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Mutational analysis of the C-terminal signal peptide of bovine liver 5′-nucleotidase for GPI anchoring: a study on the significance of the hydrophilic spacer region

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Abstract

Bovine liver 5′-nucleotidase is a GPI-anchored protein whose Ser^{523} attaches to GPI as the ω-site. For GPI-modification, pro-protein of the enzyme possesses a signal peptide at the C-terminus, comprising a hydrophilic spacer sequence of 8 amino acid residues and the following hydrophobic region of 17 amino acid residues. The C-terminal signal peptide is replaced by GPI on a luminal leaflet of endoplasmic reticulum. To characterize the C-terminal signal peptide for GPI modification, we constructed a series of deletion and elongation mutant genes, altering length of the hydrophilic spacer sequence by site-directed mutagenesis. Systematic deletion and Ala insertion of the sequence showed that the sequence of 6–14 residues were compatible for GPI modification. For GPI transfer to the pro-protein, the optimum length of spacer sequence would be 8, being consistent with natural selection. The spacer sequence may play a role for leading the ω-residue correctly to the active site of putative GPI transamidase. The elongation of the spacer is more permissible than deletion. Nevertheless, the length of the spacer sequence may influence efficiency of GPI modification by its positive or negative control. © 1997 Elsevier Science B.V.

Keywords: 5′-Nucleotidase; Glycosylphosphatidylinositol; Hydrophilic spacer sequence; Site directed mutagenesis; Post translational modification; Glycosylphosphatidylinositol anchor

1. Introduction

Glycosylphosphatidylinositol (GPI)-anchored proteins are widely distributed over eukaryotic cells [1,2], and their presence in archaebacteria was recently demonstrated [3]. Precursor polypeptides of the GPI-anchored proteins (prepro-protein) have signal peptides at its N- and C-termini. The N-terminal signal peptide for secretion is removed from the nascent polypeptide and the resulting pro-protein is transferred into the luminal side of the endoplasmic reticulum. The C-terminal signal peptide for GPI modification ends by a short stretch of hydrophobic region, and the pro-protein binds to the luminal membrane by that region. However, the pro-protein is modified immediately with GPI moiety by a putative GPI transamidase. Systematic deletion of the C-terminal hydrophobic region demonstrated the re-

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

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Table 1
The amino acid sequences of the C-terminal signal peptides of the GPI-anchored protein precursors

Protein	C-Terminal amino acid sequence					
			Spacer sequence			Hydrophobic region
		ω	ω + 1	ω + 2		
p137 (human)	GSRG	A	R	G	LMNGYRGPAMDSEED	MMVTALHSLTLQTVVIHSLSSVLPGITLAIN
E48 (human)	CCQE	D	L	C	NEKLHNAAPTRTA	LAHSALSLGLALSLLAVILAPSL
CEA (human)	ITVS	A	S	G	TSPGLSAGAT	VGIMIGVLVGVALI
ThB (mouse)	TDLC	N	E	R	LVSAAPGHA	LLSSVTLGLATSLSLLTVMALCL
Thy-1 (rat)	KLVK	C	G	G	ISLLVQNTS	WLLLLLLSLSFLQATDFISL
Thy-1 (human)	KLVK	C	E	G	ISLLAQNTS	WLLLLLLSLSLLQATDFMSL
GAS1 (yeast)	SSKK	N	A	A	TNVKANLAQ	VVFTSIISLSIAAGVGFALV
Sca-2 (mouse)	SSFC	N	F	S	AAGLGLRAS	IPLLGLGLLLSLLALLQLSP
LY-6C antigen (mouse)	EDLC	N	A	A	VPTGASTWT	MAGVLLFSLSSVILQTLL
DAF (human)	SGTT	S	G	T	TRLLSGHTC	FTLTGLLGTLVTMGLLT
Dipeptidase (pig)	NYGY	S	A	A	PSLHLPPGS	LLASLVPLLLLSLP
VSG YNat 1.1 (*T. congolense*)	SHLP	S	G	S	SHGTKAIRS	ILHVALLM
PHR1 (*C. albicans*)	GSSS	S	S	G	VKATQQMS	MVKLVSIITIVTAFVGGMSVVF
N-CAM (chicken)	TVIP	A	T	L	GSPSTSSS	FVSLLLSAVTLLLLC
Folate receptor-b (human)	AMHV	N	A	G	EMLHGTGG	LLLSLALMLQLWLLG
AP-P (pig)	EPLS	A	R	A	APTTSLGS	LMTVSALAILGWSV
GP2 (rat)	NGTP	R	N	T	GFLLAWPT	FFLPVFLAWLF
u-PAR (human)	QYRS	G	A	A	PQPGPAH	LSLTITLLMTARLWGGTLLWT
LFA-3 antigen (human)	TCIP	S	S	G	HSRHRYA	LIPIPLAVITTCIVLYMNVL
Glypican (rat)	GQKT	S	A	A	TRPEPHY	FFLLFLFTLVLAAARPRWR
CD59 (human)	EQLE	N	G	G	TSLSEKT	VLLLVTPFLAAAWSLHP
AChE (*Torpedo*)	DGEL	S	S	S	GTSSSKG	IIFYVLFSILYLIFY
Prion protein (hamster)	DGRR	S	S	A	VLFSSPP	VILLISFLIFLMVG
Brevican (rat)	APSS	G	N	S	AEGSMPA	FLLFLLLQLWDT
VSG ILTat 1.1 (*T. brucei*)	NTTG	S	N	S	FVIHKAP	LFLAFLLF
VSG TxTat 1 (*T. brucei*)	NTTA	S	N	S	FVINKAP	LLLGFLLF
VSG MIT 221 (*T. brucei*)	TTGS	S	N	S	FVISKTP	LWLAVLLF
RT6.2 (rat)	NCLY	S	S	A	GARESC	VSLFLVVLPSLLVQLLCLAEP
Melanotransferrin (chicken)	QCSG	A	G	N	KLIQQH	LLVITFVPFIILGQLQGLG
DIP (human)	YGYS	S	G	A	SSLHRH	FGLLLASLAPLVLCLSLL
5′-Nucleotidase (rat)	RIKF	S	A	A	SHYQGS	FPLIILSFWAVILVLYQ
5′-Nucleotidase (human)	RIKF	S	T	G	SHCHGS	FSLIFLSLWAVIFVLYQ
5′-Nucleotidase (bovine)	RIQF	S	A	G	SHCCGS	FSLIFLSVLAVIIILYQ
5′-Nucleotidase (mouse)	RIKF	S	A	A	SHYQGS	FPLVILSLSAVIFVLYQ
CAMPATH-1 antigen (human)	TSSP	S	A	S	SNISGG	IFLFFVANAIIHLFCFS
OX45 antigen (mouse)	DLAR	S	S	G	VCWTAT	WLVVTTLIIHRILLT
VSG MIT 118a (*T. brucei*)	EKCR	N	G	S	FLTSKQ	FAFSVVSAAFVALLF
VSG MIT 117a (*T. brucei*)	NACK	D	S	S	ILVTKK	FALTVVSAAFVALLF
PARP (*T. brucei*)	EPEP	G	A	A	TLKSVA	LPFAIAAAALVAAF
VSG MITat 1.1000 BC (*T. brucei*)	EKCC	D	G	S	FLVNKK	FALMVYDFVSLLAF
VSG MITat 1.5b (*T. brucei*)	EDCR	N	G	S	FLTSKQ	FALMVSAAFVTLLF
CNTN1 (human)	QVKI	S	G	A	PTLSPS	LLGLLLPAFGILV
AChE (human)	GFTH	G	E	A	ARRPG	LPLPLLLLHQLLLLFLSHLRRL
APase (human placenta)	AGTT	D	A	A	HPGRS	VVPALLPLLAGTLLLLETATAP
Folate receptor-a (human)	AAAM	S	G	A	GPWAA	WPFLLSLALMLLWLLS
APase (human intestinal)	ACTT	D	A	A	HP	VAASLPLLAGTLLLLGASAAP

Table 1 (continued)

Protein	C-Terminal amino acid sequence					
			Spacer sequence			Hydrophobic region
		ω	ω + 1	ω + 2		
APase (human liver)	CAPA	S	S	A	GS	LAAGPLLVALALYPLSVLF
MRC OX-45 antigen (rat)	TLAR	S	S	G	VH	WIAAWLVVTLSIIPSILLA
Gp64 (slime mold)	NNVC	S	S	A	TT	IAFNAFVVFAIVLSVLLF
PsA (slime mold)	STTT	G	S	A	ST	VVASLSLIIFSMILSLC
VCAM1 (mouse)	HLMF	A	K	S	FY	FICYLCLYLAL

p137 (human)[34], E48 (human)[35], CEA (human) [36], ThB (mouse) [37], Thy-1 (rat) [38], Thy-1 (human) [39], GAS-1 (yeast) [40], Sca-2 (mouse) [41], Ly-6C antigen (mouse) [42], DAF (human) [43], dipeptidase (pig) [44], VSG YNat 1.1 (*T. congolense*) [45], PHR1 (*C. albicans*) [46], N-CAM (chicken) [47], folate receptor-b (human) [48], AP-P (pig) [49], GP2 (rat) [50], u-PAR (human) [51], LFA-3 antigen (human) [52], glypican (rat) [53], CD59 (human) [54], AChE (*Torpedo*) [55], Prion protein (hamster) [56], brevican (rat) [57], VSG ILTat 1.1 (*T. brucei*) [58], VSG TxTat 1 (*T. brucei*) [59], VSG MIT 221 (*T. brucei*) [60], RT6.2 (rat) [61], melanotransferrin (chicken) [62], DIP (human) [63], 5′-nucleotidase (rat) [64], 5′-nucleotidase (human) [65], 5′-nucleotidase (bovine) [13], 5′-nucleotidase (mouse) [66], CAMPATH-1 antigen (human) [67], OX45 antigen (mouse) [68], VSG MIT 118a (*T. brucei*) [69], VSG MIT 117a (*T. brucei*) [60], PARP (*T. brucei*) [70], VSG MITat 1.1OOO BC (*T. brucei*) [71], VSG MITat 1.5b (*T. brucei*) [72], CNTN1 (human) [73], AChE (human) [74], APase (human placenta) [75], folate receptor-a (human) [48], APase (human intestine) [76], APase (human liver) [77], MRC OX-45 antigen (rat) [78], Gp64 (slime mold) [79], PsA (slime mold) [80], VCAM1 (mouse) [81]. The sequences are arranged according to the length of the hydrophilic spacer sequence.

quirement of length and hydrophobicity of the region for GPI modification [4–6]. The C-terminal amino acid residue of mature polypeptide covalently bound to GPI moiety is called the ω-site which is followed by the residues named ω + 1, ω + 2 and so on toward the C-terminus [7]. The transamidase cleaves the peptide bond of pro-protein at the C-terminal side of the ω-residue, transferring this terminal carboxyl to the amino group of ethanolamine in GPI. The C-terminal signal peptide includes a hydrophilic spacer sequence between the ω-site and the hydrophobic region (Table 1). Although the hydrophilic spacers are various in length and no obvious consensus is observed among their sequences, it is well known that the types of amino acid residues at the ω- and ω + 2-sites are restricted [7–10]. Mutational analysis of decay-accelerating factor (DAF) [11] and artificial spacer of CD46 [12] suggested that 9–12 amino acid residues were required as the spacer for GPI modification. On the other hand, almost a half of precursor proteins have a hydrophilic spacer sequence whose length is less than nine amino acid residues, as shown in Table 1. Thus the modification depends not simply on the number of residues in the sequence.

5′-Nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) is a GPI-anchored protein which has been shown to be an ectoenzyme present in a wide variety of eukaryotic cells [13]. Cloning and sequencing of the cDNAs coding for the enzyme was previously reported and the enzyme was expressed as a GPI-modified form on the cell surface of COS-1 transfected with the cDNA [14]. More than half of the cDNA product was released from cell surface by phosphatidylinositol-specific phospholipase C (PI-PLC), which is common feature of GPI-anchored proteins [15,16]. We have also reported the ω-site of bovine 5′-nucleotidase is Ser^{523} by analysis of GPI-linked C-terminal polypeptide [17]. Therefore, the enzyme is suitable to investigate the role of spacer sequence for GPI modification, in that the protein has a relatively short, 8 residues of spacer sequence. In this report, we constructed a series of deletion and elongation mutants of the spacer sequence of 5′-nucleotidase and examined the effects of the mutations on GPI modification.

2. Materials and methods

2.1. Materials

Plasmid pSVNT is a derivative of pSVL into which the wild-type cDNA of bovine liver 5′-nucleotidase was inserted [14]. M13-mp18 was used as a vector for mutagenesis. *Escherichia coli* JM110

(*dam*-) and TG1 were used for DNA technology. Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from the culture broth of *Bacillus thuringiensis* as described [18]. Adenosine-5′-monophosphate (AMP) was used as a substrate for assay of the 5′-nucleotidase activity.

2.2. Construction of the deletion mutant genes

A series of deletions were introduced by Eckstein's oligonucleotide-directed mutagenesis [19–21] (Sculptor in vitro mutagenesis system, Amersham International plc, UK). A 305-bp of *Hin*dIII fragment of 5′-nucleotidase cDNA encoding the anchoring signal, was inserted into the *Hin*dIII site of M13-mp18 multiple cloning site (M13-NTC) and used as a template for mutagenesis. Mutagenic oligonucleotide was designed to occupy the complementary sequence of 5′-nucleotidase cDNA. Deletions were introduced to the coding sequence just after the ω-site (Ser^{523}) as follows: 5′-AAGAAATATCAAAGAAAA/ACAGCAATGACTTCCTGC-3′ (complementary to the coding sequence of 524A–534L) for mutant Δ(530–531); 5′-AAGAAATATCAAAGAAAA/ATGACTTCCTGCAGAAAA-3′ (complementary to the coding sequence of 522L–534L) for mutant Δ(528–531); 5′-AAGAAATATCAAAGAAAA/TCCTGCAGAAAACTGGAT-3′ (complementary to the coding sequence of 520I–534L) for mutant Δ(526–531); and 5′-AAGAAATATCAAAGAAAA/AGAAAACTGGAT-CCGACC-3′ (complementary to the coding sequence of 518G–534L) for mutant Δ(524–531). The fusion point of deletion in each oligonucleotide was denoted by slash. For example, Δ(530–531) denotes the deletion mutant lacking the two residues of 530G and 531S in the hydrophilic spacer. Silent mutation destroying the *Ssp*I site by change of T to C (underlined) was also introduced to aid for screening of mutants. To construct the mutant cDNA clones, the mutated DNA fragments were cut out with *Eco*O65I and *Bcl*I digestion, and exchanged for the corresponding fragment of pSVNT.

2.3. Construction of the elongation mutant genes

Stepwise Ala insertion after Ser^{531}, the end of spacer sequence of the C-terminal signal, was performed by oligonucleotide-mediated PCR mutagenesis using M13-NTC as a template. The mutagenic, forward and reverse primers designed to insert 2, 4, 6, 7, 8 and 9 Ala residues after Ser^{531}, respectively, were as follows: forward 5′-TGTGGAAGTgctgctTTTTCTT-TGATA-3′ and reverse 5′-TATCAAAGAAAAagcagcACTTCCACA-3′ primer for mutant 531A2; forward 5′-TGCTGTGGAAGTgctgccgctgccTTTTCTTTGATA-3′ and reverse 5′-TATCAAAGAAAggcagcggcagcACTTCCACAGCA-3′ primer for mutant 531A4, forward 5′-TGCTGTGGAAGTgctgccgctgccgctgccTTTTCTTTGATA-3′ and 5′-TATCAAGAAAAggcagcggcagcggcagcACTTCCACAG-CA-3′ primer for mutant 531A6, forward 5′-TGTGGAAGTgctgctgccgccgctgccgctTTTTCTTTGATAT-TT-3′ and reverse 5′-TATCAAAGAAAAagcggcagcggcggcagcagcACTTCCACAGCA-3′ primer for mutant 531A7, forward 5′-TGTGGAAGTgctgctgccgccgctgccgctcgccTTTTCTTTGATATTT-3′ and reverse 5′-TATCAAAGAAAAggcagcggcagcggcggcagcagcACTTCCACAGCA-3′ primer for mutant 531A8 and forward 5′-TGTGGAAGTgctgctgccgccgctgccgctgccgctTTTTCTTTGATATTT-3′ and reverse 5′-TATCAAAGAAAAagcggcagcggcagcggcggcagcagcACTTCCACcAGCA-3′ primer for mutant 531A9. Nucleotide sequence corresponding to Ala insertions were denoted with small-letters. Silent mutation by change of T to C (underlined) destroyed the *Ssp*I site in order to aid screening of mutants. 531AX means the mutant gene inserted a definite number (= X) of Ala residues after Ser^{531}. The mutated PCR fragments were exchanged for the corresponding restriction fragment of pSVNT as described above.

2.4. Cell culture and DNA transfection

COS-7 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 (BIO-Whittaker, Maryland, USA). Transfection of the mutant genes was carried out in the dishes or the flasks using cationic liposome-mediated method [22] (LipofectAMINE Reagent) with serum-free medium (OPTI-MEM I Reduced Serum Medium, Gibco Life Technologies, Inc. Grand Island, NY, USA), according to the manufacturer's protocol.

2.5. Assay of 5′-nucleotidase activities

The enzyme activity expressed on the cell surface was measured as follows. The cells in each culture dish (35 mm diameter) were washed twice with 1 ml of ST buffer (0.25 M sucrose and 0.1 mM phenylmethylsulfonyl fluoride in 20 mM Tris-HCl, pH 7.5). The cells were incubated with substrate solution (600 μl) containing 5 mM $MgCl_2$ and 10 mM AMP in 50 mM glycine buffer, pH 8.5, and the hydrolysis of AMP in the supernatant was measured [23]. After cultivation of transfected cells, media were pooled by one-tenth of original volume. The cells were washed twice with ST buffer, harvested, resuspended in the same buffer containing pepstatin (5 μg/ml) and then antipain (5 μg/ml), and homogenized. The 5′-nucleotidase activity in the concentrated media and the cell homogenate was measured. An enzyme unit was defined as the amount of the enzyme catalyzing the hydrolysis of 1 μmol AMP per min at 37°C.

2.6. PI-PLC treatment of cells

Transfected cells (1×10^6 cells) were incubated with 1 U/ml of PI-PLC for 60 min at 37°C. After PI-PLC treatment, the reaction mixture was centrifuged at $19\,000 \times g$ for 60 min at 4°C. Then the enzyme activities released into the supernatant and retained on the cells were determined.

2.7. Northern blot analysis of 5′-nucleotidase mRNA in transfected cells

Total RNA was extracted with guanidium thiocyanate followed by centrifugation in lithium chloride and cesium trifluoroacetic acid [24] (Quick Prep Total RNA extraction Kit, Pharmacia Biotech), electrophoresed and analyzed by RNA blot hybridization using fluorescein-labeled probe encoding the most of 5′-nucleotidase (1.6-kb *Bam*HI fragment of cDNA). The hybridized probe was detected by alkaline phosphatase-conjugated anti-fluorescein antibody using CDP-Star as a chemiluminescent substrate [25] (CDP-Star detection module, Amersham International plc, UK), according to the manufacturer's protocol.

3. Results

As shown in Table 2, the C-terminal signal of bovine 5′-nucleotidase comprises a hydrophilic spacer sequence (8 amino acid residues) and the following hydrophobic amino acid stretch (17 amino acid residues) [4]. In order to understand how the hydrophilic spacer is responsible for attachment of GPI to the ω-site (Ser^{523}), a series of mutations within the spacer were introduced by oligonucleotide-mediated site-directed mutagenesis. The mutant cDNAs were constructed by replacement of a 147-bp *Eco*O65I–*Bcl*I fragment encoding from Gly^{195} to Ile^{244} of the wild-type cDNA with a corresponding mutated fragment (Tables 2 and 4).

3.1. The effect of deletion of the spacer sequence for GPI modification

Four deletion mutants denoted as Δ(530–531), Δ(528–531), Δ(526–531) and Δ(524–531) were devoid of 2, 4, 6 and 8 residues of the hydrophilic spacer, respectively (Table 2). The gene product of

Table 2
C-Terminal amino acid sequences of the wild-type and deletion mutant cDNAs

cDNA clones	ω-Residue (Ser^{523})	Spacer sequence	Hydrophobic region
Wild-type	-----QFS	AGSHCCGS	FSLIFLSVLAVIIILYQ
Δ(530–531)	-----QFS	AGSHCC	FSLIFLSVLAVIIILYQ
Δ(528–531)	-----QFS	AGSH	FSLIFLSVLAVIIILYQ
Δ(526–531)	-----QFS	AG	FSLIFLSVLAVIIILYQ
Δ(524–531)	-----QFS		FSLIFLSVLAVIIILYQ

Amino acid sequences of the C-terminal regions of 5′-nucleotidase (Gln^{521}–Gln^{548}) and the deletion mutants are shown in single-letter codes, and the ω-residue (Ser^{523}) is underlined.

mutant Δ(524–548) which completely lost its downstream sequence of the ω-site, was secreted into the culture medium (denoted formerly as 523S) [4].

GPI modification of the protein was evaluated by measuring the cell surface-bound and the PI-PLC-released 5′-nucleotidase activities in COS-7 cells transfected with the wild-type and mutants cDNAs. Transient expression of 5′-nucleotidase on the cell surface was measured at 48 h after transfection as described in Section 2: Materials and methods. The expressed activities associated with intact COS-7 cells were shown in Table 3. COS-7 cells also expressed an endogenous 5′-nucleotidase activity as a GPI-anchored form encoded by the chromosomal gene [26] and the activity was taken into account as a basal control. The surface-associated activity of cells transfected with wild-type plasmid, pSVNT, was elevated about three times higher than those of controls. Gene product of the mutant Δ(530–531) was also expressed on the cell surface, where the enzyme activity was about one-third that of the wild-type transfectant. In both cases, significant amounts of the enzyme activities were released by PI-PLC (Table 3), showing that the products were anchored via GPI.

On the other hand, the enzyme activities on the cells transfected with mutant genes deleted more than four residues, were not increased significantly, but comparable, with those of controls. Northern blot analysis of RNA isolated from the mutant-transfected cells did not show any difference in the transcriptional level from that of pSVNT-transfectant. Therefore, such low activities were not due to the low efficiency in transcription of the mutant cDNAs (data not shown). The result suggests that the GPI-anchoring of the 5′-nucleotidase needs more than 6 amino acid residues in the hydrophilic spacer sequence. The mutant gene products which have less than 4 residues of spacer sequence, were not able to be modified with GPI nor to be expressed on the cell surface. Therefore, we measured the enzyme activities in the cell homogenates and the culture media to locate the products by the mutant genes. As shown in Table 3, homogenates of the wild-type- and Δ(530–531)-transfected cells contained significant enzyme activities. Homogenates of transfectants with mutant cDNAs deleted more than 4 residues; however, they exhibited the activities on a level with controls. The enzyme activities in the culture media of the cells transfected by wild-type and mutant genes were of the basal level, while the activity of the Δ(524–548), a secretion mutant, was about 2.5 times higher than those of controls. The gene products lacking more than 4 residues of the spacer sequence may be degraded intracellularly.

Table 3
5′-Nucleotidase activities in the transfectants of the wild-type and deletion mutants, and their release from the cell surface by PI-PLC

Transfected DNA	PI-PLC	5′-Nucleotidase activity (mU/10^6 cells)			
		Cell-bound	Released	Media	Cell homogenate
pSVNT (wild-type)	−	3.61	0.025	0.509	1.395 [a]
	+	2.42	1.13 [a]	n.d.	n.d.
Δ(530–531)	−	1.79	0.04	0.474	0.947 [a]
	+	0.80	0.74 [a]	n.d.	n.d.
Δ(528–531)	−	1.15	0.10	0.547	0.628 [a]
	+	0.65	0.53 [a]	n.d.	n.d.
Δ(526–531)	−	0.98	0.043	0.519	0.457 [a]
	+	0.81	0.54 [a]	n.d.	n.d.
Δ(524–531)	−	1.06	0.030	0.511	0.403 [a]
	+	0.96	0.50 [a]	n.d.	n.d.
pSVL (vector)	−	1.19	0.055	0.544	0.522
	+	0.77	0.51	n.d.	n.d.
Δ(524–548)	−	n.d.	n.d.	1.151 [a]	0.412
	+	n.d.	n.d.	n.d.	n.d.

The transfected cells were incubated with PI-PLC at 37°C for 1 h, and 5′-nucleotidase activities released into the reaction supernatant and retained on the cell surface were determined. Also, 5′-nucleotidase activities in the homogenates and the media were determined. n.d., not determined.

[a] Significantly different from the control value at $P < 0.01$.

Table 4
C-Terminal amino acid sequences of the wild-type and elongation mutant cDNAs

cDNA clones	ω-Residues (Ser[523])	Spacer sequence	Hydrophobic region
Wild-type	-----QFS	AGSHCCGS	FSLIFLSVLAVIIILYQ
531A2	-----QFS	AGSHCCGSAA	FSLIFLSVLAVIIILYQ
531A4	-----QFS	AGSHCCGSAAAA	FSLIFLSVLAVIIILYQ
531A6	-----QFS	AGSHCCGSAAAAAA	FSLIFLSVLAVIIILYQ
531A7	-----QFS	AGSHCCGSAAAAAAA	FSLIFLSVLAVIIILYQ
531A8	-----QFS	AGSHCCGSAAAAAAAA	FSLIFLSVLAVIIILYQ
531A9	-----QFS	AGSHCCGSAAAAAAAAA	FSLIFLSVLAVIIILYQ

Amino acid sequences of the C-terminal regions of 5′-nucleotidase (Gln[521]–Gln[548]) and the elongation mutants are shown in single-letter codes, and the ω-residue (Ser[523]) is underlined.

3.2. The effect of elongation of the spacer sequence for GPI modification

Six elongation mutants denoted as 531A2, 531A4, 531A6, 531A7, 531A8 and 531A9, were inserted 2, 4, 6, 7, 8 and 9 residues of Ala after Ser[531], respectively (Table 4). Expression levels of 531A2, 531A8 and 531A9 gene products were comparable with that of wild-type. The cells transfected with 531A4, 531A6 and 531A7 mutant genes also expressed significant cell-surface enzyme activities, which were partially suppressed by 30, 50 and 20% that of wild-type, respectively.

To detect the mutant gene products which failed to be expressed on the cell surface, the enzyme activities of the culture media and the cell homogenates

Table 5
5′-Nucleotidase activities in the transfectants of the wild-type and elongation mutants and their release from the cell surface by PI-PLC

Transfected DNA	PI-PLC	5′-Nucleotidase activity (mU/10^6 cells)			
		Cell-bound	Released	Media	Cell homogenate
pSVNT (wild-type)	−	3.735	0.027	0.528	3.833 [a]
	+	1.775	2.054 [a]	n.d.	n.d.
531A2	−	4.137	0.034	0.548	3.562 [a]
	+	1.849	1.893 [a]	n.d.	n.d.
531A4	−	2.623	0.055	0.477	2.017 [a]
	+	1.207	1.191 [a]	n.d.	n.d.
531A6	−	1.887	0.060	0.494	1.793 [a]
	+	1.389	0.747 [a]	n.d.	n.d.
531A7	−	2.96	0.069	0.394	2.519 [a]
	+	1.779	1.427 [a]	n.d.	n.d.
531A8	−	4.448	0.056	0.444	3.205 [a]
	+	1.536	2.166 [a]	n.d.	n.d.
531A9	−	4.087	0.107	0.282	3.079 [a]
	+	1.712	1.983 [a]	n.d.	n.d.
pSVL (vector)	−	0.703	0.031	0.455	0.625
	+	0.352	0.441	n.d.	n.d.
Δ(524–548)	−	n.d.	n.d.	4.370 [a]	0.743
	+	n.d.	n.d.	n.d.	n.d.

The transfected cells were incubated with PI-PLC at 37°C for 1 h, and 5′-nucleotidase activities released into the reaction supernatant and retained on the cell surface were determined. Also, 5′-nucleotidase activities in the homogenates and the media were determined. n.d., not determined.

[a] Significantly different from the control value at $P < 0.01$.

were measured. Significant amounts of the enzyme activities were observed in the cell homogenate from the wild-type and all of the elongated mutants (Table 5). On the contrary, enzyme activities secreted into the culture media of these cells remained at the basal level, while the considerable enzyme activity of the Δ(524–548) was secreted. (Due to delay in the step of concentrating this enzyme, the secreted enzyme activity of this mutant in Table 3 became much lower than in Table 5, although the transcription levels of 5′-nucleotidase mRNA were almost comparable.) Expression of the gene products which have 12–15 residues of spacer sequence, 531A4-7, were also suppressed in the cell homogenates. By Northern blot analysis, however, appreciable difference was not detected in the transcriptional level among the wild-type and mutant gene transfectants.

To confirm the GPI anchoring of the enzyme expressed on the cell surface, transfected cells were incubated with PI-PLC, and the enzyme activities released into the supernatant were determined. In every case, significant amounts of the enzyme activities were released by PI-PLC (Table 5), showing that the products derived from transfected cDNAs were anchored via GPI. The results implicated that decreased efficiency of GPI modification occurred on the pro-proteins of mutant 531A4, 531A6 and 531A7, and the pro-proteins failed to be GPI modified were degraded immediately.

From the above experiments, the relationship between GPI modification and length of the spacer (number of amino acid residues) can be summarized in Fig. 1.

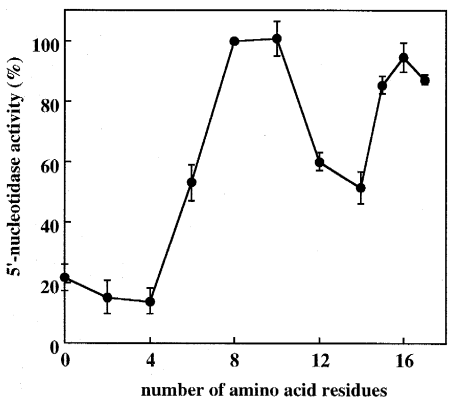

Fig. 1. Relationship between GPI modification and number of residues of the spacer sequence. The relative activities expressed on the cell surface of transfectants, were plotted against number of residues of the spacer sequence. The activity of wild-type transfectant is indicated as 100%.

4. Discussion

Amino acid compositions and sequences of C-terminal signal peptides usually vary with the precursor proteins. Sequences of the C-terminal signal peptides of GPI-anchored proteins were aligned in Table 1, according to the length of hydrophilic spacer sequences. Span of the hydrophilic spacer ranges between 4 and 17 and that of the bovine liver 5′-nucleotidase is 8.

To determine the minimum length of spacer sequence more precisely, we trimmed the spacer sequence by two amino acid units from the junction of the spacer with hydrophobic region toward the N-terminus and examined their expression in COS transfectant (Table 2). By deletion of the last two residues of the spacer, a significant amount of the enzyme activity was still expressed, and the activity was released from the cell surface by PI-PLC (Table 3). The gene products which have less than 6 residues of the spacer were not expressed on the cell surface. Thus it follows that more than 6 residues of hydrophilic spacer are required for GPI anchoring of the 5′-nucleotidase. The result is not consistent with those of DAF [11] and CD46 [12].

The gene products exhibiting lowered expression on the cell surface, Δ(528–531), Δ(526–531) and Δ(524–531), were not detected in the cell homogenates and the culture media. This suggested that the gene products were not present in the active form of 5′-nucleotidase. Moran and Caras demonstrated that incomplete pro-proteins not modified with GPI, missing to be a substrate of the transamidase, were accumulated in a sorting compartment between the endoplasmic reticulum and the medial Golgi [27]. In the mutants of bovine 5′-nucleotidase, the precursor proteins failed to accept GPI moiety were so unstable that these proteins may be degraded immediately by intrinsic proteases.

Since both GPI and the pro-proteins bind to luminal membrane of the endoplasmic reticulum, transamidase may also act on the luminal side. The first three-dimensional structure of transamidase was

resolved by X-ray crystallography of bacterial penicillin-binding protein 2x (PBP2x) [28]. The enzyme digests a terminal peptide bond of a peptidoglycan to form an acyl–enzyme intermediate, and then transfers the acyl moiety to an amino group of adjacent acceptor peptide. In the active-site pocket of the enzyme, the binding site of the peptidoglycan lies very close to that of the acceptor peptide. Mode of reaction of the putative GPI transamidase seems to be similar to that of PBP2x. The acyl–enzyme intermediate of the GPI transamidase with pro-protein was also reported and the acyl moiety of this intermediate would be transferred to GPI [29]. Hypothetical mechanism of GPI modification was schematically represented in Fig. 2. The pro-protein, a substrate of GPI transamidase, must bind to the active-site pocket of the enzyme which is proximate to the binding pocket of GPI (Fig. 2). The spacial arrangement of the terminal amino group of GPI would be restricted, because the diradyl moiety of GPI is buried in the luminal membrane. Spacial relationship of the ω-site with the hydrophobic region of pro-protein may be conserved among various pro-proteins. Binding face of the pro-protein to the enzyme is assumed to be the ω- and ω + 2-residues because of their restriction for GPI modification. Therefore, the spacer sequence

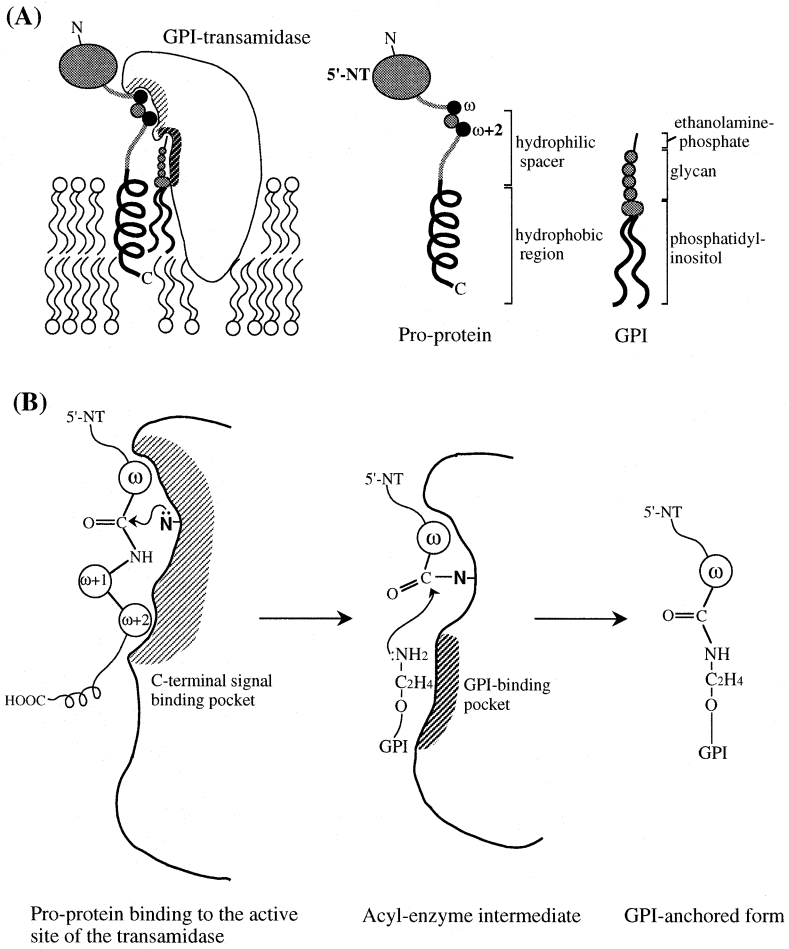

Fig. 2. Hypothetical mechanism of GPI modification. A: the pro-protein and the GPI, substrates of GPI transamidase, bind to form an E–S complex with the transamidase on the luminal side of the endoplasmic reticulum. Binding pockets of these substrates are closely located on the active-site cleft of the transamidase. B: the reaction mechanism of GPI transfer to the pro-protein was schematically represented. After formation of E–S complex, nucleophilic group of the active-site residue (showed in bold capital, N) attacks the carbonyl carbon of ω-residue, forming an acyl–enzyme intermediate. Then, the acyl moiety of the intermediate is transferred to the terminal amino group of GPI ($NH_2C_2H_4O$-), and GPI anchoring is completed.

may play a role in arranging the ω- and ω + 2-residues for correct binding to the active site of the GPI transamidase. By deletion of the spacer sequence, the gene products anchored by GPI were reduced. On the contrary, transfectant of mutant 531A2 which had 10 residues of spacer sequence expressed the comparable amount of 5′-nucleotidase activity with that of wild-type having 8 residues of spacer sequence. Thus, the spacer length of 8–10 residues is suitable for GPI modification, as shown in Fig. 1. The number of residues of the wild-type spacer sequence, 8, may suffice requirement to keep the most efficient GPI modification.

Although the transfectants of 531A4, 531A6 and 531A7 were partially suppressed in the 5′-nucleotidase activity, those of 531A8 and 531A9 having more than 16 residues of spacer sequences expressed cell-surface activity comparable with that of wild-type. The modification with GPI may occur at the alternative residue(s) located downstream of Ser^{523}, resulting in optimization of the length of spacer sequences. For example, if Ser^{531} of 531A8 mutant became a ω-residue, the length of spacer sequence would be 8 and the ω- and ω + 2-residues would be Ser and Ala, respectively, being compatible to 'the ω and ω + 2 rule'. The alteration of GPI-modified site was reported in folate receptor (FR) by Yan and Ratnam [30]. In the FR mutant having altered ω-residue, the enzyme activity was expressed only 20% that activity of wild-type, due to insufficient length of spacer sequence. The amino acid sequence of the spacer of bovine 5′-nucleotidase is AGSHCCGS, and combinations of His and Cys as well as Cys and Ser are not suitable for the ω- and ω + 2-residue [9]. In this sequence, GPI modification of the gene products of 531A4 and 531A6 were partially suppressed since His and Cys were not acceptable for a ω-residue to regulate the length of spacer sequence. Thus, the ω-residue of these mutants may be Ser^{523} and suppression of GPI modification was caused by the mutations. On the contrary, partial restoration of the suppression observed in 531A7 probably resulted from partial alteration of the ω-residue.

By deletion of more than 4 residues of the spacer, complete suppression of the GPI modification was observed. On the other hand, elongation of the spacer up to 6 residues resulted in partial reduction of anchor modification (Fig. 1), suggesting that elongation of the spacer is more permissible than deletion. In all of these elongation mutants, the spacer sequences enabled the ω-residue to enter into the active-site pocket because of its flexibility. Since the natural pro-proteins whose spacer sequences range from 4 to 17 residues are considered to be catalyzed by the similar transamidase, it will explain that each spacer makes unique conformation and thereby leads the ω-residue to the binding pocket of GPI transamidase. Nevertheless, the length of the spacer sequence may influence efficiency of GPI modification by its positive or negative control.

Recently, Gaa1p [31] and Gpi8p [32], a part of the putative GPI transamidase of yeast, were reported, and GPI-deficient mutant cell lines were found [33]. Gpi8p shows significant homology to a novel family of vacuolar plant endopeptidases, one of which is supposed to catalyze a transamidation step in the maturation of concanavalin A. Formation of the acyl–enzyme intermediate was also monitored by using nucleophilic reagents [29]. Reconstitution of reaction system of GPI transamidation using combination of these mutants and site-directed mutagenesis of the C-terminal signal sequence, would promote to clarify the molecular mechanism of GPI modification.

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