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Important Roles of the C-Terminal Portion of HPC-1/Syntaxin 1A in Membrane Anchoring and Intracellular Localization[1]

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HPC-1/syntaxin 1A (HPC-1), which plays an important role in vesicular transport to the plasma membrane, possesses a hydrophobic sequence at its C terminus. When expressed from cDNA in COS cells, wild-type HPC-1 was localized in the Golgi complex and the plasma membrane. Truncation of the hydrophobic domain resulted in the cytoplasmic localization of the mutant, thus indicating that the domain indeed functions as a membrane anchor. A fusion protein with the C-terminal glycosylation sites was glycosylated in transfected cells, providing evidence that HPC-1 has a transmembrane structure, and that the protein is first inserted into the endoplasmic reticulum and then transported to the plasma membrane. A chimeric protein consisting of *Escherichia coli* maltose-binding protein with the last 24 amino acids of HPC-1 was inserted into the endoplasmic reticulum in a transmembrane topology and localized along the exocytic pathway of transfected cells similar to HPC-1. These results indicate that the portion is important for intracellular localization of HPC-1.

Key words: ER-targeting sequence, HPC-1/syntaxin 1A, intracellular localization, tail-anchored protein, transmembrane topology.

In eukaryotic cells, proteins containing either a signal sequence or a signal/anchor sequence at their N termini are synthesized on the rough endoplasmic reticulum (ER) (*1-3*). After translocation across the ER membrane, secretory and plasma membrane proteins are transported through the Golgi complex to the cell surface. Resident proteins in the central vacuolar system are directed to and retained in their final destinations with the aid of either specific targeting (*4*) or retention signals (*5-9*).

On the other hand, proteins without a signal sequence are synthesized on free ribosomes. They remain in the cytosol, or are post-translationally localized in intracellular organelles such as mitochondria, the peroxisome, the nucleus, and the ER membrane. Therefore, the ER is a unique organelle into which membrane proteins are integrated co-translationally or post-translationally. ER-resident proteins in the latter case include microsomal aldehyde dehydrogenase (msALDH) (*10, 11*), microsomal cytochrome b_5 (*12, 13*), and heme oxygenase (*14*). All these proteins are integrated into the ER membrane through hydrophobic sequences at their C termini, resulting in the exposure of most molecular portions to the cytoplasm. Therefore, these protein are called tail-anchored proteins. In addition to these ER enzymes, tail-anchored proteins exist along the exocytic pathway (*15*). For example, HPC-1/syntaxin 1A (HPC-1), a 34-kDa protein with a hydrophobic sequence at its C terminus (*16, 17*), has been shown to be located in synaptic vesicles or chromaffin granules (*18*) in addition to the plasma membrane (*19*). This protein has been shown to play an important role in the docking or fusion of synaptic vesicles with the presynaptic active zone (*20, 21*) and of secretory vesicles with the plasma membrane (*22-24*). Recent studies have provided insight into the mechanisms underlying the formation, regulation, and function of the membrane protein complex composed of two tail-anchored proteins, this protein and synaptobrevin, and this protein and a lipid-anchored protein, synaptosome-associated protein of 25 kDa (SNAP-25), in transport vesicle targeting (*20, 25, 26*). However, little is known about the membrane topology of the C terminus or the targeting sequence of HPC-1 to the plasma membrane. Here, we show that the hydrophobic domain of HPC-1 spans the phospholipid bilayer and functions as a membrane anchor. In addition, we show that the C-terminal portion of HPC-1 is important not only for insertion into the ER membrane, but also for localization to the plasma membrane.

MATERIALS AND METHODS

Materials—Fetal bovine serum (FBS) and Dulbecco's minimal essential medium were purchased from Filtron

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Abbreviations: BFA, brefeldin A; ER, endoplasmic reticulum; endo H, endoglycosidase H; FBS, fetal bovine serum; PDI, protein disulfide isomerase; MBP, maltose-binding protein; msALDH, microsomal aldehyde dehydrogenase; WGA, wheat germ agglutinin.

(Brooklyn, Australia) and Nissui Pharmaceutical (Tokyo), respectively. Brefeldin A (BFA) and cycloheximide were obtained from Epicenter Technologies (Madison, WI, USA) and Sigma-Aldorich Chimie GmbH (Diesenhofen, Germany), respectively. [^{35}S]Methionine-cysteine was from DuPont NEN (Wilmington, DE, USA). Peroxidase-conjugated goat anti-rabbit IgG was purchased from Tago (Burlingame, CA, USA). Rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated goat anti-mouse IgG were obtained from Protos Immunoresearch (San Francisco, CA, USA) and American Qualex Antibodies & Immunochemicals (La Mirada, CA, USA), respectively. Fluorescein-conjugated wheat germ agglutinin (WGA) was purchased from E-Y Laboratories (San Mateo, CA, USA). Mouse monoclonal antibodies against human protein disulfide isomerase (PDI) and human mitochondrial 65-kDa protein were from Fuji Yakuhin Kogyo (Toyama) and Chemicon International (Temecula, CA, USA), respectively. Rabbit anti-maltose-binding protein (MBP) antiserum and pMAL-cRI were purchased from New England BioLabs (Beverly, MA, USA). Rabbit antibodies and mouse monoclonal antibodies against rat HPC-1 were obtained and characterized as described (*16*). Rabbit antibodies against rat PDI were obtained and characterized as described (*27*). Endoglycosidase H (endo H) and Protein A-Sepharose 4B were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA) and Pharmacia LKB Biotechnology (Uppsala, Sweden), respectively. Restriction enzymes and DNA-modifying enzymes were obtained from Nippon Gene (Toyama) and Takara (Kyoto). DNA sequencing kits were obtained from United States Biochemicals (Cleveland, OH, USA). Oligonucleotide primers were synthesized with an Applied Biosystem Model 381A DNA synthesizer. All other chemicals were of the highest purity commercially available. The cDNA encoding bovine opsin in the pSP vector and a 50% suspension of *Staphylococcus aureus* were kindly provided by Dr. Takashi Morimoto (New York University, NY, USA) and Dr. Shigeru Taketani (Kansai Medical University, Osaka), respectively.

Plasmid Constructions—All constructions were verified by the dideoxy chain termination method (*28*) and restriction enzyme digestion. The full-length cDNA for rat HPC-1 (*16*) was inserted into the *Eco*RV-*Pst*I sites of the mammalian expression vector, pMIW (*29*), to construct pMIW-HPC-1. The gapped duplex method of oligonucleotide-directed mutagenesis (*30*) was used for truncation or insertion of HPC-1 cDNA. Oligonucleotide nos. 1 (5′ CGCAGGAAGTAGATCATGAT 3′) and 2 (5′ GGCATCTTTGGATCTAGAGTCGACTGACCACGGCTCCATTC 3′) were used to generate an artificial stop codon after amino acid residue 265 of HPC-1 and to introduce *Xba*I and *Acc*I sites at its C terminus, respectively. The introduction of two restriction sites resulted in the addition of a Ser-Arg-Val-Asp (SRVD) sequence at the C terminus. The mutated cDNAs were inserted into the pMIW vector digested with *Eco*RV-*Pst*I to construct pMIWHPC-1⊿266-288 and pMIWHPC-1XA, respectively.

A chimeric cDNA containing the *N*-glycosylation sites of bovine opsin (*31*) was created as follows. First, a DNA fragment encoding the N-terminal region of bovine opsin was amplified by PCR using oligonucleotide nos. 3 (5′ CTGTCTAGAATGAACGGGACCGAGGG 3′) and 4 (5′ TTCACTAGTCGACCGTCTTGTTGGAGAAAG 3′), and pSPOpsin as a template. The resultant PCR fragment was then digested with *Xba*I and *Acc*I, and ligated into the *Xba*I-*Acc*I sites of pMIWHPC-1XA to construct pMIW-HPC-1/OP3.

The full-length MBP was synthesized by PCR using oligonucleotides nos. 5 (5′ GGACGATATCTTATGAAAATCGAAGAAGGTAA 3′) and 6 (5′ AATCGTTAACAGGCTGAAAATCTTCTCT 3′), and pMAL-cRI as a template. The PCR fragment was digested with *Eco*RV and *Hpa*I, and ligated into the *Eco*RV site of pMIW vector to construct pMIWMBP. Chimeric cDNAs for MBP fusion proteins were constructed by PCR. The following pairs of oligonucleotides were used: oligonucleotide nos. 7 (5′ CACCAAGAAGGAATTCAAGTACCAGA 3′) and 8 (5′ AGGGAGACCCCTCGAGAATGGAGC 3′) to amplify HPC-1(33) and HPC-1(33)OP3; oligonucleotides nos. 9 (5′ CAAGGCACGCGAATTCAAGATCATGA 3′) and 8 to amplify DNA fragments designated as HPC-1(24) and HPC-1(24)OP3; oligonucleotide nos. 10 (5′ CAAGGAATTCTGGTCGAAATTCTTCCTGC 3′) and 11 (5′ AATTCCTCGAGAAGCTTGGTGTGTTAACCATTTGG 3′) to amplify ALDH(35); and oligonucleotide nos.12 (5′ CAACGAATTCAGGCTGCAGCTGCTGCTTC 3′) and 11 to amplify ALDH(18). The PCR fragments were digested with *Eco*RI and *Xho*I, then ligated into the *Eco*RI-*Xho*I sites of pMIWMBP. The resultant plasmids were designated as pMIWMBPHPC-1(33), pMIWMBPHPC-1(33)OP3, pMIWMBPHPC-1(24), pMIWMBPHPC-1(24)OP3, pMIWMBPALDH(35), and pMIWMBPALDH(18), respectively.

Expression and Localization of HPC-1 in COS Cells—The transfection of expression plasmids, subcellular fractionation, membrane extraction, and indirect immunofluorescence microscopy were performed as described previously (*10*). Samples were analyzed by SDS-PAGE (*32*), followed by immunoblotting as described (*10*).

Biosynthetic Labeling of COS Cells and Immunoprecipitation—Forty-four hours after transfection, the cells were preincubated at 37°C with or without BFA (10 μg/ml) in Dulbecco's minimal essential medium devoid of methionine and FBS, and then pulse-labeled for 30 min in the same medium containing 200 μCi/ml of [^{35}S]methionine-cysteine. After labeling, the cells were chased with or without BFA in the complete medium containing 10% FBS for 3 h, washed three times with cold phosphate-buffered saline, then lysed in 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Nonidet P-40, 5 mM EDTA, 1% Trasyol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT for 15 min on ice. The lysates were centrifuged for 30 min in a microfuge, and the resulting supernatants were preincubated with a *S. aureus* suspension for 30 min on a rotating device at 4°C. Then HPC-1/OP3 was immunoprecipitated from the precleared medium by the addition of rabbit anti-HPC-1 antibodies, followed by incubation with Protein A-Sepharose 4B for 2 h. The immunoprecipitates were washed four times with 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 2 mM EDTA. The proteins were eluted by heating at 100°C for 2 min in 1% SDS and 1% β-mercaptoethanol. For endo H treatment, the eluate was adjusted to 50 mM sodium citrate, pH 5.5, 1% Nonidet P-40, and 1% β-mercaptoethanol, then incubated with or without 1 mU endo H overnight at 37°C. The samples were separated by SDS-PAGE, followed by fluorography. Similar procedures were

used for the immunoprecipitation and endo H treatment of MBP fusion proteins.

RESULTS

Intracellular Localization of Wild-Type and Truncated Forms of HPC-1 in COS Cells—HPC-1 has no signal sequence at the N terminus, but instead it possesses a characteristic hydrophobic domain (amino acids 266-288) at its C terminus (*16, 17*) (Fig. 1A). To determine the role of the hydrophobic domain in membrane anchoring, wild-type and truncated forms of HPC-1 (Fig. 1A) were expressed transiently in COS cells under the control of the β-actin promotor and Rous sarcoma enhancer in the pMIW expression vector (*29*). We first confirmed that wild-type HPC-1 was expressed in the total homogenate of the transfected cells with an apparent molecular mass of 34 kDa by immunoblotting using anti-HPC-1 antibodies. On the other hand, no cross-reactive protein with the apparent size of 34 kDa was detected in untransfected COS cells (data not shown), indicating the absence of endogenous HPC-1. We then analyzed subcellular localization of the expressed proteins. In this case, the postnuclear supernatant was centrifuged at 88,000 × *g* for 80 min to separate

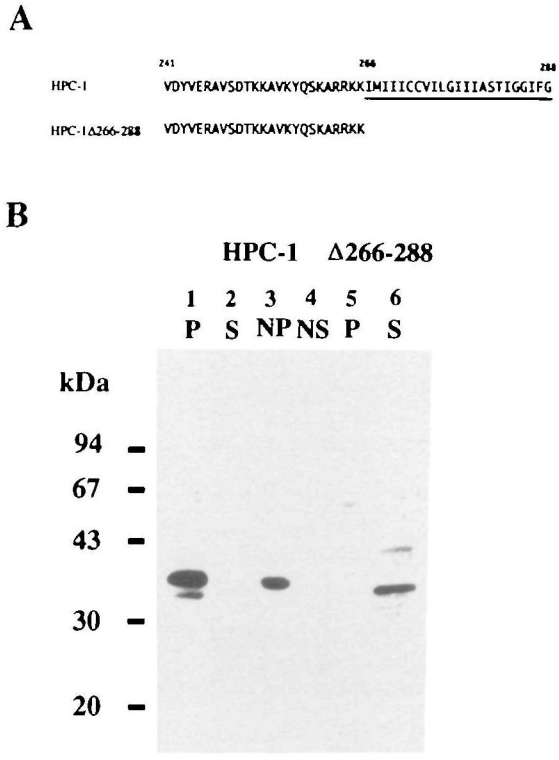

Fig. 1. **Immunoblot analysis of wild-type and truncated forms of HPC-1.** (A) The single amino acid code is used to represent the C-terminal sequences of HPC-1 and HPC-1Δ266-288, and the amino acid numbers are shown on top of HPC-1 sequence. The hydrophobic domain is underlined. (B) COS cells were transfected with cDNAs encoding wild-type or truncated forms of HPC-1 in the pMIW expression vector, and then harvested 44 h after transfection. Membrane (P) and soluble (S) fractions were prepared by centrifugation of the postnuclear fraction at 88,000 × *g* for 80 min. The membrane fraction of HPC-1 was treated with 100 mM Na_2CO_3 at 0°C for 30 min, then centrifuged at 88,000 × *g* for 80 min to separate the pellet (NP) from the supernatant (NS). Each fraction was assayed by immunoblotting using anti-HPC-1 antibodies. Lane 1, HPC-1 (P); lane 2, HPC-1 (S); lane 3, HPC-1 (NP); lane 4, HPC-1 (NS); lane 5, HPC-1Δ266-288 (P); lane 6, HPC-1Δ266-288 (S).

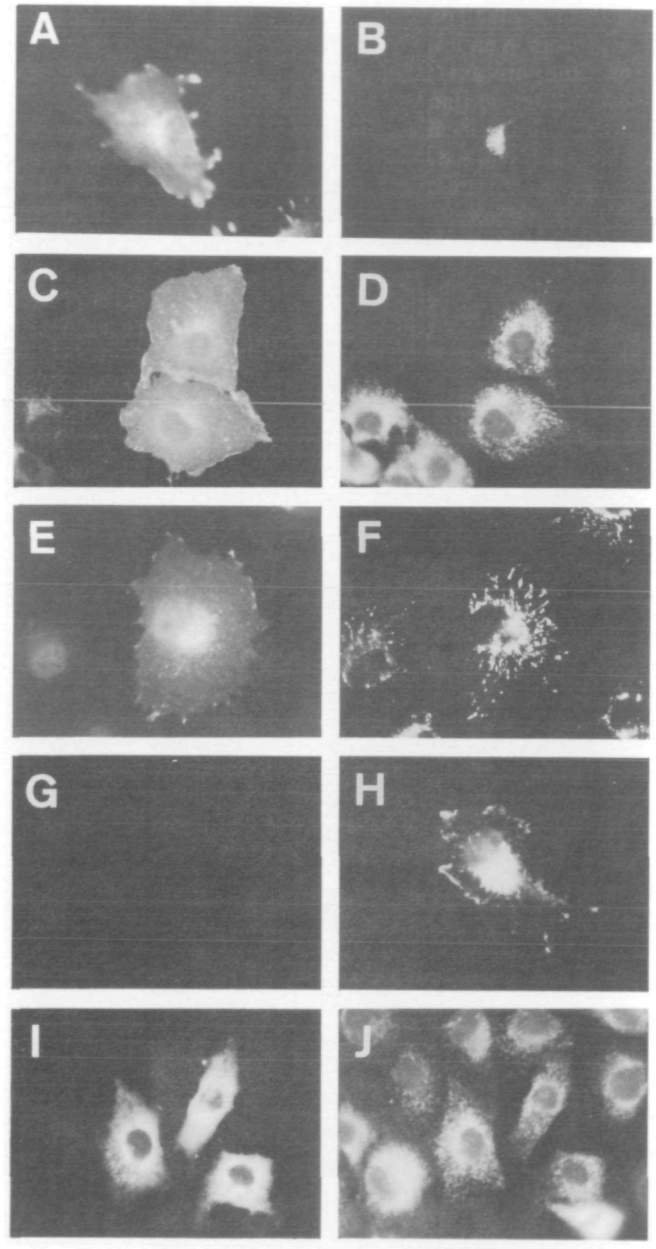

Fig. 2. **Localization of wild-type and truncated forms of HPC-1 by double indirect immunofluorescence microscopy.** COS cells were transfected with pMIWHPC-1 (A-F) or pMIWHPC-1Δ266-288 (I and J), fixed 44 h after transfection, then permeabilized. Wild-type HPC-1 (A, C, and E) and HPC-1Δ266-288 (I) were detected by incubation with rabbit anti-HPC-1 antibodies and rhodamine-conjugated anti-rabbit IgG. The Golgi complex was localized by staining with fluorescein-conjugated WGA (B). Endogenous PDI (D and J) and a mitochondrial 65-kDa protein (F) were labeled with the corresponding mouse monoclonal antibodies and fluorescein-conjugated anti-mouse IgG. For detection of cell-surface and internal HPC-1 antigens, COS cells transfected with pMIWHPC-1 (G and H) were fixed 44 h after transfection. To detect cell-surface HPC-1 antigens (G), the cells were labeled with mouse monoclonal antibodies to HPC-1 and fluorescein-conjugated anti-mouse IgG. After permeabilization with a detergent, the internal HPC-1 (H) was stained with rabbit anti-HPC-1 antibodies and rhodamine-conjugated anti-rabbit IgG.

the membrane fraction from the cytosol fraction. Immunoblotting revealed that wild-type HPC-1 was recovered exclusively in the membrane fraction (Fig. 1B, lanes 1 and 2). Additionally, HPC-1 in the membrane fraction was resistant to alkali extraction (lanes 3 and 4), indicating that the protein is an integral membrane protein. In contrast, a truncated mutant, HPC-1⊿266-288, was found in the soluble fraction (lanes 5 and 6).

Intracellular localization of the expressed proteins was further determined by double indirect immunofluorescence microscopy. In the transfected cells, HPC-1 was detected in the Golgi complex in addition to the plasma membrane (Fig. 2, A and B). The distribution of HPC-1 was different from that of endogenous PDI (Fig. 2, C and D), an ER marker protein, or that of a mitochondrial 65-kDa protein (Fig. 2, E and F). We also found that HPC-1 reacted with the corresponding antibodies not before but after permeabilization, indicating the intracellular localization of HPC-1 epitopes (Fig. 2, G and H). As expected, HPC-1⊿266-288 was distributed diffusely throughout the cytoplasm (Fig. 2, I and J). These biochemical and immunolocalization data indicate that the hydrophobic domain of HPC-1 is necessary for insertion into intracellular membranes and that HPC-1 is a tail-anchored protein distributed along the exocytic pathway.

Membrane Topology and Intracellular Transport Route of HPC-1—To demonstrate that HPC-1 localized in the Golgi complex is in transit to the plasma membrane, COS cells transfected with HPC-1 were treated with cycloheximide. After a 5-h chase in the presence of cycloheximide, the Golgi staining of HPC-1 (Fig. 3B) became extremely weak as compared with that before the chase (Fig. 3A). After a 10-h chase, the Golgi labeling disappeared and HPC-1 was concentrated at the plasma membrane (Fig. 3C). These results support the above idea that HPC-1 is transported to its final destination *via* the Golgi complex. Next, we attempted to define the membrane topology and the membrane integration site of HPC-1. For these purposes, we fused the N-terminal region of bovine opsin (the OP3 extension), which contains two *N*-glycosylation sites,

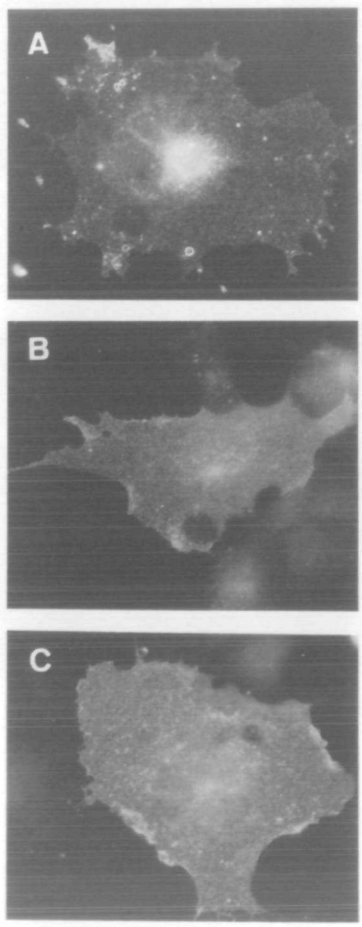

Fig. 3. **Immunofluorescence localization of HPC-1 in the presence of cycloheximide.** COS cells were transfected with pMIWHPC-1. After 44 h of transfection, the cells were fixed (A) or treated with cycloheximide (150 μg/ml). After 5 h (B) or 10 h (C) of incubation with cycloheximide, the cells were fixed and then permeabilized. HPC-1 was detected by incubation with rabbit anti-HPC-1 antibodies and rhodamine-conjugated anti-rabbit IgG.

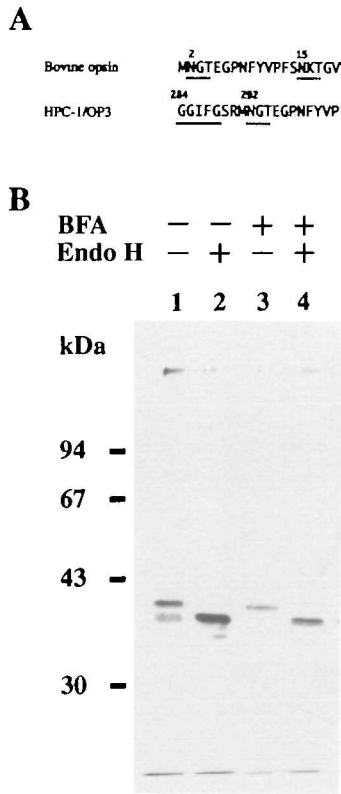

Fig. 4. **Processing of newly synthesized HPC-1/OP3 in control and BFA-treated COS cells.** (A) The single amino acid code is used to represent the N-terminal sequence of bovine opsin and the C-terminal sequence of HPC-1/OP3, and the amino acid numbers are shown on top of each sequence. The *N*-glycosylation sites of bovine opsin and HPC-1/OP3 are shown by dotted underlines, and the hydrophobic sequence of HPC-1/OP3 is underlined. (B) COS cells were transfected with HPC-1/OP3 in the pMIW expression vector. Forty-four hours later, the cells were pulse-labeled for 30 min and subsequently chased for 3 h in the complete medium. After immunoprecipitation, the protein was analyzed directly by SDS-PAGE (lane 1) or after incubation overnight with endo H (lane 2). When the effect of BFA was investigated, cells treated with BFA (10 μg/ml) were pulse-labeled for 30 min and subsequently chased for 3 h in the complete medium containing BFA. After immunoprecipitation, the protein was analyzed directly by SDS-PAGE (lane 3) or after incubation overnight with endo H (lane 4).

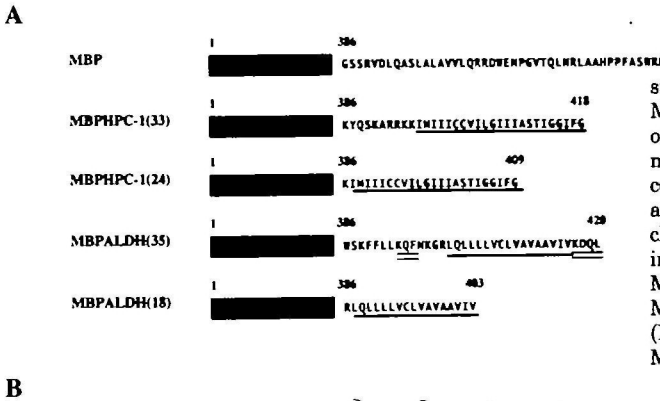

Fig. 5. **Immunoblot analysis of MBP and various MBP chimeras.** (A) The solid bar shows amino acids 1-385 of MBP. The single amino acid code is used to represent the C-terminal sequences of MBP and various MBP chimeras, and the amino acid numbers are shown on top. Each hydrophobic domain and the ER-targeting sequences of msALDH are underlined and double underlined, respectively. (B) COS cells were harvested 44 h after transfection with plasmids. Membrane (P) and soluble (S) fractions were prepared by centrifugation of the postnuclear fraction at 88,000 × *g* for 80 min. Each fraction was assayed by immunoblotting using anti-MBP antibodies. Lane 1, MBP (P); lane 2, MBP (S); lane 3, MBPHPC-1(33) (P); lane 4, MBPHPC-1(33) (S); lane 5, MBPHPC-1(24) (P); lane 6, MBPHPC-1(24) (S); lane 7, MBPALDH(35) (P); lane 8, MBPALDH(35) (S); lane 9, MBPALDH(18) (P); lane 10, MBPALDH(18) (S).

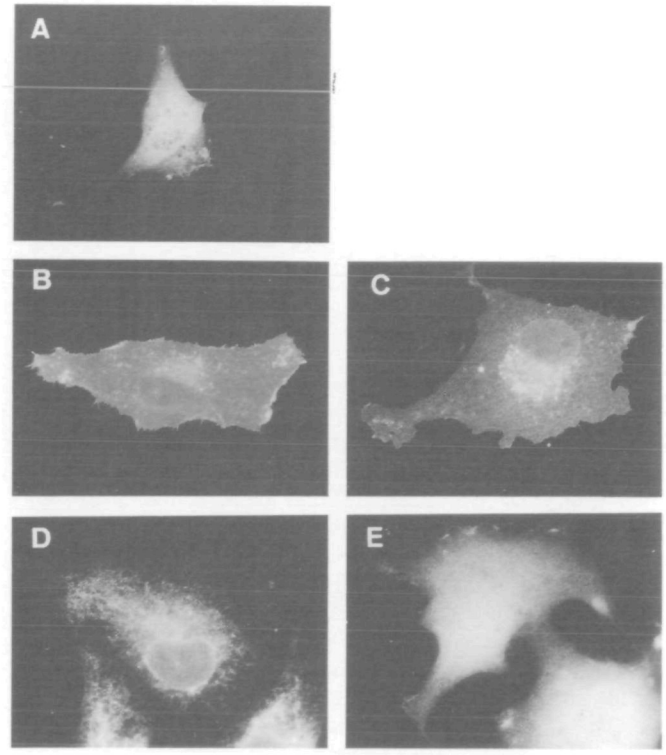

Fig. 6. **Immunofluorescence localization of MBP and various MBP chimeras.** COS cells were transfected with cDNA encoding MBP (A), MBPHPC-1(33) (B), MBPHPC-1(24) (C), MBPALDH(35) (D), or MBPALDH(18) (E) in the pMIW expression vector, fixed 44 h after transfection, permeabilized, and stained with anti-MBP antibodies, then with rhodamine-conjugated anti-rabbit IgG.

to the C terminus of HPC-1. Asn292 and Asn305 of the chimera (HPC-1/OP3) correspond to Asn2 and Asn15 of bovine opsin (*31*), respectively (Fig. 4A). First, we confirmed that the chimera was recovered in the membrane fraction upon subcellular fractionation and exhibited a similar staining pattern to that of wild-type HPC-1 on indirect immunofluorescence microscopy (data not shown). These results indicate that the short OP3 extension did not influence the intracellular localization of the chimera. We then analyzed whether or not the chimera is glycosylated in transfected COS cells. The transfected cells were pulse-labeled for 30 min with [^{35}S]methionine-cysteine, chased for 3 h, then the chimera was immunoprecipitated using antibodies against HPC-1. As shown in Fig. 4B (lane 1), two products were immunoprecipitated. Upon endo H treatment, the upper band disappeared (Fig. 4B, lane 2), demonstrating that the upper product is glycosylated. Together with cycloheximide block experiments, these suggest that HPC-1/OP3 is first inserted into the ER and transported to the plasma membrane *via* the exocytic pathway. The endo H sensitivity of glycosylated HPC-1/OP3, however, was not consistent with the plasma membrane localization observed on immunofluorescence microscopy. One possible interpretation is that newly synthesized HPC-1/OP3 is not processed to an endo H-resistant form with Golgi processing enzymes. An alternative possibility is that HPC-1/OP3 is retained in the ER even after the chase time because of extremely slow transport from the ER. To check these possibilities, metabolic labeling was performed in the presence of BFA, which effectively blocks membrane transport out of the ER (*33*) and also causes redistribution of Golgi enzymes into the ER (*34*). Upon treatment of the transfected cells with BFA, the chimera remained sensitive to endo H (Fig. 4B, lanes 3 and 4). Therefore, it is suggested that the carbohydrate structure of HPC-1/OP3 is not processed to the endo H-resistant form in spite of its transport from the ER to the plasma membrane.

Roles of the C-Terminal Portion of HPC-1 in Intracellular Localization—Since the ER-targeting sequences of msALDH, a tail-anchored ER protein, are located in the last 35 amino acids (*10*), we investigated the role of the

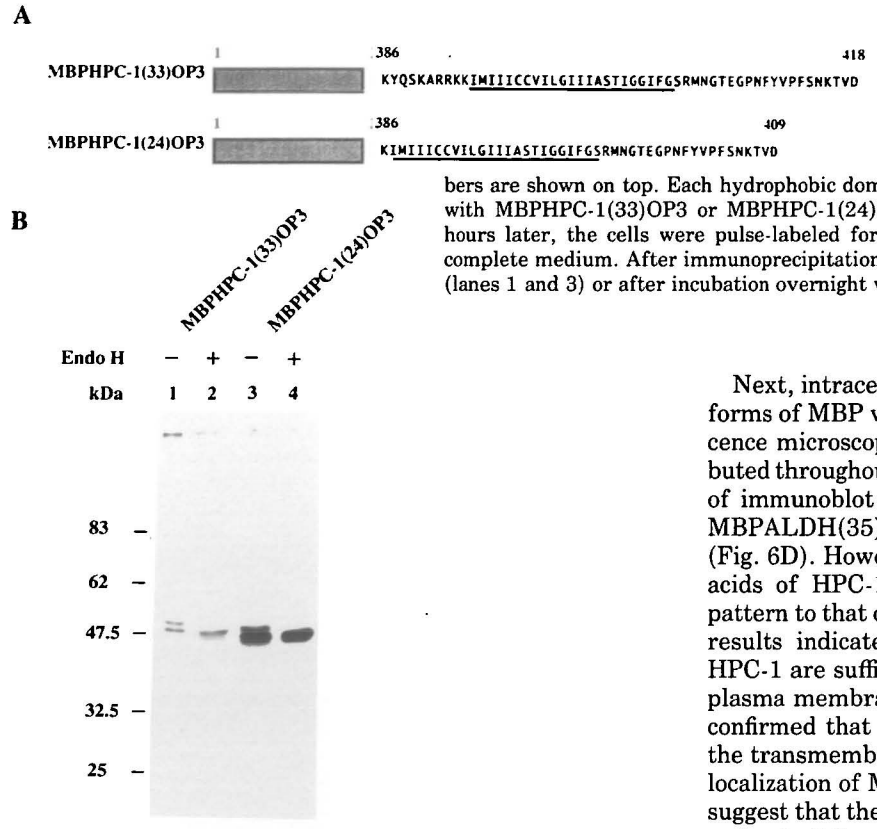

Fig. 7. **Processing of newly synthesized MBPHPC-1(33)OP3 and MBPHPC-1(24)-OP3 in COS cells.** (A) The solid bar shows amino acids 1-385 of MBP. The single amino acid code is used to represent the C-terminal sequences of MBPHPC-1(33)OP3 and MBPHPC-1(24)OP3, and the amino acid numbers are shown on top. Each hydrophobic domain is underlined. (B) COS cells were transfected with MBPHPC-1(33)OP3 or MBPHPC-1(24)OP3 in the pMIW expression vector. Forty-four hours later, the cells were pulse-labeled for 30 min and subsequently chased for 3 h in the complete medium. After immunoprecipitation, the protein was analyzed directly by SDS-PAGE (lanes 1 and 3) or after incubation overnight with endo H (lanes 2 and 4).

C-terminal portion of HPC-1 in its intracellular localization. For this purpose, we constructed various expression plasmids that encode *E. coli* MBP or MBP fusion proteins (Fig. 5A). A reporter protein, MBP, was supposed to remain in the cytoplasm when expressed in COS cells. In MBPHPC-1(33) and MBPHPC-1(24), the C-terminal sequence (amino acids 386-456) of MBP are replaced by the last 33 (amino acids 256-288) and 24 (amino acids 265-288) amino acids of HPC-1, respectively. Thus, MBPHPC-1(33) and MBPHPC-1(24) possess the positively charged region and one lysine residue in addition to the transmembrane domain of HPC-1, respectively. Furthermore, we constructed two MBPALDH chimeras for comparison. MBPALDH(35) and MBPALDH(18) contain the last 35 amino acids and amino acids 463-480 of msALDH, respectively. The former chimera possesses both the ER-targeting sequences and the membrane-spanning domain of msALDH (*10, 11*), while MBPALDH(18) lacks the ER-targeting sequences (Fig. 5A).

These chimeras as well as wild-type MBP were expressed in COS cells, and their subcellular localization was determined by immunoblotting using antibodies against MBP. As shown in Fig. 5B, wild-type MBP and MBPALDH(18) were detected in both membrane and soluble fractions. However, these proteins in the membrane fraction were recovered in the soluble fraction upon alkali extraction (data not shown). On the contrary, MBPHPC-1(33), MBPHPC-1(24) and MBPALDH(35) were detected exclusively in the membrane fraction (Fig. 5B) and were resistant to alkali extraction (data not shown). These results show that the three chimeras are integrated into intracellular membranes, in contrast to MBP and MBPALDH(18).

Next, intracellular localization of wild-type and chimeric forms of MBP was determined by indirect immunofluorescence microscopy. MBP and MBPALDH(18) were distributed throughout the cytoplasm, consistent with the results of immunoblot analysis (Fig. 6, A and E). As expected, MBPALDH(35) exhibited a typical ER-staining pattern (Fig. 6D). However, attachment of the last 33 or 24 amino acids of HPC-1 to MBP resulted in a similar staining pattern to that of wild-type HPC-1 (Fig. 6, B and C). These results indicate that the C-terminal 24 amino acids of HPC-1 are sufficient for localization of *E. coli* MBP to the plasma membrane. As for MBPALDH chimeras, we have confirmed that the ER-targeting sequences in addition to the transmembrane domain of msALDH are necessary for localization of MBP to the ER. Taken together, our results suggest that the sequence of HPC-1 for intracellular localization is different from that of msALDH.

Membrane Topology and Intracellular Transport Route of MBPHPC-1 Chimeras—To confirm the membrane topology and intracellular transport route of MBPHPC-1 chimeras, we constructed MBPHPC-1(33)OP3 and MBPHPC-1(24)OP3, which contain the OP3 extension at their C termini (Fig. 7A). These chimeras were recovered in the membrane fraction upon subcellular fractionation and detected in the Golgi complex and the plasma membrane of transfected COS cells on indirect immunofluorescence microscopy (data not shown). The transfected cells were pulse-labeled for 30 min and chased for 3 h, then two chimeras were immunoprecipitated. As shown in Fig. 7B, both MBPHPC-1(33)OP3 and MBPHPC-1(24)OP3 were glycosylated to an endo H-sensitive form similar to HPC-1/OP3. Taken together, these results show that the two chimeras have a transmembrane topology and suggest that they are transported to the plasma membrane from the ER.

DISCUSSION

Our immunostaining data demonstrate that HPC-1 is localized along the exocytic pathway when expressed from cDNA in COS cells, as is endogenous HPC-1 in rat cerebellum (*19*). Additionally, we have shown that HPC-1 is indeed anchored to intracellular membranes by the hydrophobic domain at its C terminus and that most of the molecular portion is exposed to the cytoplasm, as has been postulated (*19-21*). Thus, these results allow us to use this expression system to investigate the intracellular transport route and membrane topology of HPC-1.

To investigate these subjects, we used HPC-1/OP3 chimera, which contains the *N*-glycosylation sites of bovine opsin (the OP3 extension) at the C terminus. In the

previous study, we revealed the transmembrane topology of msALDH, a tail-anchored ER protein, using the same strategy, or the *N*-glycosylation of ALDH/OP3 containing the same OP3 extension at the C terminus of msALDH (*11*). Additionally, ALDH/OP3 was localized to the ER and formed the crystalloid ER in transfected COS cells similar to msALDH (*11*), indicating that the OP3 extension has no effect on intracellular localization of the chimera. By taking advantage of the *N*-glycosylation of HPC-1/OP3, we have shown that the hydrophobic domain of HPC-1 spans the ER membrane. This result is consistent with a three-dimensional model of HPC-1 recently obtained by Sato and Akagawa. They constructed the model based on the deduced amino acid and the known biochemical information by utilizing a special software (Discover, Homology, Molecular Simulation) and an INDIGO 2 work station. The calculating system has shown that the C terminus of HPC-1 could transverse the phospholipid bilayer (Sato, C. and Akagawa, K., manuscript in preparation). Thus, our result together with this model supports the transmembrane topology of HPC-1. These results as well as those reported for microsomal cytochrome b_5 (*13*) and synaptobrevin (*35*) suggest that the C termini of most tail-anchored proteins exhibit a lumenal orientation regardless of their intracellular localization.

The *N*-glycosylation of HPC-1/OP3 has additionally clarified that the protein is first inserted into the ER membrane. The experiments with cycloheximide demonstrated that HPC-1 localized in the Golgi complex can be chased away. In addition, we have recently shown using an *in vitro* system that HPC-1/OP3 is post-translationally integrated into rough microsomes from dog pancreas and glycosylated (Masaki, R. *et al.*, unpublished observation). Taken together, it is suggested that HPC-1 is post-translationally inserted into the ER and then transported to the plasma membrane *via* the exocytic pathway. However, the carbohydrate structure of HPC-1/OP3 remained endo H-sensitive, which is inconsistent with its intracellular localization. To find the cause of this discrepancy, we treated transfected COS cells with BFA, which effectively blocks membrane transport out of the ER (*33*) and also causes redistribution of Golgi enzymes into the ER (*34*). BFA did not change the endo H sensitivity of HPC-1/OP3. On the contrary, the carbohydrate chain of ALDH/OP3 was processed to an endo H-resistant form as a result of BFA treatment (*11*). This result indicates that the carbohydrate structure of the OP3 extension can be further processed by Golgi processing enzymes. Therefore, it seems that conformational changes in the glycosylation site of HPC-1/OP3 after *N*-glycosylation would result in inhibition of further processing with Golgi enzymes. There are several examples of glycoproteins which contain high mannose oligosaccharides in spite of passage through the Golgi complex. It has been shown that the cell surface transferrin receptor contains both complex and high mannose oligosaccharides (*36*). HSP47, a collagen-specific molecular chaperone, is retained in the ER *via* the C-terminal Arg-Asp-Glu-Leu (RDEL) sequence and has a high mannose oligosaccharide. Although mutated HSP47, which is devoid of the RDEL sequence, is secreted by transfected cells, the secreted mutant possesses an endo H-sensitive carbohydrate structure (*37*).

The intracellular transport route of HPC-1 is consistent with the current idea that tail-anchored proteins localized along the exocytic pathway are first inserted into the ER membrane in a post-translational manner and transported to their final destinations. For example, synaptobrevin in neuroendocrine cells is transported through the Golgi complex to synaptic vesicles after insertion into the ER membrane in a signal recognition particle- and Sec61p-independent fashion (*35*). Similarly, tail-anchored ER proteins such as msALDH (*10, 11*) and microsomal cytochrome b_5 (*12, 13*) are inserted into the ER post-translationally and retained in the ER. In the previous study, we showed that two hydrophilic sequences on both sides of the transmembrane domain of msALDH (see Fig. 5A) play an important role in ER targeting (*10*) and that the protein is retained in the ER by blockading exit from the ER (*11*). As for microsomal cytochrome b_5, the last 10 amino acids, which include the hydrophilic tail of this protein, are important for its targeting to the ER (*12*). In addition, the membrane-spanning domain of microsomal cytochrome b_5 functions as an ER-retention signal (*38*). Therefore, there must be targeting or retention sequences in tail-anchored proteins to ensure their correct intracellular localization.

In this study, we have demonstrated that the last 24 amino acids of HPC-1 are sufficient for localization of cytosolic MBP to the plasma membrane in addition to membrane anchoring. Since HPC-1 is first inserted into the ER membrane, this may indicate that the portion contains the ER-targeting sequence. As for MBPALDH chimeras, we have confirmed here that the ER-targeting sequences on both sides of the membrane-anchoring domain are necessary for targeting of MBP to the ER. Thus, it appears that HPC-1 belongs to a different class than msALDH with respect to the ER-targeting sequence. Two MBP chimeras behaved totally differently after integration into the ER. MBPALDH(35) was retained in the ER, while MBPHPC-1(24) exited from the ER to the plasma membrane. Recently, Pedrazzini *et al.* reported that the short transmembrane domain (17 amino acids) of microsomal cytochrome b_5 plays an important role in its ER retention by demonstrating the relocation of a mutant cytochrome b_5 with a lengthened membrane anchor (22 amino acids) to the plasma membrane (*38*). This would be the case for the two MBP chimeras, since the transmembrane domains of MBPALDH(35) and MBPHCP-1(24) are composed of 17 and 23 amino acids, respectively. The short transmembrane domain of MBPALDH(35) seems to be important for its ER residency, while the longer transmembrane domain of MBPHPC-1(24) would result in the escape of the chimera from the ER. These may indicate that the transmembrane domains of tail-anchored proteins possess targeting and/or retention sequences similar to membrane proteins synthesized in the signal recognition particle-dependent manner (*2, 7-9*).

In summary, HPC-1 is transported to the plasma membrane after insertion into the ER membrane in a transmembrane topology. Our results indicate the important role of the C-terminal portion of HPC-1 in intracellular localization in addition to membrane anchoring. Elucidation of the mechanisms of integration of HPC-1 into the ER should provide valuable clues for understanding post-translational intracellular localization of tail-anchored proteins.

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REFERENCES

1. Sabatini, D.D., Kreibich, G., Morimoto, T., and Adesnik, M. (1982) Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* **92**, 1-22
2. Sakaguchi, M., Mihara, K., and Sato, R. (1987) A short amino-terminal segment of cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence. *EMBO J.* **6**, 2425-2431
3. Zerial, M., Melancon, P., Schneider, C., and Garoff, H. (1986) The transmembrane segment of the human transferrin receptor functions as a signal peptide. *EMBO J.* **5**, 1543-1550
4. Kornfeld, S. and Mellman, I. (1989) The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* **5**, 483-525
5. Munro, S. and Pelham, H.R.B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907
6. Nilsson, T., Jackson, M., and Peterson, P.A. (1989) Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* **58**, 707-718
7. Swift, A.M. and Machamer, C.E. (1991) A Golgi retention signal in a membrane-spanning domain of coronavirus E1 protein. *J. Cell Biol.* **115**, 19-30
8. Munro, S. (1991) Sequences within and adjacent to the transmembrane segment of α-2,6-sialyltransferase specify Golgi retention. *EMBO J.* **10**, 3577-3588
9. Ahn, K., Szczesna-Skorupa, E., and Kemper, B. (1993) The amino-terminal 29 amino acids of cytochrome P4502C1 are sufficient for retention in the endoplasmic reticulum. *J. Biol. Chem.* **268**, 18726-18733
10. Masaki, R., Yamamoto, A., and Tashiro, Y. (1994) Microsomal aldehyde dehydrogenase is localized to the endoplasmic reticulum via its carboxyl-terminal 35 amino acids. *J. Cell Biol.* **126**, 1407-1420
11. Masaki, R., Yamamoto, A., and Tashiro, Y. (1996) Membrane topology and retention of microsomal aldehyde dehydrogenase in the endoplasmic reticulum. *J. Biol. Chem.* **271**, 16939-16944
12. Mitoma, J. and Ito, A. (1992) The carboxy-terminal 10 amino acid residues of cytochrome b_5 are necessary for its targeting to the endoplasmic reticulum. *EMBO J.* **11**, 4197-4203
13. Vergeres, G., Ramsden, J., and Waskell, L. (1995) The carboxyl terminus of the membrane-binding domain of cytochrome b_5 spans the bilayer of the endoplasmic reticulum. *J. Biol. Chem.* **270**, 3414-3422
14. Shibahara, S., Muller, R., Taguchi, H., and Yoshida, T. (1985) Cloning and expression of cDNA for rat heme oxygenase. *Proc. Natl. Acad. Sci. USA* **82**, 7865-7869
15. Kutay, U., Hartmann, E., and Rapoport, T.A. (1993) A class of membrane proteins with a C-terminal anchor. *Trends Cell Biol.* **3**, 72-75
16. Inoue, A., Obata, K., and Akagawa, K. (1992) Cloning and sequence analysis of cDNA for a neuronal cell membrane antigen, HPC-1. *J. Biol. Chem.* **267**, 10613-10619
17. Bennett, M.K., Calokos, N., and Scheller, R.H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257**, 255-259
18. Tagaya, M., Toyonaga, S., Takahashi, M., Yamamoto, A., Fujiwara, T., Akagawa, K., Moriyama, Y., and Mizushima, S. (1995) Syntaxin 1 (HPC-1) is associated with chromaffin granules. *J. Biol. Chem.* **270**, 15930-15933
19. Koh, S., Yamamoto, A., Inoue, A., Inoue, Y., Akagawa, K., Kawamura, Y., Kawamoto, Y., and Tashiro, Y. (1993) Immunoelectron microscopic localization of the HPC-1 antigen in rat cerebellum. *J. Neurocytol.* **22**, 995 1005
20. Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993) SNAP receptors implicated in vesicle targeting and function. *Nature* **362**, 318-324
21. Bennett, M.K., Carcia-Arraras, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D., and Scneller, R.H. (1993) The syntaxin family of vesicular transport receptors. *Cell* **74**, 863-873
22. Kushima, Y., Fujiwara, T., Morimoto, T., and Akagawa, K. (1995) Involment of HPC-1/syntaxin-1A antigen in transmitter release from PC12h cells. *Biochem. Biophys. Res. Commum.* **212**, 97-103
23. Komazaki, S., Fujiwara, T., Takada, M., and Akagawa, K. (1995) Rat HPC-1/syntaxin 1A and syntaxin 1B interrupt intracellular membrane transport and inhibit secretion of the extracellular matrix in embryonic cells of an amphibian. *Exp. Cell Res.* **221**, 11-18
24. Nagamatsu, S., Fujiwara, T., Nakamichi, Y., Watanabe, T., Katahira, H., Sawa, H., and Akagawa, K. (1996) Expression and functional role of syntaxin 1/HPC-1 in pancreatic β cells. *J. Biol. Chem.* **271**, 1160-1165
25. Calakos, N., Bennett, M.K., Peterson, K.E., and Scheller, R.H. (1994) Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* **263**, 1146-1149
26. Pevsner, J., Hsu, S.-C., Braun, J.E.A., Calakos, N., Ting, A.E., Bennett, M.K., and Scheller, R.H. (1994) Specificity and regulation of a synaptic vesicle docking complex. *Neuron* **13**, 353-361
27. Akagi, S., Yamamoto, A., Yoshimori, T., Masaki, R., Ogawa, R., and Tashiro, Y. (1988) Distribution of protein disulfide isomerase in rat hepatocytes. *J. Histochem. Cytochem.* **36**, 1533-1542
28. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
29. Suemori, H., Kadowaki, Y., Goto, K., Araki, I., Kondoh, H., and Nakatsuji, N. (1990) A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous β-galactosidase expression. *Cell Differ. Dev.* **29**, 181-186
30. Kramer, W. and Frits, H.-J. (1987) Oligonucleotide-directed construction of mutation via gapped duplex DNA. *Methods Enzymol.* **154**, 350-367
31. Kou, C.-H., Yamagata, K., Moyzis, R.K., Bitensky, M.W., and Miki, N. (1986) Multiple opsin mRNA species in bovine retina. *Brain Res. Mol. Brain Res.* **1**, 251-260
32. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
33. Misumi, Y., Miki, A., Takatsuki, A., Tamura, G., and Ikehara, Y. (1986) Novel blockade by brefeldin A of intracellular transport of secretary proteins in cultured rat hepatocytes. *J. Biol. Chem.* **261**, 11398-11403
34. Lippincott-Schwarts, J., Yuan, L.C., Bonifacino, J.S., and Klausner, R.D. (1989) Rapid distribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from the Golgi to ER. *Cell* **56**, 801-813
35. Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B., and Rapoport, T.A. (1995) Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane. *EMBO J.* **14**, 217-223
36. Omary, M.B. and Trowbridge, I.S. (1981) Biosynthesis of the human transferrin receptor in cultured cells. *J. Biol. Chem.* **256**, 12888-12892
37. Satoh, M., Hirayoshi, K., Yokota, S., Hosakawa, N., and Nagata, K. (1996) Intracellular interaction of collagen-specific stress protein Hsp47 with newly synthesized procollagen. *J. Cell Biol.* **133**, 469-483
38. Pedrazzini, E., Villa, A., and Borgese, N. (1996) A mutant cytochrome b_5 with a lengthened membrane anchor escapes from the endoplasmic reticulum and reaches the plasma membrane. *Proc. Natl. Acad. Sci. USA* **93**, 4207-4212