Retention of Cytochrome $b_5$ in the Endoplasmic Reticulum Is Transmembrane and Luminal Domain-dependent*

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Cytochrome $b_5$ (b5), a typical tail-anchored protein of the endoplasmic reticulum (ER) membrane, is composed of three functionally different domains: amino-terminal heme-containing catalytic, central hydrophobic membrane-anchoring, and carboxyl-terminal ER-targeting domains (Mitoma, J., and Ito, A. (1992) EMBO J. 11, 4197–4203). To analyze the potential retention signal of b5, mutant proteins were prepared to replace each domain with natural or artificial sequences, and subcellular localizations were examined using immunofluorescence microscopy and cell fractionation. The transmembrane domain functioned to retain the cytochrome in the ER, and the mutation of all or part of the transmembrane domain with an artificial hydrophobic sequence had practically no effect on intracellular distribution of the cytochrome. However, when the transmembrane domain was extended systematically, a substantial portion of the protein with the domain of over 22 amino acid residues leaked from the organelle. Thus, the transmembrane length functions as the retention signal. When cytochromes with mutations at the carboxy-terminal end were overexpressed in cells, a substantial portion of the protein was transported to the plasma membrane, indicating that the carboxy-terminal luminal domain also has a role in retention of b5 in the ER. Carbohydrate moiety of the glycosylatably-mutated b5 was sensitive to endoglycosidase H but resistant to endoglycosidase D. Therefore, both transmembrane and carboxyl-terminal portions seem to function as the static retention signal.

The secretory pathway in eukaryotic cells consists of discrete membrane-bound organelles such as endoplasmic reticulum (ER) (1), Golgi apparatus, plasma membrane, and lysosome. It is widely accepted that proteins destined for localization in these organelles or for secretion are co- or post-translationally inserted into the ER and transported from the ER by a default pathway, in which resident proteins are selectively retained by specific signals (1). Thus, ER proteins have to be sequestered away from proteins destined for secretion or other organelles and are excluded from transport vesicles or are specifically retrieved from the Golgi compartment, even if they escape from the ER.

A mechanism through which ER proteins are brought back from the Golgi apparatus by specific signals and by their receptors (2–6) was proposed to localize proteins to a specific organelle in the secretion pathway. The mammalian KDEL (2) and yeast HDEL (3) tetrapeptides at the carboxy-terminal ends were first identified as such a signal for ER luminal proteins. The double lysine motif (KKXX or KXXX), which is localized at the carboxy-terminal portion of type I integral ER membrane proteins, such as UDP-glucuronosyltransferase (6, 7) and Wbp1 in yeast (8) is also well characterized. The KKXX sequence binds the coatomer protein complex in vitro, and yeast coatomer mutants show defective retrieval (9, 10). The transmembrane domain has recently been demonstrated as another retrieval signal for Sec12p, since chimeric proteins containing this domain were localized in the ER and had cis-Golgi α1–6 mannosylated carbohydrates (11, 12).

In addition to these motifs, some proteins in the ER seem to require two signals in different portions in the molecule. Sönichsen et al. (13) showed that the calreticulin mutant, which lacked the Ca$^{2+}$ binding domain but included the KDEL sequence, escaped from the ER to a much greater extent. The mutant protein that changed the carboxy-terminal portion of Wbp1 from KK to QQ or SS was retained in the ER, without acquiring Golgi-specific carbohydrate modifications (8, 10). For Sec12p, an amino-terminal portion of the protein was reported to participate in static retention in the ER (12), thereby indicating that transmembrane and amino-terminal domains of Sec12p seem to function independently as retrieval and retention signals, respectively.

Cytochrome $b_5$ (b5), a typical tail-anchored protein of the ER membrane, is composed of three functionally different domains: amino-terminal heme-containing catalytic, central hydrophobic membrane-anchoring, and carboxyl-terminal ER-targeting domains (14). Although Pedrazzini et al. (15) have shown that insertion of five amino acids into the transmembrane domain of b5 resulted in localization of the mutant protein on the cell surface, it has remained to be determined how long the transmembrane domain can retain b5 in the ER, whether another portion of the cytochrome has a retention signal, and how these portions function as signals. We now report that both transmembrane and luminal domains function as static retention signals of b5 to the ER and that fewer than 22 amino acid residues of the transmembrane domain have a major role in retaining b5 in the ER.
MATERIALS AND METHODS
Reagents and Biochemicals
Restriction enzymes and DNA modifying enzymes were purchased from Nippon Gene, Toyobo, Fermentas MBII, or U. S. Biochemical Corp. The expression vectors pSVL, pSG5, and pEG were from Amersham Pharmacia Biotech, Stratagene, and Invitrogen, respectively. Fetal calf serum was from Life Technologies, Inc. Dulbecco's modified Eagle's medium was from Nissui. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Cappel Products. Peroxidase-conjugated goat anti-rabbit IgG was from Zymed Laboratories Inc. Endoglycosidase H and N-Glycosidase F were from Boehringer Mannheim, and endoglycosidase D was from Seikagaku Co. Ltd.

Construction of Mutant Proteins
cDNAs for rat b5 (14) and OMb, a b5-like heme protein of the outer mitochondrial membrane,2 were used to construct fusion proteins. Mutant proteins were constructed according to the method of Runkel (16) or polymerase chain reaction and confirmed by deoxy sequencing using Sequenase, then were inserted into eukaryotic expression vectors pSVL, pcDNA, or pSG5.

OMbC10, OMbB5, and TMOMbB5—cDNA fragments of OMb and b5 were inserted in tandem into M13mp18 to obtain M13mp18OMbB5. Deletions of nucleotides coding the last 10 amino acids of OMb and the catalytic and transmembrane domains of b5, the transmembrane and last 10 amino acids of OMb and catalytic domain of b5, and the last 22 amino acids of OMb and the 1st methionine to 115th isoleucine of b5 were done to obtain OMbC10, OMbB5, and TMOMbB5, respectively, using appropriate oligonucleotides.

TMBSOMbC10—Nucleotides coding the last 8 amino acids of OMb and the 1st methionine to 89th proline of OMb were deleted from M13mp18OMbB5C10, using an appropriate oligonucleotide.

B5AL1—4—The nucleotides coding a part of the transmembrane domain of b5 were exchanged using oligonucleotides coding amino acid sequences shown in Fig. 1C.

B5Syn18C7, B5Syn20C7, B5Syn22C7, B5Syn24C7, B5Syn26C7, and B5Syn28C7—Mutants containing an artificial hydrophobic transmembrane domain of various lengths were constructed from the M13mp18OMbB5C10 (14). Because this protein has a 22-amino acid transmembrane domain (16) and a portion of the protein lacking this domain, B5Syn22C7 was designated as B5Syn22C7 instead of B5Syn20C10. To construct B5Syn24C7, B5Syn26C7, and B5Syn28C7, amino acids AL, ALAL, and ALALAL were inserted between leucine 121 and serine 122 of B5Syn22C7, using oligonucleotides corresponding to these amino acids. To construct B5Syn20C4, B5Syn24C7, B5Syn26C7, and B5Syn28C7, amino acids AL, ALAL, and ALAL were inserted into B5Syn22C7, respectively, using oligonucleotides corresponding to these amino acids. To construct B5Syn22C7 and B5Syn18C7, LL-(120-121) and LALL-(118-121) were deleted from the B5Syn22C7, using oligonucleotides.

B5TT7Gly, B5cmycGly, B5Syn26C7TT7Gly, and B5Syn26C7 cmycGly—To construct a N-linked glycosylation site at the carboxyl-terminal portion of b5 and B5Syn26C7, TT (MASSMTGGQQMG) or c-myc (EQKLI-SEEDL) peptide and glycosylating sequence were introduced by polymerase chain reaction, using oligonucleotides corresponding to these amino acids and the M13 universal primer with pBluescriptSK(−)

Expression of b5 Derivatives in COS Cells
COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum in an atmosphere of 5% CO2 at 37 °C. DNA transfection was carried out as described (14), using cationic liposomes (17). The cells were cultured for 36 h after transfecting the plasmid into the cells.

Cell Fractionation
Cells cultured in three 10-cm diameter dishes were harvested in ice-cold STE (0.25 M sucrose, 20 mM Tris/HCl, 0.1 mM EDTA, 2 μg/ml each leupeptin and pepstatin, pH 8.0). The suspension was centrifuged at 500 g for 5 min, and the pellet was homogenized gently in 1 ml of STE buffer using a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 500 × g for 5 min to remove nuclear and unbroken cells, and the pellet was gently homogenized again in 0.5 ml of STE buffer, then centrifuged at 500 × g for 5 min. The first and second supernatants (1.5 ml) were then layerd over sucrose layers consisting of 0.5 ml of 2 M sucrose solution, 9 ml of linear gradient from 1.5 M to 5.5 M sucrose solution, and 1 ml of 0.5 M sucrose solution, and centrifuged at 25,000 rpm for 8 to 10 h in an RPS-40T rotor (Hitachi), or 1 ml of postnuclear supernatants was placed in discontinuous sucrose layers consisting of 0.5 ml of 2 M, 3 ml of 1.1 M, 5.5 ml of 0.6 M, and 1 ml of 0.5 M sucrose solution, instead of linear gradient, and centrifuged under the same conditions. Fractions of 1 ml were collected from the bottom of the tube and designated as fraction numbers 1 to 11.

Determination of b5 and Its Derivatives
The amount of b5 and derivatives expressed in the transfected cells was estimated using immunoblot analysis. The subcellular fractions were subjected to SDS-PAGE, followed by transfer of the proteins to a polyvinylidine difluoride filter. Rabbit antibodies against b5 and OMb and peroxidase-conjugated goat anti-rabbit IgG were used for the primary and secondary antibody, respectively. Amounts of proteins were measured using a densitometer.

Immunofluorescence Microscopy
After homogenization of COS cells expressing the glycosylatably-mutated b5s, membrane fractions were prepared by centrifugation of the postnuclear supernatants at 100,000 × g for 20 min. In endoglycosidase H digestion, the membrane fraction (25 μg) suspended in citrate buffer, pH 5.5, containing 0.02% SDS, 0.02 μM 2-mercaptoethanol, 1 mM phenylmethylsulfonfyl fluoride was treated overnight with 2.5 milli-units of the enzyme at 37 °C. For N-glycosidase F digestion, the membrane fraction (25 μg) suspended in Tris-HCl buffer, pH 8.0, containing 0.15% SDS, 0.03 μM 2-mercaptoethanol, 1.2% Triton X-100, and 10 μM EDTA was digested overnight with 2 milliunits of the enzyme at 37 °C. Endoglycosidase D was done at 37 °C overnight in 0.2 μM phosphate buffer, pH 6.5, containing 1% Triton X-100, 10 mM EDTA, and 1 μM phenylmethylsulfonfyl fluoride. Extent of digestion was estimated by immunoblot analysis, as described above.

Analytical Methods
Documented methods were used to assay NADPH-cytochrome P450 α-mannosidase II, alkaline phosphodiesterase I, and glutathione S-transferase activities (19, 20).

RESULTS
The Transmembrane Retention Signal of b5 Does Not Reside in a Specific Amino Acid Sequence—To determine whether a specific amino acid sequence of the transmembrane domain of b5 serves as the retention signal, we constructed several mutant proteins, in which all or half of the transmembrane portion of the cytochrome was substituted by a corresponding sequence of OMb, which is a b5-like heme protein of the outer mitochondrial membrane and has a similar molecular architecture (21) (Fig. 1A). Hydrophilic catalytic portion and both hydrophilic and transmembrane portions of b5 were replaced with the corresponding portions of OMb to obtain OMbB5 and OMbC10, respectively. While in the mutants, TMOMbB5 and TMBSOMbC10, the transmembrane domains consist of the amino-terminal half of the domain in OMb and the carboxyl-terminal half of that in b5 and vice versa, respectively. All mutant proteins were constructed to have an ER-targeting signal at the carboxyl-terminals.

Subcellular localization of the original and mutant proteins in the COS cells was examined by indirect immunofluorescence microscopy (Fig. 1B). In cells expressing the original b5, the reticular staining pattern characteristic of the ER was observed, indicating that the endogenous protein was targeted correctly in the cells. OMbB5 and TMOMbB5 had the same staining pattern as that of b5, whereas the plasma membrane, in addition to the ER, was stained in cells expressing OMbC10 and TMBSOMbC10. Thus, proteins that have the transmembrane domain of b5, B5 and OMbB5, showed a typical ER pattern, and a portion of the protein lacking this domain, OMbC10, could not be retained in the ER membrane. Our observations mean that the transmembrane domain of b5 has

2 A. Ito, unpublished data.
an important role in retaining the cytochrome in the ER.

To determine whether the ER retention signal consists of a specific amino acid sequence, the transmembrane segment of b5 was subdivided into four portions and each portion was replaced with an artificial hydrophobic sequence of ALALA (AL1–3) or AAA (AL4) (Fig. 1C). Subcellular distributions of the mutant proteins in COS cells are shown in Fig. 1D. Because all the mutant proteins are located only in the ER membrane, the retention signal obviously does not reside in a specific amino acid sequence.

**Length of the Transmembrane Domain Is Responsible for the Retention**—Although definition of the absolute length or number of amino acids of the transmembrane domain is difficult, one difference between b5 and OMbC10 or TMB5OMbC10 is length of the hydrophobic stretch; B5 and TMOMbB5 have 19 and 18 amino acids, respectively, whereas OMbC10 and TMB5OMbC10 have 21 and 22 residues, respectively. Although Pedrazzini et al. (15) found that the insertion of five amino acids into the transmembrane domain of b5 resulted in localization of the mutant protein on the cell surface, it remained unknown how long the domain can function as the retention signal. We then systematically constructed derivatives with an artificial hydrophobic sequence of various lengths of 18 to 28, instead of the transmembrane segment of b5 (Fig. 2A). A mutant with the transmembrane segment of 18 amino acids was constructed using an artificial sequence of 15 hydrophobic amino acid residues and three hydrophobic residues derived from the b5 molecule. We termed the mutant B5syn18C7, instead of B5syn15C10, to indicate length of the transmembrane domain. A typical ER pattern of fluorescence was observed with cells expressing B5syn18C7 and B5syn20C7 (Fig. 2A, a and b). In cells expressing B5syn22C7 and B5syn24C7, both the tubular network around the nucleus and edge of the cell was usually stained (Fig. 2B, c and d). In some cells, the cell surface was stained and there was little tubular net work (data not shown). When the transmembrane segment was extended further, B5syn26C7 and B5syn28C7, the cell surface was stained (Fig. 2B, e and f). When the non-permeabilized cells expressing...
B5syn24C7 and B5syn26C7 were treated with anti-peptide antibody against the carboxyl-terminal 7 amino acids located inside the ER vesicles (18), the plasma membrane was clearly stained. Thus, the mutant proteins were inserted into the membrane with a correct topology (Fig. 2g and h).

Location of b5 with the transmembrane segment of 24 amino acids in Golgi and plasma membrane was confirmed by subfractionation experiments, using sucrose density gradient centrifugation of the cell lysates, followed by immunochemical quantitation (Fig. 2C). The figure shows distributions of b5 and the derivatives as well as those of marker enzyme activities, NADPH-cytochrome P450 reductase (closed square), α-mannosidase II (open square), alkaline phosphodiesterase I (closed triangle), and glutathione S-transferase (open diamond) were measured, as the marker of the ER, Golgi apparatus, plasma membrane, and cytosol fractions, respectively. Amounts of mutant proteins and enzyme activities are shown as a relative value. a, B5; b, B5syn20C7; c, B5syn24C7; d, B5syn26C7.

Luminal Domain of b5 Also Affects Retention—The carboxyl-terminal hydrophilic domain functions as the ER-targeting signal of b5 and is exposed on the luminal side of the ER vesicle (14, 18, 22). When the c-myc peptide was attached to the carboxyl-terminal end of b5, the mutants were precisely targeted to the ER and inserted into the membrane in a topologically correct orientation (18). However, a substantial portion of the protein leaked from the ER, in case of overexpression using the pSG5 vector, instead of pSVL, although the wild type cytochrome was strictly localized to the ER (18) under the same conditions (see Fig. 3B). To confirm participation of the carboxyl-terminal portion on the retention of b5 in the ER, we constructed several mutant proteins, B5Rev and B5syn26Rev, in which the carboxyl-terminal seven amino acids were arranged in the reverse order (Fig. 3A). Cells expressing B5C7Rev stained the plasma membrane, in addition to the ER (Fig. 3B). Subcellular fractionation showed that a larger amount of B5syn26C7Rev was recovered in Golgi and plasma membrane fractions, compared with the finding for B5syn26C7 (Fig. 3C). Change in amino acid sequence of the luminal domain of B5syn26C7 did not affect resistance of proteins to alkaline treatment of the membranes (data not shown). These observations suggest that the carboxyl-terminal portion of the cyto-
chrome participates in retention in the ER, probably by a mechanism independent of that of the transmembrane domain.

To investigate amino acid residue(s) in the carboxyl-terminal portion responsible for retention of b5 in the ER, some well conserved amino acids (23) were mutated to other residues (Fig. 3D). Replacement of tyrosine 130, a residue completely conserved among cytochromes for various animal species, with serine had essentially no effect on retention of the cytochrome (Fig. 3E). Substantially the same results were obtained in the case of substitution of three carboxyl-terminal charged residues, arginine 128, glutamic acid 133, and aspartic acid 134 for neutral or counterionic residues (Fig. 3E).

**B5 Is Retained in the ER without Recycling from the Later Compartments**—The ER location of membrane proteins is achieved by retrieval or combination of static retention and retrieval mechanisms (2–6, 11, 12). To investigate whether b5 is statically retained or retrieved from the post-ER compartments, we added an Asn glycosylation site to the carboxyl-terminal portion of the mutant proteins. Y130S, mutation of tyrosine at position 130 to serine; R128D, mutation of arginine 128 to aspartic acid; E133Q/D134N, mutation of glutamic acid 133 and aspartic acid 134 residues to glutamine and asparagine; RD/EQ/DN, mutation of arginine 128, glutamic acid 133, and aspartic acid 134 residues to aspartic acid, glutamine, and asparagine, respectively. E, immunofluorescence microscopy of the COS cells transfected with Y130S (a), R128D (b), E133Q/D134N (c), and RD/EQ/DN cDNAs (d).

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**Fig. 3. Mutation in the carboxyl-terminal portion of b5 affects its ER retention.** A, amino acid sequences of the carboxyl-terminal portion of mutant proteins. B5C7Rev and B5syn26C7Rev, the carboxyl-terminal seven amino acids of b5 and B5syn26C7 were arranged in reverse order. The residues are presented in one-letter code. B, immunofluorescence microscopy of COS cells transfected with B5 (a) and B5C7Rev (b) cDNAs, using eukaryotic expression vector pSG5. C, subcellular distribution of B5syn26C7 and B5syn26C7Rev. Cells transfected with each plasmid were fractionated with discontinuous sucrose density gradient. The ER fraction was obtained from fractions 3 and 4, and Golgi apparatus and plasma membrane fractions were from fractions 9 and 10. Amounts of the expressed proteins were detected with Western blotting, and the stained bands were quantified using a NIH-image analyzer. Open circle and lozenge shapes are B5syn26C7 and B5syn26C7Rev, respectively. D, amino acid sequences in the carboxyl-terminal portion of the mutant proteins. Y130S, mutation of tyrosine at position 130 to serine; R128D, mutation of arginine 128 to aspartic acid. E133Q/D134N, mutation of glutamic acid 133 and aspartic acid 134 residues to glutamine and asparagine.
motif were fused to the carboxyl-terminal end of B5 and B5syn26C7 (Fig. 4A) and expressed in COS cells, using a pcDNA vector. The typical ER pattern of the immunofluorescence staining was observed in cells expressing B5T7Glyc (Fig. 4B). The same staining patterns were observed with B5cmycGlyc (data not shown). Therefore these modifications at the carboxyl-terminal end did not affect intracellular localization of the proteins. B5T7Glyc and B5cmycGlyc gave a single band on SDS-PAGE and were sensitive to both endoglycosidase H and N-glycosidase F and converted to a band about 3 kDa smaller (Fig. 4C). Thereby they must remain as a glycoprotein of the high mannose type. On the other hand, B5syn26C7T7Glyc and B5syn26C7cmycGlyc gave higher molecular mass bands (25) as well as a band about 3 kDa larger than the non-glycosylated form, although in these cases a non-glycosylated form was sometimes observed (Fig. 4C). The higher molecular weight bands were sensitive to N-glycosidase F but resistant to endoglycosidase H, whereas the lower band was sensitive to both endoglycosidases, as in the case of B5T7Glyc and B5cmycGlyc, indicating that proteins with higher molecular weight contain complex-type oligosaccharides. This implies that once proteins leak out from the ER to the latter compartment the proteins can be modified by the sugar chain to a complex type and that the molecule found in the ER is retained in the organelle without recycling.

To confirm static retention of b5 in the ER, the fused protein was subjected to endoglycosidase D digestion. When Asn-linked glycosylated protein is transported to the cis-Golgi compartment, the oligosaccharide is converted from GlcNAc2Man9 to GlcNAc2Man9 by mannosidase I which is located in the cis-Golgi compartment (26). Endoglycosidase D is active only to the latter structure, although endoglycosidase H digests both types (26, 27). As shown in Fig. 4C, both B5T7Glyc and B5cmycGlyc were resistant to endoglycosidase D. The finding confirms that b5 is statically retained in the ER, without recycling. We propose that both transmembrane and carboxyl-terminal portions function as the static retention signal.

**DISCUSSION**

We obtained evidence that retention of b5 in the ER membrane depends on both transmembrane and carboxyl-terminal luminal domains of the molecule and not on the cytoplasmic domain, although the transmembrane domain does have a major effect. Mutation of all or part of the transmembrane domain with an artificial hydrophobic sequence had practically no effect on the intracellular distribution of the cytochrome, whereas lengthening this domain by four or more residues greatly reduced retention in the ER. We thus concluded that length of the transmembrane serves as the retention signal and that there are no key residues responsible for the retention in the domain. Pedrazzini et al. (15) reached a similar conclusion from data that insertion of five amino acids into the transmembrane domain of b5 resulted in localization of the mutant protein to the cell surface. Although the amino acid sequence of the transmembrane domain of b5 is highly homologous among sequences from various animal species (23) there are no known common amino acid sequences in the transmembrane domains between b5 and other ER membrane proteins (28–32). On the other hand, the sequence of the transmembrane domain of OMB (33) (Fig. 1) is similar to that of b5, but the mutant with the transmembrane domain of OMB moved forward to the distal organelles in the secretory pathway. These observations lend support to our conclusion.

Although it is difficult to define the absolute length or number of amino acids of the transmembrane domain, we calculated b5 to have 19 amino acid residues (14). Progressive lengthening of the sequence resulted in an increased leakage of proteins from the ER, and a substantial portion of the protein with the domain of 22 residues was no longer retained in the ER. Addition of residues in the transmembrane domain of
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UBC6, a tail-anchored membrane protein in yeast, affected the efficiency of retention of the protein in the ER (34). Length of the transmembrane domain of membrane proteins, at least of tail-anchored proteins, seems to be a general feature required for retention.

The transmembrane domain of b5 seems to function as a static retention signal, not a retrieval one, although that of Sec12p was reported to function as a retrieval signal and the retrieval depended on Rer1p, a receptor protein (12). A lipid-based mechanism has been proposed for transmembrane domain-mediated retention, as first stated by Munro (35). The mixed lipid populations in the intracellular membrane would separate into lipid microdomains with distinct compositions, thickness, and degree of structural perturbability, and proteins would selectively partition into one domain and so be prevented from entering transport vesicles comprising the other domain by virtue of physical properties of their transmembrane domains (36, 37). The ER is the start of the secretory pathway and it has a lipid composition differing from the plasma membrane (38). The ER has low levels of cholesterol and sphingolipids, which creates a flexible environment suitable for insertion, modification, and assembly of proteins. There is no known mechanism to distinguish differences in length of transmembrane domains by three amino acid residues. When proteins moving through the pathway encounter points where the bilayer composition changes from ER-like to plasma membrane-like, proteins with a longer transmembrane domain would preferentially move forward. In this case, the shorter transmembrane domain, which is responsible for ER retention, seems to be a default signal, and the longer transmembrane domain may possibly have an affinity for cholesterol and/or sphingolipids and be selectively collected into forward-moving cholesterol and sphingolipid-rich vesicles.

The present results also show that the carboxyl-terminal luminal domain of b5, in addition to the transmembrane one, has a role in its retention in the ER membrane. Because a fused protein in which the carboxyl-terminal portion of b5 was attached to the carboxyl-terminal end of syntaxin IA (39, 40), a plasma membrane protein, could not be retained in the ER, this portion may be weak as the retention signal; the transmembrane domain seems to function as the major signal. The signal in the carboxyl-terminal portion appears to work as a static retention signal, like the transmembrane domain, since no Golgi-type carbohydrate moiety was evident in the mutant b5 present in the ER. In conclusion, both transmembrane and carboxyl-terminal luminal domains function as a static retention signal of b5 to the ER, and length of the transmembrane domain apparently has a major effect on the retention. Whether each domain functions independently or concertedy, how length of the transmembrane domain is measured, and how proteins are sorted in the ER remain to be subjects of ongoing studies.

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