Microsomal Aldehyde Dehydrogenase Is Localized to the Endoplasmic Reticulum via Its Carboxyl-Terminal 35 Amino Acids

Ryuichi Masaki, Akitsugu Yamamoto, and Yutaka Tashiro

Department of Physiology, and Cell Biology Division of Liver Research Center, Kansai Medical University, Moriguchi, Osaka 570, Japan

Abstract. Rat microsomal aldehyde dehydrogenase (msALDH) has no amino-terminal signal sequence, but instead it has a characteristic hydrophobic domain at the carboxyl terminus (Miyauchi, K., R. Masaki, S. Taketani, A. Yamamoto, A. Akayama, and Y. Tashiro. 1991. *J. Biol. Chem.* 266:19536–19542). This membrane-bound enzyme is a useful model protein for studying posttranslational localization to its final destination. When expressed from cDNA in COS-1 cells, wild-type msALDH is localized exclusively in the well-developed ER. The removal of the hydrophobic domain results in the cytosolic localization of truncated proteins, thus suggesting that the portion is responsible for membrane anchoring. The last 35 amino acids of msALDH, including the hydrophobic domain, are sufficient for targeting of *E. coli* β-galactosidase to the ER membrane. Further studies using chloramphenicol acetyltransferase fusion proteins suggest that two hydrophilic sequences on either side of the hydrophobic domain play an important role in ER targeting.

A great deal of attention has been paid to resolve the mechanism for sorting and targeting of newly synthesized proteins to their final destinations, and this is one of the fundamental problems in cell biology. Newly synthesized proteins are destined to follow two distinct routes, depending on the presence or absence of a signal sequence at their amino termini (37). The signal recognition particle (SRP)[1] in the cytosol recognizes and binds to a signal sequence of nascent peptides. The resulting SRP-ribosome-nascent peptides complexes are targeted to the ER membrane through their interactions with the docking protein complex (35, 36). After translocation across the ER membrane, secretory and plasma membrane proteins follow a common pathway from the ER, through the Golgi complex, to the cell surface by default with bulk flow of lipids (33). Resident proteins in the central vacuolar system are localized and retained in their final destinations with the aid of either specific targeting (18) or retention signals (25–27, 44).

On the other hand, proteins without a signal sequence at their amino termini are synthesized on free polysomes and are posttranslationally directed to intracellular organelles such as mitochondria, peroxisomes, the nucleus, and the ER, or they remain in the cytosol. Much is known regarding targeting signals (14, 16, 39) and cytosolic protein factors (1, 15) by which proteins are imported into these organelles. However, less is known regarding posttranslational targeting of integral membrane proteins to the outer mitochondrial membrane, the peroxisomal membrane, or the ER membrane.

Cytochrome *b5* has a hydrophobic domain instead of an amino-terminal signal sequence at its carboxyl terminus (42). For a long time, cytochrome *b5* has been noted to be a typical membrane protein inserted posttranslationally into microsomal membranes through the hydrophobic domain. Besides, it has been reported that cytochrome *b5* is localized in any membranes of intracellular organelles, including the ER membrane, the Golgi membrane, the plasma membrane, and the outer mitochondrial membrane (47). Experiments using SRP-depleted or -supplemented in vitro systems have shown that this protein does not require SRP for insertion into microsomal membranes (3). Therefore, the carboxyl-terminal hydrophobic sequence was termed an "insertion" sequence through which spontaneous integration of cytochrome *b5* into any exposed membranes could occur not only in vitro, but also in vivo (5). However, recent studies have shown that cytochrome *b5* is restricted in the ER in vivo (11), and that the last 10 amino acids of this protein adjacent to the hydrophobic domain are important for its targeting to the ER (23).

Recently, we have isolated and sequenced a full-length

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; FP2, NADPH-cytochrome P-450 reductase; msALDH, microsomal aldehyde dehydrogenase; PBS(+), PBS containing 1 mM CaCl₂ and 0.5 mM MgCl₂; PDI, protein disulfide isomerase; PTP, protein tyrosine phosphatase; STE, sucrose solution containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin A, 0.5 mM PMSF, and 10 U/ml Trasyol; SRP, signal recognition particle.

cDNA for rat microsomal aldehyde dehydrogenase (msALDH) (24). The deduced amino acid sequence of this enzyme predicts a similar molecular structure to that of cytochrome $b5$, a bulky amino-terminal domain without an amino-terminal signal sequence and a short hydrophobic domain at the carboxyl terminus. We report here that msALDH is localized exclusively in the ER in COS-1 cells when expressed from cDNA, and that expression of this protein apparently alters the structure of the ER from a reticular to large vesicular one. In addition, we report that the hydrophobic domain is responsible for membrane anchoring and that msALDH is likely to have two ER targeting sequences on either side of the membrane anchoring domain. Our results suggest a novel mechanism for the posttranslational ER targeting of this tail-anchored protein.

Materials and Methods

Materials

FBS was purchased from Filtron Co. (Brooklyn, Australia). Penicillin-streptomycin liquid was from Gibco Laboratories (Grand Island, NY). DME was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Affinity-purified goat anti–rabbit IgG and anti–mouse IgG conjugated with TRITC were purchased from Protos Immunoresearch (San Francisco, CA). Affinity-purified goat anti–rabbit IgG and anti–mouse IgG conjugated with FITC were obtained from American Qualex Antibodies & Immunochemicals Co. (La Mirada, CA). FITC-conjugated wheat germ agglutinin was from E-Y Laboratories, Inc. (San Mateo, CA). Affinity-purified goat anti–mouse IgG and anti–rabbit IgG conjugated with peroxidase were purchased from Dako A/S (Glostrup, Denmark) and Tago, Inc. (Burlingame, CA), respectively. Rabbit anti–chloramphenicol acetyltransferase (CAT) antibody was obtained from 5 Prime→3 Prime, Inc. (Boulder, CO). Mouse mAb to human mitochondrial 65-kD protein was from Chemicon International, Inc. (Temecula, CA). Mouse mAbs to *Escherichia coli* β-galactosidase and the β-subunit of human prolyl 4-hydroxylase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and Fiji Yakuhin Kogyo Co., Ltd. (Toyama, Japan), respectively. Since the β-subunit of prolyl 4-hydroxylase has been shown to be identical to protein disulfide isomerase (PDI) (34), this mAb is referred to as PDI mAb in this paper. Rabbit antibodies to rat msALDH, NADPH-cytochrome P-450 reductase (FP2), and PDI have been prepared and characterized as described (2, 21, 24). Rabbit antiserum to bovine mitochondrial complex III was a generous gift from Dr. Takamasa Ozawa (Nagoya University, Nagoya, Japan). The eukaryotic assay vectors, pCH110 and pSV2CAT, were from Pharmacia LKB Biotechnology, Inc. (Uppsala, Sweden) and Stratagene (La Jolla, CA), respectively. The eukaryotic expression vector pMIW (43) was kindly provided by Dr. Akihiro Inoue (National Institute for Physiological Sciences, Okazaki, Japan). Restriction enzymes and DNA modifying enzymes were purchased from Nippon Gene (Toyama, Japan) and Takara Co., Ltd. (Kyoto, Japan). All other chemicals were of the highest purity commercially available.

Plasmid Constructions

All constructions were verified by the dideoxy chain termination method (38). Insertion of a full-length cDNA encoding rat msALDH into the unique EcoRI site of the SV-40-based vector pCD (30) has been described previously (24). The gapped duplex method of oligonucleotide-directed mutagenesis (19) was used for truncation or deletion of msALDH cDNA. The full-length msALDH cDNA was cloned into the EcoRI site of M13tv18 prepared from an umber mutant phage, and single-stranded phage DNA was purified for use as the template for mutagenesis. Synthetic oligonucleotides used are termed as follows (with mutated nucleotides underlined): no. 1 (5' GGTCAGCTGATCGAAATTC 3'), no. 2 (5' CTCGTGTGATTGGTTGCG-GT 3'), no. 3 (5' GTGATCGTCTAGGATCAGC 3'), no. 4 (5' AAATTCTTC CTGCTGAACAAAGGAAGGCTG 3'), and no. 5 (5' CTGCTGAAACAG-TTCCTGCAGCTGCTGCTTC 3'). The oligonucleotides nos. 1, 2, and 3 were used to generate sequences that included artificial stop codons after amino acids 449, 470, and 480 of msALDH, respectively. The oligonucleotide no. 4 was used to produce the coding sequence for the mutant protein

that lacked amino acids 457–459 of msALDH, and that for the mutant protein lacking amino acids 460–463 was no. 5. After hybridization of the oligonucleotide and a PvuII fragment derived from M13mpl8 with the template DNA, second-strand synthesis was carried out with T4 DNA polymerase and *E. coli* DNA ligase, and the resultant double-stranded DNA was transfected into *E. coli* BMH71-18 mutS for amplification. Phages recovered from *E. coli* BMH71-18 mutS were then transfected into *E. coli* MV1184 to select non-umber ones, and single-stranded DNA from two to six plaques was sequenced to confirm the desired mutations. The mutated cDNAs were excised from the double-stranded replicative form DNAs and cloned into the EcoRI site of the pCD vector. The right orientation was verified by restriction enzyme digestion, and the resulting constructs were referred to as pCDALDHΔ450-484, pCDALDHΔ471-484, pCDALDHΔ-481-484, pCDALDHΔ457-459, and pCDALDHΔ460-463, respectively.

A chimeric cDNA for *E. coli* β-galactosidase fusion protein was constructed as follows. First, a DNA fragment encoding the carboxyl-terminal 53 amino acids (amino acids 994-1046) of β-galactosidase, followed by five amino acids (amino acids 450-454) of msALDH, was created by PCR using oligonucleotides no. 6 (5' AGCCATCGCCATCTGCTG 3') and no. 7 (5' GGAAGAATTTCGACCATTTTTGACACCAGACC 3'). Similarly, a second DNA fragment corresponding to the last five amino acids (amino acids 1042-1046) of β-galactosidase followed by the last 35 amino acids (amino acids 450-484) and 3' untranslated sequence of msALDH was amplified using oligonucleotides no. 8 (5' GGTCTGGTGTCAAAAATGGTCGAAAT-TCTTCC 3') and no. 9 with mutated nucleotides underlined for introduction of an EcoRI site (5' AACAACTTAGAATTCACTGCAGTTC 3'). These two fragments were then used as the templates to create a chimeric cDNA with the oligonucleotides nos. 6 and 9. The resultant PCR fragment was digested with EcoRI and ligated in-frame to the EcoRI site (amino acids 1029-1030) of pCHI10 to construct pCHI10/ALDH.

Since it was difficult to construct a series of various fusion genes using β-galactosidase cDNA according to the method described above, we used CAT cDNA for further analyses on the role of the carboxyl-terminal portion of msALDH in the intracellular localization. Chimeric cDNAs for CAT fusion proteins (CAT/ALDH chimeras) were constructed essentially by combination of oligonucleotide-directed mutagenesis and PCR. A 1.8-kbp HindIII-BamHI cDNA fragment encoding CAT in the pSV2CAT was cloned into M13tv18. Oligonucleotides no. 10 (5' AGTGGCAGGGCGGTACCT-AATTTTTTTA 3') and no. 11 (5' ATAAGTGATATCAAGCGGATGA 3') with mutated nucleotides underlined were used for generation of a KpnI site at the carboxyl terminus and an EcoRV site in the 3' untranslated sequence of CAT, respectively. The mutated cDNA was cloned into HindIII/ BamHI-digested pMIW expression vector to construct pMIWCAT. PCR was then used to generate DNA fragments corresponding to the carboxyl-terminal regions of msALDH, including the 3' untranslated sequence. PCR reactions used the following primers and templates to amplify DNA fragments termed ALDH1-7 and 9: ALDH1; oligonucleotides no. 12 (5' GAGTCCAAGGGTACCTGGTCGAAATTC 3') with mutated nucleotides underlined for introduction of a KpnI site and no. 9 (pCDALDH as template), ALDH2; oligonucleotides no. 12 and no. 9 (pCDALDHΔ481-484 as template), ALDH3; oligonucleotides no. 13 (5' AAACAGTTCAAC-GGTACCAGGCTGCAGCTG 3') and no. 9 (pCDALDH as template), ALDH4; oligonucleotides no. 13 and no. 9 (pCDALDHΔ481-484 as template), ALDH5; oligonucleotides no. 14 (5' GAAATTCTTCGGTACC-AAACAGTTCAAC 3') and no. 9 (pCDALDH as template), ALDH6; oligonucleotides no. 14 and no. 9 (pCDALDHΔ481-484 as template), ALDH7; oligonucleotides no. 12 and no. 9 (pCDALDHΔ457-459 as template), and ALDH9; oligonucleotides no. 12 and no. 9 (pCDALDHΔ460-463 as template). These amplified fragments were then digested with KpnI and HpaI and ligated into the KpnI-EcoRV site of pMIWCAT. The resultant plasmids were designated pMIWCAT/ALDH1-7, and pMIWCAT/ALDH9, respectively. For construction of pMIWCAT/ALDH8, pMIWCAT/ALDH2, which has the unique PstI site in the coding sequence for the hydrophobic domain of msALDH, was digested with PstI and BamHI, and ligated into PstI/BamHI digested pMIWCAT/ALDH7. Similarly, the same fragment was ligated into PstI/BamHI digested pMIWCAT/ALDH9 to construct pMIWCAT/ALDH10.

Cell Culture and Transfection

COS-1 cells were maintained in DME with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a 5% CO_2 incubator, and were replated the day before transfection by trypsinization. Transfection was performed 4 h after the medium was replaced by the fresh one. For subcellular fractionation experiments, cells plated in a 100-mm dish (50–70% confluent) were transfected with an expression plasmid (20 μg) using the cal-
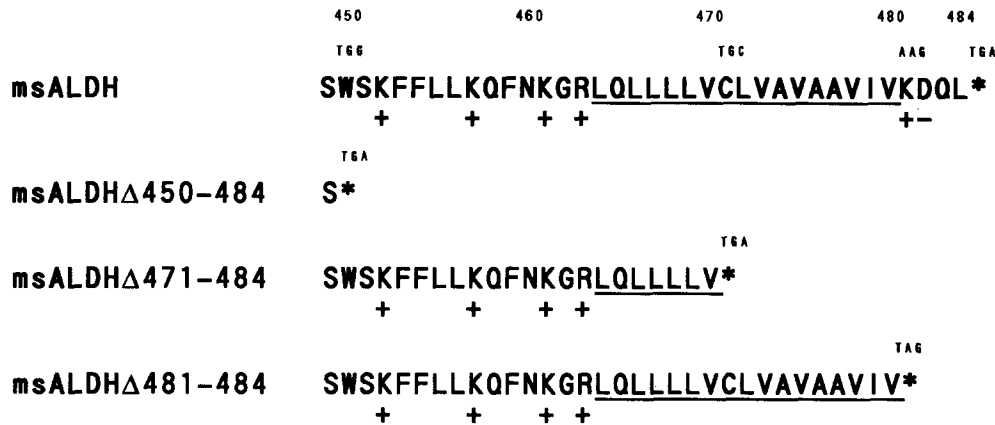
Figure 1. Deduced amino acid sequences of the carboxyl termini of wild-type and truncated forms of msALDH. The single amino acid code is used, and the amino acid numbers of msALDH are shown at the top. Positively and negatively charged amino acids are marked + and − at the bottom, respectively. The hydrophobic domains are underlined. The cDNA for msALDH was converted to code for three truncated proteins, msALDHΔ450-484, msALDHΔ471-484, or msALDHΔ481-484 by oligonucleotide-directed mutagenesis.

cium phosphate precipitation method (48). For immunofluorescent experiments, cells were grown on a 22 × 22-mm coverslip in a 35-mm dish (10–20% confluent) and were transfected with 4 μg plasmid DNA per 35-mm dish. For immunogold localization of CAT/ALDH chimeras, cells plated in a 60-mm dish (50–70% confluent) were transfected with 10 μg plasmid DNA per dish. 4 h after application of DNA-calcium phosphate precipitate at 37°C, cells were shocked with 15% glycerol for 2 min at room temperature, then incubated again at 37°C for an additional 44 h before harvesting for subcellular fractionation or fixation for indirect immunofluorescence microscopy or immunoelectron microscopy.

Subcellular Fractionation and Assays

Cell fractionation was performed essentially as described previously for COS-1 cells by Clark and Waterman (10) with slight modifications. Briefly, cells were washed once with PBS and harvested in 5 ml of ice-cold 0.5 M sucrose containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.5 mM PMSF, and 10 U/ml Trasyol (0.5 M STE). After centrifugation at 800 g for 5 min, the pellet was suspended in 0.5 ml of 0.5 M STE, homogenized with a Teflon-glass homogenizer, then diluted with an equal volume of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.5 mM PMSF, and 10 U/ml Trasyol to obtain isotonic conditions. The total homogenate (designated H) was layered over 0.5 ml of 0.5 M STE and centrifuged at 800 g for 10 min at 4°C using a swing out bucket, yielding a pellet (P1) consisting mainly of nuclei and unbroken cells. The supernatant and the interface were again layered over 0.5 M STE and centrifuged as above at 9,000 g for 10 min to isolate mitochondrial fraction (P2). The resultant supernatant was centrifuged at 88,000 g for 80 min at 4°C to sediment microsomal fraction (P3, mostly of the ER membrane). The final supernatant, consisting mostly of cytosol, was designated S3. Membrane fractions, P1, P2, and P3 were resuspended by hand homogenization in 0.25 M STE. Endogenous enzyme activities of FP2 (an ER marker) and succinate-cytochrome c reductase (a mitochondrial marker) were assayed by the methods of Omura and Takesue (31) and King (17), respectively. Protein was measured by Bradford's method (7).

Membrane Extractions

Membrane fractions (100 μg protein) were resuspended in 0.8 ml of 100 mM Na2CO3 (pH 11.5), and were incubated for 30 min at 0°C (13). The suspension was then centrifuged at 88,000 g for 80 min at 4°C, and the pellet (P) was suspended in 100 μl of SDS-PAGE sample buffer (20). The supernatant (S) was precipitated with 10% TCA, washed twice with 90% ethanol and once with diethyl ether, and dried. The resultant pellet was suspended in 100 μl of SDS-PAGE sample buffer.

Immunoblot Analysis

All procedures were done at room temperature. Proteins were separated on 8.5% polyacrylamide gels (20) and electrophoretically transferred to a dura-
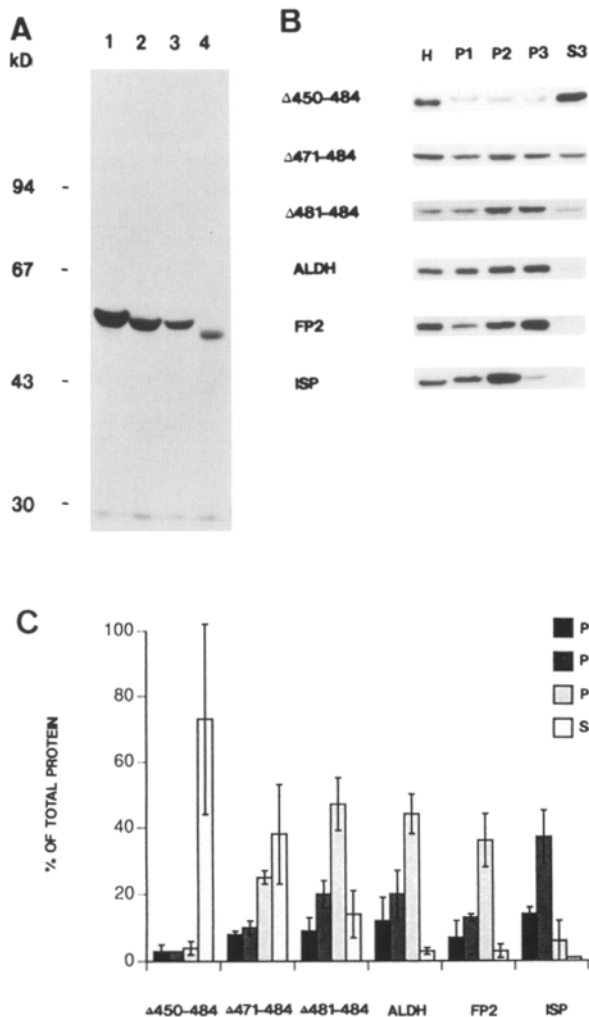
pore membrane according to the method of Burnette (8) using a semidry transfer blotter for 2.5 h at 36 V. After blocking with 3% skim milk in TBS for 1.5 h, blots were incubated with primary antibody in 3% skim milk/TBS for 1.5 h, and washed four times (5 min each) with 0.05% Tween-20 in TBS. They were then incubated with secondary antibody (peroxidase-conjugated goat anti–rabbit IgG or anti–mouse IgG) in 0.05% Tween-20/TBS for 1.5 h, followed by four washes (5 min each) with 0.05% Tween-20/TBS. Blots were stained using the enhanced chemiluminescence Western blotting detection system (Amersham Corp., Arlington Heights, IL). Protein bands were quantitated using an enhanced laser densitometer (LKB Instruments Inc., Bromma, Sweden) to evaluate the relative level of proteins in each subcellular fraction. The densitometric scan value was then estimated by multiplying the relative level by the protein yield (milligrams of protein) in corresponding subcellular fraction. In calculating the percent distribution of each immunodetectable protein, the densitometric scan value of the homogenate was defined as 100%.

Indirect Immunofluorescence Microscopy

All procedures, except for the incubation with antibodies or FITC-conjugated lectin at 37°C, were carried out at room temperature. Cells grown on coverslips were washed gently three times (5 min each) with PBS, fixed with 4% paraformaldehyde in PBS containing 1 mM CaCl2 and 0.5 mM MgCl2 [PBS(+)] for 20 min, and permeabilized with 0.1% Triton X-100 in PBS(+) for 1 h. They were then rinsed twice with PBS(+), and incubated with 2% FBS in PBS(+) to block nonspecific binding of primary antibodies for 1 h followed by 45 min incubation with primary antibody in 2% FBS/PBS(+). After washing four times (5 min each) with PBS(+), cells were incubated with secondary antibody in 2% FBS/PBS(+) for 45 min. For localization of the Golgi complex, cells were incubated with FITC-conjugated wheat germ agglutinin in 2% FBS/PBS(+) for 45 min. After washing with PBS(+), they were then mounted on glass slides with 90% glycerol in PBS containing 1 mg/ml paraphenylenediamine, examined, and photographed on a microscope (BH-2; Olympus Corp., Tokyo, Japan) with Ektachrome 400 film (Kodak, Rochester, NY).

Immunoelectron Microscopy on Frozen Ultrathin Sections

Frozen ultramicrotomy was performed as described by Tokuyasu (46). Transfected cells were harvested by centrifugation at 1,000 g for 3 min, and the pellet was fixed with 4% paraformaldehyde and 0.1% glutaraldehyde dissolved in 0.1 M cacodylate buffer, pH 7.4, for 15 min. Small pieces of the fixed pellets of COS-1 cells were incubated overnight in 0.1 M sodium phosphate buffer, pH 7.4, containing 2.3 M sucrose and 20% polyvinyl pyrrolidone, and were rapidly frozen in liquid propane at −180°C. Frozen ultrathin sections were cut with an ultramicrotome (Ultracut-N; Reichert, Wien, Australia) with a cryoattachment (FC-4D; Reichert). The sections were picked up on formvar-carbon–coated nickel grids, incubated with 2% gelatin in PBS containing 10 mM glycine, then reacted with anti-CAT antibody or control IgG prepared from a nonimmunized rabbit serum. The sections

Table I. Distribution of FP2, Succinate-Cytochrome c Reductase, and Protein in Subcellular Fractions

	FP2 (%)	SCR (%)	Protein (%)
H	100	100	100
P1	6 ± 3	9 ± 2	10 ± 4
P2	12 ± 4	48 ± 2	12 ± 3
P3	42 ± 8	7 ± 3	21 ± 3
S3	7 ± 1	0 ± 0	39 ± 6

Cell fractionation was performed as described in Materials and Methods. In calculating the percent distribution, each value of the homogenate was defined as 100%. The distribution of FP2 and succinate-cytochrome c reductase(SCR) is determined from the specific activities measured. The specific activities of the homogenate fractions are 52.3 ± 11.0 nmol cytochrome c reduced/min per mg protein (the mean ± SD n = 4) and 26.9 ± 2.8 nmol cytochrome c reduced/min per mg protein for FP2 and succinate-cytochrome c reductase, respectively.
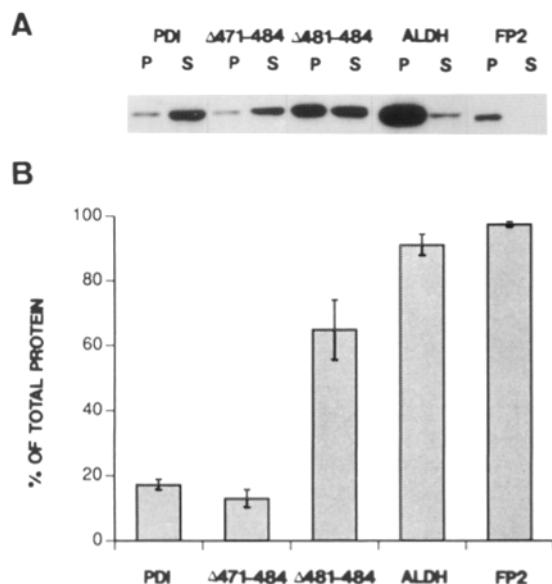
Results

Construction of Truncated Mutants of msALDH

In the previous study (24), we cloned and sequenced the full-length cDNA for rat msALDH. The nucleotide sequence predicts a polypeptide of 484 amino acids, and the most characteristic feature of this membrane-bound ALDH is carboxyl-terminal 35 amino acids, consisting of a stem region (amino acids 450-463) and a hydrophobic domain (amino acids 464-480) followed by a short hydrophilic tail region (amino acids 481-484) as shown in Fig. 1. Since rat cytosolic tumor-associated ALDH, which is 65.5% identical to msALDH (24), lacks the carboxyl-terminal portion, we asked whether this portion played an important role in the intracellular localization of msALDH. For this purpose, we constructed three mutant proteins truncated at either amino acid 450 (msALDHΔ450-484, which corresponds to the tumor-associated ALDH in size), 471 (msALDHΔ471-484, deletion of more than half of the hydrophobic domain together with the tail region), or 481 (msALDHΔ481-484, deletion of solely the tail region) by oligonucleotide-directed mutagenesis (Fig. 1).

These mutant proteins and wild-type msALDH were expressed transiently in COS-1 cells under the control of SV-40 promoter in the pCD expression vector. This transient expression system in COS-1 cells was chosen as the most rapid method for evaluating the intracellular localization of expressed proteins. Cells were allowed to express these proteins for 44 h, harvested, and the expressed proteins were analyzed by immunoblotting using anti-msALDH antibody. As shown in Fig. 2 A, msALDH and three truncated proteins were expressed efficiently in the total homogenates. As expected, molecular masses of the truncated proteins were smaller than that of msALDH (54 kD). In addition, no cross-reactive protein was detected in untransfected COS-1 cells (data not shown), indicating the absence of endogenous msALDH. These results allowed us to investigate the intracellular localization of msALDH by transfection experiments.

Deletion of the Carboxyl-terminal Portion of msALDH Abolishes ER Localization

We analyzed the intracellular distribution of msALDH and three truncated proteins by subcellular fractionation accord-

Figure 2. Immunoblot analysis of wild-type and truncated forms of msALDH. (*A*) COS-1 cells were transfected with cDNA for wild-type or truncated forms of msALDH in the pCD vector, and were harvested 44 h after transfection. Equal amounts of protein (20 μg) from each homogenate were resolved on SDS-PAGE and immunoblotted using anti-msALDH antibody. Lane *1*, msALDH; lane *2*, msALDHΔ481-484; lane *3*, msALDHΔ471-484; lane *4*, msALDH-Δ450-484. (*B*) Subcellular fractions (*P1, P2, P3,* and *S3*) were isolated from each homogenate (*H*) as described in Materials and Methods, and equal amounts of protein (12 μg, except for 6 μg of subcellular fractions from cells expressing msALDH or msALDH-Δ481-484) were immunoblotted using anti-msALDH antibody. Endogenous FP2 and mitochondrial Rieske iron-sulfur protein (*ISP*) of the complex III were detected using anti-FP2 antibody and anti-complex III antiserum, respectively. (*C*) The percent distribution in each subcellular (*P1, P1, P3,* and *S3*) fraction is shown for msALDH, three truncated proteins, FP2, or ISP. The distribution of immunodetectable proteins in each subcellular fraction was calculated as described in Materials and Methods. In calculating the percent distribution, the densitometric scan value of the homogenate (*H*) was defined as 100%. The bars show the mean ± SD (*n* = 3).

were then washed six times with gelatin solution, and were reacted with goat anti-rabbit IgG gold conjugate (10 nm in diameter) (British BioCell, Cardiff, UK). After washing with sodium cacodylate buffer, pH 7.4, sections were postfixed in 2% glutaraldehyde, then in 1% OsO₄, stained with uranyl acetate, embedded in LR white resin (Bio Rad Microscience Division, Watford, UK), and observed with an electron microscope (H-7000; Hitachi, Tokyo, Japan).

A

PDI Δ471-484 Δ481-484   ALDH   FP2
P   S   P   S   P   S   P   S   P   S

B

Figure 3. Effect of sodium carbonate treatment on the membrane association of wild-type msALDH and two truncated proteins. (A) Each P3 fraction was treated with 100 mM Na₂CO₃ for 30 min at 0°C, and centrifuged at 88,000 g for 80 min to separate the pellets (P) from the supernatants (S). The distribution of msALDH, msALDHΔ481-484, msALDHΔ471-484, FP2, or PDI in the P and S fractions was assayed by immunoblotting. Polyclonal anti-PDI antibody was used for immunodetection of endogenous PDI. (B) The relative level of each protein in the P and S fractions was quantitated by a scanning densitometer. In calculating portions recovered in the P fraction, the total level of each protein recovered in the P and S fractions was defined as 100%. The bars show the mean ± SD (n = 3).

ing to the method of Clark and Waterman (10). The separation of ER membranes from mitochondria was checked by assaying two typical marker enzymes, FP2 (NADPH-cytochrome P-450 reductase), which has been shown to be an integral ER membrane protein (22), and a mitochondrial marker succinate-cytochrome c reductase. As shown in Table I, FP2 and succinate-cytochrome c reductase were enriched in the P3 fraction and the P2 fraction, respectively. In addition, when equal amounts of protein were immunoblotted, the highest levels of FP2 and mitochondria Rieske iron-sulfur protein were found in the P3 fraction and the P2 fraction, respectively (Fig. 2 B). The percent distribution of the two proteins in each subcellular fraction also confirmed a good separation of ER membranes from mitochondria (Fig. 2 C). The subcellular distribution of msALDH and msALDHΔ481-484 was almost identical to that of FP2 (Fig. 2, B and C), suggesting the ER localization of these proteins in transfected COS-1 cells. On the other hand, msALDHΔ450-484 was found exclusively in the S3 fraction. This result indicated that the mutant protein lacking the last 35 amino acids of msALDH was no longer associated with intracellular membranes. Curiously, msALDHΔ471-484 was recovered not only in the S3 fraction, but also in the P3 fraction, showing an intermediate distribution between msALDH and msALDHΔ450-484.

We explored the nature of the association of the expressed

proteins with microsomal membranes. Upon treatment of the P3 fraction with 100 mM Na₂CO₃ (pH 11.5) (13), FP2 (the integral ER membrane protein) remained attached to microsomal membranes as judged by immunoblotting (Fig. 3, A and B). Wild-type msALDH and msALDHΔ481-484 were also resistant to alkali extraction, although ∼35% of msALDHΔ481-484 was released. Under the same conditions, >80% of msALDHΔ471-484 and PDI (a luminal ER resident) were released, indicating a loose association of these proteins with microsomal membranes. Similar results were obtained upon Triton X-114 cloud point extraction (6) (data not shown). These data, together with those from subcellular fractionation, suggested that the carboxyl-terminal portion of msALDH including the hydrophobic sequence (amino acid 464-480) was necessary for both its ER localization and the tight association with the ER membrane.

We next determined the intracellular localization of msALDH and the truncated proteins using indirect immunofluorescence microscopy. Cells transfected with cDNAs in the pCD vector were fixed and permeabilized 44 h after transfection, and the expressed proteins were detected by incubation with anti-msALDH antibody followed by TRITC-conjugated secondary antibody. As shown in Fig. 4 A, anti-msALDH antibody stained a number of large vesicular structures that surrounded the nucleus, in addition to diffuse reticular ones in COS-1 cells transfected with msALDH cDNA. No staining was seen at the plasma membrane and in the nucleoplasm. A similar pattern of staining for msALDH was observed either 24 h after transfection in COS-1 cells or when expressed in HeLa cells or baby hamster kidney cells (data not shown). To explore this strange structure in more detail, we used double indirect immunofluorescence microscopy using two mAbs or FITC-conjugated lectin. Expressed msALDH was found to colocalize with endogenous PDI (Fig. 4 B, arrow), although PDI displayed a characteristic reticular staining pattern in cells not expressing msALDH (Fig. 4 B, arrowhead). In contrast, msALDH colocalized neither with a mitochondrial 65-kD protein (Fig. 4, C and D) nor with the perinuclear Golgi complex visualized by FITC-conjugated wheat germ agglutinin (Fig. 4, E and F). These results strongly suggest the ER localization of msALDH and the drastic morphological change of the ER by expression of msALDH. A similar ER staining pattern was observed for msALDHΔ481-484 (Fig. 4, G and H), whereas both msALDHΔ471-484 and msALDHΔ450-484 were found distributed diffusely throughout the cytoplasm, and they did not colocalize with PDI (Fig. 4, I-L). In addition, a significant amount of msALDHΔ450-484 appeared to enter the nucleus (Fig. 4 K). These immunolocalization data were consistent with those from subcellular fractionation, suggesting strongly the important role of the carboxyl-terminal portion of msALDH in its ER localization.

The Carboxyl-Terminal 35 Amino Acids of msALDH Are Sufficient for ER Localization

We focused our attention on the role that the carboxyl-terminal portion of msALDH might play in ER targeting, and we asked whether this portion could direct a heterologous protein to the ER membrane. For this purpose, we constructed an expression plasmid shown in Fig. 5 A. The control vector (pCH110) contains E. coli β-galactosidase, which
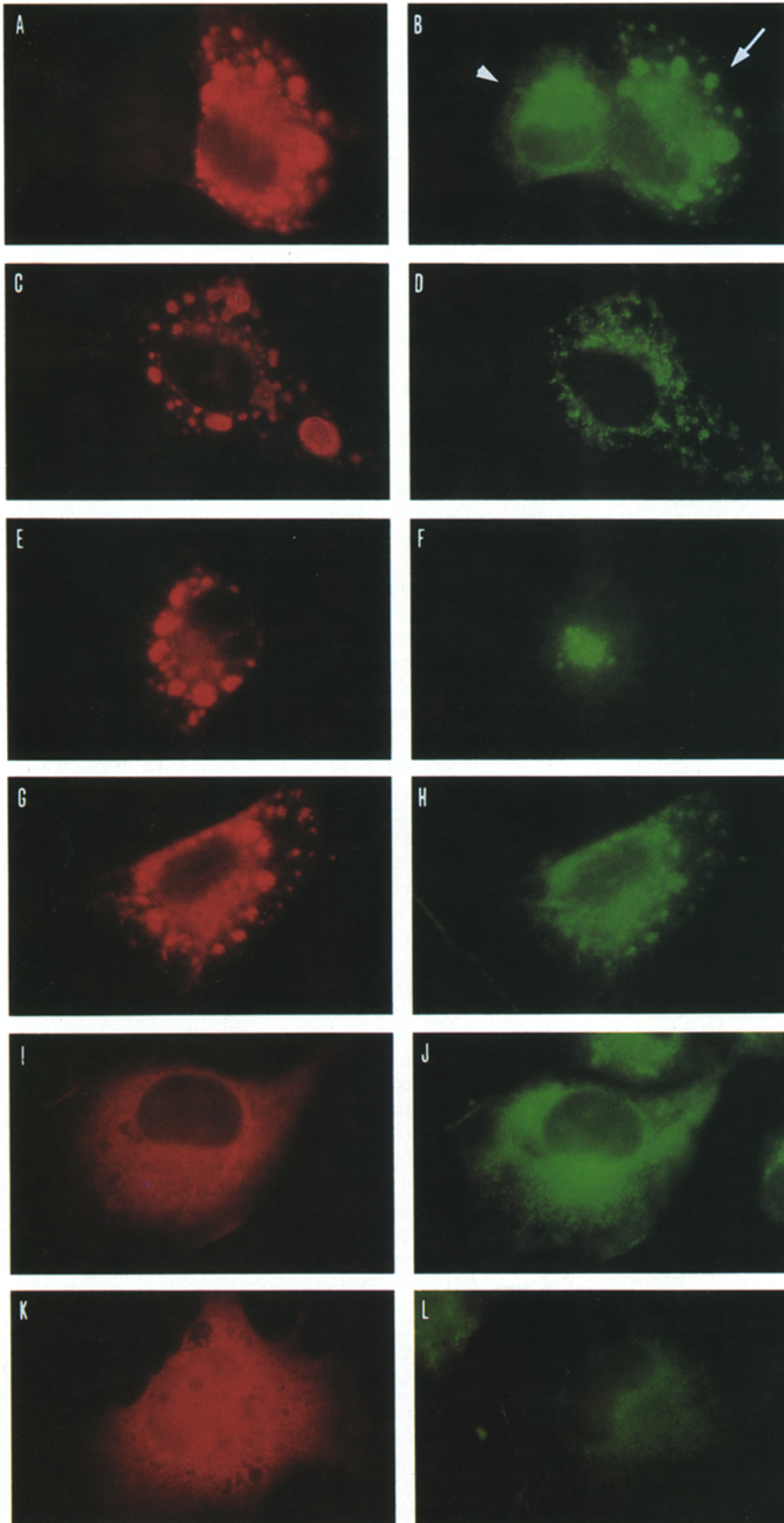
Figure 4. Localization of wild-type msALDH and three truncated proteins in transfected cells by double indirect immunofluorescence microscopy. COS-1 cells grown on coverslips were transfected with pCDALDH (*A-F*), pCDALDHΔ481-484 (*G* and *H*), pCDALDHΔ471-484 (*I* and *J*) or pCDALDHΔ450-484, (*K* and *L*), fixed 44 h after transfection, and permeabilized. Wild-type msALDH (*A*, *C*, and *E*) and the truncated proteins (*G*, *I*, and *K*) were then detected by incubation with anti-msALDH antibody followed by TRITC-conjugated secondary antibody. For visualization of endogenous PDI (*B*, *H*, *J*, and *L*) or a mitochondrial 65-kD protein (*D*), cells were stained with corresponding mAbs followed by FITC-conjugated secondary antibody. The Golgi complex was localized by staining with FITC-conjugated wheat germ agglutinin (*F*). The arrow in *B* indicates COS-1 cell expressing msALDH, whereas the arrowhead in the same figure indicates the cell that does not express msALDH.

A

B
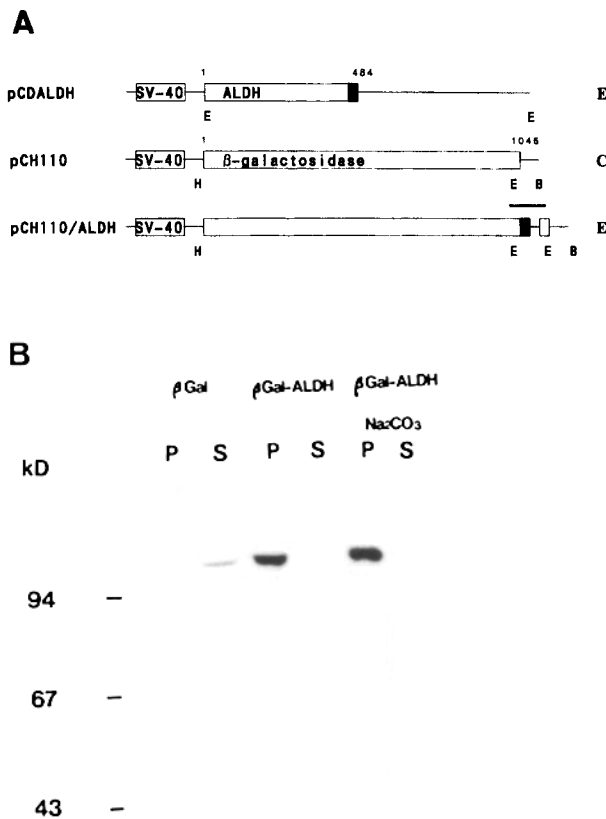
Figure 5. Immunoblot analysis of β-galactosidase and β-galactosidase/ALDH in transfected cells. (*A*) Schematic diagrams of msALDH, β-galactosidase, and β-galactosidase/ALDH in the expression vectors are shown. SV-40 implies the SV-40 early promoter. The expression plasmids pCDALDH, pCH110, and pCH110/ALDH contain msALDH, *E. coli* β-galactosidase, and β-galactosidase/ALDH, respectively. The amino acid numbers of each protein are shown at the top, and the relevant restriction endonuclease sites are at the bottom. *E*, EcoRI; *H*, HindIII; *B*, BamHI. The last 35 amino acids of msALDH are indicated by the solid bar. The chimeric cDNA fragment with EcoRI sites shown by the bar was amplified by PCR and cloned in-frame to the unique EcoRI site in pCH110 as described in Materials and Methods. To the right, the intracellular location of each protein is noted as *E* for ER or *C* for cytosolic. (*B*) COS-1 cells were transfected with pCH110 (*βGal*) or pCH110/ALDH (*βGal-ALDH*), and harvested 44 h after transfection. The membrane (*P*) and cytosol (*S*) fractions were prepared by centrifugation of the postnuclear fractions at 88,000 *g* for 80 min. The membrane fraction containing βGal-ALDH was treated with 100 mM Na₂CO₃ at 0°C for 30 min, and was centrifuged at 88,000 *g* for 80 min to separate the pellet (*P*) from the supernatant (*S*). Each fraction was assayed by immunoblotting using mAb to β-galactosidase.

was supposed to remain in the cytoplasm when expressed in COS-1 cells under the control of the SV-40 promoter. The constructed expression plasmid (pCH110/ALDH) contains the last 35 amino acids of msALDH cloned in-frame to the 3′ end of β-galactosidase. COS-1 cells were transfected with these DNAs and subjected to a crude subcellular fractionation. In this case, the postnuclear supernatant was centrifuged at 88,000 *g* for 80 min to separate the membrane

fraction (P, containing mostly mitochondria and microsomes) from the cytosol fraction (S). Immunoblotting using mAb to *E. coli* β-galactosidase revealed that wild-type β-galactosidase was recovered, as expected, in the S fraction, whereas β-galactosidase/ALDH chimera was found concentrated in the P fraction (Fig. 5 *B*). In addition, the chimera in the P fraction was resistant to alkali extraction, indicating a tight anchoring of this protein to intracellular membranes (Fig. 5 *B*).

Indirect immunofluorescence microscopy showed that β-galactosidase was distributed diffusely throughout the cytoplasm (Fig. 6 *A*). However, attachment of the carboxyl-terminal 35 amino acids of msALDH to β-galactosidase resulted in the reticular pattern of staining that surrounded the nucleus and extended throughout the cytoplasm (Fig. 6 *B*). Double indirect immunofluorescence microscopy using polyclonal anti-PDI antibody showed the colocalization of β-galactosidase/ALDH chimera and PDI in the ER (Fig. 6 *C*). These results suggested that the carboxyl-terminal 35 amino acids were sufficient for targeting of *E. coli* β-galactosidase to the ER membrane.

Both Stem and Tail Regions of msALDH Contain ER-targeting Sequences

We attempted to define the sequence requirement for ER targeting within the last 35 amino acids of msALDH, which is composed of three regions as shown in Fig. 1, and we constructed a series of CAT/ALDH chimeras (CAT/ALDH1-4) (Fig. 7). CAT/ALDH1 contains the last 35 amino acids of msALDH at the carboxyl terminus of CAT, while CAT/ALDH2-4 chimeras lack either the tail, stem, or both regions of msALDH. COS-1 cells were transfected with these cDNAs in the pMIW expression vector that possesses β-actin promoter and Rous sarcoma enhancer. The intracellular localization of the expressed proteins were determined by indirect immunofluorescence microscopy using anti-CAT antibody. Wild-type CAT was distributed, as expected, throughout the cytoplasm. In addition, it was also detected in the nucleoplasm (Fig. 8 *A*). CAT/ALDH1 showed the characteristic ER staining pattern (Fig. 8 *B*), which was confirmed by double indirect immunofluorescence microscopy using PDI mAb (Fig. 8 *C*). This result was similar to that obtained with β-galactosidase/ALDH (Fig. 6, *B* and *C*). Both CAT/ALDH2 and CAT/ALDH3 were also found to colocalize with endogenous PDI in the ER (Fig. 8, *D-G*). On the other hand, CAT/ALDH4 containing only the hydrophobic domain of msALDH at its carboxyl terminus showed a similar pattern of staining to that of wild-type CAT. This chimera remained not only in the cytoplasm, but in the nucleoplasm as well (Fig. 8 *H*), and it did not colocalize with PDI (Fig. 8 *I*). The simplest interpretation of these results is that both the stem and tail regions appear to contain ER-targeting information of msALDH.

To elucidate more narrowly the sequence requirement within the stem region for ER targeting, we constructed an additional series of CAT/ALDH chimeras (CAT/ALDH5-10) (Fig. 9), in which three to seven amino acids of the stem region of either CAT/ALDH1 or CAT/ALDH2 are deleted. The intracellular localization of these chimeras expressed in COS-1 cells was analyzed by indirect immunofluorescence microscopy. Three chimeras, CAT/ALDH5, CAT/ALDH7,
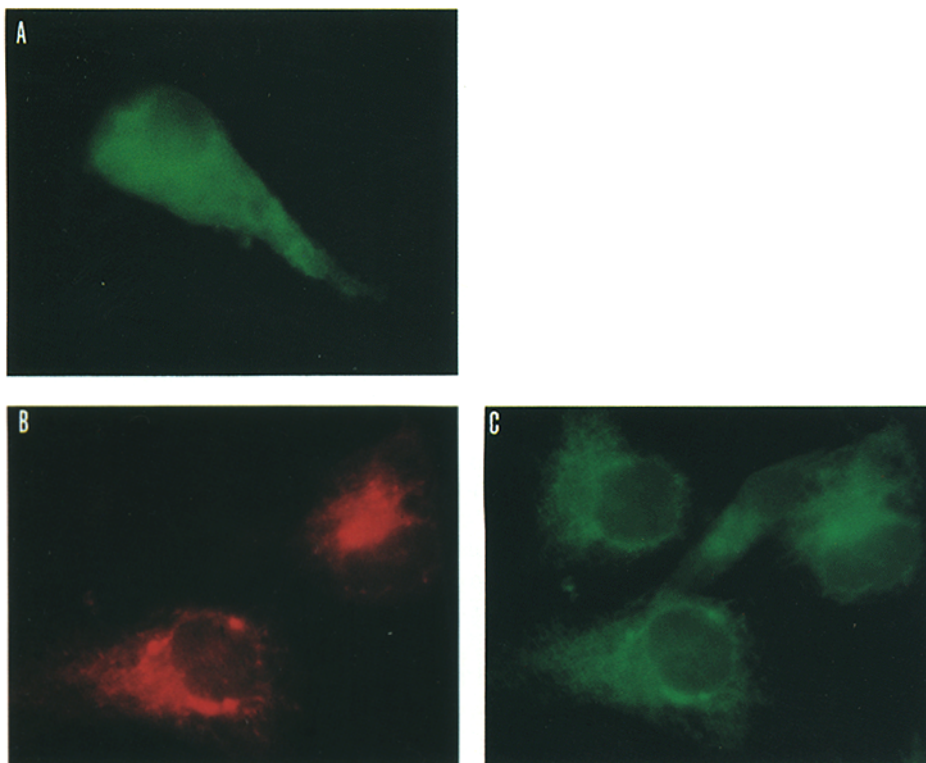
Figure 6. Localization of β-galactosidase and β-galactosidase/ALDH in transfected cells by double indirect immunofluorescence microscopy. COS-1 cells were transfected with pCH110 (*A*) or pCH110/ALDH (*B* and *C*), fixed 44 h after transfection, and permeabilized. mAb to β-galactosidase was used to stain β-galactosidase (*A*) or β-galactosidase/ALDH (*B*). Polyclonal anti-PDI antibody was used for detection of endogenous PDI (*C*).

and CAT/ALDH9, were supposed to be directed to the ER by virtue of the tail region, even if they lack an ER targeting sequence within the stem region, and indeed they showed the reticular staining pattern including the nuclear membrane (Fig. 10, *A*, *C*, and *E*). Among the mutant proteins lacking the tail region, CAT/ALDH6 and CAT/ALDH10 showed the reticular pattern (Fig. 10, *B* and *F*). These reticular compartments were confirmed to be the ER by colocalization with PDI (data not shown). In contrast, CAT/ALDH8, which lacks the Lys-Gln-Phe sequence (amino acids 457-459) within the stem region and the tail region, was found distributed diffusely throughout the cytoplasm and the nucleoplasm (Fig. 10 *D*).

We investigated in more detail the intracellular localization of CAT and CAT/ALDH chimeras in transfected COS-1 cells using an IgG-gold immunoelectron microscopic technique on frozen ultrathin sections. Gold particles were distributed in the cytoplasm and in the nucleoplasm in COS-1 cells transfected with pMIWCAT (Fig. 11 *A*). A similar distribution of gold particles was observed in COS-1 cells expressing either CAT/ALDH4 (data not shown) or CAT/ALDH8 (Fig. 11 *D*). In marked contrast, gold particles were predominantly detected on the cytoplasmic surface of the ER membrane in COS-1 cells expressing CAT/ALDH1 (Fig. 11 *B*), CAT/ALDH7 (Fig. 11 *C*), or the other CAT/ALDH chimeras (data not shown). These immunoelectron micro-
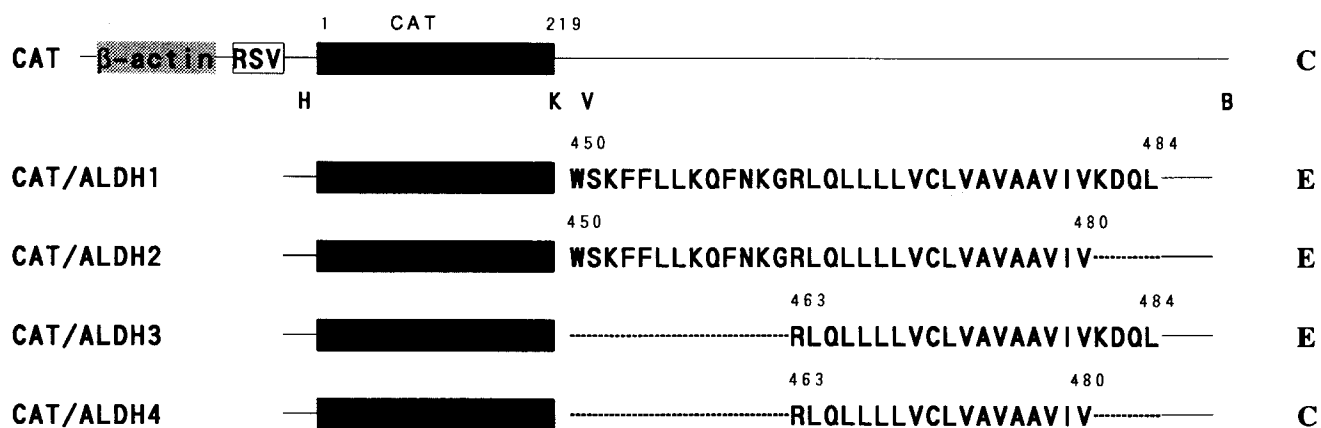
Figure 7. Schematic diagrams of CAT and CAT/ALDH1-4 chimeras in the pMIW expression vector. β-actin and RSV mean the β-actin promoter and Rous sarcoma enhancer, respectively. The coding sequence of CAT is indicated by the solid bar. The single amino acid code is used to represent the carboxyl-terminal sequences of msALDH. Deleted sequences are indicated by broken lines. The amino acid numbers of each protein are shown at the top, and the relevant restriction endonuclease sites at the bottom. *H*, HindIII; *K*, KpnI; *V*, EcoRV; *B*, BamHI. To the right, the intracellular location of each protein is noted as *E* for ER or *C* for cytosolic.
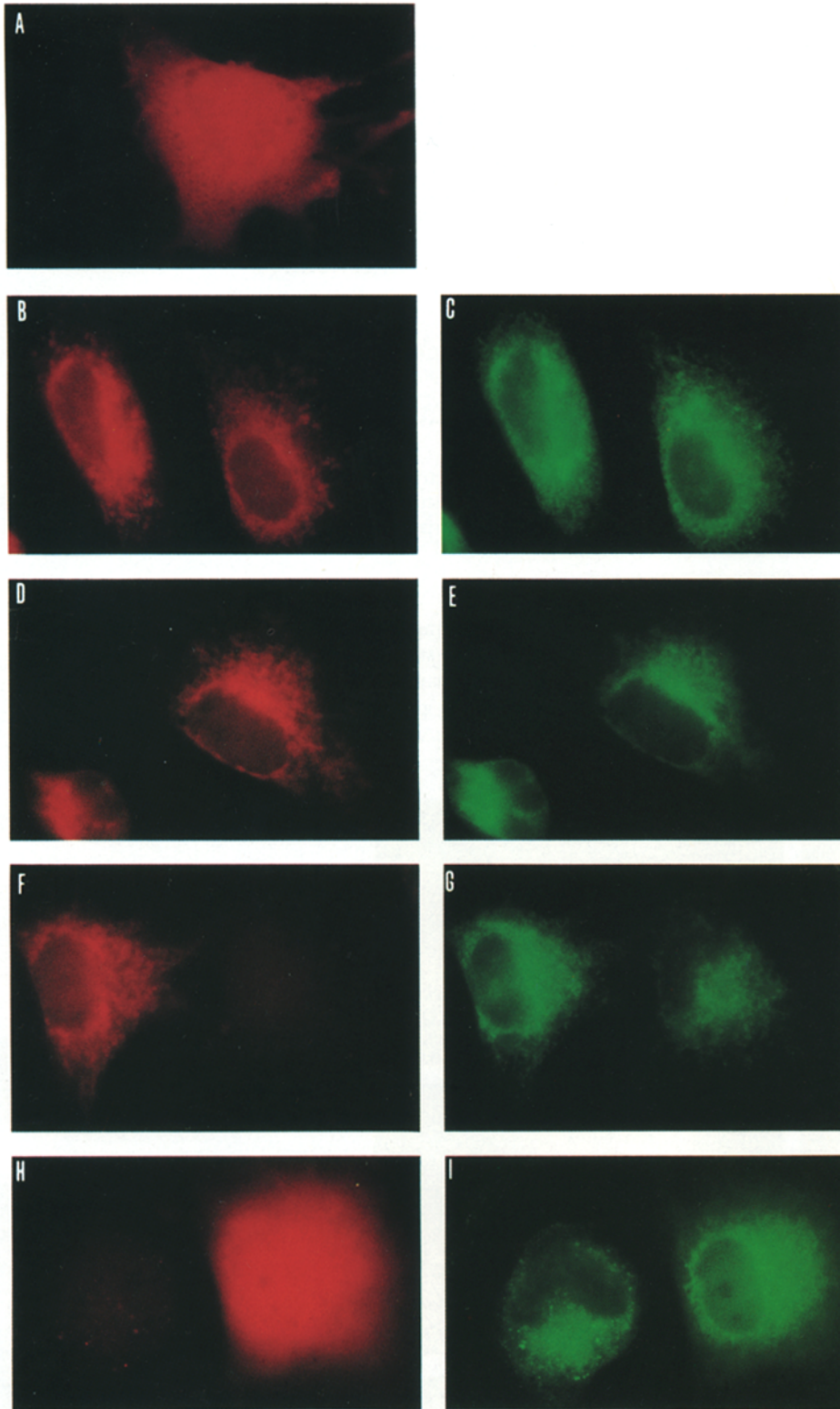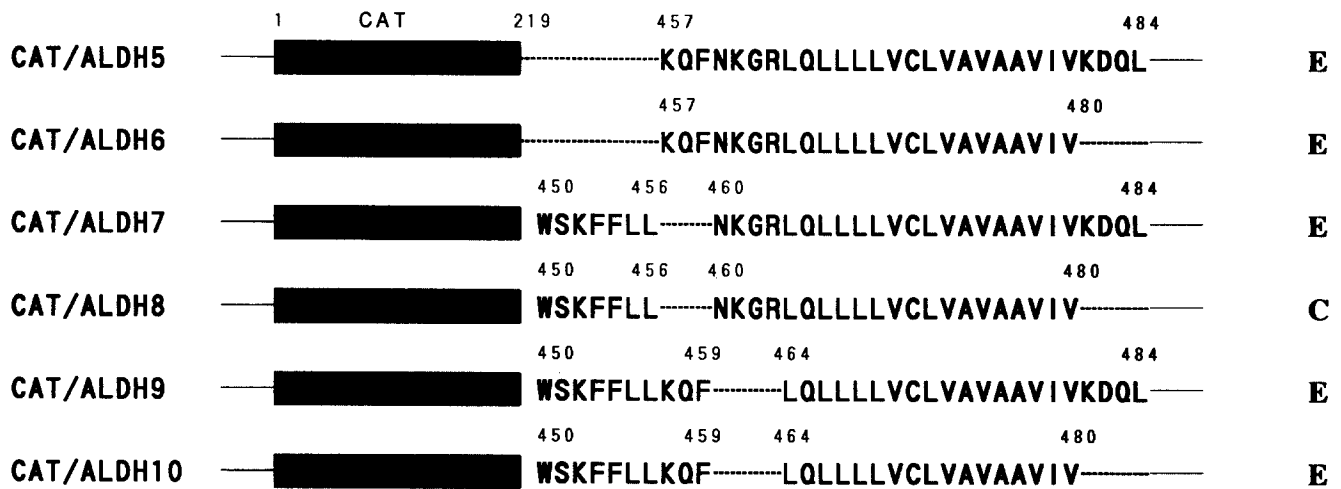
Figure 8. Localization of CAT and CAT/ALDH1-4 chimeras in transfected cells by double indirect immunofluorescence microscopy. COS-1 cells were transfected with pMIW-CAT (*A*), pMIWCAT/ALDH1 (*B* and *C*), pMIWCAT/ALDH2 (*D* and *E*), pMIWCAT/ALDH3 (*F* and *G*), or pMIWCAT/ALDH4 (*H* and *I*), fixed 44 h after transfection, and permeabilized. Wild-type CAT (*A*) and CAT/ALDH chimeras (*B, D, F,* and *H*) were detected by anti-CAT antibody. Endogenous PDI (*C, E, G,* and *I*) was stained with mAb to PDI.

scopic data are consistent with indirect immunofluorescent data, and these immunolocalization data together strongly suggest that the ER-targeting sequences of msALDH exist within the Lys-Gln-Phe sequence in the stem region and within the Lys-Asp-Gln-Leu sequence of the tail region.

Discussion

### Expression of msALDH in COS-1 Cells Alters the ER Structure

We have shown here by subcellular fractionation and indirect

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                 1      CAT        219      457                                        484
CAT/ALDH5    ————■■■■■■■■■■■■——————————KQFNKGRLQLLLLVCLVAVAAVIVKDQL——         E

                                         457                                    480
CAT/ALDH6    ————■■■■■■■■■■■■——————————KQFNKGRLQLLLLVCLVAVAAVIV—————         E

                 450     456    460                                        484
CAT/ALDH7    ————■■■■■■■■■■■■  WSKFFLL———————NKGRLQLLLLVCLVAVAAVIVKDQL——         E

                 450     456    460                                        484
CAT/ALDH8    ————■■■■■■■■■■■■  WSKFFLL———————NKGRLQLLLLVCLVAVAAVIV—————         C

                 450     459    464                                        484
CAT/ALDH9    ————■■■■■■■■■■■■  WSKFFLLKQF———————LQLLLLVCLVAVAAVIVKDQL——         E

                 450     459    464                                        480
CAT/ALDH10   ————■■■■■■■■■■■■  WSKFFLLKQF———————LQLLLLVCLVAVAAVIV—————         E
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Figure 9. Schematic diagrams of CAT/ALDH5-10 chimeras in the pMIW expression vector. The coding sequence of CAT is indicated by the solid bar. The single amino acid code is used to represent the carboxyl-terminal sequences of msALDH. Deleted sequences are indicated by broken lines. The amino acid numbers of each protein are shown at the top. To the right, the intracellular location of each protein is noted as *E* for ER or *C* for cytosolic.

immunofluorescence experiments that not only msALDH, but also msALDHΔ481-484, colocalizes with two ER marker proteins, FP2 and PDI, when expressed from cDNAs in COS-1 cells. Surprisingly, indirect immunofluorescence microscopy revealed the apparent alteration of the ER structure in cells expressing either msALDH or msALDH-Δ481-484. The altered ER structure was characterized by large vesicular structures mainly located near the nucleus. Since the unusual staining pattern was observed by expression of msALDH or msALDHΔ481-484, it appeared that the ER an-
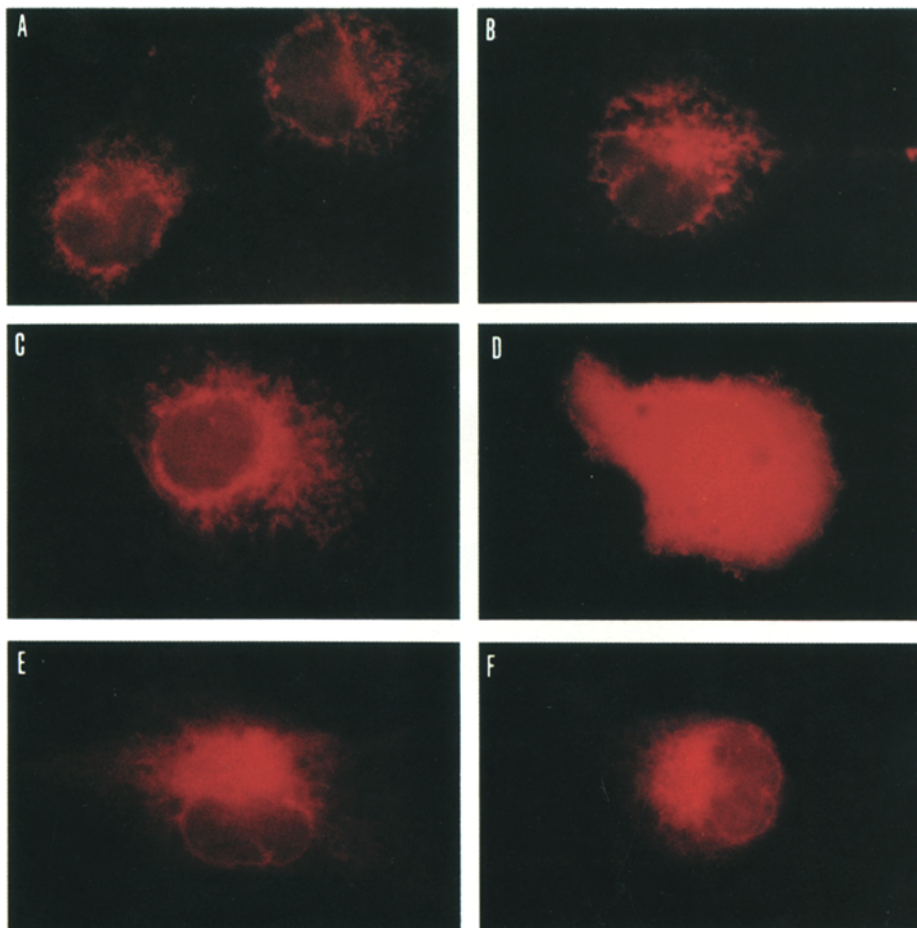
Figure 10. Localization of CAT/ALDH5-10 chimeras in transfected cells by indirect immunofluorescence microscopy. COS-1 cells were transfected with pMIWCAT/ALDH5 (*A*), pMIWCAT/ALDH6 (*B*), pMIWCAT/ALDH7 (*C*), pMIWCAT/ALDH8 (*D*), pMIWCAT/ALDH9 (*E*), or pMIWCAT/ALDH10 (*F*), fixed 44 h after transfection, permeabilized, and stained with anti-CAT antibody.
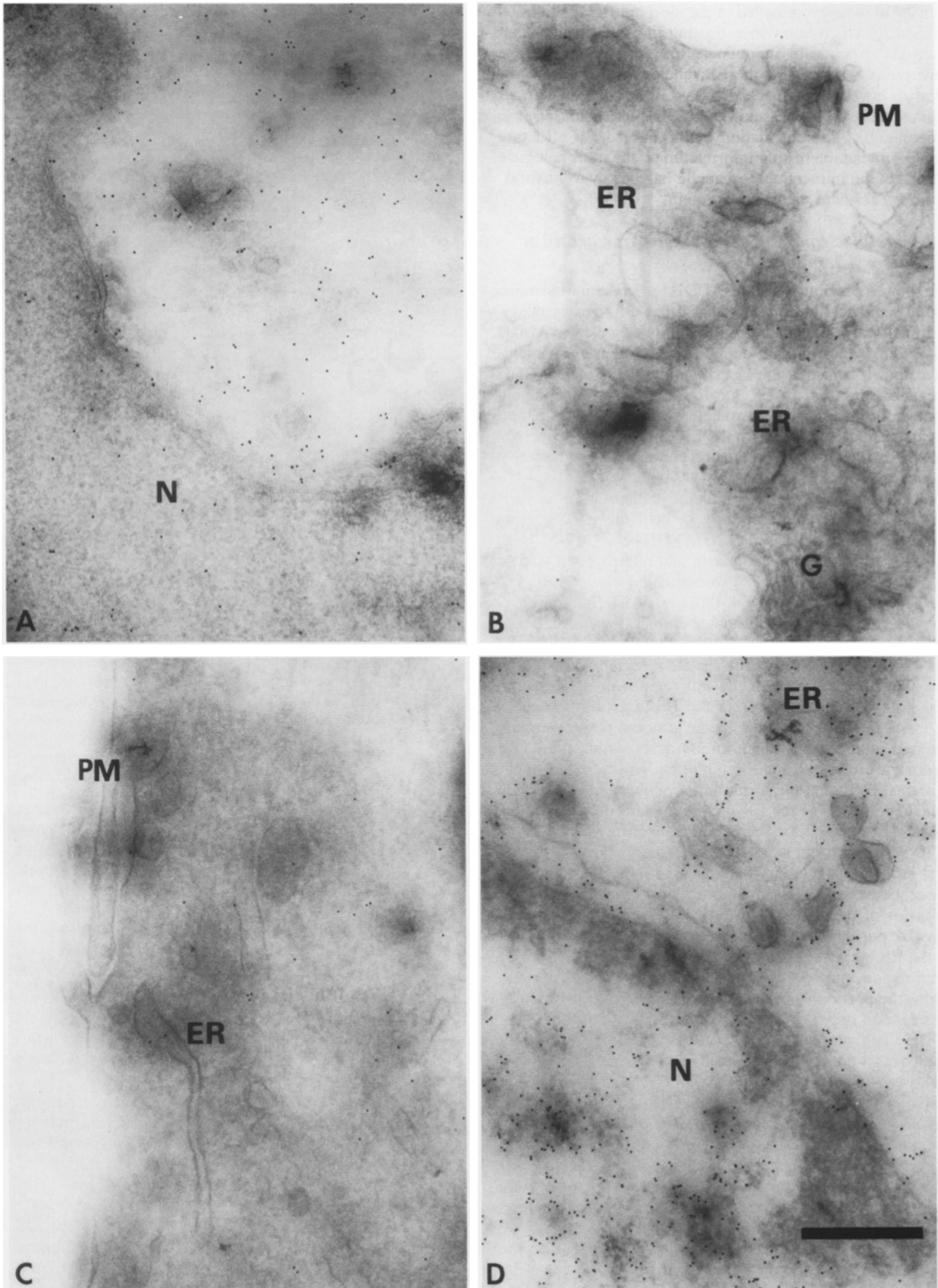
Figure 11. Immunogold electron microscopic localization of CAT and CAT/ALDH chimeras in transfected cells. COS-1 cells were transfected with pMIWCAT (*A*), pMIWCAT/ALDH1 (*B*), pMIWCAT/ALDH7 (*C*), or pMIWCAT/ALDH8 (*D*), and fixed 44 h after transfection. Ultrathin cryosections of COS-1 cells were reacted with anti-CAT antibody and subsequently with goat anti-rabbit IgG gold conjugate (10 nm in diameter). *ER*, endoplasmic reticulum; G, Golgi apparatus; *PM*, plasma membrane; *N*, nucleus. Bar, 500 nm.

choring of these proteins led to the morphological alteration of the ER. We are now investigating in more detail the altered ER structure by immunoelectron microscopy, and we have recently found that the ER tubules in COS-1 cells expressing msALDH are packed in crystalloid hexagonal arrays (Yamamoto, A., R. Masaki, and Y. Tashiro, manuscript in preparation). The elucidation of the altered ER structure would provide interesting information on the morphological change of the intracellular organelle induced by overexpression of a resident membrane protein.

ER-targeting Sequences of msALDH Are Located at Its Carboxyl Terminus

We showed previously that msALDH has no amino-terminal signal sequence, but instead, that it has a longer carboxyl-terminal portion than cytosolic tumor-associated ALDH (24). Our truncation and domain replacement experiments have shown here that the last 35 amino acids of msALDH are necessary for its ER localization and also sufficient enough for localization of two heterologous proteins, E. coli β-galactosidase and CAT, to the ER. We therefore conclude that the carboxyl-terminal portion contains an ER-targeting sequence of msALDH.

Further analyses on the intracellular distribution of various CAT/ALDH chimeras have defined the ER targeting sequence of msALDH. Both CAT/ALDH4 and CAT/ALDH8 were distributed in the cytoplasm and nucleoplasm similar to wild-type CAT in spite of possessing the whole hydrophobic domain (amino acids 464-480) of msALDH at their carboxyl termini, whereas the other chimeras were predominantly localized to the ER. The two chimeras are lacking the Lys-Gln-Phe sequence within the stem region and the Lys-Asp-Gln-Leu sequence of the tail region, thus suggesting that both sequences represent the ER-targeting sequences of msALDH. We are now in the process of determining through mutagenesis which amino acids within these two regions are required for ER targeting. On the other hand, it seems likely that the hydrophobic sequence of msALDH functions mainly as the ER membrane anchor because this portion is essential for membrane anchoring.

To the best of our knowledge, our data characterize the first intracellular protein that appears to have two targeting sequences separated by a membrane anchor. The ambiguous behavior of msALDHΔ471-484 could be explained as follows. A portion of this truncated protein could be targeted to the ER by virtue of the Lys-Gln-Phe sequence within the stem region, but because of the shortened hydrophobic domain, the portion would be either released into the cytosol or maintain only a loose association with the membrane.

The significance of the presence of two separated targeting sequences is not yet clear. A small portion of msALDHΔ481-484 was, in fact, recovered in the S3 fraction, whereas only insignificant amounts of msALDH or FP2 (Fig. 2, B and C), which is efficiently targeted to the ER through the amino-terminal signal sequence, were recovered in the S3 fraction. Therefore, it is likely that the presence of two targeting sequences in the same protein might be a means to ensure the more efficient ER localization of msALDH. In addition, the hydrophilic tail region might also serve to increase the strength of the membrane anchoring, as indicated by the partial membrane extraction of msALDHΔ481-484 with Na_2CO_3 (Fig. 3). Although it is not yet clear whether the

A

B
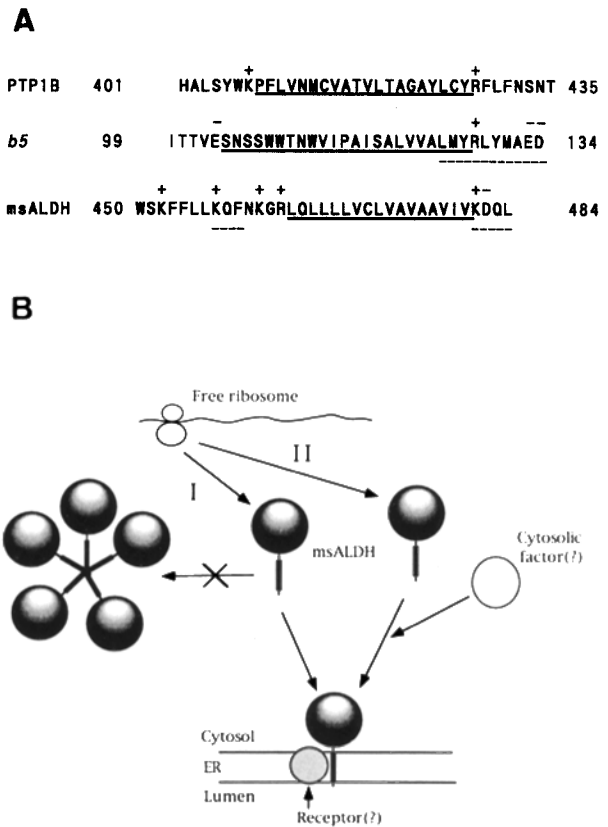
Figure 12. Possible mechanisms for ER targeting of msALDH. (A) Alignment of the sequences of the carboxyl-terminal 35 amino acids of human PTP 1B (amino acids 401-435) (9), rat cytochrome b5 (amino acids 99-134) (32), and rat msALDH (amino acids 450-484) (24). The single amino acid code is used. Positively and negatively charged amino acids are marked + and − at the top, respectively. Each hydrophobic domain is underlined and the ER targeting sequences of either cytochrome b5 (23) or msALDH are indicated by broken lines. (B) Two possible models for ER targeting of msALDH. In model I, the aggregation of the newly synthesized msALDH polypeptides in the cytoplasm is prevented by the hydrophilic ER targeting sequences adjacent to the hydrophobic anchoring sequence. The monomeric msALDH would be inserted into the ER membrane with the aid of a receptor protein on the ER membrane. In model II, the complex of msALDH and a cytosolic receptor protein would be targeted to the ER, and msALDH would be inserted correctly into the ER membrane spontaneously or through the interaction with a receptor protein on the membrane.

hydrophobic segment fully transverses the membrane, this transmembrane disposition seems more likely to explain the tight anchoring of the protein in the ER membrane than a loop model where the hydrophilic tail remains on the cytoplasmic surface.

The Lys-Asp-Gln-Leu sequence of the tail region strongly resembles the well-characterized Lys-Asp-Glu-Leu retention signal found in the ER luminal proteins (25), suggesting that the Lys-Asp-Gln-Leu sequence, if translocated into the lumen of the ER, serves as an ER retention signal of msALDH. However, Andres et al. have shown that proneuropeptide Y mutant bearing the Lys-Asp-Gln-Leu sequence at the carboxyl terminus is processed and secreted like wild-type proneuropeptide Y when expressed in AtT-20 cells, despite the intracellular retention of the unprocessed proneuropep-

tide Y with the Lys-Asp-Glu-Leu sequence (4), thus suggesting the important role of the acidic Glu residue in ER retention. We are now studying the membrane topology of msALDH and the intracellular localization of both msALDH and msALDHΔ481-484 in more detail by immunoelectron microscopy to check the potential role of the tail region for ER retention.

Novel ER targeting sequences have recently been reported for two other tail-anchored proteins. Frangioni et al. have found that the ER targeting sequence of protein tyrosine phosphatase 1B (PTP 1B) exists within the carboxyl-terminal 35 amino acids (12). Mitoma and Ito have shown that the last 10 amino acids of cytochrome b5, which include the hydrophilic tail of this protein, are important for its targeting to the ER (23). Fig. 12 A shows an alignment of the last 35 amino acids of PTP 1B (9), cytochrome b5 (32), and msALDH (24), where each hydrophobic domain is underlined and the ER-targeting sequences of cytochrome b5 and msALDH are indicated by the broken line. Although the sequence required for ER targeting within the last 35 amino acids of PTP 1B has not been defined, PTP 1B and cytochrome b5 have the common Leu-X-Tyr-Arg motif (Fig. 12 A) within their last 10 amino acids. In addition, PTP 1B and cytochrome b5 have tails of similar length (seven and eight amino acid residues, respectively), which are longer than that (four amino acid residues) of msALDH. It therefore seems likely that PTP 1B possesses a similar ER-targeting sequence to that of cytochrome b5. We have not found in the carboxyl-terminal portion of msALDH a motif similar to that in those two proteins, despite the fact that the ER-targeting sequences of cytochrome b5 and msALDH are both hydrophilic and adjacent to the membrane anchors. In addition, the presence in msALDH of two ER-targeting sequences separated by the membrane-anchoring domain suggests that this protein, with respect to the ER-targeting sequence, belongs to a different class than the other two.

Possible Mechanisms for ER Targeting

In addition to the three proteins described above, several other ER membrane proteins are known that also have carboxyl-terminal anchors, such as heme oxygenase (40) and ribosome-binding protein p34 (28), but ER-targeting sequences of these proteins have not yet been identified. Cytochrome b5, heme oxygenase, and msALDH are synthesized on free polysomes (29, 41, 45) and posttranslationally targeted to the ER. Indeed, the SRP-independent integration of cytochrome b5 into microsomal membranes has been demonstrated (3). Our present study, together with these findings, suggests a novel pathway by which a class of tail-anchored proteins with a novel targeting sequence is directed to the ER.

How might the ER-targeting sequence function? Fig. 12 B shows two possible models to explain the ER targeting of msALDH. One possibility (I) is that the hydrophilic targeting sequences serve to prevent the aggregation of the newly synthesized msALDH polypeptides through their hydrophobic anchoring sequences, which makes it easier for them finding a specific receptor on the ER membrane. Another possibility (II) is that a cytosolic receptor binds to the targeting sequences of msALDH to form a complex that effects the correct insertion of the carboxyl-terminal anchor into the ER membrane spontaneously or through their interactions with

a receptor on the membrane. The elucidation of the mechanisms by which ER localization of msALDH occurs should provide valuable clues for an understanding of the posttranslational targeting and insertion of a class of membrane proteins with a carboxyl-terminal anchor.

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