Microheterogeneity in Glycosylphosphatidylinositol Anchor Structures of Bovine Liver 5′-Nucleotidase

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ABSTRACT: In our study, 5′-nucleotidase was released from bovine liver by the treatment with *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C and purified to a homogeneous state by concanavalin A–Sepharose and (diethylaminoethyl)-Toyopearl column chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Purified 5′-nucleotidase were then cleaved by cyanogen bromide (CNBr), and then inositol phosphoglycan-containing C-terminal peptides (IPG peptides) were separated by C_{18} reverse-phase liquid chromatography and analyzed by peptide sequencer, amino acid analyzer, gas chromatography (GC), and GC–mass spectrometry (MS). Ser523 of the amino acid sequence deduced from 5′-nucleotidase cDNA [Suzuki *et al.* (1993) *J. Biochem. (Tokyo) 113,* 607–613] is revealed to be the C-terminal amino acid to which a glycosylphosphatidylinositol is anchored. Separated peaks of CNBr-cleaved IPG peptides were then analyzed by electron spray ionization (ESI)–MS. Eight different molecular weight (MW) species of CNBr-cleaved IPG peptides were detected. Three fractions of CNBr-cleaved IPG peptides were separately treated by trypsin, and trypsinized IPG peptides were purified by C_{18} reverse-phase liquid chromatography. Finally, five different MW species of trypsinized IPG peptides (1629.4, 1752.7, 1791.8, 1832.8, and 1994.5) were detected by ESI–MS. Together with sequential exoglycosidase treatment and quantitative analysis of sugar moieties by GC and GC–MS, microheterogeneity in the structures of these five glycosylphosphatidylinositol (GPI) anchor species was determined. The common core structure was ethanolamine phosphate–mannose–mannose–mannose(–ethanolamine phosphate)–glucosamine–*myo*-inositol phosphate. Variations observed in additional mannose, *N*-acetylhexosamine, and ethanolamine phosphate moieties form this heterogeneity. One additional ethanolamine phosphate residue connected to the mannose next to glucosamine was common to these five GPI anchor structures.

More than 100 extracellular membrane proteins in various eucaryotic cells have been reported to be anchored by glycosylphosphatidylinositol (GPI),[1] linked covalently to the protein C-terminus (Cross, 1990; Field & Menon, 1993). The core structure of GPI anchor was first determined in *Trypanosoma* variant surface glycoprotein (VSG); i.e., ethanolamine phosphate–$(mannose)_3$–glucosamine–*myo*-inositol phosphate is shown as a core structure attached to the C-terminal GPI-anchored peptides of these proteins (Ferguson *et al.*, 1988). Then, it was revealed that in the structural study of GPI anchors of rat Thy-1 (Homans *et al.*, 1988) and human acetylcholinesterase (Roberts *et al.*, 1988), this core structure is also conserved in the mammalian GPI-anchored proteins. In the structural study of GPI anchors, GC, GC–MS, FAB–MS, and two-dimensional NMR were used in combination with HPLC elution profiles after hydrolysis by glycosidases. In the structural study of *Trypanosoma* VSG, some heterogeneity in galactose side chains has been reported (Ferguson *et al.*, 1988). This kind of microheterogeneity was also reported in the study of rat Thy-1 as the presence or absence of an additional mannose side chain (Homans *et al.*, 1988). Recently, in the studies of GPI structures of human acetylcholinesterase (Deeg *et al.*, 1992) and human brain scrapie prion protein (Stahl *et al.*, 1991), the existence of a limited amount of microheterogeneity has been also reported.

In our previous paper, we reported the cDNA structure of 5′-nucleotidase of bovine liver (Suzuki *et al.*, 1993). In this paper we determined the C-terminal amino acid to which a GPI precursor should be transferred. Also, we tried to elucidate the possibility of microheterogeneity in the GPI anchor structure of bovine liver 5′-nucleotidase by electron spray ionization (ESI)–MS. We identified five different species of GPI anchor structures of 5′-nucleotidase. An additional ethanolamine phosphate commonly exists in all of these five structures. This additional ethanolamine phosphate does not exist in GPI anchor structures in *Trypanosoma* VSG (Ferguson *et al.*, 1988). Recently, it was suggested that the GPI anchor structure is important in interaction with other membrane components for targeting these GPI anchor proteins to the apical surface of the plasma membrane (Brown & Rose, 1992). Thus, for discussing the physiological meaning of GPI anchor structures, more precise analytical data of microheterogeneity in GPI structure seem to be essential.

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[1] Abbreviations: PI, phosphatidylinositol; GPI, glycosylphosphatidylinositol; IPG peptide, inositol phosphoglycan-containing C-terminal peptide; GC, gas chromatography; MS, mass spectrometry; FAB, fast atom bombardment; ESI, electron spray ionization; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Man, mannose; GlcN, glucosamine; HexNAc, *N*-acetylhexosamine; PVDF, poly(vinylidene difluoride); VSG, variant surface glycoprotein; NMR, nuclear magnetic resonance; Con A, concanavalin A; PIPLC, phosphatidylinositol-specific phospholipase C; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; PARP, procyclic acidic repetitive protein.

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MATERIALS AND METHODS

Materials. α-1,2-D-Mannosidase of *Aspergillus saitoi* was kindly supplied by Dr. Tamao Endo (Institute of Medical Science, University of Tokyo). β-*N*-Acetylhexosaminidase and α-mannosidase of jack bean were purchased from Sigma and Seikagaku Kogyo (Japan), respectively. Affinity adsorbent Con A–Sepharose was purchased from Pharmacia, and DEAE-Toyopearl was from Tosoh (Japan). All chemicals used were of analytical reagent grade unless otherwise stated.

Assay of 5′-Nucleotidase. Assay of 5′-nucleotidase was performed by the method of Emmelot and Bos (1966), with 5′-AMP as a substrate. The amount of inorganic phosphate produced was determined by the method of Baginski (1974).

Phosphatidylinositol-Specific Phospholipase C. Phosphatidylinositol-specific phospholipase C (PIPLC) was purified in a homogeneous state from the culture broth of *Bacillus thuringiensis* IAM 12077 (Ikezawa *et al.*, 1983). Assay of PIPLC was performed with phosphatidylinositol (PI) as a substrate as reported previously (Taguchi *et al.*, 1980).

Purification of 5′-Nucleotidase. Solubilization and purification of 5′-nucleotidase from bovine liver was performed by the methods as described in our previous paper (Suzuki *et al.*, 1993) with some modifications. All the processes for preparation and purification of 5′-nucleotidase were operated at 4 °C unless otherwise stated. Bovine liver (wet weight 1.5 kg) was cut with scissors into large pieces and washed with 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose and 100 μM PMSF. These slices were further cut into small pieces with a mixer in the same isotonic buffer. Pellets consisting mainly of small slices and unbroken cells were collected by centrifugation at 2000*g* for 15 min and then suspended in the same isotonic buffer and incubated with 10 milliunits/mL PIPLC at 37 °C for 30 min. Solubilized 5′-nucleotidase was collected in the supernatant by centrifugation at 40000*g* for 30 min. We have already shown that 5′-nucleotidase was certainly released from the cell surface without cell lysis by this procedure (Taguchi & Ikezawa, 1978). Thus higher specific activity of the enzyme was obtained by PIPLC treatment from intact cells than from membrane preparations, because of low contamination of cytosolic proteins and minor nonspecific protein release from the membrane.

Crude extracts of 5′-nucleotidase were precipitated by 40–80% ammonium sulfate and dialyzed against 10 mM Tris-HCl, pH 7.5. A dialyzed sample was applied to a Con A–Sepharose column (60 × 60 mm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. 5′-Nucleotidase activity was eluted by the starting buffer containing 200 mM methyl α-D-glucoside at a flow rate of 2.5 mL/min. Fractions containing 5′-nucleotidase activity were dialyzed and then applied to a DEAE-Toyopearl column (10 × 120 mm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM $MgCl_2$. The enzyme activity was eluted by a linear gradient of 0–1 M NaCl in the same buffer. Active fractions were concentrated and then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) with a 12% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970). After electrophoresis, protein bands were transferred to an Immobilon PVDF membrane (Millipore, Co. Ltd.) with a Bio-Rad electroblotting system. Separated protein bands on PVDF membrane were detected by staining with Ponseau S. Identification of 5′-nucleotidase was performed by direct peptide sequencing analysis of the protein band on the PVDF membrane. Purified single bands of 5′-nucleotidase were cut out and collected for further experiments. Approximately 0.8 mg of purified 5′-nucleotidase was obtained.

Preparation and Purification of CNBr-Cleaved Peptides from 5′-Nucleotidase. PVDF membranes were stained with Ponseau S after electroblotting. The stained band of 5′-nucleotidase on the PVDF membrane was treated with 1 M CNBr, and then the cleaved peptides were eluted from the membrane by 70% formic acid and dried under N_2 gas. The sample was dissolved in 0.1% TFA containing 10% acetonitrile and then applied to a C_{18} reverse-phase column. Peptides were eluted with a gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid (TFA). Peaks of separated peptides were subjected to further analysis.

For analysis of larger peptides, 5′-nucleotidase was directly eluted from the PVDF membrane (Szewczyk & Summers, 1988), and then *S*-pyridylethylated by 4.2 μM vinylpyridine. After *S*-pyridylethylation, the protein was cleaved with CNBr and resulting peptides were separated by Tricine–SDS–PAGE. Tricine–SDS–PAGE was performed by the method of Schagger and von Jagow (1987) with a 13.5% polyacrylamide gel containing 0.1% SDS. After electrophoresis, the peptide bands were transferred to PVDF membranes by electroblotting.

Preparation and Purification of Trypsinized Inositol Phosphoglycan-Containing C-Terminal Peptides (Trypsinized IPG Peptides). CNBr-cleaved inositol phosphoglycan-containing C-terminal peptides (CNBr-cleaved IPG peptides) I, II, and III + IV were separately treated with 75 ng of trypsin in 10 mM $CaCl_2$/100 mM Tris-HCl (pH 8.2) at 37 °C for 24 h. Trypsinized IPG peptides were dissolved in 0.1% TFA/2.5% acetonitrile and then applied to a C_{18} reverse-phase column. HPLC was performed with a gradient of 2.5–100% acetonitrile in 0.1% TFA at a flow rate of 200 μL/min.

Protein Sequencing and Amino Acid Analysis. Purified peptides were analyzed with an Applied Biosystems Model 477A protein sequencer/Model 120A PTH analyzer on-line system.

Amino acid analysis was performed with a Waters Pico-Tag analyzing system after hydrolysis with 6 N HCl at 110 °C for 24 h and treatment with phenyl isothiocyanate (PITC).

Electron Spray Ionization (ESI)–Mass Spectrometry (MS) Analysis. CNBr-cleaved IPG peptides were analyzed by JMS-LX2000 (JOEL, Japan) equipped with an electron spray ion source at 2.0 kV. Samples in water/methanol were directly injected into the ESI chamber at a flow rate of 1 μL/min. Trypsinized IPG peptides were analyzed by JMS-SX102A (JOEL, Japan) equipped with an ESI source at 7.0 kV.

Preparation of HF-Cleaved Inositol Glycans and Their [^{14}C]Acetylation. A mixture of trypsinized IPG peptides was treated with 25% HF at 0 °C for 40 h and the reaction was terminated by adding LiOH. After deionization with Dowex 50W-X8 and AG2-X8, the dried sample was dissolved in 200 μL of 0.2 M carbonate buffer (pH 10.5), mixed with 50 μL of [^{14}C]acetic anhydride and incubated at 20 °C for 1 h. After incubation, the sample was deionized on a Sephadex G-15 column. The sample eluted in the void volume was concentrated and applied to a Cosmosil 5-NH_2 (4.6 × 150 mm) column. HPLC was performed at 50 °C at a flow rate of 0.5 mL/min. Glucose oligomers (G1–G7) were used as standards for the calculation of glucose units of the samples. Labeled peak fractions obtained around glucose units 5–7 were separately collected and concentrated.

Determination of Glycosyl Moiety of HF-Treated Inositol Glycans by the Combination of Glycosidase Treatment with HPLC Analysis. HF-treated inositol glycans were treated with several exoglycosidases such as α-mannosidase (jack bean), α-1,2-mannosidase (*A. saitoi*), and β-*N*-acetylhexosaminidase (jack bean). Then glucose units of the reaction

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                                -21        -11        -1
                          MNPGAA RTPALRILPL GALLWPAARP
                                    Signal peptide
        10         20         30         40         50
WELTILHTND VHSRLEQTSE DSSKCVNASR CVGGVARLAT KVHQIRRAEP
P-1
        60         70         80         90        100
HVLLLDAGDQ YQGTIWFTVY KGTEVAHFMN ALGYESMALG NHEFDNGVEG
                              ^P-2   @A^P-3
       110        120        130        140        150
LIDPLLKEVN FPILSANIKA KGPLASKISG LYSPYKILTV GDEVVGIVGY

       160        170        180        190        200
TSKETPFLSN PGTNLVFEDE ITALQPEVDK LKTLNVNKII ALGHSGFEVD

       210        220        330        340        350
KLIAQKVKGV DVVVGGHSNT FLYTGNPPSK EVPAGQYPFI VTSDDGRKVP

       260        270        280        290        300
VVQAYAFGKY LGYLKVEFDE KGNVVTSHGN PILLNSSIPE DPNIKADINK

       310        320        330        340        350
WRVKLDNYST QELGKTIVYL DGTAQSCRFR ECNMGNLICD AMINNNLRHP
                                  ^P-4     ^P-5
       360        370        380        390        400
DEMSWNHVSM CILNGGGIRS PIDERNNGTI TWENLAAVLP FGGTFDLVQL
  ^P-6   ^ P-7
       410        420        430        440        450
KGSTLKKAFD DTVHRYGQRT GEFLQVGGIH VVYDISRNPG DRVVKLEVLC

       460        470        480        490        500
TQCRVPSYEP LRMDKVYKVI LPSFLVSGGD GFQMIKDEKI KHDSGDQDIN
            ^P-8                  ^P-9
       510        520        530        540        550
VVSGYISKMK VLYPAVEGRI QFSAGSHCCG SFSLIFLSVL AVIIILYQ*
        ^P-10                 ↑
                  Cleavage/GPI attachment site
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FIGURE 1: Amino acid sequence of bovine liver 5′-nucleotidase deduced from cDNA, displayed in single-letter code. Amino acids are numbered above the sequences. Amino acids −26 to −1 comprise the signal peptide. Methionine (M) residues cleaved by CNBr are marked as ^. All the peptides cleaved theoretically by CNBr, P-1–P-10, were numbered from the N-terminus. CNBr-cleaved peptide fragments purified by C_{18} reverse-phase HPLC (P-2, P-4, P-5, P-6, P-8, P-9, and P-10) or Tricine–SDS–PAGE (P-1, P-3, and P-7) were subjected to sequencing analysis. Underlined amino acids were identified by peptide sequence analysis. Amino acids indicated by a star were the site of discrepancy from those deduced from cDNA.

products by glycosidase treatments were calculated from the elution peaks of Cosmosil 5-NH_2 HPLC.

GC and GC–MS Analysis. Samples were hydrolyzed with 4 N HCl at 80 °C for 6 h or with 6 N HCl at 110 °C for 24 h. Then, to the dried samples, 10 μL of *N*-(trimethylsilyl)imidazole, 2 μL of trimethylchlorosilane, and 10 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide were added. After vigorous mixing and standing for 60 min, 2–4-μL samples were subjected to GC or GC–MS analysis. GC–MS was performed using a JEOL DX300 mass spectrometer fitted with a JMA-3500 mass data analysis system. Both sugar and inositol derivatives were separated by a glass column (2 mm × 2 m) packed with 3% silicon SE30 on a Uniport HP 60/80 mesh. Standard *myo*-inositol was eluted at a retention time of 19.5 min in the GC spectrum and exhibited the typical mass spectrum pattern at m/z 612, 507, 432, and 305. In GC and GC–MS analysis, *scyllo*-inositol was used as an internal standard for quantitative determination.

RESULTS

Peptide Sequence Analysis of CNBr-Cleaved IPG Peptides of 5′-Nucleotidase. Figure 1 shows the amino acid sequence deduced from the cDNA sequence of 5′-nucleotidase reported in our previous paper (Suzuki *et al.*, 1993), starting from the N-terminus of the mature protein. By CNBr treatment, 10 different peptides must be theoretically formed as shown in Figure 1. These peptides were numbered as P-1–P-10, in order from the N-terminus to the C-terminus of mature 5′-nucleotidase. Figure 2 shows the chromatogram of a C_{18} reverse-phase column of CNBr-cleaved peptides of 5′-nucleotidase. Major peaks obtained were subjected to peptide sequencing. Figure 1 shows the sequence data of P-1–P-10 peptides obtained from peptide sequence analysis as underlined amino acids. Sequences of three relatively large peptides, P-1, P-3, and P-7, were obtained by direct sequencing of spots on PVDF membrane (Millipore Co. Ltd.) after the peptide samples separated by Tricine–SDS–PAGE were blotted. A discrepancy was observed in the sequence of P-2 peptide, i.e., D-A in place of E-S in the cDNA as shown in Figure 1. It seems to be a genetic variation among individuals in the same species.

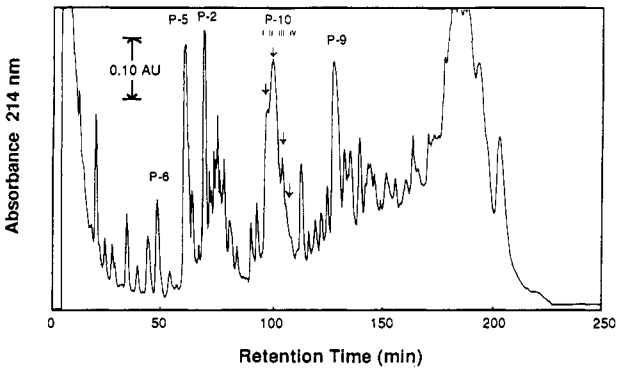

FIGURE 2: HPLC of CNBr-cleaved peptides of 5′-nucleotidase. CNBr-cleaved peptides of 5′-nucleotidase were purified by C_{18} reverse-phase HPLC. Fractions I–IV obtained as subfractions of the P-10 peptides were marked by the arrows. The programs for cleavage and acetonitrile gradient are shown in the text.

Table 1: Subclass Distribution of CNBr-Cleaved IPG Peptides Obtained by ESI–MS Analysis[a]

detected m/z $(M + 3H)^{3+}$	calculated molecular weight[b]	fraction I	II	III	IV
915.3	2742.7 (X)		+++++	+++++	++
924.5	2770.6 ($X + 28$)		++	+++	+++
956.3	2865.9 ($X + 123$)	++	+		
969.3	2904.8 ($X + 162$)	++++	++		
978.6	2932.8 ($X + 162 + 28$)	++	+	+	+
983.0	2945.8 ($X + 203$)	+	++	+	+
991.8	2973.3 ($X + 203 + 28$)		+	+	+
1037.1	3106.9 ($X + 162 + 203$)	+			

[a] Four subfractions (I–IV) of CNBr-cleaved IPG peptides obtained by C_{18} reverse-phase HPLC were separately subjected to ESI–MS analysis, and the data obtained are summarized. The number of positive symbols is roughly proportional to the amount of components. [b] The value for molecular weight of X is calculated to be 2742.7. Other components were expressed as $X + n$, i.e., some modified form of X.

As shown in Figures 1 and 2, peaks 10 (I–IV) were revealed to be C-terminal peptides. In these subfractions, the peptides were shown to contain 1 mol of inositol in each molecule by GC and GC–MS analysis. As shown in Figure 2, CNBr-cleaved IPG peptides consists of several different subpeaks in the C_{18} reverse-phase chromatogram. This suggests the microheterogeneity of GPI anchor structures in CNBr-cleaved IPG peptides. Thus, these four separated fractions (I–IV in Figure 2) were separately subjected to ESI–MS analysis.

ESI–MS Analysis of CNBr-Cleaved IPG Peptides. Figure 4A,B shows ESI–MS spectra of fractions I (Figure 4A) and II (Figure 4B) of CNBr-cleaved IPG peptides. For each molecule, ion peaks of $(M + 3H)^{3+}$ and $(M + 4H)^{4+}$ were detected. ESI–MS analysis data of four peaks are summarized in Table 1. Mass values of major components were X, $X + 28$, $X + 123$, $X + 162$, and $X + 203$, in which the mass value

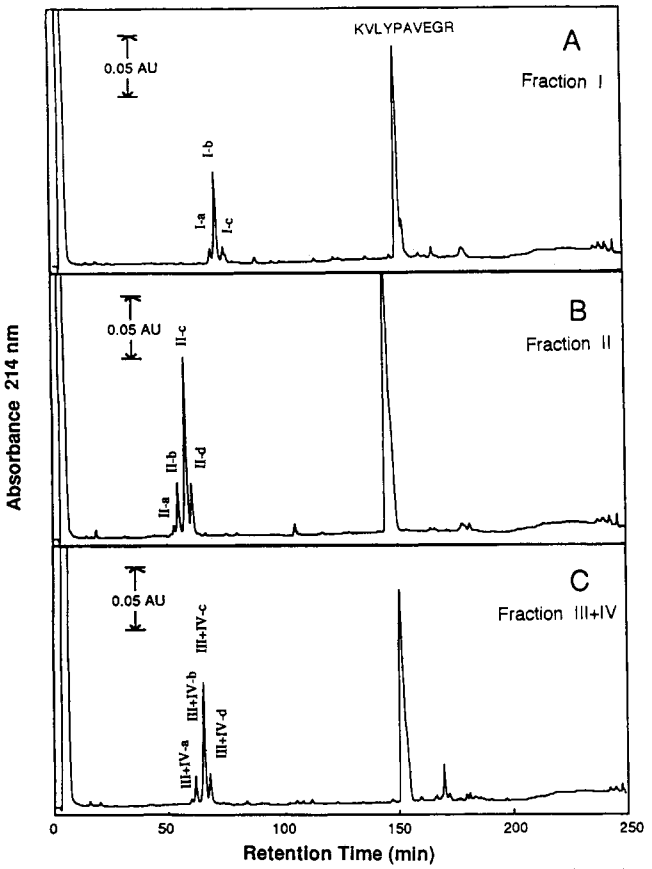

FIGURE 3: HPLC of trypsinized IPG peptides obtained from CNBr-cleaved IPG peptides (P-10). Three CNBr-cleaved peptide fractions (I, II, and III + IV) were separately treated with trypsin as described in the text. Trypsinized peptides were then purified with C_{18} reverse-phase HPLC. (A) Fraction I; (B) fraction II; (C) fraction III + IV.

Table 2: Molecular Weights of Purified Trypsinized IPG Peptides

fraction	detected m/z $(M + 2H)^{2+}$	detected m/z $(M + 3H)^{3+}$	calculated molecular weights[a,b]
I-a	877.2	585.3	1752.7 (Y + 123)
I-b	896.9		1791.8 (Y + 162)
I-c	998.4	665.7	1994.5 (Y + 162 + 203)
II-a	877.2	585.3	1752.7 (Y + 123)
II-b	896.9	598.3	1791.9 (Y + 162)
II-c	815.7		1629.4 (Y)
II-d	917.4		1832.8 (Y + 203)
III + IV-a	877.2	585.3	1752.7 (Y + 123)
III + IV-b	896.9		1791.8 (Y + 162)
III + IV-c	815.7		1629.4 (Y)
III + IV-d	917.4		1832.8 (Y + 203)

[a] Molecular weights of trypsinized IPG peptides revealed from the 2+ and/or 3+ charge states on the mass spectrum. [b] The value for molecular weight of Y is calculated to be 1629.4. Other components were expressed as $Y + n$, i.e., some modified form of Y.

of X was postulated as 2742.7. The values of 123, 162, and 203 are coincident with those for ethanolamine phosphate, mannose, and *N*-acetylhexosamine, respectively. The value of 28 was not expected theoretically. Totally, eight different subpopulations of C-terminal GPI anchor structure were detected.

ESI–MS Analysis of Trypsinized IPG Peptides. Figure 3 shows chromatograms of the second C_{18} reverse-phase HPLC of trypsinized fractions I (Figure 3A), II (Figure 3B), and III + IV (Figure 3C) obtained in Figure 2. Individual peaks in Figure 3 were analyzed separately by ESI–MS. Figure 4C,D shows mass spectra of trypsinized IPG peptides II-b (Figure 4C) and II-c (Figure 4D). In ESI–MS analysis of trypsinized IPG peptides, mainly $(M + 2H)^{2+}$ ions of these molecules

Table 3: Sequential Exoglycosidase Digestion of the Radioactive Inositol Glycan Fractions Obtained by HF Treatment of Trypsinized IPG Peptides of 5′-Nucleotidase[a]

glycosidases	glucose units in ^{14}C-labeled inositol glycans A	B	C
none	5.5	6.5	7
α-1,2 mannosidase (*A. saitoi*)	4.5	4.5	6
↓	↓	↓	↓
α-mannosidase (jack bean)	3.5	3.5	5
β-*N*-acetylhexosaminidase (jack bean)	5.5	6.5	5.5
↓	↓	↓	↓
α-mannosidase	3.5	3.5	3.5
α-mannosidase	3.5	3.5	5
↓	↓	↓	↓
β-*N*-acetylhexosaminidase	3.5	3.5	3.5

[a] The radioactive oligosaccharides at each digestion step were analyzed by Cosmosil 5-NH_2 HPLC. Glucose units were calculated from the elution profile of standard oligosaccharides (G1–G7).

Table 4: Analysis of Sugar Components by GC and GC–MS[a]

(A) Mannose Analysis of the Trypsinized IPG Peptides

trypsinized IPG peptides	molar ratio mannose/peptide
Y	3.0
Y + 162	3.9

(B) Composition Analysis of the Inositol Glycan Obtained by HF Treatment of the Trypsinized IPG Peptide (Y)

component	amount (mol)
2,5-anhydromannitol	1
mannose	3.1[b]
myo-inositol	1.1[b]

[a] Mannose, glucosamine, and inositol contents in the trypsinized IPG peptides and the HF-treated inositol glycan were analyzed by GC and GC–MS. Mannose was detected after hydrolysis with 4 N HCl at 80 °C, and *myo*-inositol after hydrolysis with 6 N HCl at 110 °C. Glucosamine was detected as 2,5-anhydromannitol after deamination with HNO_2 and reduction with $NaBH_4$. [b] The value was expressed relative to 2,5-anhydromannitol (AHM) as 1 mol.

were detected (Table 2). Results of all the separated peaks are summarized in Table 2. MW values of these peptides were Y, Y + 123, Y + 162, Y + 203, and Y + 162 + 203, in which the value of Y is postulated as 1629.4. Finally, five different subpopulations of trypsinized IPG peptides were identified (Figure 5). The value of $X - Y = 1113$, which just coincides with the MW of the peptide (KVLYPAVEGR) (Figure 3) which should be cleaved by trypsin. Also, peptide sequence analysis of these trypsinized peptides revealed that these are of the expected amino acid sequence, IQFS and KVLYPAVEGR (Figure 5).

Amino Acid Composition Analysis of CNBr-Cleaved IPG Peptides and Trypsinized IPG Peptides. Amino acid components were analyzed for CNBr-cleaved IPG peptides (fraction II in Figure 2, mainly containing X) and trypsinized IPG peptides (fraction II-c in Figure 3, mainly containing Y). Amino acid components of the CNBr-cleaved IPG peptide (X) is exactly consistent with the data from peptide sequence analysis, and this sample contained 2 mol of ethanolamine. By analysis of the trypsinized IPG peptide (Y), 152 pmol of Glu/Gln, 181 pmol of Ser, 181 pmol of Ile, 175 pmol of Phe, and 310 pmol of ethanolamine (1:1:1:1:2) were detected. The other amino acids including alanine were not detected in trypsinized IPG peptides, as expected from peptide sequence analysis data (Ile-Gln-Phe-Ser). From these results, the amino acid Ser523 in cDNA is determined as a C-terminal amino acid to which the GPI anchor precursor should be transferred. Ala524 was not detected by amino acid analysis and peptide

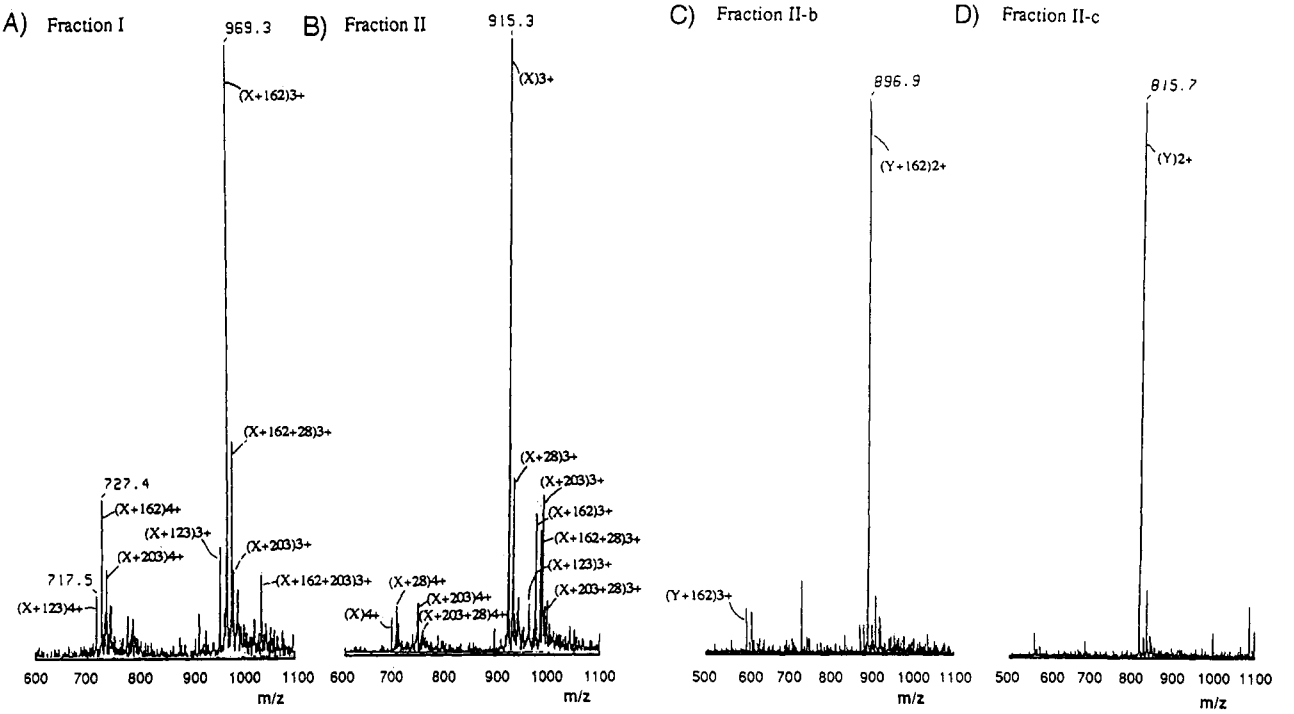

FIGURE 4: Electron spray ionization (ESI)–mass spectrum (MS) of CNBr-cleaved IPG peptides and trypsinized IPG peptides. Fractions I (A) and II (B) of CNBr-cleaved IPG peptides were subjected to ESI–MS analysis. $(M + 3H)^{3+}$ and $(M + 4H)^{4+}$ ions were detected for each C-terminal peptide. Trypsinized IPG peptides, peak II-b (C) and peak II-c (D), derived from CNBr-cleaved IPG peptides (fraction II) were analyzed by ESI–MS. $(M + 2H)^{2+}$ and $(M + 3H)^{3+}$ ions were detected for each trypsinized C-terminal peptide.

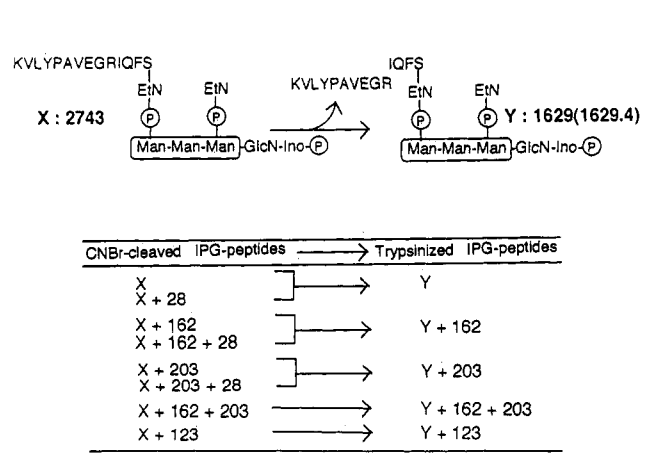

FIGURE 5: Possible degradation diagram obtained by ESI–MS analysis of CNBr-cleaved IPG peptides to trypsinized IPG peptides by trypsin. (A) Tryptic degradation process of CNBr-cleaved IPG peptides. (B) Each species of CNBr-cleaved peptide was digested by trypsin, and the molecular mass peak was detected by ESI–MS. CNBr-cleaved IPG peptides are illustrated as derivatives of the *X* series, and trypsinized IPG peptides of the *Y* series. Mass values of 123, 162, and 203 were expected to ethanolamine phosphate, mannose, and *N*-acetylhexosamine, respectively.

sequence analysis. From these results, the molecular mass of CNBr-cleaved IPG peptides is calculated as 1589.9 and that of trypsinized IPG peptides as 478.6. These data used in the calculation of molecular mass of GPI anchor structures were obtained in ESI–MS analysis.

HPLC Analysis of the HF-Cleaved Inositol Glycans from Trypsinized IPG Peptides in Combination with Several Exoglycosidase Treatments. HF-cleaved inositol glycans were digested by α-1,2-mannosidase, α-mannosidase, and β-*N*-acetylhexosaminidase and analyzed by Cosmosil 5-NH_2 HPLC. Major samples obtained after [^{14}C]acetylation were divided into three fractions by Cosmosil 5-NH_2 HPLC. Fractions A, B, and C were treated with each glycosidase and reaction products were analyzed on HPLC. These results are summarized in Table 3. It revealed that the glycan B contains two α-1,2-mannosyl linkages per molecule, at the nonreducing terminus and the glycan C contains an additional *N*-acetylhexosamine connected by a β-1,4 linkage with the mannose next to glucosamine. From these results, the structures of the glycans A, B, and C were supposed to be Manα1–2Manα1–6Man–GlcN–*myo*Ins, Manα1–2Manα1–2Manα1–6Man–GlcN–*myo*Ins, and Manα1–2Manα1–6(HexNAcβ1–4)Man–GlcN–*myo*Ins, respectively. These three glycan components correspond to those of three major trypsinized IPG peptides 1, 2, and 3 as shown in Figure 6.

Sugar Analysis by GC and GC–MS of the Trypsinized IPG Peptides. The trypsinized IPG peptides *Y* and *Y* + 162 were subjected to GC analysis after treatment with 4 N HCl. Table 4 shows that *Y* contains three mannose residues (R. T. 12.8 and 15.2) whereas (*Y* + 162) contains four. GC analysis shows that the molar ratio of mannose, 2,5-anhydromannitol, and *myo*-inositol in deaminated *Y* is 3:1:1 as shown in Table 4. These data give us a good support to deduce the final structure of each GPI anchor species described in Figure 6.

DISCUSSION

Recently, some microheterogeneities in the sugar side chains attached to the core of GPI-anchored proteins have been reported in the analysis of GPI structures of *Trypanosoma* VSG, rat Thy-1, scrapie prion protein, and human erythrocyte acetylcholinesterase (Ferguson *et al.*, 1988; Homans *et al.*, 1988; Stahl *et al.*, 1991; Deeg *et al.*, 1992). In our reports, five different molecular species of GPI anchors of 5′-nucleotidase were purified separately, and their structures were analyzed mainly by ESI–MS and GC. Finally, Figure 6 shows the proposed structure and percentage contents of these five GPI anchor peptides obtained.

The C-terminal amino acid to which the GPI anchor precursor should be transferred is shown to be Ser523. This result is coincident with those obtained in the studies on 5′-

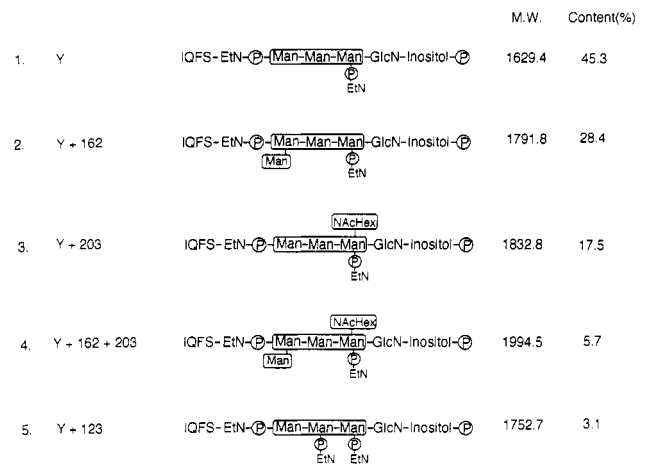

FIGURE 6: Proposed structures of the trypsinized IPG peptides from bovine liver 5′-nucleotidase. Amino acids were denoted as a single-letter code. Man, mannose; GlcN, glucosamine; HexNAc, *N*-acetylhexosamine; EtN-P, phosphoethanolamine; Inositol-P, inositol phosphate. Percent contents were calculated from the peak area of each species of trypsinized C-terminal peptide in HPLC.

nucleotidases from other mammalian species (Misumi *et al.*, 1990a,b; Ogata *et al.*, 1990) and electric ray electric lobe (Volknandt *et al.*, 1991).

Results obtained from ESI–MS analysis also indicated that *myo*-inositol exists as a monophosphate ester in all IPG peptides. But it is not clear whether the 1,2-cyclic form was destroyed during the treatment with an excess amount of PIPLC or by relatively low pH in the purification procedures.

Recently, in the GPI anchor structure of scrapie prion protein (Stahl *et al.*, 1991), the existence of sialic acid in the side chain was reported. The same kind of modification by sialic acid was also reported in the GPI anchor structure in procyclic acidic repetitive protein (PARP) of *Trypanosoma brucei* (Ferguson *et al.*, 1993). We could not find this kind of modification by sialic acid in the IPG structure of bovine 5′-nucleotidase.

Essentially, these five structures include most of the heterogeneous structures reported in mammalian GPI proteins such as Thy-1 and acetylcholinesterase. Especially, an additional phosphoethanolamine residue is common to all five structures of 5′-nucleotidase anchor attached to the protein C-terminus by another phosphoethanolamine residue, as are all other mammalian GPI anchors. The additional phosphoethanolamine had been supposed to be attached to the mannose residue next to the glucosamine in the GPI core structure. Recently, this additional modification by phosphoethanolamine has been suggested to occur during the biosynthesis of GPI core precursor before its transfer to the proteins (Hirose *et al.*, 1992; Ueda *et al.*, 1993). We supposed that this ethanolamine residue must be essential for the physiological function of the GPI-anchored proteins. Recent reports indicated that the accumulation of GPI-anchored proteins is a key step during vesicle formation in the process of apical targeting of these proteins (Brown & Rose, 1992). We consider that the free amino group of phosphoethanolamine together with that of glucosamine is very important for interaction with other membrane components such as glycosylceramides (Simons & Meer, 1988).

Our results also proved that ESI–MS analysis is a useful method, especially for the detection of posttranslational modification of proteins by the lipid anchors. In our experiments, less than 10 pmol of components can be efficiently detected by this method.

In this report, we detected the five different molecular species of GPI anchors. One of them is exactly the same structure which was reported by Ogata *et al.* (1990) in rat liver 5′-nucleotidase. We consider that the variations of the structures in the several mammalian GPI anchors may also exist in each GPI-anchored protein as in bovine liver 5′-nucleotidase reported here. In this point, ESI–MS seems to be a most useful method for this purpose.

The physiological meaning of the additional sugar side chain is not clear now, but one possible explanation is that this side chain plays an important role in the recognition of other membrane components, in the process of transfer or on the plasma membrane.

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