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In Situ Topology of Cytochrome b_5 in the Endoplasmic Reticulum Membrane

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Cytochrome b_5 is tail-anchored in the ER membrane and is composed of three functionally different portions; amino-terminal heme-containing catalytic, central hydrophobic membrane-anchoring, and carboxy-terminal ER-targeting portions [Mitoma, J. and Ito, A. (1992) *EMBO J.* 11, 4197-4203]. *In situ* topology of cytochrome b_5 in the ER membrane was studied using immunofluorescence microscopy. Antibodies were raised against the hydrophilic portion (anti-b_5) and the carboxy-terminal seven amino acid residues (anti-peptide) of cytochrome b_5 and used for detection of the cytochrome in COS cells which expressed the rat cytochrome. Anti-b_5 antibody detected the cytochrome in a reticular staining pattern characteristic of the ER, even when the cell plasma membrane was permeabilized with Streptolysin O. The anti-peptide displayed a fluorescence signal only with Triton-permeabilized cells in which the antibody was able to penetrate into the ER lumen. In a double immuno-staining of the cell using the antipeptide antibody and the antibody against protein disulfide isomerase, both antibodies showed the same staining pattern in the presence of either Triton X-100 or Streptolysin O. The results indicate that the carboxy-terminal hydrophilic stretch is exposed to the luminal side. Cytochrome b_5 was tagged with c-myc peptide at the carboxy-terminal end and the topology of the c-myc peptide was analyzed by the same method. Anti c-myc monoclonal IgG detected the tagged cytochrome b_5 only after Triton treatment of the fixed cells, suggesting that the addition of c-myc peptide to the carboxy-terminal end does not affect insertion or orientation of the cytochrome in the ER membrane.

Key words: cytochrome b_5, endoplasmic reticulum, immunofluorescence microscopy, membrane topology, tail-anchored protein.

There has been increasing interest in the mechanism of targeting and insertion of "tail-anchored" proteins which are characterized by an amino-terminal cytosolic domain and a short carboxy-terminal anchoring domain. Cytochrome b_5 is a typical ER protein of such a class. The cytochrome has 134 amino acid residues and the mild proteolysis of the protein showed that it is composed of two domains, amino-terminal hydrophilic and carboxy-terminal hydrophobic ones (*1-3*). The amino-terminal domain of about 100 amino acid residues is exposed to the cytoplasm, contains a protoheme, and participates in the electron transfer function, whereas the carboxy-terminal "tail," consisting of about 30 amino acids, has two distinct parts, a hydrophobic membrane-anchoring part (about 20 residues) and a terminal hydrophilic part (about 7 residues). The carboxy-terminal ten amino acid residues contain sufficient information for targeting the cytochrome to the ER membrane (*4*). The protein is synthesized on free polysomes and is inserted into the ER membrane without the participation of the SRP/SRP receptor system (*5-8*).

In order to elucidate the mechanism of targeting and insertion of cytochrome b_5 into the ER membrane, information on the topology of the cytochrome in the membrane is essential. However, contradictory data on the topology of the cytochrome in the ER membrane have been derived from proteolytic studies. Ozols (*9*) showed that the peptide consisting of the carboxyl-terminal 6 amino acids of cytochrome b_5 was released from the intact microsomal vesicles by externally added trypsin, suggesting that the membrane-anchoring domain of the cytochrome turns in the ER membrane, forming a hairpin-like loop. On the other hand, Vergeres *et al.* (*10*) reported that cytochrome b_5 inserted *in vitro* into microsomal membranes was sensitive to carboxypeptidase Y treatment only in the presence of a detergent, Tergitol, suggesting that the transmembrane domain of the cytochrome incorporated *in vitro* spans the membrane with its carboxy-terminus located in the lumen of the ER.

In this study, instead of an *in vitro* system, we applied an immunocytochemical method to determine the *in situ* orientation of the carboxy-terminal end of the cytochrome in the ER membrane, and here we present evidence of its existence on the luminal side.

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Abbreviations: ER, endoplasmic reticulum; PDI, protein disulfide isomerase; DMEM, Dulbecco's modified Eagle's medium; SLO, Streptolysin O.

MATERIALS AND METHODS

Reagents and Biochemicals—Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui and fetal calf serum from Gibco BRL and Boehringer Mannheim. Keyhole limpet hemocyanin and Streptolysin O (SLO) were from Sigma. Monoclonal antibodies against c-myc peptide (9E10) and protein disulfide isomerase were from Santa Cruz Biotechnology and Fuji Yakuhin Kogyo, respectively. The goat anti-rabbit IgG and its peroxidase-conjugated antibodies were from Cappel Products. FITC-conjugated goat anti-rabbit IgG and anti-mouse IgG were from EY Laboratory. Rhodamine-conjugated donkey anti-goat IgG was from Chemicon. Restriction enzymes and modifying enzymes were purchased from Takara, Nippon Gene, and Toyobo. The expression vector pSVL was from Pharmacia LKB.

Construction and Expression of Wild Type and Mutated Cytochrome b_5 in COS Cells—The cDNA for the mutant cytochrome b_5 tagged with c-myc peptide at the carboxy-terminal end was constructed by a single primer method (*11*) from a cDNA of cytochrome b_5 (*4*), using an oligonucleotide corresponding to a ten-amino-acid sequence (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) of c-myc (*12*) (5′-AGACAGGTTACAAGTCCTCTTCAGAAATAAGC-TTTTGCTCATCTTCTGCC3′).

The resultant plasmid containing the mutated fragment of cytochrome b_5 was digested with *Eco*RI and *Bam*HI, and then ligated into pBluescript SK$^+$. To express the mutated cytochrome b_5 in cultured COS-7 cells, pBluescript SK$^+$ containing the mutated fragment was digested with *Xho*I and *Xba*I, and the excised fragment of mutant cytochrome b_5 was inserted into pSVL, a mammalian cell expression vector.

COS-7 cells, which were grown in DMEM containing 10% fetal calf serum in an atmosphere of 5% CO_2 at 37°C, were transfected as described (*13*). A synthetic anionic amphiphile, *O,O′*-didodecyl-*N*-[*p*-(2-trimethylammonioethoxy)-benzoyl]-(L)-glutamate bromide was used to form cationic liposomes (*13*). Plasmid DNA (4 μg) containing cytochrome b_5 or tagged mutant coding fragment in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and liposome suspension (20 μg) prepared by sonication of the amphiphile were mixed in 20 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl. The mixture was incubated for 5 min at room temperature and then poured gently onto the cells in a dish of 35 mm diameter, and the cells were incubated for 3 to 6 hours at 37°C in an atmosphere of 5% CO_2. After the addition of DMEM containing 10% fetal calf serum, the cells were incubated for 48-72 h.

Preparation of Antibodies—Anti-b_5 antibody was raised against the amino-terminal hydrophilic portion of cytochrome b_5 which was obtained by trypsin treatment of the whole molecule. Anti-peptide antibody was raised against the peptide corresponding to the seven amino acid sequence of the carboxyl-terminal end. The peptide, which has an additional cysteine residue at the amino-terminal end (Cys-Arg-Leu-Tyr-Met-Ala-Glu-Asp), was manually synthesized by the Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] solid-phase method (*14*). After purification of the peptide by a reversed phase HPLC, it was conjugated to the carrier protein keyhole limpet hemocyanin as described (*15*). Rabbits were immunized by injection of the antigen as an emulsion with complete Freund's adjuvant. Anti-sera obtained were affinity-purified by the use of antigen-conjugated columns.

Immunofluorescence Microscopy—Immunofluorescence microscopy was carried out as described previously (*4*). Selective permeabilization of cells using SLO was performed by the method described by Otto and Smith (*16*). COS-7 cells expressing the wild type or mutated cytochrome b_5 were grown on coverslips in a dish of 35 mm diameter. The cells on the coverslips were washed with PBS (10 mM phosphate buffer pH 7.2, 0.15 M NaCl), and fixed with 2% paraformaldehyde-0.1% glutaraldehyde in PBS for 15 min. They were washed several times with PBS, then permeabilized with the SLO for selective permeabilization of plasma membranes or with Triton X-100 for permeabilization of all cellular membranes nonselectively. For selective permeabilization, the cells were incubated for 15 min on ice with 200 units/ml of the SLO which had been preincubated with 10 mM dithiothreitol in PBS for 10 min at 0°C. Unbounded SLO was washed off with PBS containing 10 mM dithiothreitol, and the coverslips were incubated at 37°C for 20 min in the PBS-dithiothreitol solution. On the other hand, the other portion of the cells was treated with 1% Triton X-100 for 2 min for nonselective permeabilization. After permeabilization of the cells, excess aldehydes were quenched by incubation with 1 mg/ml $NaBH_4$ for 10 min. The fixed and permeabilized cells were treated with rabbit anti-b_5 antibody, anti-peptide antibody, or mouse anti-c-myc antibody as the primary antibody, and then treated with FITC-conjugated goat anti-rabbit or anti-mouse IgG in PBS containing 10 mM glycine and 0.1% bovine serum albumin.

For double staining, the cells expressing cytochrome b_5 were treated with rabbit anti-peptide and mouse anti-PDI antibodies. For detection of cytochrome b_5, goat anti-rabbit IgG and rhodamine-conjugated donkey anti-goat IgG were added as secondary and tertiary antibodies, respectively. PDI protein was detected with FITC-conjugated goat anti-mouse IgG after addition of an excess amount of goat anti-rabbit IgG to prevent the binding of the FITC-conjugated IgG to the donkey IgG.

Preparation of Microsomal Fraction from COS Cells and Treatment with Trypsin and Alkali—The cells expressing the wild type or mutated cytochrome b_5 were washed with PBS and harvested in ice-cold STE buffer (0.25 M sucrose, 20 mM Tris-HCl, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, pH 8.0). After centrifugation at 500 × *g* for 5 min, the pellet was suspended in ice-cold STE buffer and homogenized gently with a Teflon-glass homogenizer. To precipitate the nucleus and unbroken cells, the homogenate was centrifuged at 500 × *g* for 5 min at 4°C. The post nucleus lysate was centrifuged at 4,500 × *g* 5 min at 4°C to isolate the mitochondria and lysosomes, and the resultant supernatant was centrifuged at 40,000 rpm 15 min at 4°C in an RP100AT4 rotor (Hitachi). The final pellet contained mostly the ER membrane. The ER membrane containing about 100 μg protein were incubated in 100 mM Na_2CO_3 (pH 11.5) for 30 min at 0°C or digested with trypsin at 0°C overnight. To separate the pellet from supernatant, treated membranes were centrifuged at 80,000 rpm for 30 min in an RP100AT4 rotor (Hitachi).

RESULTS

Characterization of Antibodies—Antibodies against the amino- and carboxy-terminal portions of cytochrome b_5 were prepared as described in "MATERIALS AND METHODS" and their specificity was checked by immunoblot analysis (Fig. 1). The anti-b_5 antibody exhibited a single band with rat liver microsomes and the mutant cytochrome b_5, B5⊿C31D, which lacks the carboxyl-terminal 30 amino acids (*4*), as well as cytochrome b_5. The original and mutant cytochromes were expressed in *Escherichia coli* and purified to homogeneity. The anti-peptide antibody, however, showed no immuno-reactive band with the mutant protein, though the antibody gave a single band with both rat liver microsomes and the intact cytochrome. These results indicate that the antibodies are specific to the amino-terminal and carboxy-terminal portions of the molecule, respectively.

Topology of the Carboxy-Terminal Portion of Cytochrome b_5 in the ER Membrane—To investigate the *in situ* topology of the carboxy-terminal portion of cytochrome b_5, COS cells expressing the cytochrome were subjected to indirect immunofluorescence microscopy under membrane-selective permeabilizing conditions. SLO was used to obtain selective permeabilization of the plasma membrane in the fixed cells, and consequently antigenic sites exposed to the cytoplasmic side of the ER membrane were able to react with antibodies. On the other hand, Triton X-100 can permeabilize all cellular membranes so that antibody can react with the antigens in the luminal side of the ER. As can be seen in Fig. 2, the anti-b_5 antibody detected the cytochrome in a reticular staining pattern characteristic of the ER, even when only the plasma membrane was permeabilized with SLO (Fig. 2, A and C). The anti-peptide displayed a fluorescence signal only with the Triton-permeabilized cells and no signal was observed in the cells treated with SLO (Fig. 2, B and D). Since no immunofluorescence signal was observed in the untransfected cells (cells near the central transfected cell in Fig. 2), the antibodies against b_5 and the peptide used in this experiment cannot cross-react with any component, even the resident cytochrome b_5, of the COS cells. The results indicate that the carboxy-terminal hydrophilic stretch is exposed to the luminal side of the ER.

To check the methodological reliability of our analysis, we applied a double immunostaining method to detect protein disulfide isomerase (PDI), a luminal protein of the ER, as well as the carboxy-terminal peptide of cytochrome b_5 in the same cell (Fig. 3). When the cells were treated with Triton X-100 and stained with anti-PDI antibody to detect PDI protein, a reticular pattern of fluorescence was observed in all the cells, though the cell in the center of the figure was more clearly detected because it was in focus (Fig. 3B). The same cell was co-stained with the anti-peptide antibody in exactly the same pattern (Fig. 3A), indicating the co-localization of both antigens. On the other hand, the SLO-treated cells were not stained with either of the antibodies (Fig. 3, C and D). Thus, the protein localized in the ER lumen could be detected by the specific antibodies only in the Triton X-100-treated cells, indicating the

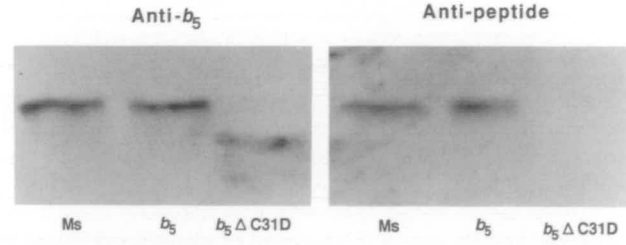

Fig. 1. **Characterization of anti-b_5 and anti-peptide antibodies.** Rat liver microsomes (2 μg) and the purified cytochromes, the original (b_5, 10 ng) and the mutated cytochrome b_5 (b_5⊿C31, 200 ng) were electrophoresed and subjected to Western blot analysis using anti-b_5 antibody. For detection with the anti-peptide antibody, 20 μg, 100 ng, and 2 μg of proteins were used, respectively. Ms, rat liver microsomes.

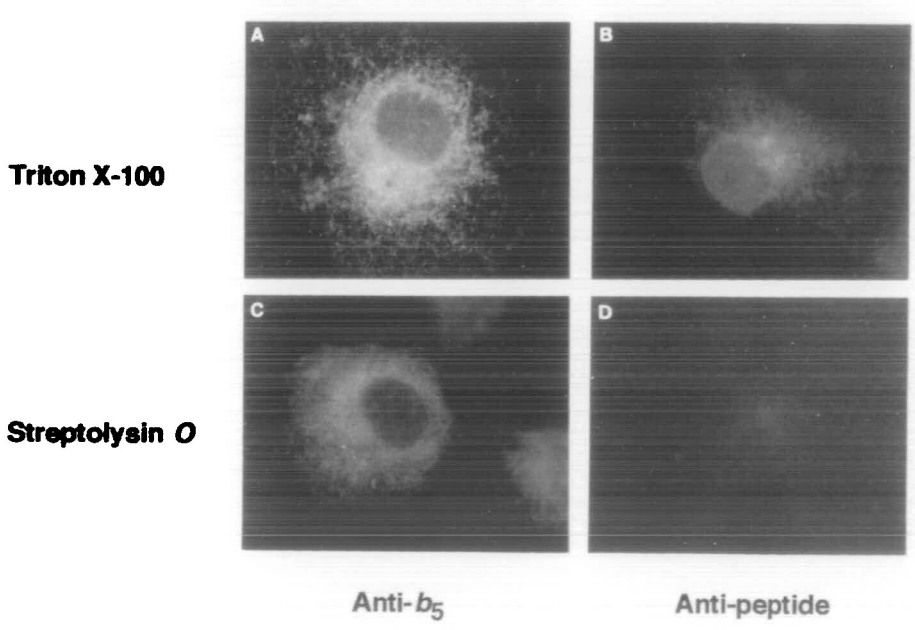

Fig. 2. **Indirect immunofluorescence microscopy of COS cells expressing cytochrome b_5.** Immunofluorescence stainings were carried out in COS-7 cells transfected with rat cytochrome b_5 cDNA. Staining was done using either anti-b_5 (A and C) or anti-peptide antibody (B and D). The cells were permeabilized with Triton X-100 (A and B) or SLO (C and D) as described under "MATERIALS AND METHODS."

reliability of our analytical methods.

Topology of c-myc Peptide Attached to the Carboxy-Terminal End of Cytochrome b_5—Attachment of a tag to proteins is a useful method to detect the protein or a portion of the protein. C-myc peptide is one of the most useful tags because a specific antibody against the peptide is commercially available. To examine whether we could use cytochrome b_5 tagged with c-myc peptide at the carboxy-terminal end to study the mechanism of membrane insertion of the cytochrome, we examined if the mutant cytochrome has the same orientation as the wild type cytochrome. The c-myc-tagged cytochrome b_5 was expressed in COS cells as and the topology of the c-myc peptide was analyzed by immunofluorescence microscopy, using anti-c-myc peptide antibody, 9E10 (Fig. 4). When COS cells expressing the cytochrome b_5 tagged with c-myc peptide were permeabilized by Triton X-100, the reticular network staining pattern of the ER was observed after incubation with both anti-b_5 and anti-c-myc antibody (Fig. 4, A and B), indicating the localization of the mutant protein in the ER. The targeting of the c-myc-tagged cytochrome b_5 to the ER suggests either that the targeting signal of the cytochrome need not be localized at the carboxy-terminal end of the molecule (*4*), or that the c-myc peptide functioned as the targeting signal, because the signal for targeting does not reside in a specific amino acid sequence, but instead in a balance of polar amino acid residues (Mitoma *et al.*, unpublished results), and the amino acid sequence of c-myc peptide is similar to that of the carboxy-terminal portion of the cytochrome (*12*). When only the plasma membrane was permeabilized with SLO, the anti-c-myc antibody was unable to detect the peptide conjugated to cytochrome b_5 (Fig. 4D). Thus, the c-myc peptide at the carboxy-terminal end of cytochrome b_5 is exposed to the luminal side of the

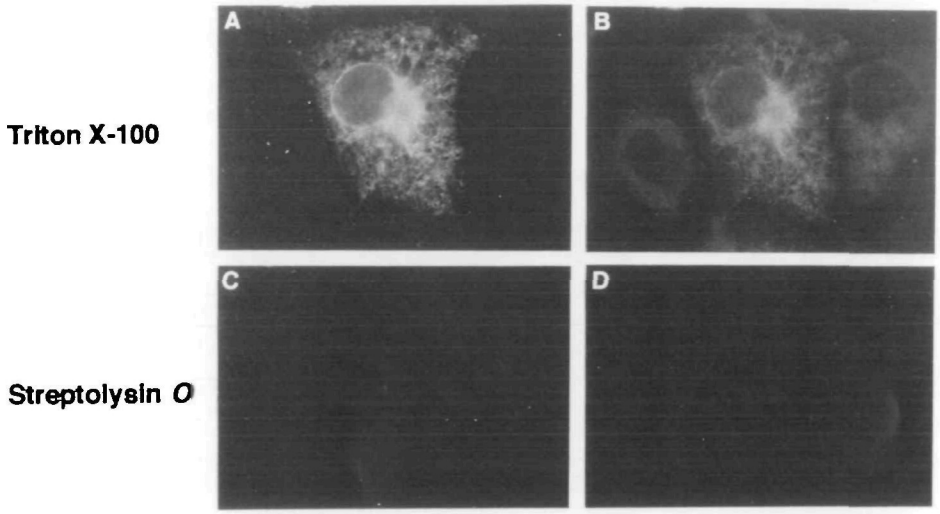

Fig. 3. **Double immunofluorescence labeling of COS cells expressing cytochrome b_5.** Immunofluorescence stainings were carried out in COS-7 cells transfected with rat cytochrome b_5 cDNA. For double immuno-staining, anti-peptide (A and C) or anti-PDI antibodies (B and D) was used as the primary antibody. The cells were permeabilized with Triton X-100 (A and B) or SLO (C and D) as described under "MATERIALS AND METHODS."

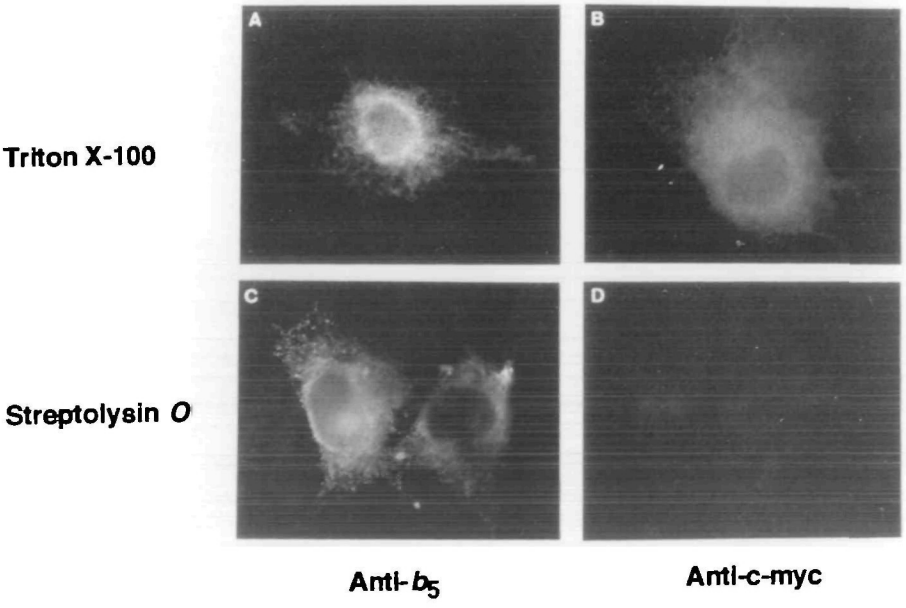

Fig. 4. **Indirect immunofluorescence microscopy of COS cells expressing cytochrome b_5 tagged with c-myc peptide.** Immunofluorescence stainings were carried out in COS-7 cells transfected with the tagged cytochrome b_5 cDNA. Staining was done using either anti-b_5 (A and C) or anti-c-myc antibody (B and D). The cells were permeabilized with Triton X-100 (A and B) or SLO (C and D) as described under "MATERIALS AND METHODS."

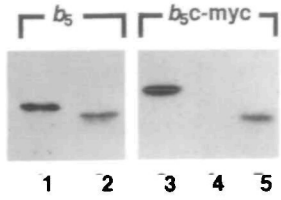

Fig. 5. **Trypsin treatment of the original and mutated cytochrome b_5 in the ER membranes.** Microsomal membranes (about 100 μg protein) prepared from the cells expressing the original rat cytochrome b_5 (b_5) or the cytochrome tagged with c-myc peptide (b_5 c-myc) were treated with trypsin (about 0.5 μg) as described under "MATERIALS AND METHODS." After centrifugation of the samples, cytochrome b_5 in the supernatant and membrane was detected by Western blot analysis. 1, cytochrome b_5 before the treatment; 2, cytochrome b_5 in the supernatant fraction; 3, b_5c-myc before the treatment; 4, b_5c-myc in the membrane fraction; 5, b_5c-myc in the supernatant fraction.

ER vesicle.

The same disposition of the mutated cytochrome in the ER membrane as that of the wild type cytochrome b_5 was confirmed by their sensitivity to sodium carbonate and trypsin treatments. Microsomal membranes obtained from the COS cells expressing the wild-type and mutated cytochrome b_5 were treated with sodium carbonate (0.1 M) or trypsin. Most cytochrome b_5 protein was recovered in the membrane fraction after treatment with Na_2CO_3 (data not shown), indicating that the mutated protein is an integral membrane protein, like the wild-type one. After the trypsin treatment of the microsomal membranes, a trypsin resistant hydrophilic core was detected at the same position in SDS PAGE as that of "trypsin b_5" from rat liver microsomes (Fig. 5). Thus, the addition of c-myc peptide to the carboxy-terminal end does not affect insertion or orientation of the cytochrome b_5 in the ER membrane.

DISCUSSION

The present results clearly indicate that the membrane-anchoring domain of cytochrome b_5 spans the ER membrane and its carboxy-terminal hydrophilic stretch is exposed to the luminal side. This is the first clear indication of the *in situ* topology of a tail-anchored protein in membranes. Since cytochrome b_5 has a proline residue in the middle of the membrane-anchoring domain, two types of conformation are possible in the ER membrane: loop and transmembrane conformations. The former might be possible if the proline exists in the *cis* configuration, whereas if the proline takes the *trans* configuration, the membrane-anchoring domain could form a conventional α-helix and span the entire membrane. The present *in situ* results support the transmembrane conformation of the membrane-anchoring domain of cytochrome b_5. The conclusion is confirmed by the result showing that mutation of the proline to alanine has no effect on the enzyme activity or the sensitivity to trypsin treatment of cytochrome b_5 in the ER membrane (*17*). The existence of a proline residue in the transmembrane domain of cytochrome b_5 seems not to be essential for formation of the topology of the cytochrome in the ER membrane.

Since *in vitro* studies have shown that the purified cytochrome b_5 can be spontaneously incorporated into various membranes, including liposomal membranes (*18-20*) as well as the ER membranes (*21-23*), cytochrome b_5 had been considered as a typical protein targeted through the receptor-independent, hydrophobic-insertion pathway and thus able to insert into any available hydrophobic environment (*24, 25*). However, we have shown that a hydrophilic sequence of about 10 amino acids at the carboxy-terminal end of cytochrome b_5 is the signal responsible for targeting of the cytochrome to the ER membranes (*4*). Although the minimum requirements of the amino acids in the carboxy-terminal sequence for the ER-targeting signal still remain unknown, the cytochrome b_5 molecule may be first targeted to the ER membrane by a specific interaction of its carboxy-terminal signal with a receptor-like protein(s) and then anchored into the membrane by its hydrophobic portion. Since the carboxy-terminal end of cytochrome b_5 has a guanidyl group and three carboxy groups within the last seven amino acids, there might be specific machinery for the translocation of the highly polar end of the cytochrome across the ER membrane. Microsomal aldehyde dehydrogenase, another tail-anchored protein of the ER with a highly polar carboxy-terminal end (*26, 27*), might share the same machinery for the insertion into the ER membrane.

Insertion of the cytochrome to the ER membranes has been studied for years (*5-8, 21-23*), but none of these studies determined the topology of the *in vitro*-inserted cytochrome. Vergeres *et al.* (*10*) have recently shown that *in vitro*-inserted cytochrome b_5 is sensitive to carboxypeptidase Y treatment only in the presence of Tergitol and that the solubilized cytochrome was cleaved at both lysine(93), which is in the amino-terminal portion, and arginine(127), which is in the carboxy-terminal hydrophilic portion, while the membrane-bound cytochrome was cleaved only at lysine(93). Their results, taken together with the present results, demonstrate the correct disposition of the cytochrome inserted *in vitro* into membranes. Kutay *et al.* (*28*) have recently indicated the transmembrane insertion of the hydrophobic tail of synaptobrevin, a tail-anchored protein of synaptic-like vesicles, into the ER membrane, by examining the *N*-glycosylation of the protein with a glycosylation site introduced at the carboxy-terminal portion, and they suggested participation of protein(s) in the insertion. In addition to these methods, the immunochemical method using antibodies against the carboxy-terminal sequence and the tag peptide will be a useful and reliable tool to analyze the topology of *in vitro*-inserted proteins.

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