Membrane-Anchoring Domain of Rat Liver 5′-Nucleotidase: Identification of the COOH-Terminal Serine-523 Covalently Attached with a Glycolipid†

Shigenori Ogata, Yukari Hayashi, Yoshio Misumi, and Yukio Ikehara*

Department of Biochemistry and Joint Laboratory for Pathological Biochemistry, Fukuoka University School of Medicine, Jonan-ku, Fukuoka 814-01, Japan

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ABSTRACT: The involvement of glycosylphosphatidylinositol (GPI) in membrane anchoring of 5′-nucleotidase was investigated by chemical analyses. 5′-Nucleotidase purified from rat liver microsomes was subjected to BrCN cleavage, hexane extraction, and high-performance liquid chromatography, resulting in the purification of a single fragment with M_r 2300. Chemical analyses revealed that the purified fragment contains the tetradecapeptide Lys-Val-Ile-Tyr-Pro-Ala-Val-Glu-Gly-Arg-Ile-Lys-Phe-Ser and characteristic components of GPI including ethanolamine, glucosamine, mannose, inositol, palmitic acid, and stearic acid. In addition, it was confirmed that digestion of 5′-nucleotidase with lysyl endopeptidase yielded a fragment containing the dipeptide Phe-Ser and the same GPI components as above. The sequences of the tetradeca- and dipeptides thus determined are identified at positions 510–523 and 522–523, respectively, in the primary structure deduced from the cDNA sequence, which predicts a further extension to position 548, containing a hydrophobic amino acid sequence [Misumi, Y., Ogata, S., Hirose, S., & Ikehara, Y. (1990) *J. Biol. Chem. 265*, 2178–2183]. Taken together, these results indicate that the mature 5′-nucleotidase molecule lacks the predicted COOH-terminal peptide extension and is attached at serine-523 with GPI, which functions as the membrane anchor of 5′-nucleotidase.

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) is a glycoprotein of the plasma membrane which has been shown to be an ectoenzyme present in a wide variety of mammalian cells (Evans, 1980; Luzio et al., 1987). Although the precise physiologic function of 5′-nucleotidase is unknown, the enzyme might play a role in the production of extracellular nucleosides, of which adenosine can cause vasodilation (Arch & Newsholme, 1978) and may act as a neurohumoral agent (Pull & McIlwain, 1972). It is also of interest to note that 5′-nucleotidase is a maturation marker for human T and B lymphocytes (Thompson et al., 1986) and its expression is reduced in lymphocytes from patients with a variety of immunodeficiencies (Thompson et al., 1984; Salazar-Gonzalez et al., 1985). The protein has been purified from several tissues as a detergent-solubilized form and found to be composed of two identical subunits of M_r 72 000–74 000 (Evans & Gurd, 1973; Naito & Lowenstein, 1981; Bailyes et al., 1984).

5′-Nucleotidase was first thought to be a conventional integral membrane protein. It was, however, reported that the enzyme could be released from the plasma membrane of several tissues by treatment with phosphatidylinositol- (PI)[1] specific phospholipase C (Low & Finean, 1978; Taguchi & Ikezawa, 1978), suggesting that PI is involved in the attachment of 5′-nucleotidase to the membrane. Since then, the involvement of PI has been suggested for an increasing number of membrane proteins, although for most of them this is based on their sensitivity to PI-specific phospholipase C (Low, 1987). Recently, hydrophobic COOH-terminal domains were isolated from the variant surface glycoprotein (VSG) of trypanomes (Ferguson et al., 1985), Thy-1 (Tse et al., 1985), acetylcholinesterase (Roberts et al., 1988), and alkaline phosphatase (Micanovic et al., 1988; Ogata et al., 1988). Chemical analyses revealed that they contain a unique glycosyl phospholipid, including ethanolamine, hexoses, hexosamines, and PI, which is proposed as the membrane anchor (Ferguson & Williams, 1988). However, no detailed chemical data have been provided for the membrane-anchoring domain of 5′-nucleotidase.

Most recently, we have cloned the cDNA for rat liver 5′-nucleotidase and demonstrated that the predicted primary structure contains a hydrophobic domain at the COOH terminus, a possible signal for posttranslational modification by glycosylphosphatidylinositol (GPI) (Misumi et al., 1990). In this study we prepared a membrane form of rat liver 5′-nucleotidase, from which the COOH-terminal fragment was isolated and analyzed for its chemical compositions. Identification of the COOH-terminal residue attached with GPI confirmed the absence of the predicted COOH-terminal hydrophobic peptide extension in the mature form.

Experimental Procedures

Materials. Concanavalin A–Sepharose, Sephacryl S-300, and myoglobin and its fragments were obtained from Pharmacia-LKB Biotechnology Inc. Lysyl endopeptidase (*Achromobacter lyticus* protease I, EC 3.4.21.50) was obtained from Wako Junyaku (Osaka, Japan). Thyroglobulin, ferritin, and IgG were from Sigma. PI-specific phospholipase C was purified from *Bacillus cereus* as described previously (Kominami et al., 1985). Anti-(rat liver 5′-nucleotidase) IgG–Sepharose was prepared as described (Misumi et al., 1990).

Purification of Rat Liver 5′-Nucleotidase. A membrane form of 5′-nucleotidase was purified from rat liver by the same

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* Address correspondence to this author at the Department of Biochemistry.

[1] Abbreviations: PI, phosphatidylinositol; GPI, glycosyl-PI; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; VSG, variant surface glycoprotein.

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method as described previously (Misumi et al., 1990). In brief, the purification steps are summarized as follows. An aqueous phase obtained by 1-butanol extraction (at pH 8.5) of crude microsomes was subjected to chromatography through a concanavalin A–Sepharose column (2.5 × 20 cm), and fractions with the enzyme activity eluted from the column were applied to the immunoaffinity column (2 × 8 cm), followed by elution with 50 mM diethylamine containing 0.1% Triton X-100. The sample thus obtained was found to contain a single protein with M_r 73000 when analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (Misumi et al., 1990). Repeating these purification steps, we finally obtained 52 mg of the purified enzyme from about 3.5 kg of rat liver.

Removal of Noncovalently Attached Lipids. The purified 5′-nucleotidase was treated as follows to remove completely any lipid components noncovalently attached to the protein and Triton X-100 contained in the sample (Ferguson & Cross, 1984; Ogata et al., 1987). Four volumes of ethanol was added to the enzyme preparation and mixed well. The mixture was left overnight at −20 °C; then, the resultant precipitates were collected by centrifugation at 18000*g* for 30 min and washed twice with 80% ethanol and once with 100% ethanol. The precipitates were then treated with chloroform/methanol (2:1), and the resultant residues were washed twice with 100% ethanol. The protein residue thus obtained was resuspended in deionized water, freeze-dried, and used for the following analyses.

Isolation of Amphipathic Peptide Fragment. The purified protein (10 mg), after being delipidized as above, was cleaved with 2.5 M BrCN in 70% (v/v) formic acid for 24 h at room temperature (Ogata et al., 1989). The digest was diluted with 10 volumes of H_2O and freeze-dried. The residues were dissolved in 1.6 mL of 45% formic acid and mixed well with 2 mL of hexane, followed by centrifugation at 5000*g* for 10 min. An emulsion like layer obtained between the hexane and water phases was carefully taken, washed twice with water, and freeze-dried. The dried sample was dissolved in 0.5 mL of 66.7% acetonitrile containing 23.3% formic acid and 0.067% trifluoroacetic acid (solvent A) and subjected to high-performance liquid chromatography (HPLC) through a Superose 6 column (1 × 30 cm) with solvent A at a flow rate of 0.5 mL/min. Fractionated samples were analyzed by SDS–PAGE. Fractions containing a major peptide with M_r 2300 were subjected to a second HPLC on a Superose 12 column (1 × 30 cm) under the same conditions as above.

The purified 5′-nucleotidase was also cleaved with lysyl endopeptidase (Tsunasawa et al., 1987). The protein (10 mg) in 0.9 mL of 50 mM Tris-HCl (pH 9.0) was incubated at 30 °C for 8 h with lysyl endopeptidase (enzyme/substrate ratio by weight 1:200). The digest was subjected to hexane extraction, followed by separation by HPLC, as described above.

Analysis of Amino Acid Composition and Sequence. Primary amino acids, hexosamines, and ethanolamine were quantitated by a Hitachi amino acid analyzer (Model 835) as described previously (Ogata et al., 1988). Samples were hydrolyzed in 6 M HCl at 110 °C in vacuo for 24 h. For correction of hexosamine values, samples were hydrolyzed in 3 M *p*-toluenesulfonic acid at 110 °C for 24 h. Samples were also analyzed for their peptide sequences by an Applied Biosystems Model 477A gas-phase sequencer with an on-line Model 120A phenylthiohydantoin derivative analyzer using the manufacturer's programming (Misumi et al., 1988).

Analysis of Fatty Acids. Purified samples were hydrolyzed under N_2 in 1 mL of 6 M HCl at 100 °C for 5 h (Ferguson & Cross, 1984; Ogata et al., 1987). The hydrolysates were extracted with hexane, and the extracted samples were treated with 12% BF_3 in methanol for 5 min at 100 °C to obtain fatty acid methyl esters. The samples were analyzed by gas-liquid chromatography on a Shimadzu GC-14A apparatus equipped with a CP-Sil 88CB column (0.22 mm × 50 m) (Chrompack Inc.). The integrator areas were corrected to weight percents relative to the heptadecanoic acid internal standard. The molar quantities of each fatty acid were calculated on the basis of the amount of internal standard used.

Analysis of Neutral Sugars. For analysis of inositol, samples were hydrolyzed under N_2 in 1 mL of 6 M HCl at 100 °C for 24 h (Tse et al., 1985) with L-arabinose as internal standard. Neutral sugars were separated from amino sugars as described previously (Ikehara et al., 1981). Inositol was identified and quantitated by gas–liquid chromatography after monosaccharides were reduced and trifluoroacetylated (Ikehara et al., 1981; Ogata et al., 1987). For determination of other neutral sugars, samples were hydrolyzed in 2.5 M trifluoroacetic acid at 100 °C for 8 h (Imanari et al., 1969). Neutral sugars were separated from amino sugars and analyzed by the same procedures as above.

Polyacrylamide Gel Electrophoresis. SDS–PAGE (12.5% gels containing 8 M urea) was carried out according to Burr and Burr (1983), followed by silver staining of gels for protein (Oakley et al., 1980). Molecular weight markers used are myoglobin and its fragments (M_r 17200–1695).

Sephacryl S-300 Chromatography. 1-Butanol extracts of liver microsomes were adjusted to contain 20 mM Tris-HCl (pH 7.5)–50 mM NaCl–1 mM $MgCl_2$ (buffer A) with or without 0.1% Triton X-100, and 5 mL of each sample was applied onto a Sephacryl S-300 column (5 × 90 cm) which had been equilibrated with buffer A in the presence or absence of 0.1% Triton X-100 (Miki et al., 1986). Fractions of 7.4 mL were collected and used for determination of 5′-nucleotidase activity.

Other Methods. 5′-Nucleotidase activity was determined with 5′-AMP as substrate (Ikehara et al., 1977). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Phase separation of 5′-nucleotidase in a Triton X-114 solution was carried out according to Bordier (1981) with a slight modification (Miki et al., 1986).

Results

Preparation of a Membrane Form of 5′-Nucleotidase. 1-Butanol extraction of liver microsomes yielded about 70% of total 5′-nucleotidase activity into an aqueous phase. 5′-Nucleotidase thus extracted was found to have a hydrophobic nature characteristic of membrane-bound proteins. When being subjected to gel filtration through a Sephacryl S-300 column, the enzyme was eluted at a position of M_r 150000 in the presence of Triton X-100, whereas it was eluted at the void volume of the column in the absence of the detergent, possibly due to its aggregation (Figure 1). Its hydrophobic property was also confirmed by phase separation in a Triton X-114 solution (data not shown). 5′-Nucleotidase extracted as a membrane form was then purified, in the presence of 0.1% Triton X-100, to a single protein, which was identified to consist of two identical subunits with M_r 73000 (Misumi et al., 1990).

Isolation of an Amphipathic Peptide Fragment. Purified 5′-nucleotidase was subjected to isolation of a hydrophobic fragment, possible the membrane-anchoring domain of the protein. After removal of any noncovalently attached lipids, the purified protein was cleaved with BrCN. When the resultant fragments were extracted with hexane and centrifuged, an emulsionlike layer was obtained at the interface between

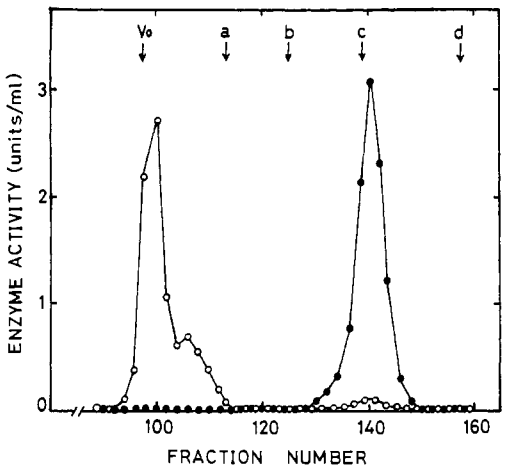

FIGURE 1: Chromatographic behaviors of 5'-nucleotidase through a Sephacryl S-300 column in the absence or presence of Triton X-100. A butanol extract of liver microsomes (5 mL each) was subjected to the Sephacryl S-300 column with buffer A in the absence (○) or presence (●) of 0.1% Triton X-100. Fractions of 4.7 mL were collected and determined for the enzyme activity. Arrows a, b, c, and d indicate elution positions of thyroglobulin (670 kDa), ferritin (460 kDa), IgG (160 kDa), and albumin (68 kDa), respectively. V_0, void volume of the column.

the hexane (top) and water (bottom) phases. The sample thus obtained in the interface was subjected to HPLC through a Superose 6 column, resulting in the separation of eight major peaks, when monitored by absorbance at 280 nm (Figure 2A). SDS-PAGE of each major peak fraction indicated that peak 5 contains a major component with M_r 2300 (Figure 2A, inset). Preliminary chemical analysis demonstrated that this fraction contains glucosamine, ethanolamine, and inositol, characteristic components of the GPI anchor. Fractions of peak 5 were pooled and then subjected to HPLC through a Superose 12 column. As shown in Figure 2B, the fragment with M_r 2300 was eluted as a single component at the first peak.

The yield of this component was about 60 nmol (based on its phenylalanine content, see below), when 10 mg of the purified 5'-nucleotidase was subjected to these purification steps. We finally obtained about 300 nmol of this fragment from about 50 mg of the protein by repeating these steps.

Amino Acid Composition and Sequence of the Isolated Fragment. The purified fragment with M_r 2300 was analyzed for its amino acid composition, demonstrating that it contains 11 different amino acids. In addition, it was found to contain glucosamine and ethanolamine. Molar ratios of these residues were calculated by taking the phenylalanine content as 1 mol (Table I).

The same sample was analyzed by automated Edman degradation for its amino acid sequence, demonstrating that the sequence starts with lysine and is identified to serine at position 14 (Table II). No amino acid residue could be identified after cycle 14. Molar ratios of amino acids identified in the sequence are completely compatible with those determined by the amino acid composition analysis (Tables I and II). These results indicate that the purified fragment contains 14 residues of 11 different amino acids. Furthermore, this sequence is identified at positions 510–523 in the primary structure of rat liver 5'-nucleotidase predicted from the cDNA sequence (Misumi et al., 1990), as shown in Figure 3. This sequence cannot be seen in any other part of the structure.

Identification of the Characteristic Components of the GPI Anchor. The purified fragment was also analyzed for other chemical components which are proposed for the GPI anchor (Ferguson & Williams, 1988). All the compositions were

Table I: Chemical Composition of Purified Fragments[a]

component	molar ratio: BrCN-cleavage fragment	molar ratio: lysyl endopeptidase cleavage fragment
serine	1.1 (1)	1.2 (1)
glutamic acid/glutamine	1.3 (1)	
proline	1.0 (1)	
glycine	1.4 (1)	
alanine	1.0 (1)	
valine	1.7 (2)	
isoleucine	1.6 (2)	
tyrosine	0.8 (1)	
phenylalanine	1.0 (1)	1.0 (1)
lysine	1.8 (2)	
arginine	0.9 (1)	
ethanolamine	1.9 (2)	1.8 (2)
glucosamine	0.6 (1)	0.7 (1)
galactosamine	ND[b]	ND
mannose	2.8 (3)	2.7 (3)
galactose	ND	ND
inositol	1.1 (1)	0.9 (1)
myristic acid ($C_{14:0}$)	0.15	0.12
palmitic acid ($C_{16:0}$)	0.44	0.51
stearic acid ($C_{18:0}$)	1.35	1.28
oleic acid ($C_{18:1}$)	0.05	0.03

[a] Each fragment purified from BrCN- cleaved or lysyl endopeptidase cleaved peptides was analyzed for chemical composition as described under Experimental Procedures. Each value was normalized by taking the phenylalanine content in the same sample as 1 mol. Values are the means of three separate experiments. Proximal integral numbers are shown in parentheses. [b] ND, not detectable.

Table II: Amino Acid Sequence of Purified Fragments[a]

cycle	BrCN-cleavage fragment: amino acid	BrCN-cleavage fragment: yield (pmol)	lysyl endopeptidase cleavage fragment: amino acid	lysyl endopeptidase cleavage fragment: yield (pmol)
1	Lys	288	Phe	145
2	Val	272	Ser	46
3	Ile	252	X	
4	Tyr	236	X	
5	Pro	118		
6	Ala	302		
7	Val	266		
8	Glu	118		
9	Gly	166		
10	Arg	128		
11	Ile	154		
12	Lys	130		
13	Phe	110		
14	Ser	72		
15	X			
16	X			

[a] Each purified fragment was subjected to amino acid sequencing as described under Experimental Procedures. X, no amino acid was identified.

normalized for their molar ratios by taking the phenylalanine content in the same sample as 1 mol, as summarized in Table I. It is evident that the fragment contains ethanolamine (2 mol), glucosamine (1 mol), inositol (1 mol), and mannose (3 mol). The fragment also contains one major fatty acid (stearic acid) and three other minor fatty acids (myristic acid, palmitic acid, and oleic acid), the total amounting to about 2 mol/mol of the fragment. Considering that these components comprise the GPI covalently linked to the tetradecapeptide, the molecular mass of the fragment is estimated to be about 3300 Da, slightly larger than the 2300 Da of the fragment actually determined by SDS–PAGE (Figure 2). The mass difference is not unexpected since a precise molecular mass of such a small molecule, especially when being attached with non-

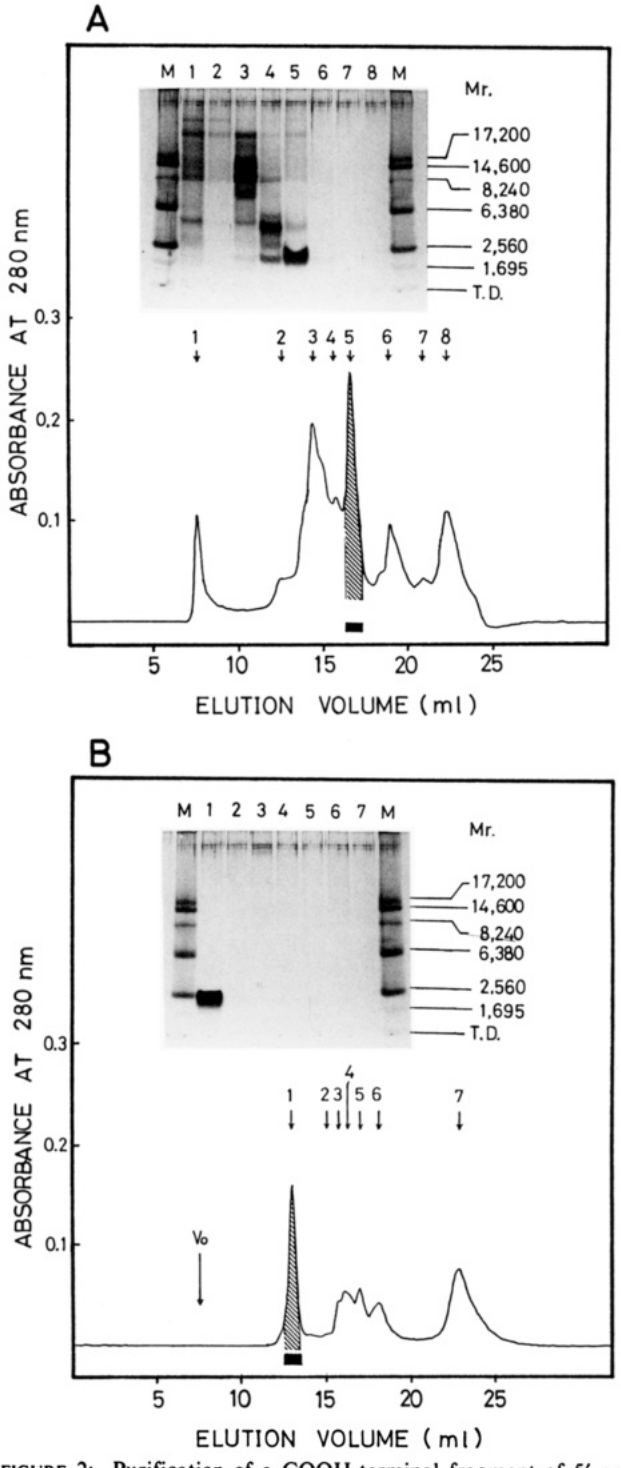

FIGURE 2: Purification of a COOH-terminal fragment of 5′-nucleotidase by HPLC. (A) BrCN-cleaved/hexane-extracted fragments of 5′-nucleotidase were prepared and subjected to HPLC through a Superose 6 column. Fractions of 0.4 mL were collected and analyzed by SDS–PAGE, followed by silver staining of the gels (inset). Lanes 1–8 in panels A and B correspond to samples with the same number of peaks eluted, respectivley. Lane M, molecular mass markers. T. D., tracking dye front. (B) Pooled fractions of peak 5 (hatched) in (A) were freeze-dried, redissolved in solvent A, and subjected to HPLC through a Superose 12 column under the same conditions as above. Each major peak was also analyzed by SDS–PAGE.

peptide components, cannot be determined by SDS–PAGE.

Isolation of Lysyl Endopeptidase Cleaved Fragment. A second peptide cleavage method was used for isolation and identification of the COOH terminus of the mature 5′-nucleotidase. The presence of the COOH-terminal sequence Lys-Phe-Ser in the tetradecapeptide (Table II and Figure 3)

Purified fragment: K-V-I-Y-P-A-V-E-G-R-I-K-F-S

COOH-Terminal region predicted by the cDNA sequence:

510 520
-Y-I-S-K-M-K-V-I-Y-P-A-V-E-G-R-I-K-F-S-A-A-S-H-

530 540 548
Y-Q-G-S-F-P-L-I-I-L-S-F-W-A-V-I-L-V-L-Y-Q

FIGURE 3: Amino acid sequence of purified fragment and comparison with cDNA-predicted peptide sequence. The determined sequence of the purified fragment was compared with the COOH-terminal sequence predicted by the cDNA sequence of rat liver 5′-nucleotidase (Misumi et al., 1990). The determined sequence and a stretch of hydrophobic amino acids are indicated by double and single underlines, respectively. Amino acid residues are shown by single-letter code.

suggests that treatment of the protein with lysyl endopeptidase will release a fragment containing the dipeptide Phe-Ser and GPI. Fragments obtained by treatment with lysyl endopeptidase were subjected to extraction with hexane, followed by HPLC, in the same manner as above. Six major peaks were obtained by the first HPLC through the Superose 6 column, although the elution profile detected at 280 nm was different from that of the BrCN-cleaved sample (data no shown). Amino acid sequencing of each major peak revealed the presence of the dipeptide Phe-Ser in three fractions of a peak which appeared between peaks 5 and 6 of Figure 2A. The fractions were collected and then applied to the second HPLC (Superose 12). The Phe-Ser fragment was eluted at a position corresponding to the third peak of Figure 2B. Chemical analysis of the fragment confirmed that it contains the same GPI components as determined for the BrCN-cleavage fragment, in addition to the dipeptide (Table I).

Discussion

It has been suggested that 5′-nucleotidase is anchored to the membrane via GPI, on the basis of the observation that the enzyme is released from the membrane by PI-specific phospholipase C (Low & Finean, 1978; Taguchi & Ikezawa, 1978). To obtain more direct evidence for this unique linkage of 5′-nucleotidase to the membrane, we have tried to isolate and characterize the membrane-anchoring domain. The BrCN-cleaved fragment of 5′-nucleotidase (membrane form) was extracted with hexane, resulting in the separation of three layers. Of the three layers, an interface layer containing amphipathic materials was used for purification of the membrane-anchoring domain. It was important to note that in order to obtain the fraction enriched with the amphipathic fragment, it was necessary to dissolve the fragments in formic acid as completely as possible before the extraction with hexane. Other extraction procedures tested, including a chloroform/methanol extraction, were not so effective in obtaining this fragment.

Once an effective extraction method was established, the fragment of M_r 2300 was purified in a good yield [about 40%, 60 nmol of the fragment from 10 mg (=147 nmol) of the protein] by relatively simple HPLC steps (Figure 2). The purified fragment contained a tetradecapeptide with the sequence Lys-Val-Ile-Tyr-Pro-Ala-Val-Glu-Gly-Arg-Ile-Lys-Phe-Ser. This sequence corresponds to positions 510–523 in the primary structure of rat liver 5′-nucleotidase predicted by the cDNA sequence (Figure 3) (Misumi et al., 1990). The presence of methionine at position 509 accounts for the release of this peptide by cleavage with BrCN. In addition, lysyl endopeptidase treatment released the dipeptide Phe-Ser, which is aligned at positions 522–523, since no other part of the

primary structure contains this sequence (Misumi et al., 1990). Although the cDNA sequence predicts a further peptide extension to position 548, including a hydrophobic stretch (Figure 3), the data presented here demonstrate that serine-523 is the COOH-terminal residue of the mature protein. Identification of the GPI components in both the tetradecapeptide and dipeptide fragments indicates that serine-523 is covalently attached with GPI. Thus, it is concluded that proteolytic removal of the predicted COOH-terminal extension (524–548) is accompanied by replacement with GPI at serine-523, as proposed for other GPI-anchored proteins such as VSG of *Trypanosoma brucei* (Ferguson et al., 1985), rat brain Thy-1 (Tse et al., 1985), human placental alkaline phosphatase (Micanovic et al., 1988; Ogata et al., 1988; Takami et al., 1988a), rat MRC OX-45 antigen (Killeen et al., 1988), and human carcinoembryonic antigen (Hefta et al., 1988; Takami et al., 1988b).

Rat liver 5′-nucleotidase contains neither galactose nor *N*-acetylgalactosamine in its GPI anchor, as in the case of human placental alkaline phosphatase (Ogata et al., 1988). This is in contrast to the observations that the GPI moiety of VSG contains galactose (two to four residues) (Ferguson et al., 1985, 1988) and that of rat Thy-1 contains *N*-acetylgalactosamine (Tse et al., 1985; Homans et al., 1988). The presence of ethanolamine at 2 mol/mol suggests that one residue is involved in the linkage of GPI to the α-carboxyl group of serine-523 and the other may be phosphodiester-linked to a mannose residue of the glycan core, as demonstrated for Thy-1 (Homans et al., 1988) and acetylcholinesterase (Roberts et al., 1988). The latter ethanolamine having a free amino group is considered to be a common feature of GPI anchors from species other than single-cell eukaryotes, of which VSG contains only the bridging ethanolamine in the anchor (Ferguson et al., 1985, 1988).

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Registry No. L-Ser, 56-45-1; 5′-nucleotidase, 9027-73-0.

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