0.6 μmol of sodium metaperiodate, and the mixture was kept at 4 °C for 40 h in the dark. Excess oxidant was destroyed by adding 10 μl of ethylene glycol. After standing at room temperature for 1 h, 10 mg of NaBH₄ in 500 μl of 0.1 M sodium borate buffer, pH 9.5, was added, and the mixture was left at 30 °C for 3 h. After adding acetic acid, the reduced sample flowed through a column of Dowex AG-50 (H⁺ form) (2-ml bed volume). The eluates were evaporated, and the sample was freed from boric acid by repeated evaporation with methanol. The sample was then hydrolyzed in 1 ml of 2 N HCl at 100 °C for 2 h and thoroughly dried up. The sample was radiolaabeled with NaB₃H₄ in 100 μl of 0.1 M sodium borate buffer, pH 9.5, at 30 °C for 3 h, and the excess NaB₃H₄ was consumed by 10 μl of benzaldehyde. After passing through with C18 Sep-Pak column (Waters Co.), the sample was evaporated and freed from boric acid by repeated evaporation with methanol. The oxidized oligosaccharide methanol solutions were then separated with paper electrophoresis. High voltage paper electrophoresis was performed by using pyridine/acetate buffer, pH 5.4 (pyridine/ acetic acid/water, 3:1:387), at a potential of 73 V/cm for 40 min. The acidic components were extracted and digested with 50 μl of 1 mg/ml acetic phosphatase in 0.2M citrate buffer, pH 5.0, at 37 °C overnight, then purified using ion exchange gels AG-50 and AG-3 (Bio-Rad). Then, the ascending paper chromatography was performed using the solvent butanolethanol/water (4:1:1) for 5 h. NaB₃H₄-reduced glyceraldehyde, erythrose, and mannose were used as standards. The developed paper was cut into 1-cm pieces and measured with a scintillation counter.

**RESULTS**

**PVL Column Chromatography of sAP and Its GPI-anchored Glycan**—sAP has been reported to be a GPI-anchored membrane glycoprotein and has one N-glycan, (NeuAcα2→3)₂GalβGlcNAc-Man₃GlcNAc (±Fuc)GlCNac (7), and a GPI-anchored glycan, EtN-HPO₃₋₆Man₃→2Man₁→6Man₁→4GlcN-inositol-HPO₄ which bears an ethanalamine phosphate group as a side chain (8).

In preliminary experiments, we observed that asialo-sAP bound to a PVL-Sepharose column, which interacts with β-GlCNac residues (21), and was eluted with 0.3 M GlCNac (Fig. 1A). Because all the N-glycans of asialo-sAP are galactosylated, nonreducing terminal β-GlCNac residues must be present in the GPI-anchored glycan of sAP.

To determine how the β-GlCNac residue is attached as a side chain of the GPI-anchored glycan, several analytical methods were applied. After Pronase digestion of sAP, glucosamine residues in GPI-anchored glycopeptides were deaminated by nitrous acid treatment and reduced with NaBH₄. [³H]Anhydro- mannitol was confirmed by monosaccharide composition analysis (data not shown). The [³H]labeled GPI-anchored glycopeptides were also bound to a PVL column and were eluted with 0.3 M GlCNac (Fig. 2A). The PVL binding ability of [³H]labeled GPI-anchored glycopeptides was abolished by digestion with dipeptidyl β-N-acetylamidase (Fig. 2B). Mild acid hydrolysates of [³H]labeled GPI-anchored glycopeptides also failed to bind the PVL column (Fig. 2C), suggesting that the β-GlCNac residue does not bind to GPI-anchored glycopeptides via O-glycosidic bonding. Because N-acetylglucosaminyl phosphate diesters can be easily hydrolyzed by mild acid treatment (20), the existence of a GlCNacβ1→phosphodiester residue (GlCNac-P) as a GPI-anchored glycan side chain was suggested. Because [³H]labeled GPI-anchored glycopeptides were easily adsorbed to paper or to a high performance liquid chromatography column, we investigated whether asialo-sAP itself contained a GlCNac-P residue.

**Chromatofocusing of sAP**—sAP used in this study flowed through a RCA-I-agarose column, indicating that all N-acetylactosamine moieties within its glycan are replaced by sialic acid. Purified sAP was eluted from the chromatofocusing column at pH 4.6 (peak a) and pH 4.8 (peak b), as shown in Fig. 3A. sAP forms a homodimer and contains 4 mol of sialic acid/molecule (7). Because sialidase-treated APs were eluted at pH 5.2 and 5.0 (Fig. 3, B and E), one negatively charged sialic acid corresponded to an approximate ΔpH of 0.1. Asialo- sAPs were resistant to wheat germ acid phosphatase. However, after di-
gestion with diplococcal β-N-acetyhexosaminidase, sAPs were eluted at pH 5.0 and 4.8 (Fig. 3, C and F). It was suggested that the negative charges exposed by β-N-acetyhexosaminidase digestion contribute to the delay in retention time. Subsequent to that digestion step, sAPs were digested thoroughly by wheat germ acid phosphatase and were eluted at pH 5.4 and 5.2 (Fig. 3, D and G), suggesting that they had lost their negatively charged phosphate residues. Mass spectroscopy indicated that the GPI-anchored glycan consisted of two isoforms, one with and one without an ethanolamine phosphate. Because ethanolamine phosphate diester has a single negative charge at pH 5.0, isoform b must contain both a GlcNAc-P residue and ethanolamine phosphate as side chains, whereas isoform a contains only the GlcNAc-P residue. These results suggested that β-N-acetyglucosaminyl phosphate binds to the GPI-anchored glycans of AP.

**LC/ESI-MS of Trypsin-digested sAP—**To confirm the structures of the sAP GPI-anchored glycans, LC/ESI-MS analysis was applied. Trypsin-digested sAP was analyzed by LC/ESI-MS with an Xterra MS-18 column using trifluoroacetic acid/acetonitrile as an eluent. The total ion chromatogram, which is presented in Fig. 4A, showed the respective peptides and GPI-anchored glycans corresponding to trypsin-digested sAP. Peaks e and d, at retention times 25.9 and 61.3 min, were assumed to represent peptides containing GPI-anchored glycans from the mass chromatograms monitored at m/z 79, 162, and 447 (Fig. 4, B, C, and D), which are characteristic fragment ions, HPO₂⁻high−, GlcN⁻, and (EtN-PO₄⁻high−)Man-GlcN⁻, derived from GPI anchors. From the mass spectra of peak e (Fig. 4B), the observed molecular masses of peak e were deduced as 3,033 and 3,156 Da. When the cone voltage of peak e was increased to 70 eV, several fragment ions were obtained from the GPI-anchored glycan (Fig. 4C). These were assigned as GlcN (m/z 161.9), Man-PO₄⁻EtN (m/z 286.1), PO₄ⁿ-Man-GlcN (m/z 404.1), EtN-PO₄ⁿ-Man-GlcN (m/z 447.2), and PO₄ⁿ-Man-(PO₄ⁿ-Man-GlcN (m/z 646.3). The detection of a m/z 646.3 peak indicated that β-N-acetyglucosaminyl phosphate binds to the second mannosyl residue of the core structure. Accordingly, the peptide portion of peak e should correspond to a peptide comprising Ala⁴⁶⁶⁻Asp⁴⁸⁴ (1,980 Da). Although sAP was digested with trypsin (sequence grade), peptide Ala⁴⁶⁶⁻Asp⁴⁸⁴ was suggested to be digested by a trace amount of contaminating chymotrypsin. Because other sites, which can be theoretically digested by chymotrypsin, were not digested, it is possible that the Ph₄⁺⁶₅-Ala⁴⁶⁶ site may be easily hydrolyzed by a slight chymotrypsin contamination. In fact, several charged positive ions of peak d in Fig. 4 were detected, and these were calculated to be of molecular mass 5,433 and 5,565 Da. Thus, they were assumed to represent peptide Gly⁴⁴⁶⁻Asp⁴⁸⁴ with GPI-anchored glycans carrying either GlcNAc-P or both GlcNAc-P and EtN-P as side chains, which should be theoretically released by trypsin (Fig. 4D).

**Fig. 3. Chromatofocusing of sAP sequentially treated with sialidase, β-N-acetyhexosaminidase (β-HexNAc’ase), and phosphatase.** The flow rate was 0.5 ml/min. A, isoforms a and b of sAP separated by chromatofocusing; B and E, isoforms a and b treated with Arthrobacter sialidase, respectively; C and F, diplococcal β-N-acetyhexosaminidase digests of peaks in B and E, respectively; D and G, wheat germ acid phosphatase digests of peaks in C and F, respectively.
hydrolyzed with 4 M HCl at 100 °C for 3 h. After N-acetylation, the monosaccharide composition was analyzed on an SP0810 column, and this indicated that the 3H-labeled monosaccharide corresponded to authentic N-acetylglucosaminitol (Fig. 5C). These results suggested that the reducing terminal residue of sAP is a glucosamine moiety, which may be cleaved, between glucosamine and inositol of membrane-bound AP, by endo-α-glucosaminidase.

**Periodate Oxidation of the Tritium-labeled Pronase Digest**—We determined which hydroxyl group at C-6, C-3, or C-4 of the mannose is replaced by the β-linked GlcNAc phosphate using the periodate oxidation method (25). The tritium-labeled

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**Fig. 4.** A, LC/ESI-MS of peptides from trypsin-digested sAP. Released peptides were analyzed by a ZQ mass spectrometer with an electrospray ion source. Samples dissolved in 0.1% trifluoroacetic acid were applied to an Xterra MS-18 column. The eluates were introduced into the ESI chamber at a flow rate of 2 μl/min. ESI-MS data were analyzed directly. I, total ion chromatograms; II, negative ion chromatogram at m/z 79; III, positive ion chromatogram at m/z 447. B, ESI-MS analysis of peak e in A. (M + 2H)⁺ and (M + 3H)⁺ ions were detected for each glycopeptide of 3,033 and 3,156 Da. C, ESI-MS analysis of peak e from sAP trypsin digests at 70 eV of cone voltage in positive mode. Fragment ions caused by fragmentation at glycosyl or phosphoryl linkage within the GPI structure were detected. D, ESI-MS analysis of peak d in A. (M + 3H)⁺, (M + 4H)⁺, and (M + 5H)⁺ were detected for each glycopeptide of 5,433 and 5,556 Da.
GPI-anchored glycans were periodate oxidized, and the excess oxidant was consumed by ethylene glycol. After reducing with NaBH₄, the products were hydrolyzed and then reduced with NaB₃H₄. When the [³H]-labeled components were separated by pH 5.4 paper electrophoresis phosphorylated mannitol was not detected (Fig. 5D). These results indicated that GlcNAc-P is replaced at C-6 position of the mannose residue. If GlcNAc-P is replaced at C-3 or C-4 of the mannose residue, the periodate-oxidized phosphorylated monosaccharide should be identified as phosphorylated mannitol. On the basis of the combined results of LC/ESI-MS, nitrous acid deamination, periodate oxidation, ΨVL column chromatography, chromatofocusing, and tritium labeling with NaB₃H₄ reduction, the structures of the GPI-anchored glycans derived from sAP were determined and are summarized in Fig. 6.

GlcNAc-P Residues of Several Human GPI-anchored Glycoproteins—To investigate whether GlcNAc-P residues are also contained within other GPI-anchored glycoproteins, human serum cholinesterase, CEA, and T-H glycoprotein were applied to a ΨVL column. Most of these glycoproteins effectively bound to the column (Fig. 1). Because their N-glycan structures have been determined to be mature, at least to the point of incorporating N-acetyllactosamine residues (9–12), the nonreducing terminal N-acetylglucosamine residue of these glycoproteins should be identified as phosphorylated mannitol. On the basis of the combined results of LC/ESI-MS, nitrous acid deamination, periodate oxidation, ΨVL column chromatography, chromatofocusing, and tritium labeling with NaB₃H₄ reduction, the structures of the GPI-anchored glycans derived from sAP were determined and are summarized in Fig. 6.

**Fig. 5.** ΨVL column chromatography of directly [³H]-labeled sAP (A) and its GPI-anchored glycan (B), monosaccharide composition analysis of the reducing terminal residue (C), and paper electrophoresis at pH 5.4 after periodate oxidation (D). The black arrows (a–g) show the eluted positions of authentic sugars. a, void volume; b, N-acetylmannosaminitol; c, N-acetylgalactosaminitol; d, N-acetylglucosaminitol; e, mannitol; f, glycerol; g, mannitol 6-phosphate.

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**Fig. 6.** Proposed structures of sAP isoforms a and b on chromatofocusing in Fig. 3.

GlcNAc-P Attached to GPI Anchor
In binding assays using plates coated with sAP or asialo-sAP, biotin-labeled proaerolysin bound to sAP and asialo-sAP in the same dose-dependent manner (Fig. 7, $f$ and $H_18554$, respectively). GlcNAc-P residues were released from the GPI-anchored glycan of sAP by sequential mild acid hydrolysis and acid phosphatase digestion. Mild acid hydrolysis (0.01M HCl at $100^\circ$C for 30 min) should release both GloGlcNAc of GlcNAc-P diester residues as well as sialic acids of the sialylated biantennary glycan. The binding ability of proaerolysin to mild acid-treated sAP was decreased by approximately one-third (Fig. 7, $G$). Diplococcal Glo-N-acetylhexosaminidase digestion also affected the binding ability of proaerolysin in the same manner (data not shown). Moreover, the release of GlcNAc-P residues from sAP completely abolished its binding to proaerolysin (Fig. 7, $\bullet$), although mild acid- and phosphatase-treated sAP showed the same molecular mass as that of intact sAP on SDS-PAGE (data not shown). These results suggested that the GloGlcNAc-P residue in the GPI-anchored glycan is required for its high affinity binding to proaerolysin.

The binding of proaerolysin to sAP was also assayed using the SPR method. To compare the binding of proaerolysin with intact sAP and treated sAPs, the surface densities of the immobilized sAPs were kept at $500$ RU. Proaerolysin binding profiles to sAP and its derivatives are shown at various concentrations up to 300 nM (Fig. 8). Although the binding of proaerolysin to intact sAP and asialo-sAP increased in similar dose-dependent manners (Fig. 8, $A$ and $B$), the binding to mild acid-treated sAP was decreased substantially (Fig. 8$C$), and the binding to GlcNAc-P-released sAP was extinguished (Fig. 8$D$). These results corresponded to those obtained using the plate method, and the $K_D$ value for binding of proaerolysin to sAP ($5.6 \times 10^{-8}$ M) was similar to the $K_D$ values of Thy-1, variant surface glycoprotein, and contactin reported by MacKenzie et al. (17) (Fig. 8). These results indicated that GlcNAc-P residues of GPI-anchored glycans are targets of proaerolysin.

**DISCUSSION**

A variety of membrane proteins are anchored to the cell surface via GPI (26). All GPI anchors, from yeast to mammals, have the same core structure consisting of EtN-PO$_4$-6Man$_1$–2Man$_1$–6Man$_1$–4GlcNH$_2$–6myo-D-inositol-PO$_4$ linked to a lipid moiety (27). Using a hydrogen fluoride treatment method, which cleaves both GlcNAc-phosphodiester and ethanolamine phosphodiester, Redman et al. (8) reported that AP also has the same core structure. However, we observed that asialo-AP bound to a PVL column that recognizes GloGlcNAc, even though the N-glycan structures of AP are (NeuAc$_2$–3)$_3$Gal$_3$GlcNAc$_2$Man$_3$GlicNAc$_2$–FucGlicNAc (7). Accordingly, we investigated whether the GPI glycan of AP has GloGlcNAc residues. After sequential pronase digestion and nitrous acid deamination of sAP, the GPI-anchored glycan was labeled with NaB$_3$H$_4$. The reducing terminal residue was identified as 2,5-$[^3]$H]anhydromannitol (data not shown). Because the tritium-labeled GPI-anchored glycan of sAP bound to a PVL column, a GloGlcNAc residue must be attached to this glycan. Moreover, its binding diminished after mild acid hydrolysis or...
β-N-acetylhexosaminidase digestion. If a βGlcNAc residue is attached directly to mannose residues of the core structure, it should not be hydrolyzed under mild acid conditions. It was suggested, therefore, that the βGlcNAc residue is linked to mannose via phosphate, as has been reported for lysosomal GPI-anchored glycoproteins bound to a PVL-Sepharose column of various mammalian, yeast, and fish proteins because various may be a common feature not only of human proteins but also of various mammalian, yeast, and fish proteins because various GPI-anchored glycoproteins bound to a PVL-Sepharose column and were eluted with 0.3 mM GlcNAc (data not shown).

Moreover, it was demonstrated by LC/ESI-MS analysis that sAP is cleaved between glucosamine and inositol. These results suggest that GPI-anchored AP in human placenta is not hydrolyzed by PI-phospholipase C or D but by an endo-α-glucosaminidase, although this enzyme remains to be identified. We are currently investigating whether this enzyme exists on the plasma membrane of human placental cells.

Although GPI-anchored glycans of surface glycoproteins had been reported to function as aerolysin receptors (28), the precise carbohydrate binding specificity remained unclear. We have demonstrated in this paper that the GlcNAc-P residue in the GPI-anchored glycan is a high affinity determinant for aerolysin binding. Hong et al. (29) also reported that N-glycans on GPI-anchored proteins influence aerolysin binding (29), but at least biantennary sugar chains of sAP were not bound to proaerolysin in our data. Although β-linked GlcNAc phosphate is replaced at C-6 of the mannose, and mild acid-phosphatase digestion abolished the interaction between sAP and proaerolysin, the binding could not be inhibited by 10−6 M mannose 6-phosphate, inositol phosphate, GlcNAc phosphate, or ethanolamine phosphate (data not shown). These results suggest that the minimum structure of the GPI-anchored glycan which is required for recognition by aerolysin is a larger sized component than mannose 6-phosphate.
A β-N-Acetylglucosaminyl Phosphate Diester Residue Is Attached to the Glycosylphosphatidylinositol Anchor of Human Placental Alkaline Phosphatase: A TARGET OF THE CHANNEL-FORMING TOXIN AEROLYSIN
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