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Integration of Cytochrome b_5 into Endoplasmic Reticulum Membrane: Participation of Carboxy-Terminal Portion of the Transmembrane Domain

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Integration of cytochrome b_5 (b5), a tail-anchored protein located in the endoplasmic reticulum (ER) membrane, into the membrane was studied. Mutation of three amino acids, -Leu-Met-Tyr, at the carboxy-terminal end of the transmembrane segment of b5 to alanines resulted in localization of the mutated protein, b5LMY/AAA, in the cytosol as well as in the ER membrane. When an N-glycosylation site was introduced at the carboxy-terminal end of b5LMY/AAA, a substantial amount of the glycosylated form of the mutant protein was recovered in the cytosol fraction. A portion of the mutant protein recovered in the ER was released from the membrane by incubation with the cytosol fraction, but no further release was observed in the second incubation, suggesting that b5 is present in two different states, loosely-bound and firmly-integrated forms, in the ER membrane. These results suggest that b5 is integrated into the ER membrane via the loosely bound state, in which the carboxy-terminal end of the molecule is inserted into the luminal side of the vesicle but is easily translocated back to the cytosol, and that the three amino acids are important for conversion of the loosely-bound state to the firmly-integrated state.

Key words: cytochrome b_5, endoplasmic reticulum, membrane protein, protein integration into membrane, transmembrane domain.

Abbreviations: b5, cytochrome b_5; ER, endoplasmic reticulum.

Newly synthesized proteins are transported to the endoplasmic reticulum (ER) by co- and post-translational systems. In co-trans1ational transport systems, the integration of membrane proteins into the ER membrane is initiated by a signal sequence usually located at the amino-terminus of nascent polypeptides or by the first transmembrane domain of the proteins. After their recognition by signal recognition particle (SRP) and targeting of the nascent peptide to the ER, the nascent chain is released from the SRP by the function of the SRP receptor on the ER membrane, and then the amino-terminal sequence is inserted into the membranes (*1*). The membrane integration processes proceed via the protein-conducting channel, trans1ocon, and its major components have been elucidated to be the heterotrimeric Sec61p complex (*2–4*). On the other hand, proteins without the signal sequence at the amino-terminus are post-translational1y transported to the ER without participation of an SRP. Many tail-anchored proteins, that have a short hydrophobic transmembrane domain near the carboxyl-terminus, including cytochrome b_5 (b5) (*5*, *6*), microsoma1 aldehyde dehydrogenase (*7*), heme oxygenase (*8*), synaptobrevin (*9*), are post-translationally transported to the ER. The carboxy-terminal domain of these tail-anchored proteins was reported to be important for their localization to the ER membrane (*9–14*). Analyses using reconstituted proteoliposomes showed that the post-translational transport of preprolactin requires the complex of Sec proteins and Kar2p (*15*). However, the integration of synaptobrevin into the ER membrane does not require any Sec complex proteins but seems to involve trypsin-sensitive factor(s) (*9*). Thus, little is known about the mechanism of targeting and integration of the tail-anchored proteins into the ER membrane.

B5 is a typical tail-anchored protein of the ER membrane, which is composed of three functionally different domains: an N-terminal heme-containing catalytic domain, a central hydrophobic membrane–anchoring domain, and a carboxyl-terminal ER-targeting domain. The hydrophobic segment near the carboxy-terminal portion spans the membrane (*16*, *17*) and functions as the ER retention signal (*18*, *19*). The carboxy-terminal 10 amino acids of b5 have sufficient information to target the protein to the ER (*10*), and charged amino acids in this portion are responsible for the targeting function (*20*).

In the course of studying the essential amino acids in the carboxy-terminal portion for targeting and insertion of b5, we noticed that mutation of hydrophobic residues just before the hydrophilic terminal of the molecule affected intracellular localization of the cytochrome. In this report, we studied the role of this portion and found

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that it plays a key role in the post-translational integration of b5 into the ER membrane.

MATERIALS AND METHODS

Reagents and Biochemicals—Restriction and DNA modifying enzymes were purchased from Nippon Gene, Toyobo, Takara, Fermentas MBI, and U.S. Biochemical Corp. The expression vector pSG5 and quick-change site-directed mutagenesis kit were from Stratagene. Fetal calf serum was from Life Technologies, Inc. Dulbecco's modified Eagle's medium was from Nissui. Fluorescein-conjugated and peroxidase-conjugated goat anti–rabbit IgGs were from Cappel Products and Zymed Laboratories Inc, respectively. *N*-Glycosidase F was from Boehringer Mannheim. Anti-b5 antibody was obtained as described previously (*21*).

Plasmid Construction—Mutation of rat b5 cDNA (*10*) was performed using a quick-change site-directed mutagenesis kit, and the resultant cDNA was inserted into eukaryotic expression vector pSG5. An *N*-linked glycosylation site sequence, MSMTGGQQMGNSTQI, was introduced at the carboxyl-terminal end of b5 by polymerase chain reaction, using oligonucleotides corresponding to these amino acids.

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. DNA transfection was carried out as described previously (*10*), using cationic liposomes (*22*), and the cells were cultured for 60 h.

Immunofluorescence Microscopy—Immunofluorescence microscopy was carried out as described previously (*9*, *19*). The b5 derivatives were detected using rabbit anti-b5 antibody and fluorescein-conjugated goat anti rabbit IgG as the primary and secondary antibody, respectively.

Subcellular Fractionation—The cells expressing the b5 derivatives were harvested in ice-cold STE buffer (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 cell pellet was homogenized gently in STE buffer using a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 500 ×*g* for 5 min to remove nuclei and unbroken cells. The supernatant was then centrifuged at 100,000 ×*g* for 20 min to separate the membrane fraction from the cytosolic materials. To obtain the microsomal fraction, the post-nuclear supernatant was centrifuged at 9,000 ×*g* for 7 min before separating cytosolic materials. All procedures were done at 4°C.

Determination of b5 and Its Derivatives—The amount of b5 and its derivatives expressed in the transfected cells was estimated by immunoblot analysis with rabbit anti-b5 antibody and peroxidase-conjugated goat anti–rabbit IgG as the primary and secondary antibody, respectively. Amounts of proteins were measured using Nikon scantough and NIH-Image as a densitometer.

Glycosidase Digestion—After homogenization of COS cells expressing the mutated b5 derivatives with an *N*-glycosylation site introduced at the carboxy-terminal end of the molecules, membrane fractions were prepared by centrifugation of the post-nuclear supernatant at 100,000 ×*g* for 20 min. For *N*-glycosidase F digestion, the membrane fraction was suspended in Tris-HCl buffer, pH 8.0, containing 30 mM 2-mercaptoethanol, 10 mM EDTA, and 1.2% Triton X-100, then digested overnight with 2 m units of the enzyme at 37°C. To examine the membrane topology of the carboxy-terminus of the mutated b5s, *N*-glycosidase F digestion was done in the absence and presence of 1.2% Triton X-100. The samples were analyzed by SDS-PAGE and immunoblotting.

Alkaline Flotation Centrifugation—Alkaline flotation was done essentially as described (*22*). In brief, a 40-μl aliquot of membrane fraction was added to 160 ml of 0.1 M sodium carbonate and 2 M sucrose. The mixture was placed in an ultracentrifugation tube. Two sucrose layers, 400 ml each of 1.46 M sucrose and 0.25 M sucrose, were then overlayed on the mixture. Both sucrose layers contained 0.1 M sodium carbonate. The tube was centrifuged at 350,000 ×*g* for 3 h, then the fraction in the top layer was recovered as a membrane fraction and that in the bottom layer was as a soluble fraction. The samples were analyzed by SDS–PAGE and immunoblotting.

Release of b5 Derivatives from Microsomes—The microsomal fraction prepared from COS cell expressing b5gly or b5LMY/AAAgly was diluted with the cytosol prepared from the untransfected COS cell. The mixture was incubated for 60 min at 30°C, then centrifuged at 100,000 ×*g* for 20 min to separate microsomes from the soluble materials. The samples were analyzed by SDS–PAGE and immunoblotting.

RESULTS

Effect of Mutation of Carboxy-Terminal Portion of b5 on Its Subcellular Localization—The C-terminal 10 amino acids of b5 have been shown to be necessary for its targeting to the ER (*10*). In the course of studying the essential amino acids in this portion for targeting and insertion of the cytochrome, we found an interesting phenomenon. When three amino acids just inside the luminal side of the membrane, Leu-Met-Tyr, were replaced with three alanines (Fig. 1A), immunofluorescence microscopy showed that the wild type and mutant b5, b5 and b5LMY/AAA, respectively, exhibited the same staining pattern of the typical ER type (Fig. 1B). In subcellular fractionation study, however, a large portion of b5LMY/AAA was recovered in the cytosol fraction as well as in the membrane fraction, though b5 was recovered exclusively in the membrane fraction (Fig. 1C). This finding was not the result of prolonged incubation of the cells, because the expression level of the mutant b5 was even lower than that of b5. The results suggest that the mutant b5 found in the cytosol fraction has either remained in the cytosol because of failure in transport to the ER or been released from the ER in the cell or during the cell fractionation procedure after transport to the ER. The mutation of Leu-Met-Tyr at the carboxy terminal portion of b5 to three alanines seems to affect transport or integration of b5 into the ER.

Insertion and Release of b5LMY/AAA from the ER Membrane—To examine if the carboxy-terminus of b5LMY/AAA is inserted into the luminal side of the ER, as in the case of b5, T7 antigen peptide containing a consensus *N*-glycosylation site (-MASMTGGQQMGN-

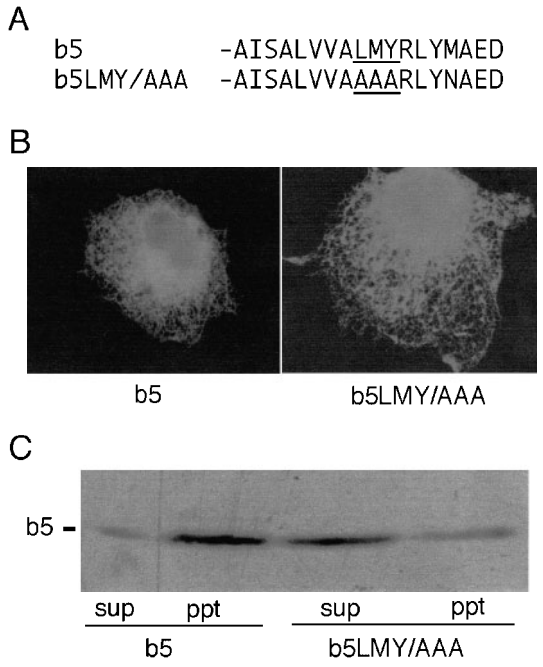

Fig. 1. **Subcellular distribution of b5 and b5LMY/AAA.** A: Amino acid sequence of carboxy-terminal region of b5 and its derivatives, b5LMY/AAA. Three consecutive amino acids, Leu-Met-Tyr, in the carboxy-terminal portion of b5 were replaced with three alanines, as underlined. B: Immunofluorescence microscopy of COS-7 cells expressing b5 and b5LMY/AAA. cDNA encoding each protein in plasmid pSG5 was transfected into COS-7 cells. After culture for 36 h, cells were processed as described under "MATERIALS AND METHODS", using anti–rat b5 antibody and fluorescein-conjugated goat anti-rabbit IgG as primary and secondary antibody, respectively. C: Subcellular distribution of b5 and b5LMY/AAA. Cells expressing b5 and b5LMY/AAA were homogenized in STE buffer. Post-nuclear supernatants of the homogenates were centrifuged at 100,000 ×*g* for 20 min to separate membrane (ppt) and cytosol (sup) fractions. The samples were analyzed by SDS-PAGE and immunoblotting.

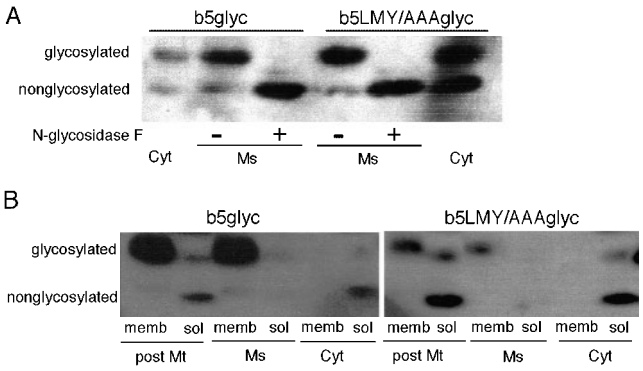

Fig. 2. **Insertion of b5 and b5LMY/AAA with glycosylation site at their carboxy terminals to the ER membrane.** A: Glycosylation and membrane insertion of b5glyc and b5LMY/AAAglyc were examined. The microsomal (Ms) and cytosol (Cyt) fractions were isolated from the cells expressing b5glyc and b5LMY/AAAglyc, as described in Fig. 1. The membrane fractions were treated further with *N*-glycosidase F, as described under Materials and Methods. The b5 derivatives were detected by immunoblotting. B: Alkaline flotation experiment of the membrane fractions. The post-mitochondrial (postMt), microsomal (Ms), and cytosol (Cyt) fractions obtained from the COS-7 cells expressing b5glyc and b5LMY/AAAglyc were subjected to alkaline flotation centrifugation in 0.1 M sodium carbonate, as described under "MATERIALS AND METHODS". Membrane (memb) and soluble (sol) fractions were separated, and b5 derivatives were detected by immunoblotting.

STQI) was added at the carboxy-terminal end of b5 and b5LMY/AAA. The wild-type b5 and b5LMY/AAA with the glycosylation site, b5glyc and b5LMY/AAAglyc, respectively, were expressed in COS-7 cells and extent of glycosylation of these mutant proteins was analyzed as described in "MATERIALS AND METHODS" (Fig 2A). Two bands, one corresponding to authentic b5 and another about 30 kDa larger in molecular size, were observed in both microsomal and cytosol fractions of the cells expressing either b5glyc or b5LMY/AAAglyc. The larger band was converted into that corresponding to b5 upon treatment with *N*-glycosidase F.

Although most b5glyc was recovered in the microsomal fraction as a glycosylated form, a small but definite amount of the cytochrome was detected in the cytosol fraction, most of it in a glycosylated form. This phenomenon was more clearly demonstrated by b5LMY/AAAglyc. In this case, about one-third of the cytochrome was recovered in the cytosol, and about half of this was in the glycosylated form. Most of the protein recovered in the microsomal fraction was glycosylated. Since glycosylation occurs on the luminal side of the ER membrane, the glycosylated form of the proteins recovered in the cytosol must derive from the ER. Non-glycosylated proteins in the cytosol seem also to be from the ER, but the possibility remains that introduction of glycosylated sequence at the end of the protein prevent its transport to the ER, because the carboxy-terminal stretch of about 10 amino acids is known to function as the targeting signal of b5 to the ER (*10*).

To confirm that the protein found in the cytosol was a soluble form, and not derived from small membrane fragments remaining in the cytosol fraction after ultracentrifugation, the microsomal and cytosol fractions were subjected to flotation centrifugation under alkaline condition, as described in Materials and Methods (Fig. 2B). Upon flotation centrifugation, proteins associated with the membrane vesicle were recovered in the top layer of lower density, whereas soluble proteins with the higher density were recovered in the bottom layer. The glycosylated proteins recovered in the microsomal membrane floated with membrane, whereas the proteins recovered in the cytosol, whether they are glycosylated or non-glycosylated, remained in the bottom layer, indicating that the proteins recovered in the cytosol were in soluble form, not associated with the membrane fragments. These results indicate that b5glyc and b5LMY/AAAglyc had been inserted into the ER membrane, glycosylated at the carboxy-terminal end of the protein on the luminal side of the membrane, then in part translocated back to the cytosol from the ER membrane vesicle. Since a larger amount of b5LMY/AAAglyc than b5glyc was recovered in the cytosol, the carboxy-terminus of the transmembrane domain, LMN, seems to be important for stable insertion of the protein into the membrane.

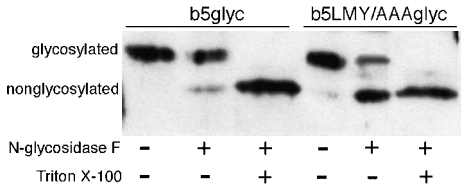

Fig. 3. **Topology of b5 derivatives in the ER membranes.** The microsomal fractions of COS-7 cells expressing b5glyc and b5LMY/AAAglyc were obtained and digested with *N*-glycosidase F in the absence and presence of 1.2% Triton X-100, as described under "MATERIALS AND METHODS".

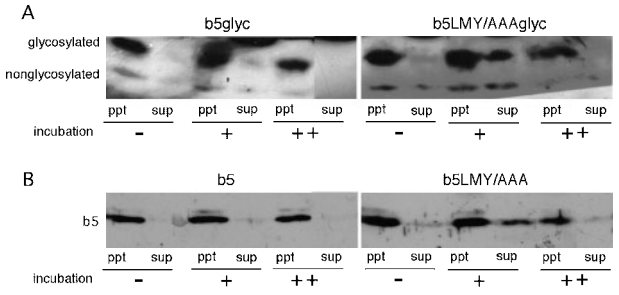

Fig. 4. **Release of b5 derivatives from the ER membranes.** The microsomal fractions obtained from COS-7 cells expressing b5glyc and b5LMY/AAAglyc were diluted with the cytosol fraction derived from non-transfected COS-7 cells, and incubated for 60 min at 37°C. After the incubation, microsomal and supernatant fractions were separated by centrifugation. For the second incubation, the first precipitate was subjected to the same procedure as the first one. Release of b5 derivatives from the membrane was analyzed as described under "MATERIALS AND METHODS" –, no incubation; +, first incubation; ++, second incubation.

Topology of b5LMY/AAAglyc in the ER Membrane— Topology and integration of b5glyc and b5LMY/AAAglyc into the ER membrane were next investigated. When microsomes were treated with *N*-glycosidase F in the presence or absence of Triton X-100 as described in "MATERIALS AND METHODS" (Fig. 3), b5glyc was resistant to the treatment in the absence of the detergent, whereas most of the b5LMY/AAAglyc in the ER was converted to non-glycosylated form in the absence of the detergent. In the presence of the detergent, both were completely converted. These data indicate that carboxy-terminal portion of b5LMY/AAAglyc is exposed to the cytosol or easily translocated back to the cytosol side during the glycosidase digestion.

To examine whether b5LMY/AAAglyc in the ER membrane is easily releasable from the membrane, microsomes were incubated with the cytosol fraction of the control COS cells for 60 min at 30°C, then the mixture was centrifuged to separate the membrane and cytosol fractions (Fig. 4A). All the b5glyc remained in the membrane fraction, whereas a portion of b5LMY/AAAglyc, either glycosylated or non-glycosylated, was released into the supernatant fraction. When the membrane recovered from the incubation was subjected to a second incubation and centrifugation, none of the protein was released from the membrane. In the absence of the cytosol, a small portion was released (data not shown). The data indicate that b5LMY/AAAglyc is in two states, easily releasable and firmly attached forms, and that b5glyc is exclusively in the latter form. Essentially the same results were obtained with b5 and b5LMY/AAA (Fig. 4B), indicating that the mutation of Leu-Met-Tyr to alanines, not the presence of a glycosyl group at the carboxy-terminal end of the molecule, is the major reason for the release of the b5 molecule from the ER membrane.

DISCUSSION

We have presented evidence that the carboxy-terminal portion of the transmembrane segment of b5 is important for stable integration of the cytochrome into the ER membrane. The experiments using b5 derivatives with a glycosylation site showed that the carboxy-terminal portion of the b5 molecule was inserted into lumen of the ER vesicles, but that some of molecules were released into the cytosol.

Although we have little information about the molecular mechanism of recognition of the targeting signal of b5 and the initial insertion of b5 into the ER membrane, retention of the cytochrome in the membrane can be explained by a lipid-partitioning process through the protein-conducting channel, like Sec61 complex (*4*). In co-translational integration into the ER membrane, the Sec61p channel allows the transmembrane domain to bypass the barrier posed by the polar head groups of the lipid bilayer and come into contact with the hydrophobic interior of the membrane. Sec61p provides a site in the membrane, at the interface of channel and lipid, through which a transmembrane domain can dynamically equilibrate between the lipid and aqueous phases, depending on the hydrophobicity of the domain.

A similar mechanism seems to work in the post-translational integration of b5 into the ER membrane. B5 is first inserted into the ER membrane in a loosely bound state, in which the carboxy-terminal end of the molecule spans the membrane to the luminal side but can be translocated back to the cytoplasmic side, and it is then converted to the firmly integrated state. In the loosely-bound state, the carboxy-terminal region of the b5 molecule would be in a channel-like structure, where the polypeptide inserted would be in equilibrium and then be converted to the firmly-integrated form or translocated back to the cytosol. In b5, the equilibrium would lie far toward the firmly integrated form in the lipid phase, because its transmembrane domain is highly hydrophobic. Mutation of LMY to AAA at the carboxy-terminal end of the transmembrane segment would affect this equilibrium slightly, resulting in release of b5 to the cytosol from the membrane. In b5LMY/AAA, 40 to 50% of the molecule was recovered in the microsomal fraction, and out of this 50 to 65% was in firmly-integrated form and 35 to 50% was in loosely-bound form, indicating that about 20% of the total molecule was in the loosely-bound intermediate form under the conditions examined. In a preliminary experiment in which leucine in LMY was mutated to alanine, a substantial portion of the mutant b5 was recovered in the cytosol fraction as well as the microsomal fraction. Thus, the presence of a detectable amount of the loosely-bound form of the mutated protein as a result of the alanine substitution of LMY seemed not

to be caused by just shortening of the overall length of the transmembrane segment of the molecule. Attachment of an oligosaccharide chain at the carboxy terminus could also produce a similar effect and stabilize loosely bound form. This could result in detection of release of a small portion of even the wild-type b5. Since the release of the b5 was stimulated by the cytosol fraction, some protein(s) in the fraction may participate in the release and even in the insertion and equilibration processes. We presently have no information about any such protein(s). The mechanism involving a loosely-bound form, probably in a channel, could be proposed for integration of b5 into the ER membrane, though properties of the channel and cytosolic factor(s) remain unknown.

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