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Mutational analysis of the COOH-terminal hydrophobic domain of bovine liver 5′-nucleotidase as a signal for glycosylphosphatidylinositol (GPI) anchor attachment

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Abstract

In order to address the minimum domain of the COOH-terminal hydrophobic region responsible for GPI modification of bovine liver 5′-nucleotidase, we constructed a series of the deletion mutants of the COOH-terminus and expressed them in COS cells. Cells transfected by the deletion mutant of 6 amino acids (-IIILYQ) from the hydrophobic domain (-FSLIFLSVLAVIII-LYQ) did not show any elevation of cell surface-associated 5′-nucleotidase activity, whereas the 2 (-YQ) or 4 (-ILYQ) amino acid deletion mutant retained the bovine liver-derived activity on the cell surface as a GPI-anchored protein. Loss of half the hydrophobic domain (6 or 8 amino acids) resulted in accumulation of the activity in the cell. On the other hand, deletion of the whole hydrophobic domain (17 amino acids) or the entire cleaved-off domain (25 amino acids) made the product secreted into the medium. In conclusion, the hydrophobicity of 13 amino acids in length was enough for the GPI modification of the bovine liver 5′-nucleotidase.

Key words: 5′-Nucleotidase; Glycosylphosphatidylinositol anchor; COS cell

1. Introduction

A number of cell-surface proteins are known to be anchored to the plasma membrane by a COOH-terminal glycosylphosphatidylinositol (GPI) moiety [1,2]. Analyses of cDNAs of several GPI-anchored proteins revealed that the 20–30 amino acid hydrophobic peptide at the COOH-terminus is removed from the nascent chain. The cleaved-off region is now considered as a signal for the posttranslational modification by GPI [2]. Mutational analyses of the COOH-terminal signals of alkaline phosphatase [3–5], decay accelerating factor (DAF) [6] and others [1,2] demonstrated that the length of the hydrophobic domain and the amino acids next to the cleavage site are important determinants for the GPI modification.

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) is a membrane-bound glycoprotein which has been shown to be an ectoenzyme present in a wide variety of eukaryotic cells [7]. Although the precise physiological function of 5′-nucleotidase is still unclear [8], its interaction with membrane via phosphatidylinositol (PI) was demonstrated earlier by us [9] and others [10]. We and others have cloned and sequenced cDNAs coding for 5′-nucleotidases of mammalians and electric ray [11–14]. Serine has been identified as the attachment site of GPI-anchor common to rat and bovine liver 5′-nucleotidase [14,15]. However, no direct information about the function of the hydrophobic COOH-terminus is available so far.

Previously, we have constructed an expression vector of the enzyme [14]. By transfecting COS cells with the construct, the 5′-nucleotidase activity was expressed as a GPI-anchored protein. In this report, we constructed a series of deletion mutants of the hydrophobic domain, and expressed them in COS cells to characterize the COOH-terminal signal for GPI modification.

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Abbreviations: DAF, decay accelerating factor; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

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2. Materials and methods

2.1. Materials

An expression vector for bovine liver 5′-nucleotidase, pSVNT, was described previously [14]. DEAE-dextran (M_r 1 500 000) was purchased from Pharmacia. Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from the culture broth of *B. thuringiensis* as described [16]. *Escherichia coli dam*$^-$ strain was used for preparation of plasmid DNA subjected to *Bcl*I or *Eco*O65I digestion.

2.2. Construction of deletion mutants of 5′-nucleotidase cDNA

A series of COOH-terminal deletion mutants were constructed by deleting a part of the COOH-terminal hydrophobic region of the cDNA encoding 5′-nucleotidase, and by inserting a synthetic DNA fragment into the deleted site as follows. Plasmid pSVNT was digested with *Eco*O65I and *Bcl*I to remove a 146 bp fragment corresponding to the 49 amino acid residues of the COOH-terminus of bovine 5′-nucleotidase, including the hydrophobic domain. DNA fragments coding shortened hydrophobic domains of varying length were synthesized by PCR using mutated primers and pSVNT as a template. A forward primer was 5′-CATGACTCTGGTGACCAAGATAT (corresponding to residues 494G-498I; numbered taking the NH_2-terminal amino acid of the mature protein as 1). Reverse primers were designed to create a stop codon after codons of residues 523S, 531S, 540L, 542V, 544I, and 546L, respectively; 5′-TTTGATCA*CCCGGG*T-CAAGAAAACTGGATCCGA (519R-523S) for mutant 523S, 5′-TGATCATCAACTTCCACAG (528C-531S) for mutant 531S, 5′-TGATCA*CCCGGG*TCA-CAAAACTGAAAG (537L-540L) for mutant 540L, 5′-TGATCA*CCCGGG*TCACACTGCCAAAACTGAA-AG (539V-542V) for mutant 542V, 5′-GTGATCA*CC-CGGG*TCAAATAATGACTGCCAA (540L-544I) for mutant 544I, and 5′-GTGATCA*CCCGGG*TCA-TAAAATAATAATCACTGC (541A-546L) for mutant 546L. (*Sma*I sites to aid for screening of mutants are italic.) Template (approx. 30 ng pSVNT) was mixed with 5 μg of each primer, and the other components of the reaction were added to the mixture according to the manufacturer's protocol (Takara, Japan). DNA thermal cycler was programmed for a 94°C denaturation for 2 min, a 37°C annealing for 2 min, a 72°C polymerization for 3 min, and 30 cycles. PCR fragments were ligated into the 6.6 kb *Eco*O65I-*Bcl*I fragment of pSVNT.

2.3. Cell culture and DNA transfection

COS-1 cells, a transformed cell line of African green monkey kidney cells, were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% fetal calf serum (Boehringer) at $(2–3) \cdot 10^6$ cells per 35-mm dish. Transfection of COS cells with various plasmid DNAs and the detection of transiently expressed 5′-nucleotidase activity associated with the cells were performed as described previously [14].

2.4. Detection of 5′-nucleotidase activities in the homogenates and the media of the transfected cells

Transfected cells were washed twice with ST buffer (0.25 M sucrose and 0.1 mM phenylmethylsulfonyl fluoride in 20 mM Tris-HCl, pH 7.5), then scraped off and resuspended in 1 ml of ST buffer containing pepstatin (5 μg/ml) and antipain (5 μg/ml). The suspensions were homogenized (cell homogenates). One ml of each medium was centrifuged for 10 min at $8000 \times g$ to remove cell debris, and concentrated by Ultrafree (Millipore) to 200 μl. The 5′-nucleotidase activities of the homogenates and the concentrated media were determined as described below.

2.5. Assay of 5′-nucleotidase activity

5′-Nucleotidase activity was determined with 5′-AMP as a substrate. The reaction mixture (200 μl) containing 50 mM glycine buffer (pH 8.5), 5 mM $MgCl_2$ and 10 mM AMP was incubated for 30 min at 37°C, then the reaction was terminated by addition of 1 ml of 0.02% ascorbic acid and 10% tricholoroacetic acid. After centrifugation, phosphorus in the supernatant was determined by the method of Baginski et al. [17]. An enzyme unit was defined as the amount of 5′-nucleotidase that catalyzed the hydrolysis of 1 μmol AMP per min at 37°C.

2.6. Detection of 5′-nucleotidase mRNA in transfected cells

Total RNA was isolated from transfected cells ($1 \cdot 10^6$ cells) by the method of Chomczynski and Sacchi [18], electrophoresed and analyzed by RNA blot hybridization using digoxigenin-labeled 5′-nucleotidase probe. Detection of the hybridized probe was performed using DIG luminescent detection kit (Boehringer), according to the manufacturer's protocol.

3. Results

The COOH-terminal hydrophobic region of bovine 5′-nucleotidase, which is cleaved off during the GPI processing, comprises 25 amino acids following the GPI attachment site, Ser-523, as shown in Fig. 1 [14]. The region is composed of a hydrophilic 8 amino acid

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                cleavage site
     N          ↓ hydrophilic ←——— hydrophobic ——→ C
WT   -----QFS¦ AGSHCCGSFSLIFLSVLAVIIILYQ
546L -----QFS AGSHCCGSFSLIFLSVLAVIIIL
544I -----QFS AGSHCCGSFSLIFLSVLAVII
542V -----QFS AGSHCCGSFSLIFLSVLAV
540L -----QFS AGSHCCGSFSLIFLSVL
531S -----QFS AGSHCCGS
523S -----QFS
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Fig. 1. The amino acid sequences of the COOH-termini of the wild-type and the constructed 6 deletion mutants of bovine liver 5′-nucleotidase. Amino acid sequences of the COOH-terminal regions of 5′-nucleotidase (521Q-548Q) and the deletion mutants are shown in single-letter codes. The hydrophilic spacer and the hydrophobic domains are indicated above the sequences. Cleavage site of the wild type is indicated by a vertical dashed line.

spacer and a hydrophobic 17 amino acid stretch. The cleaved-off hydrophobic domain has been shown as a signal for GPI modification of several GPI-anchored proteins [1,2]. In order to know how the hydrophobic region is responsible for GPI modification of the bovine liver 5′-nucleotidase, we constructed a series of deletion mutants of the region using pSVNT, the expression vector of bovine 5′-nucleotidase, by in vitro mutagenesis as described in Materials and methods. A 146 bp *Eco*O65I-*Bcl*I portion of the wild-type cDNA was replaced by a PCR product encoding various length COOH-terminus (Fig. 1). We constructed 6 deletion mutants as shown in Fig. 1. Mutants 546L, 544I, 542V and 540V had a various length hydrophobic domain from 9 to 15 residues, respectively. Mutant 531S lost almost all the hydrophobic domain (17 residues), comprising 8 neutral and basic amino acids at the end of the product, and mutant 523S ended at Ser-523, lacking the entire COOH-terminal hydrophobic region.

Fig. 2A shows the transiently expressed 5′-nucleotidase activities associated with intact COS cells which were transfected with the 6 mutant DNAs and controls. With the wild-type, pSVNT, the activity was elevated about 2.5-times higher than those of controls (endogenous activities). As reported previously [14], 73.8% of the activity on the surface of pSVNT-transfected cells was released by PI-PLC treatment (0.5 unit/ml), indicating that the transiently expressed 5′-nucleotidase was GPI-anchored. Cells transfected with the mutants 546L and 544I also showed elevated activities comparable to that with the wild-type. The activities produced by the two mutants were also susceptible

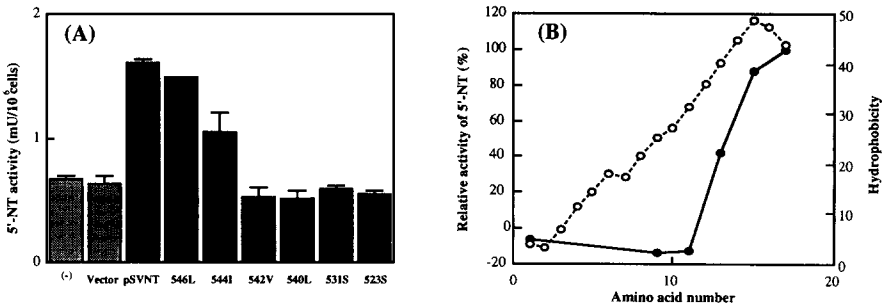

Fig. 2. The 5′-nucleotidase activities associated with intact COS-1 cells which were transfected with the wild-type cDNA of bovine liver enzyme (pSVNT) and its 6 deletion mutants. (A) COS-1 cells were transfected with each plasmid DNA (2 μg/ml), and the cell-associated 5′-nucleotidase activity was determined after 48 h incubation. (−) and Vector stand for mock- and pSVL-transfection, respectively. The values were obtained as the averages ± deviation of 2 dishes. (B) Relative 5′-nucleotidase activities by the wild-type and the mutants were plotted against the numbers of the amino acids of the hydrophobic domain (solid line). The value of the control was subtracted from each value and the activity in the wild-type was expressed as 100%. The hydrophobicity of the truncated hydrophobic region was calculated by summing the hydrophobic units of the amino acids composing the region, according to Kyte and Doolittle [26] (dashed line).

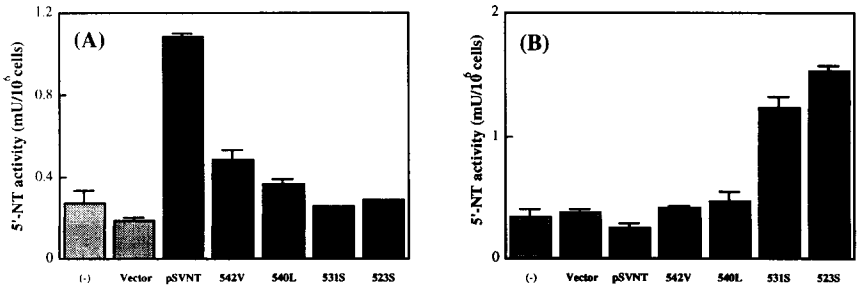

Fig. 3. Localization of the 5′-nucleotidase activities of the cells transfected by those mutants, in which the cell surface activities were not elevated. After transfection with various plasmid DNAs, the media were collected and cells were homogenized as described in Materials and methods. The 5′-nucleotidase activities in the homogenates (A) and the media (B) were determined. (−) and Vector represent mock- and pSVL-transfection. The values were obtained as the averages ± S.D. of 4 dishes.

to the PI-PLC treatment; 78.2% (546L) and 74.4% (544I) of the activities were released by the treatment, respectively, showing that the products were anchored via GPI. On the other hand, the cells transfected with 4 other deletion mutants having the domain less than 11 hydrophobic amino acids, did not show such elevated, cell-associated activities (Fig. 2A and B); the values were comparable to those of controls. These low activities were not due to the low efficiency in transcription of the mutant cDNA, since a northern blot analysis of RNA isolated from the mutant-transfected cells showed no difference in the transcriptional level from that of pSVNT-transfected cells (data not shown). Fig. 2B shows the relative activities of the cell-associated 5′-nucleotidase in the transfected cells and plotted against the amino acid number of the COOH-terminal hydrophobic region, with the hydrophobicity of the domain calculated by summing the hydrophobicity unit of each amino acid obtained by Kyte and Doolittle [23]. With hydrophobicity more than 40, the product was GPI-anchored, whereas the product with hydrophobicity lower than 32 was not modified by GPI.

To localize the products by the mutants which did not show additional cell-associated activity, the 5′-nucleotidase activities in the cell homogenates and the media were measured. As shown in Fig. 3, the enzyme activities in the homogenates of 523S-and 531S-transfected cells were of the basal level, while the activities in the media of these cells were 5–6-times higher than those of other transfected cells. On the other hand, the activities in the cell homogenates of 540L- and 542V-transfected cells were approx. 2-times higher than that of the control with no additional activities in the media. These results indicate that the 5′-nucleotidase produced by the 4 mutants (523S, 531S, 540L and 542V) were not sorted to the cell surface, instead, two of them (540L and 542V) tended to stay in the cell and the other two (523S and 531S) were forced to be secreted into the medium, probably depending on the hydrophobicity of their COOH-termini as illustrated in Fig. 4.

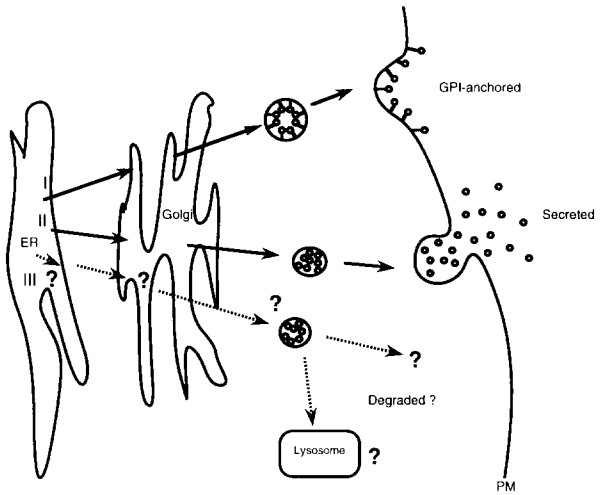

Fig. 4. The fate of the wild-type and the mutant products of 5′-nucleotidase after the entry into the ER. The products of the wild-type and mutant 544I and 546L are expressed as a GPI-anchored protein along the pathway I. Secreted mutants (523S and 531S) might utilize the pathway II. The flow of the intracellularly localized mutants (540L and 542V) is to be solved but yet unknown (III, dashed lines). PM, plasma membrane.

4. Discussion

We constructed a series of deletion mutants differing in the COOH-terminal hydrophobic domain of bovine liver 5′-nucleotidase (Fig. 1) and analyzed the fate of the products by these mutants. Similar experiments were carried out by Berger et al. [3], using human placental alkaline phosphatase (PLAP) as a probe for GPI-anchored protein. Although they tried to determine the minimal length of the COOH-terminal peptide, actually they could not fulfil their purpose perfectly, since they cut the peptide chain of this part by 5–6 amino acid intervals as AGTTDAAHP-GRSVVPALL | PLLAG | TLLLL | ETATAP. In the present study, we have shortened the COOH-terminal peptide chain more deliberately by two amino acid units as QFSAGSHCCGSFSLIFLS | VL | AV | II | IL | YQ and succeeded in getting the exact minimal length of the hydrophobic peptide required for GPI processing. Thus, the cells transfected with the mutants deleted more than 6 amino acids of the domain did not show any additional cell-associated activity of 5′-nucleotidase, whereas deletion of 2 (-YQ) or 4 (-ILYQ) amino acids from the end gave essentially the same results as the wild-type (Fig. 2). These elevated activities were released by PI-PLC, showing the presence of GPI modification of the products. These results indicated that the hydrophobic domain of at least 13 amino acid length is enough for GPI modification of bovine 5′-nucleotidase, although the slightly lower activity of 544I-transfected cells may reflect the lower efficiency of GPI-modification compared to the wild-type. Recently, Coyne et al. have reported that a hydrophobic domain of at least 11 Leu residues could function as a signal for GPI modification of truncated CD46 [19]. The difference of the amino acid numbers between 5′-nucleotidase and CD46 required for GPI modification may be due to the difference in the amino acid compositions between these two hydrophobic domains, i.e., they used synthetic Leu-11 as a signal that is highly hydrophobic whereas the 13 amino acid domain of 5′-nucleotidase contained several neutral amino acids such as Ser.

Trimming of the hydrophobic domain from 17 amino

acids to 9 or 11 (mutants 542V and 540L) kept the activity intracellularly (Fig. 3A). If the cleavage and the addition of GPI moiety at Ser-523 occurred in accordance by a putative transamidase as suggested by Amthauer et al. [20–22], the proteins produced by these mutants (540L and 542V) will not be recognized by the transamidase because of the insufficient hydrophobicity of the domain, and then will retain short hydrophobic ends which may weakly associate with the membranes of endoplasmic reticulum (ER) and Golgi. With such mutants, transiently expressed 5′-nucleotidase might be unable to be transported to the cell surface, instead, the products might be distributed randomly with the bulk of the membrane flow. Some amount of these products will be degraded by endogenous proteinase(s) probably in lysosomes (Fig. 4, route III). This may explain why almost all the activities in the mutants with 540L and 542V were detected within the cell and why the activities were relatively low compared to those of the GPI-anchored products. Of human alkaline phosphatase, a 5 amino acid deletion from the 16 amino acid hydrophobic domain caused the product to be secreted into the medium [3], whereas the activity still resided within the cell with a 6 amino acid removal of the 15 amino acid hydrophobic domain of 5′-nucleotidase (540L). The lower hydrophobicity of the uncleaved, shortened COOH-terminus of alkaline phosphatase mutant (16.5, calculated according to Kyte and Doolittle [23]) than that of 5′-nucleotidase mutant (25.3) may explain the difference of their localizations.

Using human growth hormone-DAF fusion protein containing a mutated, noncleavable signal, Moran and Caras demonstrated that uncleaved products accumulated in a sorting compartment between the ER and the medial Golgi [24]. Using the cells defective in GPI biosynthesis, Delahunty et al. also reported that the uncleaved COOH-terminus caused retention of precursor proteins in the ER [25]. It has been postulated that there exists a mechanism to retain proteins containing the uncleaved GPI signal [24]; however, it has not been well documented yet whether a product with shortened GPI signal is also retained in a specialized compartment of the cell. Analysis of subcellular localization of the intracellular products from the mutants 540L and 542V will prove this problem.

With mutants 523S and 531S, the COOH-terminal ends of their products were hydrophilic enough to be secreted into the medium (Fig. 3C), as in the case of the deletion mutants having a hydrophilic end of human alkaline phosphatase and DAF [4–6]. This indicates that there is no additional signals other than GPI-anchor in the mature protein to retain the enzyme within the cell. Fig. 4 shows the postulated pathways through which the mutant proteins pass to their locations, although the localization of these intermediates within the cell must be precisely analyzed.

The function of the hydrophobic region of GPI-anchored proteins in the posttranslational processing is not fully understood yet, however, the roles in the association with a putative transamidase in the ER and/or in the transport of the nascent protein to the proper position where the processing occurs, have been postulated [4–6]. It has been reported that the hydrophilic spacer region, especially $\omega + 2$ site, were also an important determinant for the appropriate GPI-modification as well as the GPI attachment site (ω) [21,22,26]. Therefore, this spacer region remains to be clarified in the case of 5′-nucleotidase.

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