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The Transmembrane Region of Microsomal Cytochrome P450 Identified as the Endoplasmic Reticulum Retention Signal[1]

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Microsomal-type cytochrome P450s are integral membrane proteins bound to the membrane through their N-terminal transmembrane hydrophobic segment, the signal anchor sequence. To elucidate the determinants that enable the P450s to be located in the ER, we constructed cDNAs encoding chimeric proteins in which a secretory form of carboxyesterase, carboxyesterase Sec, was connected to the N-terminus of the full-length or truncated forms of a microsomal-type P450, P450(M1), and the constructed plasmids were expressed in COS cells. Since carboxyesterase Sec is an *N*-glycosylated secretory protein, endo H treatment could be used to determine whether these chimeric proteins were located in the ER or not. Carboxyesterase Sec with the N-terminal 20 amino acids, containing the transmembrane region, of P450(M1), was located in the ER, as determined from the endo H sensitivity of the expressed protein and immunofluorescence staining of the cells. As the expressed protein exhibited carboxyesterase activity, it was not retained in the ER through the BiP-dependent quality control system recognizing unfolded proteins. Another chimeric protein construct in which carboxyesterase Sec was connected to the C-terminal region of rat UDP-glucuronosyltransferase (UDP-GT), that contained a double-lysin ER retention motif, was also located in the ER, as determined from the endo H sensitivity and immunofluorescence staining. On the other hand, the sugar moiety of the carboxyesterase Sec connected to the transmembrane segment of UDP-GT, Sec/GTd, was partially resistant to the endo H treatment. From the results of immunofluorescent staining and cell fractionation, it was concluded that the Sec/GTd product was located in the Golgi apparatus. These observations indicated that the N-terminal hydrophobic segment of P450(M1) is sufficient for the ER membrane retention, whereas the transmembrane segment of UDP-GT is not. To determine whether microsomal P450s are recycled between the ER and Golgi compartments or not, a DNA construct encoding cathepsin D connected to the N-terminus of P450(M1) was prepared and expressed in COS cells. The fusion protein was phosphorylated, but the phosphorylation was sensitive to alkaline phosphatase. As a control, authentic cathepsin D was subjected to phosphorylation of its oligosaccharide chain that was resistant to the alkaline phosphatase treatment. Since GlcNAc-P-transferase, which forms the alkaline phosphatase-resistant phosphodiester in the sugar chains of lysosome-targeting proteins, is located in the Golgi apparatus, it was concluded that the oligosaccharide chain of the cathepsin D portion of the fusion protein was not phosphorylated, and that the chimeric protein did not go to the Golgi apparatus. These results indicate that P450(M1) is not recycled from the Golgi compartments to the ER in cells.

Key words: cytochrome P450, endoplasmic reticulum, retention.

Secretory proteins and most of the membrane proteins of the organelles of the secretory pathway are synthesized on ribosomes bound to the endoplasmic reticulum (ER), translocated across or inserted into the ER membrane, and then transported to their final destinations through the process of membrane vesicle budding and fusion. It is generally believed that there is a nonselective bulk flow of proteins along the secretory pathway from the ER to the cell surface, and the proteins with sorting signals are diverted from the flow and localized to specific organelles, whereas those without such signals are transported to the cell surface to be secreted out of the cell or to be localized in the cell membrane. Thus, the sorting signals assure the residency of each protein in a specific organelle (*1, 2*). The mannose-6-phosphate marker of soluble lysosomal enzymes for lysosomes (*3, 4*), and the C-terminal tetrapeptide, KDEL, retention signal of the soluble ER luminal proteins for the ER, are classical examples of these sorting signals.

The retention of most luminal proteins and some trans-

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Abbreviations: BFA, brefeldin A; DMEM, Dulbecco's modified Eagle medium; ER, endoplasmic reticulum; endoH, endoglycosidase H; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; UDP-GT, UDP-glucuronosyltransferase.

membrane proteins of the ER depends on the presence of the C-terminal KDEL sequence. These proteins exit the ER but they are captured by the KDEL receptors in the Golgi apparatus or in the ER-Golgi intermediate compartment (*5, 6*). Much less is known, however, about the retention mechanism for the other ER membrane proteins. The ER residency of a few membrane proteins with the type I orientation, such as UDP-glucronosyltransferase, is guaranteed by the presence of the C-terminal KKXX or KXKXX motif, the double lysine motif, in their short cytoplasmic tail (*7*). They exit the ER to be retrieved from the subsequent compartments probably through a receptor-mediated retrieval process (*8*). However, a number of type I ER membrane proteins have no double-lysine motif in their C-terminus. The retention mechanism for these proteins and the proteins with the type II orientation or with multiple transmembrane segments remain largely unknown.

To study the retention mechanism for the ER membrane proteins without the KDEL or double-lysine motif, we chose microsomal cytochrome P450s (P450s). They are integral membrane proteins localized in the ER, and carry the type I signal-anchor sequence at their N-terminus, which functions both as a classical signal sequence and as a stop-transfer sequence (*9–11*). The concerted action of this signal-anchor sequence and the signal recognition particle targets P450s to the ER, and the P450s are inserted into the membranes in the type I (Nin-Ccyt) orientation (*12*).

Although the insertion of P450s into the ER has been studied extensively, the mechanism by which P450s become ER residents is not yet clear. Although P450s assume the type I orientation in the ER membrane, no double-lysine motif is present in their C-terminus. To understand the retention of P450s in the ER membrane, in this study we defined the region of the P450(M1) molecule that determines P450s' ER residency. We constructed chimeric proteins, in which the whole or portions of the P450(M1) molecule were attached to the C-terminus of carboxyesterase Sec, a secretory protein used as the reporter. As a control, other types of fusion proteins were constructed in which the C-terminal portion of UDP-glucronosyltransferase, which contains the double-lysine motif for the ER retention, was attached to carboxyesterase Sec. The fusion proteins were expressed in COS cells, and the subcellular localization of the fusion proteins was determined. To determine whether P450s are retained permanently in the ER or recycled between the ER and post-ER compartments through the "export and retrieval" process, we chose a lysosomal enzyme, cathepsin D, as a reporter, which was fused to the N-terminus of P450 at the DNA level and the fusion protein was expressed in COS cells. The results with the fusion protein suggested that the transmembrane segment of P450(M1) was sufficient for the ER retention, which diverted P450s from the export and recycling pathways to be retained in the ER.

MATERIALS AND METHODS

Plasmids—The COS cell expression vectors were based on plasmid pSG5, which was kindly provided by Dr. Y. Ikehara of Fukuoka University. This plasmid contains the SV40 origin and SV40 early promoter. A cDNA fragment carrying the entire coding sequence of carboxyesterase Sec was excised from a clone (8-1/2-1) (*13*) by *Eco*RI-*Hin*dIII digestion. The fragment was blunt-ended and ligated with *Eco*RI linkers, and then inserted into the *Eco*RI site of pSG5 to obtain Sec. To construct Sec/M1, the cDNA fragment of P450(M1) was excised by *Bam*HI digestion from pD2M1 (*14*), and then the fragment was inserted into the *Bcl*I site of Sec. To construct Sec/M1-Pro, Sec/M1-TM, Sec/M1-H, and Sec/M1-Q, the fragments encoding P450(M1) amino acids 1-37, 1-20, 1-11, and 1-7 were amplified from pM1 (*15*) by the polymerase chain reaction (PCR) using a common 5′ PCR primer, 5′-GACTCTAGAGGATCCAGTCCTAGT-3′ (a *Bam*HI site is underlined), and 3′ PCR primers, 5′-CCTCTCCCATAATGATCAAACAACCTT-3′ for the 1-37 fragment, 5′-TCACTCTGGTAATGATCATTTGGGAGA-3′ for the 1-20 fragment, 5′-CTCACTCTCTAATGATCACTTCTCCTC-3′ for the 1-11 fragment, and 5′-CTCACTCTCTAATGATCACTCTCCTCT-3′ for the 1-7 fragment (*Bcl*I site is underlined). Each resulting fragment was digested with *Bam*HI and *Bcl*I, and then inserted into the *Bcl*I site of Sec.

To construct Sec/GT and Sec/GTd, the fragments containing UDP-glucronosyltransferase amino acids 435-476 and 435-467 were amplified from pYGT (a gift from Dr. Yuasa of Rakuno Gakuen University), which contains the cDNA sequence encoding the C-terminal 476 amino acids of rat UDP-GT, using a 5′ PCR primer for both fragments, 5′-ACCTCCCCTGGGATCCGTACCACT-3′, and a 3′ PCR primer, 5′-CATTGTCATGGATCCCTACTCATTCTT-3′ for the 435-476 fragment, and 5′-CTTTTGCTTTTAGGATCCGAATCGGTA-3′ for the 435-467 fragment (*Bam*HI site is underlined). Each resulting fragment was digested with *Bam*HI and then inserted into the *Bcl*I site of Sec.

To construct M1 and CathD, P450(M1) and cathepsin D cDNA fragments were excised by *Eco*RI digestion from pD2M1 and pCat-1, and then inserted into the *Eco*RI site of pSG5. To construct CathD/M1, the *Bam*HI site of pCat-1 was shifted from bp position 1143 to 1144 by *in vitro* site-directed mutagenesis. A cDNA fragment coding the N-terminal 378 amino acids of cathepsin D was excised from the mutated pCat-1 by digestion with *Eco*RI and *Bam*HI. The fragment was then ligated into pSG5 digested with *Eco*RI and *Bam*HI. The resultant plasmid was digested with *Bam*HI, and then ligated with the DNA fragment containing P450(M1) that had been obtained from pD2M1 by *Bam*HI digestion.

Antibodies—Rabbit anti-carboxyesterase (anti-Est) antibodies were prepared as described previously with purified rat liver microsomal carboxyesterase E1 as the antigen (*13*). Anti-Est antibodies recognize carboxyesterase Sec as well as other microsomal-type carboxyesterases, including carboxyesterase E1. Rabbit anti-P450(M1) antibodies were previously described (*16*). Rabbit anti-cathepsin D antibodies were a gift from Dr. Himeno of Kyushu University.

Transfections, Labeling, and Immunoprecipitation—COS-7 cells were seeded on the day before the transfection at a density of 1.2×10^6 cells per 10 cm culture dish in Dulbecco's modified Eagle medium (GIBCO, Gent, Belgium), which contained 10% fetal calf serum. The dish was incubated at 37°C under an atmosphere of constant humidity (95%) comprising 10% CO_2 in air. The cells were approximately 80% confluent after 24 h.

The plasmid DNA (30 μg) in 1.5 ml of phosphate-

buffered saline (PBS) was mixed with 1.5 ml of a cationic liposome solution in PBS (1 mg/ml) prepared with *O,O'*-didodecyl-*N*-[*p*-(2-trimethylammoniumethoxyl)benzoyl]-L-glutamate bromide (*17*). The DNA-liposome complex, which was formed by mixing, was added to the cell culture. The cells were incubated at 37°C for 3 h, and then 10 ml of DMEM supplemented with 10% fetal calf serum was added. Forty hours later, the cells were washed with a methionine-free medium and then incubated in the same medium for 2 h. [^{35}S]Methionine (200 μCi/ml) was then added and the cells were incubated for 3 h. For pulse and chase studies, the cells were labeled as above, and then washed with fresh culture medium and further incubated in fresh medium containing 2 mM non-radioactive methionine for 3 h. For ^{32}P labeling, the cells were pre-incubated for 1 h in a phosphate-free medium, and then labeled in the same medium with 1 mCi $^{32}PO_4$ per dish for 2 h.

For immunoprecipitation, the culture medium was separated from the cells by decantation. The cells were washed with PBS, and then scraped off with a rubber policeman into 1 ml of PBS. A 0.1 volume of 10% SDS was added to the culture medium, and the mixture was heated at 100°C for 5 min. It was then diluted with 9 volumes of dilution buffer [0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 5 mM EDTA]. The cells were homogenized with 1 ml of PBS containing 1% SDS by 50 strokes through a syringe needle, and then diluted with 9 volumes of the dilution buffer. Equivalent amounts of the culture medium and cell extracts were incubated with the appropriate antibodies at 4°C for 1 h. The immunocomplexes were recovered using Protein A-Sepharose as described previously (*18*).

Cell Fractionation and Alkali Extraction of the Membrane Fraction—Cells were washed with PBS and harvested in ice-cold STE buffer [0.25 M sucrose, 20 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, and 2 μg/ml each of leupeptin and pepstatin]. The suspension was centrifuged at 500×*g* for 5 min and the pellet was suspended in a hypotonic buffer [0.025 M sucrose, 20 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, and 2 μg/ml each of leupeptin and pepstatin]. The suspension was kept on ice for 30 min and then centrifuged. The precipitated cells were suspended in 1 ml of STE buffer and then homogenized with a Dounce homogenizer. The homogenate was centrifuged at 500×*g* for 5 min to precipitate the nuclei and undisrupted cells. The supernatant (crude membrane fraction) was layered on a discontinuous sucrose gradient consisting of 1 ml of 2 M sucrose, 3.4 ml of 1.3 M sucrose, 3.4 ml of 1 M sucrose, and 2.75 ml of 0.6 M sucrose (*19*), and then centrifuged for 4 h at 40,000 rpm in an RPS-40T rotor (Hitachi). Fractions were collected from the top of the tube.

For alkali extraction, the crude membrane fraction was treated with 100 mM Na_2CO_3 on ice for 30 min (*20*).

Western Blotting—SDS-PAGE was carried out using 10% polyacrylamide gels containing 0.1% SDS. The gels were blotted onto nitrocellulose filters as described by Towbin *et al.* (*21*), and the filters were incubated in PBS containing 1% skim milk powder and 0.1% Tween-20, and then treated with appropriate antibodies for 1 h. The filters were washed with PBS and then treated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies in PBS for 2 h. After washing 5 times for 5 min each, the immunoreacted bands were visualized with an ECL Western blotting analysis system (Amersham, U.K.).

Enzyme Digestion—For endoglycosidase H and *N*-glycosidase F digestions, protein samples were precipitated with 9 volumes of ice-cold acetone. After centrifugation, the pellets were dissolved in 50 mM sodium citrate buffer (pH 5.0) containing 2% SDS, and then the solutions were heated at 100°C for 3 min. Nine volumes of 50 mM sodium citrate buffer (pH 5.0) containing 2% Triton X-100 was then added to each sample. Endo H (7.5 mU/ml) (Boehringer Mannheim GmbH, Germany) was added to the samples, which were then incubated at 37°C for 24 h. *N*-Glycosidase F (Boehringer Mannheim GmbH) digestion was performed as described for endo H digestion except for the following modifications; sodium phosphate buffer (pH 7.5) was used instead of sodium citrate buffer (pH 5.0), and the enzyme concentration was 20 mU/ml.

For alkaline phosphatase digestion, the protein samples were heated at 100°C in 0.1% SDS for 3 min and then incubated with calf intestinal phosphatase (1 U/ml) (Boehringer Mannheim GmbH) at 30°C for 1.5 h.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed by the method described by Munro and Pelham (*5*). Five micrograms each of the plasmid pSG5 harboring various cDNA constructs was transfected into COS cells in a 3.5 cm dish. After incubation for 48 h, the cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS for 10 min, and then permeabilized with 1% Triton X-100 for 10 min. The fixed cells were treated with appropriate antibodies, and then FITC-conjugated goat anti-rabbit IgG antibodies (Cappel England) in PBS containing 1% bovine serum albumin.

In Vitro Translation—*In vitro* transcription and translation were performed as described (*11*). Messenger RNAs transcribed with T7 polymerase from the pSG5 plasmids harboring various cDNA constructs were translated in a wheat germ cell-free system in the presence of dog pancreas rough microsomes for 30 min at 26°C.

Analytical Methods—Esterase activity was measured spectrophotometrically using *p*-nitrophenyl acetate in 100 mM Tris-HCl buffer (pH 8.2) at 405 nm according to Krisch (*22*). NADPH-cytochrome *c* reductase activity was measured as described (*23*). α-Mannosidase II activity was measured as described (*24*).

RESULTS

N-Terminal 20 Amino Acids of P450(M1) Are Sufficient for the Retention to ER—To identify the regions that are responsible for the retention of P450(M1) in the ER, we used carboxyesterase Sec as a reporter protein. Carboxyesterase Sec, an isozyme of liver microsomal carboxyesterases, contains no KDEL-type sequence at the C-terminus and is secreted into the blood as a glycoprotein with complex type sugar chains. Thus, the type of the carbohydrate modification occurring during the transit through the secretory pathway is a good diagnostic clue as to the localization of the enzyme. Precarboxyesterase Sec was fused at the cDNA level to the amino terminus of intact P450(M1) or modified P450(M1), with various deletions from the C-terminal region, into the transmembrane segment (Fig. 1).

The chimeric cDNAs were transfected into COS cells. Forty hours later, the cells were labeled with [^{35}S]meth-

ionine for 3 h and then incubated in the chase medium containing non-radioactive methionine for a further 3 h. The labeled proteins in the cells and medium were immunoprecipitated with anti-Est antibodies, which cross-reacted with carboxyesterase Sec, and then analyzed by SDS-PAGE.

When Sec DNA was transfected into COS cells, a single immunoprecipitable product was detected in the cells after 3 h pulse-labeling (Fig. 2A, lane 1), and a significant amount of another Sec product with lower mobility appeared in the medium (Fig. 2A, lane 7). After a 3 h chase, the amount of the Sec product in the cells decreased and that in the medium increased significantly (Fig. 2A, lanes 4 and 10).

The Sec product in the cells was sensitive to endoglycosidase H treatment (Fig. 2A, lanes 2 and 5) and migrated with slightly lower mobility in the gel than the unglycosylated Sec product synthesized in the presence of tunicamycin (Fig. 2A, lanes 3, 6, 9, and 12). The slight difference in the mobility between these products was probably due to the fact that endo H cleaved the GlcNAc-GlcNAc bond of the high mannose type sugar chains leaving GlcNAc-Asn in the substrate molecule. On the other hand, the Sec product in the medium was more resistant to endo H digestion than that in the cells, although its resistancy was not complete (Fig. 2A, lanes 8 and 11). The partial resistancy of the sugar chains of the secreted Sec product to the endo H treatment was considered to be evidence for its transit through the Golgi apparatus, where the complex type sugar modifications occur.

Taking advantage of this difference in the endo H sensitivity of the reporter protein, we examined the subcellular localization of the expression products of Sec/M1, Sec/M1-Pro, and Sec/M1-TM cDNAs in COS cells. When the cDNA coding for the carboxyesterase Sec–entire P450(M1) fusion protein (Sec/M1) was transfected into COS cells and pulse-labeled with [^{35}S]methionine for 3 h, the radiolabeled product was detected in the cells (Fig. 2B, lane 1), but not in the medium (Fig. 2B, lane 7). The product remained stably in the cells even after a 3 h chase (Fig. 2B, lane 4) and was not secreted into the medium (Fig. 2B, lane 8). The chimeric protein in the cells remained in an entirely endo H-sensitive form during the pulse and chase periods (Fig. 2B, lanes 2 and 5). These results indicated that the P450(M1) molecule, when fused to the C-terminus of the reporter protein, exhibited the ability of retaining the reporter protein in the ER. To identify the region that is responsible for the ER retention of the reporter protein, we constructed cDNAs in which the C-terminal cytoplasmic portion of P450(M1) in the fusion protein was deleted, leaving either the transmembrane domain or both the transmembrane and proline-rich (PR) domains, Sec/M1-TM (Fig. 2D) and Sec/M1-Pro (Fig. 2C), respectively. The transfection products of these chimeric cDNAs were detected only in the cells, *i.e.*, not in the medium (Fig. 2, C and D, lanes 1, 4, 7, and 8). Both the products detected in the cells were sensitive to endo H digestion throughout the pulse and chase periods (Fig. 2, C and D, lanes 2 and 5). These results indicated that the transmembrane segment of P450(M1) was sufficient for retention of the chimeric proteins in the ER. The C-terminal flanking region of the transmembrane segment of microsomal P450s contains clustered multiple basic amino acid residues followed by proline and glycine clusters, the PR region. The present

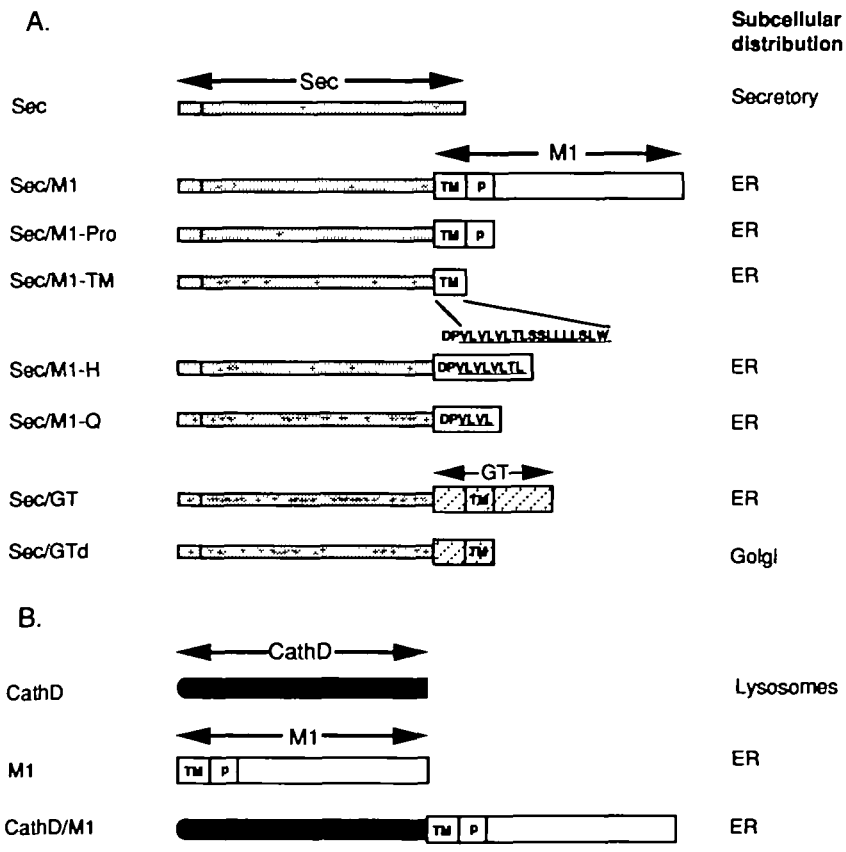

Fig. 1. **Summary of construction of carboxyesterase Sec fusion proteins (A) and cathepsin D fusion proteins (B), and their subcellular distribution in COS cells.** The dotted boxes indicate carboxyesterase Sec (Sec), the open boxes P450(M1) (M1), the striped boxes UDP-GT (GT), and the solid boxes cathepsin D (CathD). The amino acid sequence of the signal anchor portion of P450(M1) is shown in the figure and the putative transmembrane regions are underlined. S, signal sequence; TM, transmembrane region; P, proline-rich region. The names of the constructed fusion proteins are shown on the left side and their subcellular distributions are indicated on the right.

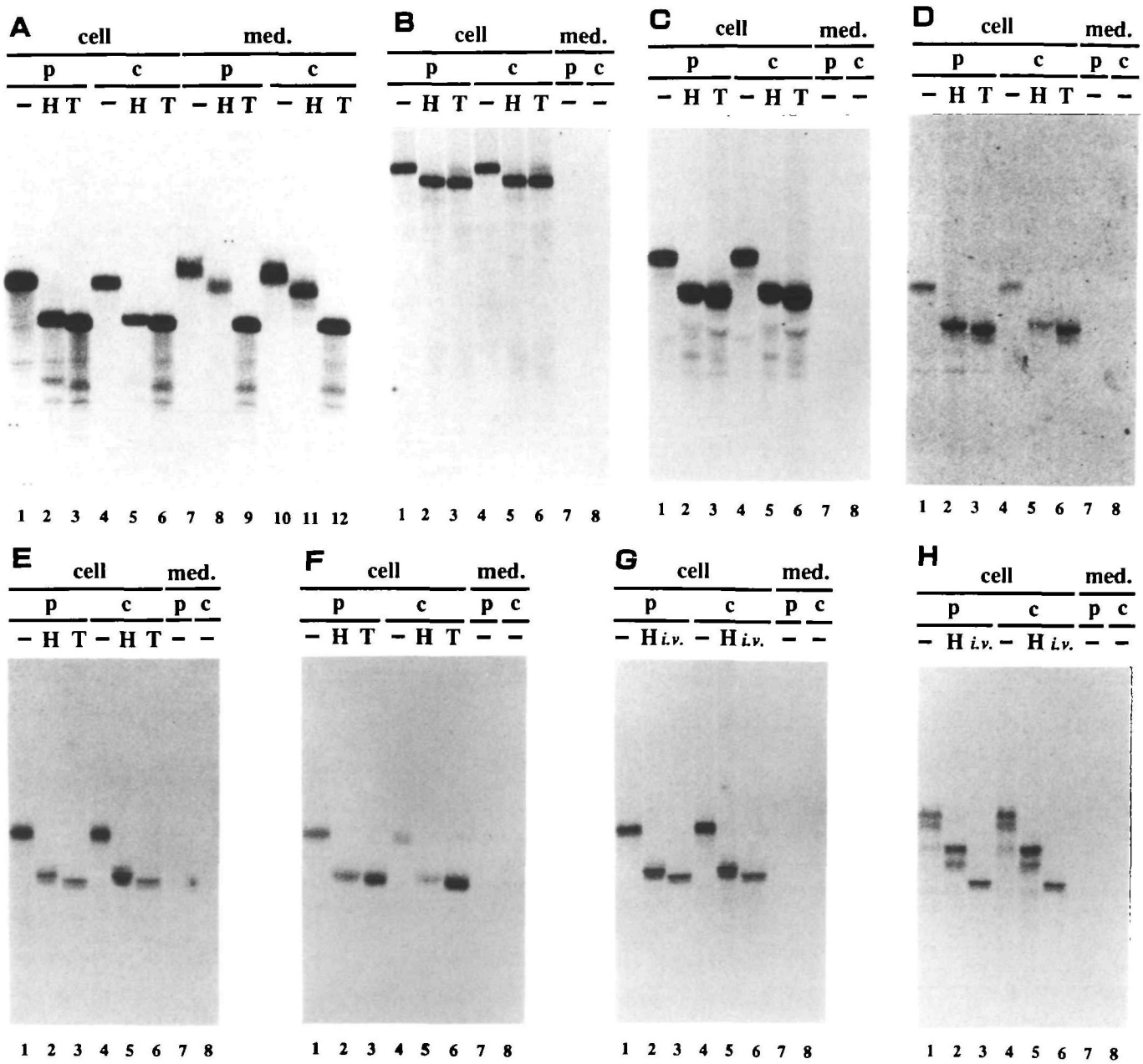

Fig. 2. **Endo H sensitivity of carboxyesterase Sec fusion proteins.** COS cells were transfected with Sec (A), Sec/M1 (B), Sec/M1-Pro (C), Sec/M1-TM (D), Sec/M1-H (E), Sec/M1-Q (F), Sec/GT (G), and Sec/GTd (H). After 40 h, the cells were labeled for 3 h (p) with 200 μCi/ml of [^{35}S]methionine in the presence (T) or absence of tunicamycin and then chased for 3 h (c). The immunoprecipitates with anti-Est antibodies from the cell extracts (cell) and media (med.) were analyzed by SDS-PAGE and fluorography, either directly, or after endo H digestion (H). In the experiments shown in G and H, *in vitro* translation products (*i.v.*) synthesized in the presence of dog pancreas rough microsomes and then digested with *N*-glycosidase F were used as the non-glycosylated protein standard.

experiment clearly indicated that the PR region as well as the basic amino acid cluster had nothing to do with the retention of P450s in the ER.

However, there remained a possibility that the oligosaccharide chains of the reporter protein portion of the chimeric proteins had lost the potential to undergo the complex type sugar modifications. To rule out this possibility we performed Brefeldin A (BFA) treatment of the transfected cells. BFA shuts off the anterograde membrane traffic of the Golgi complex while enhancing the retrograde pathway to the ER and, as a result, causes disassembly of the Golgi apparatus and its mixing with the ER. Therefore, the high mannose type glycoproteins retained in the ER are rapidly processed by the *cis*/medial Golgi enzymes into complex type glycoproteins. As shown in Fig. 3, the transfection products of Sec/M1, Sec/M1-Pro, and Sec/M1-TM cDNAs gained partial resistancy to the endo H treatment when BFA was present during the pulse period (Fig. 3, lanes 2, 4, and 6), excluding the above possibility.

To define the retention signal in the transmembrane segment of P450(M1) further, we constructed Sec/M1-H and Sec/M1-Q cDNAs (see Fig. 1), in which either the

N-terminal one-half or one-quarter of the transmembrane segment of P450(M1) was fused to the C-terminus of carboxyesterase Sec. The transfection products of these cDNAs were detected only in the cells, *i.e.*, not in the medium, after the pulse or the pulse and chase periods (Fig. 2, E and F, lanes 1, 4, 7, and 8). The products in the cells were sensitive to endo H digestion (Fig. 2, E and F, lanes 2 and 5), again indicating that both chimeric proteins are retained in the ER. It should be noted that the intracellular stability of Sec/M1-Q seemed to be lower than that of other Sec constructs, since the recovery of the immunoprecipitation products decreased significantly after a 3 h chase (compare lanes 1 and 4 in Fig. 2F).

Assessment of the Correct Folding of the Sec Portion of the Chimeric Proteins, as Monitored as Carboxyesterase Activity—It is well known that correct folding and assembly are necessary for the efficient export of proteins from the ER. Unfolded or malfolded proteins tend to bind to the chaperones in the ER or to form aggregates and thereby are retained in the ER to be degraded. It is, therefore, necessary to know if the carboxyesterase Sec fusion proteins expressed in the COS cells described above attained the correct conformations or not. For this purpose, we assayed the carboxyesterase activity of the reporter molecule, carboxyesterase Sec, and the results are shown in Table I. The basal activity of the carboxyesterase of the COS cells was about equal to the activity of the expressed Sec fusion proteins. The expressed products of the cDNAs of Sec/M1, Sec/M1-Pro, and Sec/M1-TM exhibited significant carboxyesterase activities. In contrast, however, no activity was detected for the Sec/M1-H or Sec/M1-Q protein.

These results indicate that the Sec/M1, Sec/M1-Pro, and Sec/M1-TM proteins had attained the correct conformations in the COS cells, whereas the Sec/M1-H and Sec/M1-Q proteins were in malfolded states and therefore retained in the ER, probably through interaction with the chaperones in the ER or formation of aggregates. We wanted to know whether the Sec/M1-H and Sec/M1-Q proteins were firmly anchored to the ER membranes or translocated the membranes to bind loosely to the luminal surface, since the Sec/M1-H and Sec/M1-Q proteins carried the shorter truncated transmembrane segments of P450(M1). The whole cells expressing the Sec, Sec/M1, Sec/M1-H, and Sec/M1-Q proteins were treated with 100 mM sodium carbonate (pH 11.5), which extracts both peripheral membrane proteins and luminal proteins, and the alkali-soluble and -insoluble fractions were examined by Western blotting with anti-Est antibodies. As shown in Fig. 4, the original Sec protein was efficiently recovered in the alkali-soluble fraction (lane 2), whereas other fusion proteins were mainly recovered in the alkali-insoluble membrane fractions (Fig. 4, lanes 3, 5, and 7). These results indicated that the Sec/M1-H and Sec/M1-Q proteins were both stably anchored to the ER membrane, even though their C-terminal hydrophobic domains seemed to be too short for stable anchoring to the membrane.

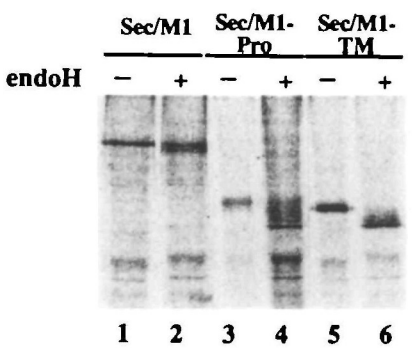

Fig. 3. **BFA treatment of Sec/M1, Sec/M1-Pro, and Sec/M1-TM products expressed in Brefeldin A (BFA)-treated cells.** After 40 h, the cells were labeled for 3 h with 200 μCi/ml of [^{35}S]methionine in the presence or absence of 10 μg/ml Brefeldin A. The immunoprecipitates with anti-Est antibodies from cell extracts were analyzed by SDS-PAGE and fluorography, either directly, or after endo H digestion.

"Double Lysine Motif"-Dependent Retention of a Carboxyesterase Sec-GT Fusion Protein—The above described data strongly suggested that the transmembrane segment of P450(M1) was sufficient to retain the reporter protein as well as P450(M1) itself in the ER. However, there remains the possibility that the carboxyesterase Sec portion of the fusion proteins, when expressed as the membrane bound form, somehow inhibits the exit of the proteins from the ER. To exclude this possibility, we constructed cDNAs in which the P450-derived transmembrane domain of the fusion protein, Sec/M1-TM, was replaced either with the transmembrane domain and the cytoplasmic tail of UDP-glucronosyltransferase (Sec/GT in Fig. 1) or with the same region from which the C-terminal "double lysine motif," the ER retention signal, had been deleted (Sec/GTd in Fig. 1). COS cells were transfected with these cDNAs, and then pulse and chase experiments were performed. As shown in Fig. 2G, the Sec/GT protein was detected exclusively in the cells (Fig. 2G, lanes 1, 4, 7, and 8) and the carbohydrate moiety of the pulse-labeled chimeric protein was sensitive to endo H digestion (Fig. 2G, lane 2), and the sensitivity was not altered even after the chase (Fig. 2G, lane 5), indicating that the double-lysine motif functioned to retain the Sec/GT protein in the ER. On the other hand, transfection with Sec/GTd cDNA gave heterogeneous products shortly after the pulse-labeling or after the chase (Fig. 2H, lanes 1 and 4), whose mobilities in the gel were obviously lower than that of the *in vitro* synthesized Sec/GTd protein (Fig. 2H, lanes 3 and 6). Of particular significance was the finding that the carbohydrate chains of the Sec/GTd protein gained partial resistance to endo H digestion (Fig. 2H, lanes 2 and 5), clearly indicating that the fusion protein was exported from the ER and its carbohydrate moiety underwent the complex type sugar modifications during the

TABLE I. **Carboxyesterase activity of carboxyesterase Sec fusion proteins.**

Transfected plasmid	Specific activity per membrane protein (mU/mg)	Specific activity[a] per Sec protein
Sec	100	344
Sec/M1	72	275
Sec/M1-Pro	88	271
Sec/M1-TM	86	250
Sec/M1-H	39	0
Sec/M1-Q	40	0
Sec/GT	77	271
Sec/GTd	82	261
Mock	41	—

[a]Specific activity was normalized as to the expression level of carboxyesterase Sec fusion proteins.

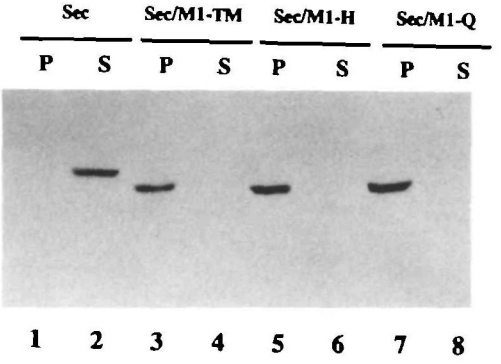

Fig. 4. **Alkali extraction of the membrane fractions containing expressed Sec, Sec/M1-TM, Sec/M1-H, and Sec/M1-Q products.** COS cells were transfected with Sec, Sec/M1-TM, Sec/M1-H, and Sec/M1-Q. After 40 h, the cells were harvested and crude membrane fractions were prepared from the cells as described under "MATERIALS AND METHODS." The membrane fractions were treated with a 0.1 M Na_2CO_3 solution, and then separated to the supernatant (S) and membrane precipitates (P). The samples were analyzed by SDS-PAGE, followed by Western blotting with anti-Est antibodies.

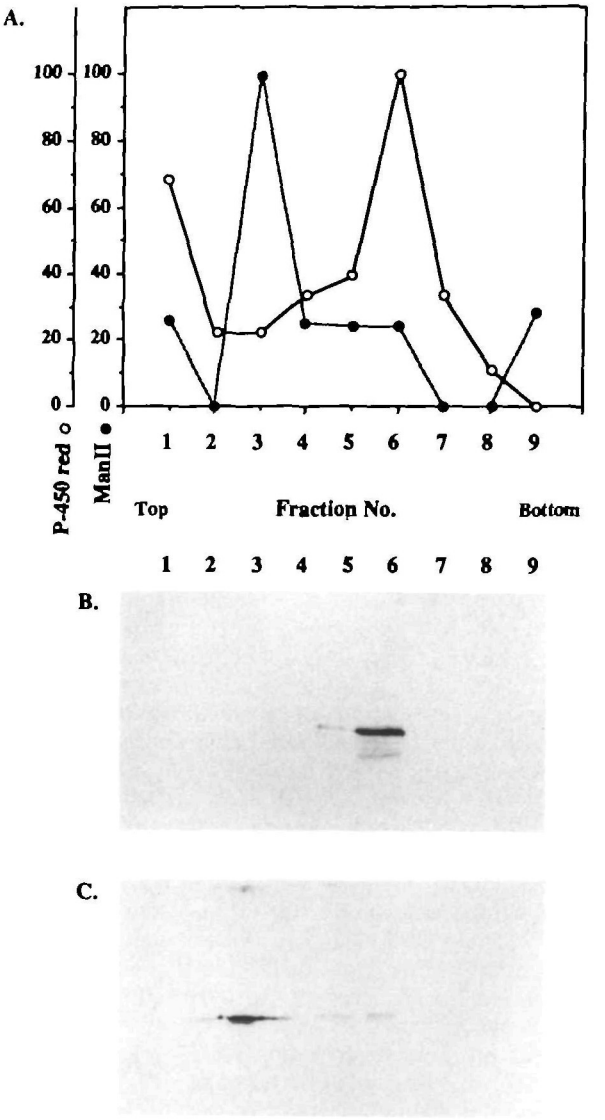

Fig. 5. **Subcellular distribution of Sec/GT and Sec/GTd.** COS cells were transfected with Sec/GT (B) and Sec/GTd (C). After incubation for 40 h, the cells were harvested, homogenized and then subfractionated as described under "MATERIALS AND METHODS." Aliquots of the fractions were collected from the top of the tubes, and then analyzed by SDS-PAGE. These samples were examined by Western blotting with anti-Est antibodies. The activities of NADPH-cytochrome P450 reductase (open circles) and α-mannosidase II (closed circles) were also measured as markers of the ER and Golgi apparatus, respectively (A), using a mixture of equivalent amounts of Sec/GT-transfected and Sec/GTd-transfected cells. The enzyme activities are shown as a percentage of the maximum activity observed.

transit through the Golgi apparatus. These results indicate that the Sec portion of the fusion proteins did not prevent the proteins from being transported out of the ER.

To determine the subcellular localization of the Sec/GT and Sec/GTd proteins in the transfected cells, we fractionated the cell lysate by sucrose density gradient centrifugation, followed by immunochemical detection of the fusion proteins by Western blotting (Fig. 5, B and C). Each of the fractions was also assayed for NADPH-cytochrome *c* reductase and α-mannosidase II activities to locate the ER and Golgi membranes, respectively (Fig. 5A). The Sec/GT protein was detected in fractions 5 and 6, which was coincident with NADPH-cytochrome *c* reductase activity, but not with that of α-mannosidase II (Fig. 5B). In marked contrast, Sec/GTd exhibited a diffuse distribution, but was mainly detected in fractions 2, 3, and 4, where the Golgi marker enzyme, α-mannosidase II, was present, although a small amount was present in the ER fractions (Fig. 5C). These results confirmed that the Sec portion of the fusion proteins did not prevent the export of the proteins from the ER, providing further support for the conclusion that the transmembrane segment of P450(M1) was sufficient for the retention in the ER.

Intracellular Localization of Carboxyesterase Sec Fusion Proteins Detected by Immunofluorescence Microscopy—In order to confirm the above biochemical observations morphologically, we next compared the intracellular localization of P450(M1) and various carboxyesterase Sec fusion proteins by immunofluorescence microscopy. P450(M1) expressed in COS cells showed a tubular network extending throughout the cytoplasm, the typical ER pattern (Fig. 6A). When Sec cDNA was expressed in COS cells, the intracellular Sec protein also exhibited an ER distribution (Fig. 6B), although it is a secretory protein. The exit of the Sec protein from the ER was probably rate-limiting due to the high expression of the protein (*25*).

Similar fluorescence patterns were observed with the cells expressing Sec/M1, Sec/M1-Pro, Sec/M1-TM, Sec/TM-H, Sec/TM-Q, and Sec/M1-GT (Fig. 6, C-H). In contrast, the cells expressing Sec/GTd showed intensely stained Golgi-like juxtanuclear structures (Fig. 6I), supporting the above observations that the sugar chains of the Sec/GTd protein were resistant to endo H digestion and the expressed Sec/GTd protein cofractionated with a Golgi marker enzyme on sucrose density gradient centrifugation of the cell lysates.

Expression and Localization of Cathepsin D Fusion Proteins—To determine whether or not P450(M1) is recycled from the Golgi compartments, we used cathepsin D as the reporter protein. The carbohydrate side chains of

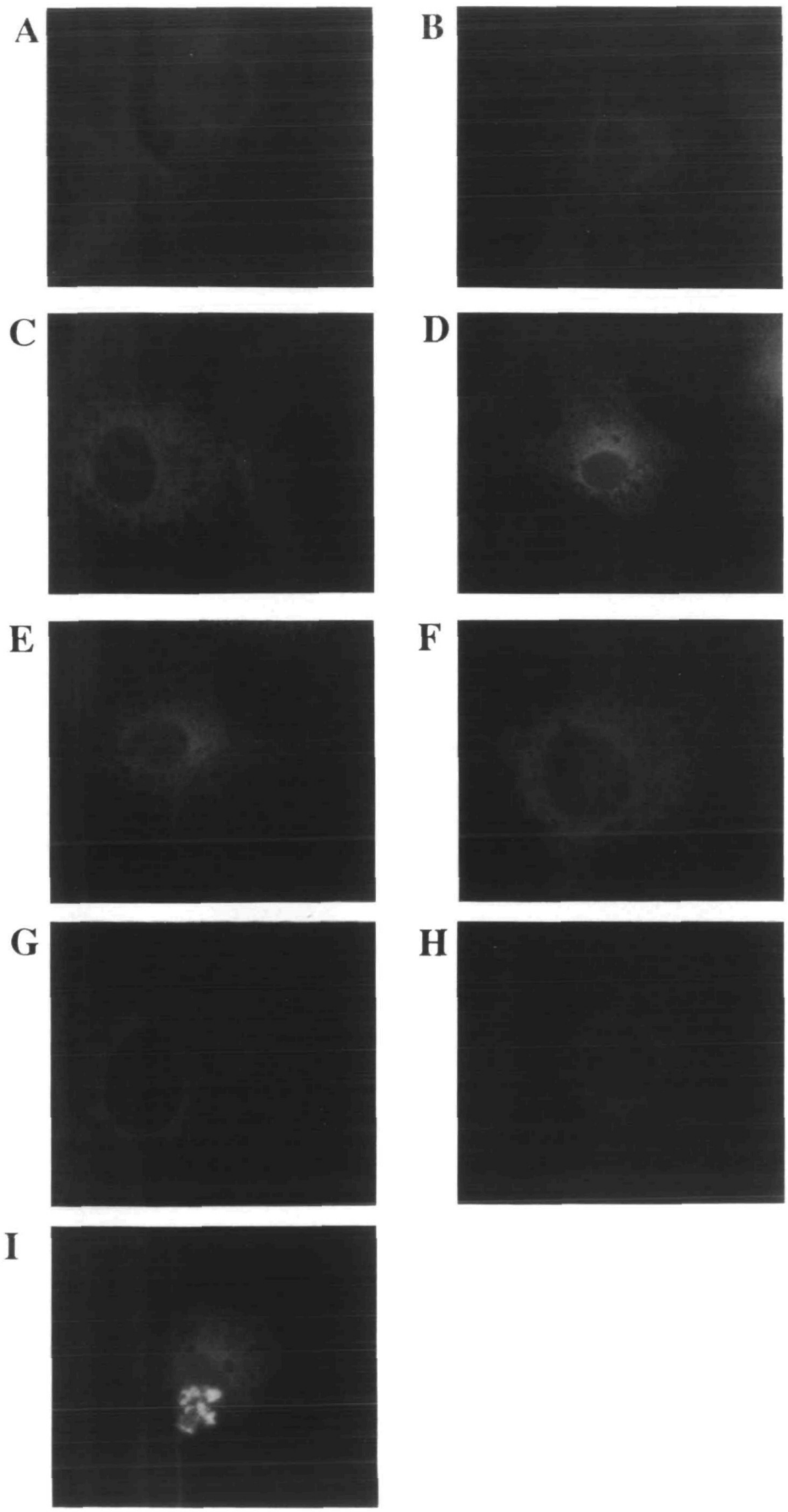

Fig. 6. **Immunofluorescence microscopy of COS cells expressing carboxyesterase Sec fusion proteins.** COS cells were transfected with M1 (A), Sec (B), Sec/M1 (C), Sec/M1-Pro (D), Sec/M1-TM (E), Sec/M1-H (F), Sec/M1-Q (G), Sec/GT (H), and Sec/GTd (I). After 40 h, the cells were examined by immunofluorescence microscopy with anti-Est antibodies and FITC conjugated 2nd antibodies, as described under "MATERIALS AND METHODS." Magnification, ×400.

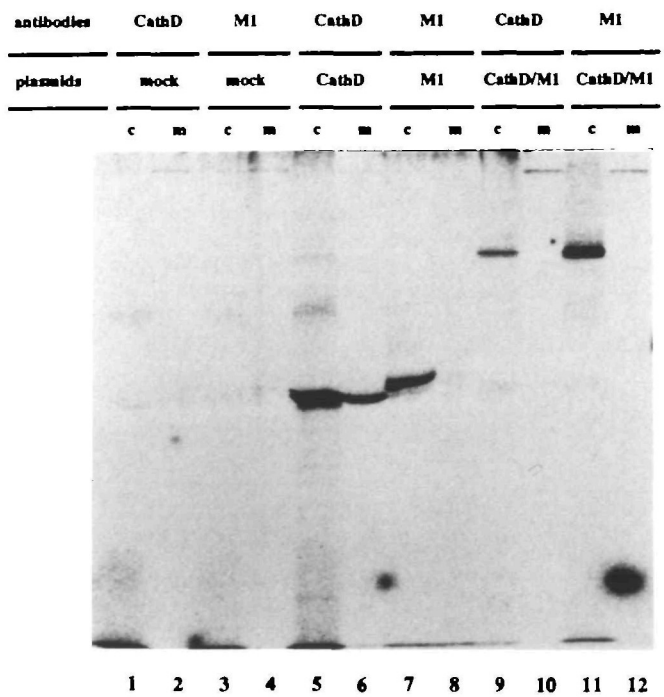

Fig. 7. **Expression of CathD, M1, and CathD/M1 and the pSG5 vector (mock).** COS cells were transfected with CathD, P450(M1), CathD/M1, and the pSG5 vector (mock). After 40 h, the cells were labeled with 200 μCi/ml of [^{35}S]methionine for 3 h. The cell extracts (c) and culture media (m) were immunoprecipitated with appropriate antibodies as shown in the figure. The immunoprecipitates were analyzed by SDS-PAGE and fluorography.

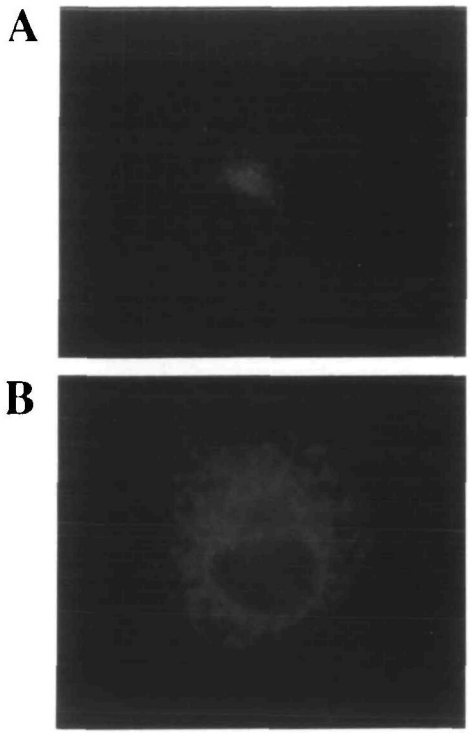

Fig. 8. **Immunofluorescence microscopy of COS cells expressing CathD and CathD/M1.** COS cells were transfected with CathD (A) and CathD/M1 (B). After 40 h, the cells were examined as described in the legend to Fig. 6 using anti-CathD antibodies instead of anti-Est antibodies. Magnification, ×400.

cathepsin D are subject to phosphorylation of the mannose residues at the 6th position, this modification targeting lysosomal enzymes to their destination (*3, 4*). The mannose-6-phosphate of the sugar chains is formed through two steps, which occur in separate subcellular compartments. As the first step, GlcNAc-P is linked to mannose to give a phosphodiester. The GlcNAc residue is subsequently removed by the second enzyme to form a mannose-6-phosphate residue, whose phosphomonoester bond is sensitive to alkaline phosphatase. The first enzyme is located in the *cis*-Golgi compartment, whereas the second step occurs in the medial Golgi compartment (*26-29*). Investigations on the formation of the mannose-6-phosphate residue of the sugar chains of the fusion protein, P450(M1)-cathepsin D, will show us whether the fusion protein goes to a Golgi compartment or not.

The fusion protein (CathD/M1 in Fig. 1) was expressed in COS cells. To confirm the expression of cathepsin D, P450(M1) and the CathD/M1 protein, the cells were labeled with [^{35}S]methionine for 3 h, and then the labeled proteins in the cells and the medium were analyzed by immunoprecipitation with appropriate antibodies and SDS-PAGE of the immunoprecipitates (Fig. 7). Since no detectable band of cathepsin D or P450(M1) was observed with the mock-transfected cells, the antibodies against rat cathepsin D and P450(M1) used in this experiment did not cross-react with the endogenous cathepsin or P450 of the COS cells (Fig. 7, lanes 1-4). Two bands with close mobilities were observed with the intracellular products of the transfected CathD cDNA (Fig. 7, lane 5). A band with the same mobility as the lower band was also detected for the medium (Fig. 7, lane 6). The lower band was probably the pro-form of cathepsin D, but the nature of the upper band was not clear. The secretion of the pro form of cathepsin D was possibly caused by the overexpression of the enzyme in the COS cells. The M1 (Fig. 7, lanes 7 and 8) and CathD/M1 (Fig. 7, lanes 9-12) products accumulated in the cells, but were not detected in the medium.

When examined morphologically by means of immunofluorescence microscopy with the antibodies to cathepsin D and p450(M1), the cells expressing the original cathepsin D showed intensely stained lysosomes. The Golgi apparatus near the nucleus was also stained (Fig. 8A). On the other hand, the cells expressing cathD/M1 showed a typical ER pattern of staining with no detectable fluorescence in the Golgi region (Fig. 8B). This confirms the ER localization of the fusion protein.

To determine whether or not the CathD/M1 fusion protein was recycled from a post-ER compartment, cells transfected with CathD, M1 or CathD/M1 were labeled with $^{32}PO_4$ for 2 h. The expressed proteins were immunoprecipitated with anti-M1 for M1, and with anti-CathD for the CathD and CathD/M1 products. The CathD, M1, and CathD/M1 products were all phosphorylated (Fig. 9, lanes 1, 3, and 5).

To define the structures of the phosphorylated products, they were treated with alkaline phosphatase. Mannose-6-P-GlcNAc was resistant to the alkaline phosphatase treatment whereas mannose-6-phosphate was sensitive (*3, 4*). The CathD product was partially resistant to the alkaline

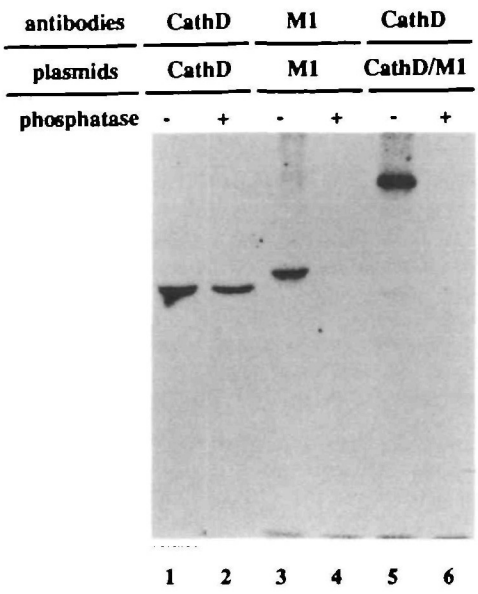

Fig. 9. **Alkaline phosphatase treatment of the CathD, M1, and CathD/M1 products.** The transfected COS cells were labeled with $^{32}PO_4$ for 2 h. The cell extracts were immunoprecipitated with appropriate antibodies. The immunoprecipitates were analyzed by SDS-PAGE and fluorography, either directly (lanes 1, 3, and 5) or after treatment with alkaline phosphatase (lanes 2, 4, and 6).

phosphatase treatment (Fig. 9, lane 2). In contrast, the radioactive phosphate incorporated into the CathD/M1 product was completely removed by the alkaline phosphatase treatment (Fig. 9, lane 6). This result apparently indicated that the fusion protein did not undergo the GlcNAc-P modification of the mannose in the sugar chain: the fusion protein did not reach the *cis*-Golgi compartment. However, there is a possibility that the conversion of the GlcNAc-P moiety of the fusion protein to M6P was very rapid in the transfected cells, resulting in the undetectable amount of the GlcNAc-P intermediate. However, such a possibility seems to be very low since the expressed cathepsin D carried a detectable amount of the GlcNAc-P intermediate. Incidentally, the radioactive phosphate incorporated into the M1 product was also removed by the alkaline phosphatase treatment. It is known that some P450s, including P450(M1), are phosphorylated by cAMP dependent protein kinase (*30*). Therefore, the phosphate incorporation into the CathD/M1 and M1 products was probably due to the phosphorylation of the protein moiety by the protein kinase.

DISCUSSION

The retention mechanism for the ER resident membrane proteins is insufficiently understood. As a step to elucidate the retention mechanism, we examined microsomal P450-(M1) to locate the region in the polypeptide chain responsible for its ER retention, and found that the 17 amino acid-long transmembrane signal-anchor region of the membrane-bound hemoprotein was sufficient for the retention. This conclusion was based on the expression of the fusion proteins consisting of carboxyesterase Sec, a secretory protein used as a reporter, and various portions of P450-(M1) in COS cells. The reporter portion of the expressed fusion proteins attained the correct conformation judging from the fact that the fusion proteins were enzymatically active. It was therefore concluded that the fusion proteins were retained in the ER by the retention signal in the fusion proteins and not through the action of the quality control system for unfolded proteins in the ER lumen. Several highly conserved structural motifs have been noted in microsomal P450s; the basic amino acid-rich region at the C-terminus of the signal-anchor sequence, the proline-rich region following the basic amino acid-rich region, the heme binding region containing a conserved cysteine residue, and the threonine-serine cluster in the middle portion of the molecule. However, these common structural motifs seem to have nothing to do with the ER retention of the P450s.

When carboxyesterase Sec was fused with the carboxy-terminal portion of microsomal UDP-glucuronosyltransferase (GT) consisting of a transmembrane segment and a short cytoplasmic tail, the fusion protein expressed in COS cells was retained in the ER, indicating that the double-lysine motif in the cytoplasmic tail functioned as the ER retention signal. In contrast, another fusion protein in which the double lysine motif was deleted, Sec/GTd, was transported from the ER and retained in the Golgi apparatus. It is known that some ER transmembrane proteins retained in the ER by the double lysine motif are transported to the plasma membrane on deletion of the retention motif (*7*). The reason why the Sec/GTd product was not transported to the plasma membrane is not clear. Anyhow, these results indicate that the carboxyesterase Sec portion of the fusion proteins did not prevent the proteins from being transported out of the ER.

It is well documented that the ER luminal proteins are retained in the ER through the "export and retrieval" mechanism (*31, 32*). A similar mechanism is probably operative in the ER retention of type I integral membrane proteins with the C-terminal double lysine motif, although the detailed mechanism remains obscure in the latter cases. We addressed this point for microsomal P450s using lysosomal cathepsin D as the reporter protein to monitor the ER to Golgi traffic in the cell. The carbohydrate moiety of cathepsin D underwent mannose-6-phosphate modification in the Golgi compartments, but the cathepsin D-P450(M1) fusion protein expressed in COS cells was retained in the ER and showed no Golgi-type modification of the sugar chains, providing evidence that the fusion protein, and probably P450s too, is not recycled through the Golgi compartments. These results seem to be consistent with the previous observation that P450(PB) was not detected in the Golgi membrane immunoelectronmicroscopically, even after maximal induction of the cytochrome by phenobarbital administration (*33*).

Recently, Szczesna-Skorupa and Kemper (*34*) reported that P450 2C2, which was localized in the ER when expressed in COS cells, did not show any shift in intracellular distribution when the cells were incubated at low temperatures, at which some membrane bound ER proteins have been shown to shift to the intermediate or *cis*-Golgi apparatus. They also showed that a modified P450 2C2 with an artificially added *N*-glycosylation site at its N-terminus received a sugar chain which was sensitive to treatment with endo H or endo D, which indicated that the sugar chain had not been processed by the Golgi-associated enzymes. Their observations are consistent with our conclusion that

microsomal P450s are not recycled between the ER and Golgi compartments.

Thus, the ER residency of P450s seems to be attained through a unique transmembrane segment-mediated mechanism, indicating the functioning of the transmembrane segment as the ER retention signal. There have been several reports that the retention of membrane proteins in the Golgi compartments is dependent on the transmembrane segments (*35-39*) and the charged residues flanking the segments (*40-42*). The signal-anchor region of P450-(M1) has no conserved sequence, as compared with the corresponding proteins of all known microsomal P450s. The N-terminal transmembrane region of microsomal P-450s and the transmembrane region of Golgi membrane proteins comprise similar hydrophobic stretches, but the former retains the proteins in the ER whereas the latter keeps the proteins in the Golgi membrane. These two types of transmembrane segments must contain unique information for the retention of membrane proteins in different intracellular membranes, and do not show any homology to each other. The transmembrane region of microsomal P450s may associate together in the membrane to form homo-oligomers or interact with other membrane proteins to assemble into a network of membrane proteins, which cannot enter the transport vesicles exiting from the ER. The function of the transmembrane region in the ER retention of P450s has to be clarified in the future.

After completion of this study, Ahn *et al.* (*43*) reported that the amino-terminal 29 amino acids of cytochrome P450 (2C1) are sufficient for its retention in the ER.

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