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Post-Translational Targeting of a Tail-Anchored Green Fluorescent Protein to the Endolpasmic Reticulum

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Microsomal aldehyde dehydrogenase (msALDH) is a tail-anchored protein localized to the cytoplasmic face of the endoplasmic reticulum (ER). The carboxyl-terminal 35 amino acids of msALDH possess ER-targeting sequences in addition to a hydrophobic membrane-spanning domain. To study the mechanism for ER targeting of this protein *in vivo*, we took advantage of a green fluorescent protein-msALDH fusion protein containing the last 35 amino acids of msALDH [GFPALDH(35)]. When expressed from cDNA in COS-7 cells, the fusion protein was localized to the ER. We then prepared a recombinant fusion protein and injected it into the cytoplasm of COS-7 cells. The injected protein was correctly localized to the ER after a 30-min incubation at 37°C. However, a recombinant fusion protein that contained only the transmembrane domain of msALDH failed to be targeted to the ER. When the assay was carried out at 4°C, the recombinant GFPALDH(35) remained in the cytoplasm. Moreover, incubation of COS-7 cells under conditions of ATP depletion resulted in the cytoplasmic distribution of the injected protein. These results indicate that GFPALDH(35) is targeted to the ER post-translationally *via* an ATP-dependent pathway. This microinjection system worked effectively in different mammalian cell types, suggesting a common mechanism for ER targeting of the tail-anchored protein.

Key words: GFP fusion protein, microinjection, msALDH, post-translational ER targeting, tail-anchored protein.

Abbreviations: DMEM, Dulbecco's modified Eagle medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; GFP, green fluorescent protein; msALDH, microsomal aldehyde dehydrogenase; PDI, protein disulfide isomerase; PB, sodium phosphate buffer; PBS, phosphate-buffered saline; SRP, signal recognition particle; Vamp, vesicle-associated membrane protein; 6×His tag, tag consisting of six consecutive histidine residues.

In eukaryotic cells, membrane proteins possessing either a signal sequence or a signal/anchor sequence at their amino termini are localized along the exocytic pathway. These proteins are targeted to the endoplasmic reticulum (ER) *via* a signal recognition particle (SRP)–dependent pathway and co-translationally integrated into the ER membrane (*1–3*). In addition, mechanisms for intracellular sorting of these proteins after integration into the ER membrane have been well elucidated (*4–6*).

On the other hand, membrane proteins without a signal sequence are found in the cytoplasmic face of intracellular organelles such as the ER, Golgi, vacuole/lysosome, intracellular vesicle, plasma membrane and mitochondria (*7–16*). A common characteristic of these proteins is a carboxyl-terminal hydrophobic sequence, which functions as a membrane anchor. Thereby, these membrane proteins display large functional domains in the cytoplasm and are referred to as tail-anchored proteins (*7*). These proteins are classified into two categories according to their intracellular localization. One includes proteins localized along the exocytic pathway, and the other includes proteins localized to the mitochondrial outer membrane. Recent studies have shown that tail-anchored proteins found in the exocytic pathway are first inserted into the ER membrane and subsequently sorted to their final destinations by vesicle-mediated trafficking (*17–19*), similar to membrane proteins synthesized on the rough ER. Therefore, the ER is a unique organelle into which membrane proteins with or without a signal sequence are integrated. However, little is known about ER targeting and integration of tail-anchored proteins.

Microsomal aldehyde dehydrogenase (msALDH) is a typical tail-anchored protein localized to the ER (*11*, *20*). We demonstrated that the carboxyl-terminal 35 amino acids of msALDH contain both a membrane-binding domain and ER-targeting sequences (*21*). We also showed that the membrane-binding domain of msALDH spans the ER membrane and suggested that the protein is retained in the ER by a static retention mechanism (*22*). However, it remains unsolved how msALDH is targeted to and integrated into the ER membrane in a transmembrane topology. In this study, we have used *Aequorea victoria* green fluorescent protein (GFP) as a reporter protein to investigate the mechanism for ER targeting of msALDH. Our microinjection experiments using a

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recombinant GFP-msALDH fusion protein have shown that the protein is post-translationally targeted to the ER in living cells and that the ER-targeting reaction is dependent on both temperature and ATP. Our results also suggest a common mechanism for ER targeting of the tail-anchored protein among mammalian cells.

MATERIALS AND METHODS

Materials—Fetal bovine serum (FBS) was obtained from Filtron (Brooklyn, Australia). Dulbecco's modified Eagle medium (DMEM) and Ham's F12 medium were from GIBCO-BRL (Rockville, MD, USA). Peroxidase-conjugated goat anti-rabbit IgG and a fluorolink Cy3-labeled anti-rabbit IgG were purchased from Tago (Burlingame, CA, USA) and Amersham Biosciences (Uppsala, Sweden), respectively. Rabbit anti-GFP antiserums for immunoblotting and immunoelectron microscopy were from Molecular Probes (Leiden, Netherlands) and Clontech (Palo Alto, CA, USA), respectively. Rabbit antibodies against rat protein disulfide isomerase (PDI) were prepared and characterized as described (*23*). A carboxyl-terminal enhanced GFP vector, pEGFPC-1, was obtained from Clontech. A bacterial expression vector, pQE-30, and Ni-NTA agarose were from Qiagen (Hilden, Germany). Restriction enzymes and DNA-modifying enzymes were obtained from Takara (Kyoto). CELLocate 5245 and Celldesk LF coverslips were from Eppendorf (Hamburg, Germany) and Sumitomo Bakelite (Tokyo), respectively. Immersible CX-10 ultrafilters and Ultrafree-MC centrifugal filter units were purchased from Millipore (Bedford, MA, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka).

Plasmid Construction—Chimeric cDNAs for GFP-msALDH fusion proteins were constructed by PCR. The following pairs of oligonucleotides and templates were used: 5′-GAGTCCAAGGGTACCTGGTCGAAATTC-3′ and 5′-AATTCCTCGAGAAGCTTGGTGTGTTAACCATTTGG-3′, and pMIWALDH (*22*) to amplify the ALDH(35) fragment; 5′-AAACAGTTCAACGGTACCAGGCTGCAGCTG-3′ and 5′-AATTCCTCGAGAAGCTTGGTGTGTTAACCATT-TGG-3′, and pCDALDHΔ481–484 (*21*) to amplify the ALDH(18) fragment. The amplified fragments were digested with *Kpn*I and *Hpa*I, then ligated into the *Kpn*I–*Sma*I sites of pEGFPC-1. The resultant plasmids were designated as pEGFPALDH(35) and pEGFPALDH(18), respectively.

To construct pQEGFPC-1, a full-length cDNA for GFP was isolated from pEGFPC-1 by PCR using the following oligonucleotides: 5′-GCCACCGCATGCAGCAAGGGCG-AGGAGCTGTTCA-3′ and 5′-GTATGGCTGATATCGAT-CAGTTATCT-3′. The obtained DNA was digested with S*ph*I and *Eco*RV, then subcloned into the *Sph*I–*Sma*I sites of pQE-30. The resultant plasmid encodes GFP with an additional MGGSHHHHHHGS sequence at its amino terminus. Two bacterial expression plasmids, pQEGF-PALDH(35) and pQEGFPALDH(18), were produced according to the methods for construction of pEGF-PALDH(35) and pEGFPALDH(18), respectively, as described above. All constructions were screened by restriction enzyme mapping and confirmed by DNA sequencing.

Purification of Recombinant Proteins—Three recombinant proteins with a tag consisting of six consecutive histidine residues (6×His tag), referred to as rGFP, rGF-PALDH(35) and rGFPALDH(18), were prepared according to the manufacturer's protocol (Qiagen) with some modifications. *Escherichia coli* JM109 cells were transformed with pQEGFPC-1, pQEGFPALDH(35) or pQEGF-PALDH(18), and grown in terrific broth medium containing ampicillin at 37°C overnight. The culture was diluted 50-fold with fresh medium and allowed to grow for 2 h. The recombinant proteins were induced by incubation with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. The cells were harvested by centrifugation at 4,000 × *g* and 4°C for 20 min and resuspended in lysis buffer [50 mM sodium phosphate buffer (PB), pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100]. They were then disrupted by sonication for 5 min on ice. The following procedures were carried out at 4°C. The extract was clarified by centrifugation at 10,000 × *g* for 20 min, diluted 4-fold with wash buffer (50 mM PB, pH 8.0, 300 mM NaCl, and 20 mM imidazole) containing 1% Triton X-100, then applied to Ni-NTA resin equilibrated with the same solution. The column was washed with 6 column volumes of wash buffer containing 0.2% Triton X-100 and further washed with 15 column volumes of wash buffer to remove Triton X-100. The recombinant proteins were then eluted with elution buffer (50 mM PB, pH 8.0, 150 mM NaCl, and 250 mM imidazole). The protein-containing fractions were pooled and concentrated with Immersible CX-10 Ultrafilters.

Cell Culture and Transfection—Cells were incubated at 37°C under 5% CO_2 in the following medium. COS-7 cells, normal rat kidney (NRK) cells and A431 cells were cultured in DMEM (high glucose) supplemented with 10% FBS and 10 mM HEPES-KOH, pH 7.2. Chinese hamster ovary (CHO)-K1 cells were cultured in Ham's F12 medium with 10% FBS and 10 mM HEPES-KOH, pH 7.2. Cells were grown on a 22 × 22-mm glass coverslip and on a Celldesk coverslip in a six-well dish for fluorescence and immunoelectron microscopic analyses, respectively. For subcellular fractionation experiments, COS-7 cells were grown in a 100-mm dish. The expression plasmids were transfected into cells with a SuperFect transfection reagent (Qiagen). Forty-four hours after transfection, the cells were fixed for microscopic analysis or harvested for subcellular fractionation.

Subcellular Fractionation—Membrane and soluble fractions of COS-7 cells were prepared as described previously (*21*). Membrane extraction using 100 mM sodium carbonate, pH 11.5, was performed according to the method of Fujiki *et al*. (*24*). Protein was assayed by the method of Bradford (*25*). Samples were separated by SDS-PAGE (10%) and electroblotted to a durapore membrane, then immunostained using rabbit anti-GFP antiserum and peroxidase-conjugated goat anti-rabbit IgG as described (*21*).

Microinjection—For microinjection experiments, cells were grown on a CELLocate coverslip in a 35-mm dish to identify injected cells. The medium was replaced just before injection. The recombinant proteins were diluted to the concentration of 0.5 mg/ml with phosphate-buffered saline (PBS) and filtered through an Ultrafree-MC centrifugal filter unit before use. Microinjection experiments were conducted using an Eppendorf Microinjector

5242 with Microloader (Eppendorf). The cells were fixed immediately after injection or incubated either at 37°C or at 4°C before fixation.

Immunofluorescence Microscopy—All procedures, except for incubation with antibodies at 32°C, were carried out at room temperature. After incubation at 37°C or 4°C, transfected or injected cells were fixed with 4% paraformalehyde in PBS for 20 min and permeabilized in 0.1% Triton X-100/PBS for 30 min. They were then blocked in 2% FBS/PBS for 30 min, followed by a 30-min incubation with rabbit anti-PDI antibodies in 2% FBS/PBS. After washing four times (5 min each) with PBS, the cells were incubated with a fluorolink Cy3-labeled anti-rabbit IgG in 2% FBS/PBS for 30 min. After washing four times (5 min each) with PBS, glass or CELLocate coverslips were examined using an Olympus BX50 microscope (Olympus, Tokyo) equipped with a Quantix 1400 digital cooled CCD camera (Photometrics, Tucson, AZ, USA).

Immunoelectron Microscopy—The pre-embedding silver enhancement immunogold method was performed as described (*26*) with a slight modification. Unless otherwise stated, all procedures were carried out at room temperature. Transfected or injected cells were fixed with 4% paraformaldehyde and 1% glutaraldehyde in 100 mM PB, pH 7.4, for 20 min. The cells were washed three times (5 min each) with 100 mM PB, treated with 0.5% $NaBH_4$ in 100 mM PB for 20 min, and washed again with 100 mM PB. The cells were soaked in 100 mM PB containing 14% glycerol and 35% sucrose, frozen in liquid N_2, and thawed. They were then blocked in 100 mM PB containing 0.005% saponin, 10% BSA, 10% normal goat serum, and 0.1% cold water fish skin gelatin (Sigma, St Louis, MO, USA) for 30 min, and reacted with rabbit anti-GFP antiserum in the blocking solution overnight. After washing six times (10 min each) with 100 mM PB containing 0.005% saponin, the cells were incubated with goat anti-rabbit IgG conjugated to colloidal gold (1.4 nm diameter) in the blocking solution for 2 h. They were then washed six times (10 min each) with 100 mM PB containing 0.005% saponin, and fixed with 1% glutaraldehyde in 100 mM PB for 10 min. Gold labelings were intensified using a silver enhancement kit (Nanoprobes, Yaphank, NY, USA) at 20°C for 6 min in the dark. After washing with distilled water, the cells were postfixed in 0.5% OsO_4 at 4°C for 90 min, washed with distilled water, incubated with 50% ethanol for 10 min, and stained with 2% uranyl acetate in 70% ethanol for 1 h. The cells were further dehydrated with a graded series of ethanol and embedded in an epoxy resin. For observation of the injected cells, positions of the cells of interest were located on an embedded block by referring to grid patterns of CELLocate coverslips, and ultra-thin sections were cut from the position. The sections were doubly stained with uranyl acetate and lead citrate, and observed with a Hitachi H-7000 electron microscope (Hitachi Koki, Tokyo).

ATP Depletion—ATP-depletion experiments were performed as described by Podbilewicz and Mellman (*27*) with some modifications. Briefly, COS-7 cells were incubated in DMEM without glucose and FBS containing 10 mM 2-deoxyglucose, 1 μM antimycin A, and 10 mM HEPES-KOH, pH 7.2, at 4°C for 1 h. The metabolic inhibitors were present during subsequent incubations at 37°C. Control cells were incubated under the same conditions except for elimination of the inhibitors. ATP concentrations were determined using an ATP assay kit (Toyo Ink Mfg., Tokyo) and quantified using a Lumat LB 9507 spectrofluorometer (EG&G Berthold, Bad Wildbad, Germany).

Analytical Ultracentrifuge—Sedimentation velocity measurements were carried out on a Beckman XL-A analytical ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) using An-60ti rotor. The recombinant proteins were diluted to about 0.6 mg/ml with PBS and loaded into centerpieces made of charcoal-filled Epon. The rotor speed was controlled at 30,000 rpm. The samples were scanned at a wavelength of 488 nm.

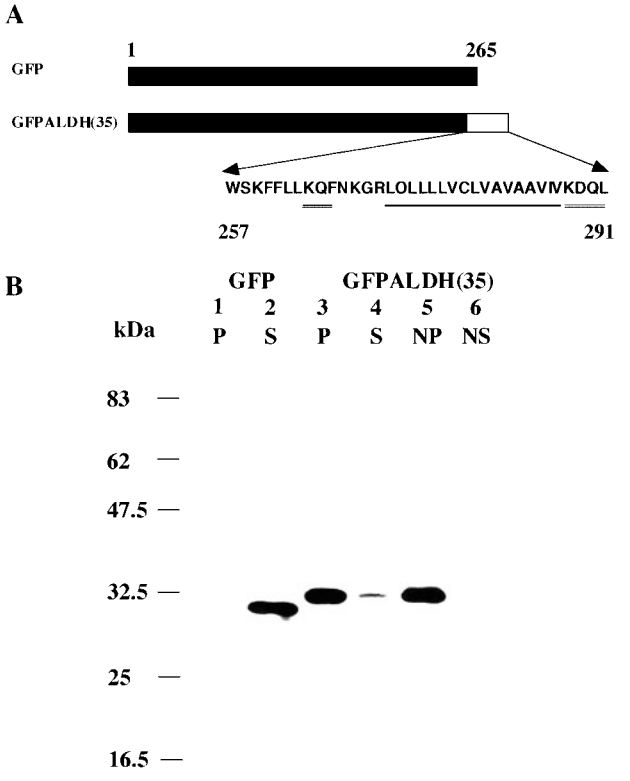

Fig. 1. **Expression constructs and immunoblot analysis of GFP and GFPALDH(35) expressed in COS cells.** (A) Schematic diagrams of GFP and GFPALDH(35) are shown. The solid bars show amino acids 1–265 and 1–256 of GFP and GFPALDH(35), respectively. The single amino acid code is used to represent the carboxyl-terminal sequence of GFPALDH(35), and the amino acid numbers are shown at the bottom. The membrane-binding domain and the ER-targeting sequences of msALDH (*21*) are underlined and double underlined, respectively. (B) COS-7 cells were grown in 10-cm dishes at 37°C, transfected with pEGFPC-1 or pEGFPALDH(35), and harvested 44 h after transfection. Membrane (P) and soluble (S) fractions were prepared by centrifugation of the postnuclear fraction at 88,000 × *g* and 4°C for 100 min. The membrane fraction containing GFPALDH(35) was treated with 100 mM sodium carbonate, pH 11.5, at 0°C for 30 min, then centrifuged at 88,000 × *g* and 4°C for 100 min to separate the pellet (NP) from the supernatant (NS). Each fraction was assayed by immunoblotting using rabbit anti-GFP antibodies and peroxidase-conjugated goat anti-rabbit IgG. Lane 1, GFP (P); lane 2, GFP (S); lane 3, GFPALDH(35) (P); lane 4, GFPALDH(35) (S); lane 5, GFPALDH(35) (NP); lane 6, GFPALDH(35) (NS). Numbers on the left indicate the size (in kDa) of molecular weight markers.

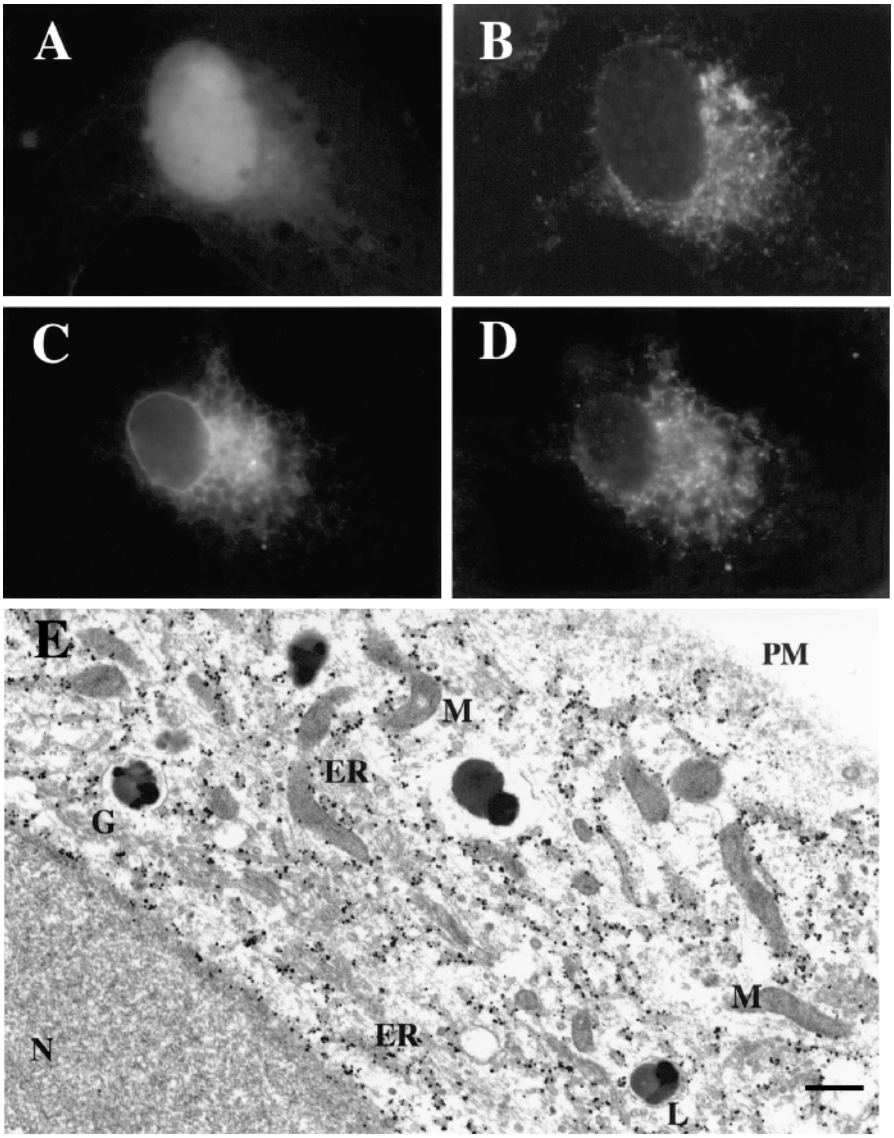

Fig. 2. **Localization of GFP and GFP-ALDH(35) expressed in COS cells.** COS-7 cells were grown on glass coverslips at 37°C and transfected with pEGFPC-1 (A) or pEGFPALDH(35) (C). The cells were fixed 44 h after transfection, permeabilized, then stained with rabbit anti-rat PDI antibodies and a fluorolink Cy3-labeled anti-rabbit IgG (B and D). For immunogold localization, COS-7 cells were grown on Celldesk coverslips at 37°C, transfected with pEGFPALDH(35) (E), and fixed 44 h after transfection. The cells were permeabilised and reacted with rabbit anti-GFP antibodies and immunogold particles. ER, endoplasmic reticulum; G, Golgi complex; L, lysosome; M, mitochondria; N, nucleus; PM, plasma membrane; bar, 500 nm.

RESULTS

Localization of GFP and GFPALDH(35) Expressed in COS Cells—We have previously shown that the carboxyl-terminal 35 amino acids of msALDH contain both a membrane-binding sequence and ER-targeting sequences (*21*). To confirm this, we used GFP as a reporter protein and constructed a mammalian expression plasmid, pEGFPALDH(35), in which the cDNA fragment encoding the last 35 amino acids of msALDH was cloned downstream of pEGFP-C1 (Fig. 1A). These plasmids were transiently transfected into COS-7 cells, and subcellular localization of the expressed proteins was analyzed by immunoblotting. In this experiment, the postnuclear supernatant was centrifuged at 88,000 × *g* for 100 min to separate the membrane fraction from the soluble fraction. As shown in Fig. 1B, lanes 1 and 2, GFP was detected in the soluble fraction of COS-7 cells with an apparent molecular mass of 30 kDa. On the contrary, GFPALDH(35) was recovered in the membrane fraction with an apparent molecular mass of 33 kDa (Fig. 1B, lanes 3 and 4). In addition, GFPALDH(35) in the membrane fraction was resistant to sodium carbonate extraction at pH 11.5 (Fig. 1B, lanes 5 and 6), indicating that the fusion protein is integrated into intracellular membranes.

Subcellular localization of the two proteins was further examined by fluorescence microscopy. As shown in Fig. 2A, GFP was distributed throughout the cytoplasm and also detected in the nucleoplasm. Thus, localization of GFP was different from that of endogenous PDI, an ER marker protein (Fig. 2B). This result was consistent with that of immunoblot analysis. On the other hand, GFPALDH(35) exhibited a typical ER-staining pattern and was indeed co-localized with PDI (Fig. 2, C and D). Immunoelectron microscopy was performed to investigate intracellular localization of GFPALDH(35) in more detail. Gold particles for the fusion protein selectively labeled the cytoplasmic face of the ER and the nuclear membrane, whereas essentially no labeling was seen in the cytoplasm, nucleoplasm, Golgi, plasma membrane, lysosome and mitochondria (Fig. 2E). In marked contrast, gold particles were barely detectable in untrans-

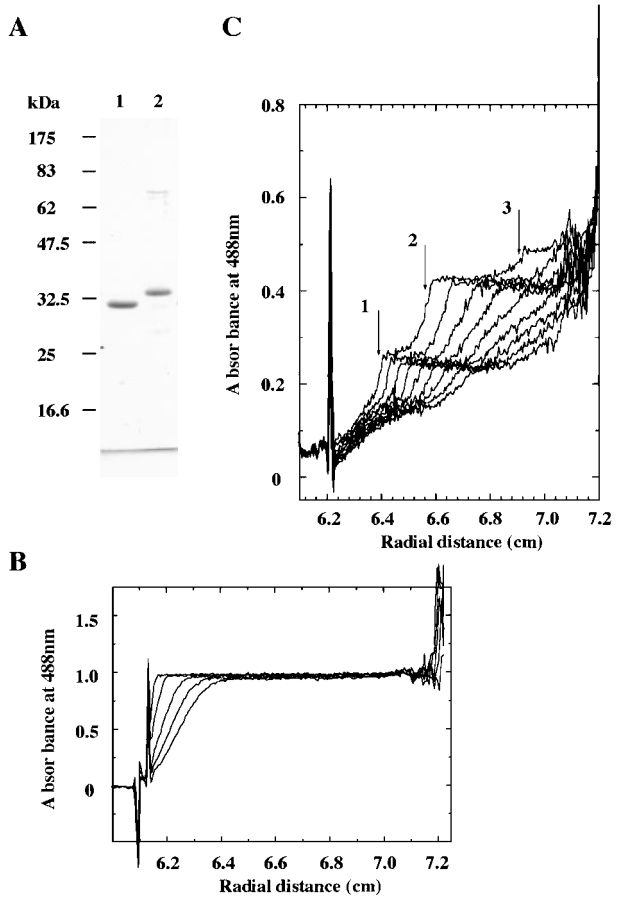

Fig. 3. **Purified recombinant GFP and GFPALDH(35).** (A) rGFP and rGFPALDH(35) were expressed in *Escherichia coli* JM109 cells as 6×His-tagged proteins and purified as described in "MATERIALS AND METHODS." The purified proteins were subjected to SDS-PAGE and stained with Coomassie Blue. Lane 1, rGFP (5 μg); lane 2, rGFPALDH(35) (5 μg). Numbers on the left indicate the size (in kDa) of molecular weight markers. (B) Sedimentation velocity run for rGFP was performed at 30,000 rpm and 25°C. The sample was scanned at a wavelength of 488 nm. Recorded times were 684, 1,269, 2,469, 3,666, and 4,864 s from left to right, respectively. (C) Sedimentation analysis of rGFPALDH(35) was carried out as in (B). Recorded times were 1,734, 2,025, 2,327, 2,625, 2,923, 3,222, 3,518, 4,121, and 4,416 s from left to right, respectively. Arrows with numbers indicate three major boundaries.

fected cells (data not shown). These results clearly indicate that the fusion protein is localized to the ER in transfected COS-7 cells, similar to msALDH (*11*, *20*, *21*). Taken together, these biochemical and immunolocalization data confirm an important role of the carboxyl-terminal 35 amino acids of msALDH in ER targeting in addition to membrane anchoring. These results allowed us to investigate the ER-targeting mechanism of msALDH using this fusion protein.

Localization of rGFP and rGFPALDH(35) Injected into COS Cells—Since the carboxyl-terminal hydrophobic sequence of msALDH should not emerge from ribosomes until translation is complete, it is likely that both GFPALDH(35) and msALDH are targeted to the ER *via* an SRP-independent pathway. To test this *in vivo*, we attempted microinjection experiments of two recombinant proteins, rGFP and rGFPALDH(35). For this purpose, the bacterial expression plasmids, pQEGFPC-1 and pQEGFPALDH(35), were constructed and transformed into *Escherichia coli* JM109 cells. The recombinant proteins were induced by addition of isopropyl-β-D-thiogalactopyranoside and purified. On electrophoresis, apparent molecular masses of rGFP and rGFPALDH(35) were estimated to be 31 kDa and 34 kDa, respectively (Fig. 3A). The increases in molecular masses compared to GFP and GFPALDH(35) are due to the amino-terminal extension of 12 amino acids including 6×His tag. We subsequently investigated molecular dimensions of the purified proteins in PBS by the sedimentation velocity method. As shown in Fig. 3B, sedimentation of rGFP was observed as a single boundary. The sedimentation coefficient was determined to be 5.9S. In sedimentation of rGFPALDH(35), three major boundaries were observed (Fig. 3C, boundary 1–3). The sedimentation coefficients estimated from the three boundaries were 19S, 35S and 49S, respectively. This result indicates that rGFPALDH(35) forms molecular assemblies in PBS.

The recombinant proteins were then injected into the cytoplasm of COS-7 cells, and intracellular localization was examined by fluorescence microscopy. As expected, both rGFP and rGFPALDH(35) were detected in the cytoplasm just after injection (Fig. 4, A, C, and D). However, punctate signals were observed in the case of rGFPALDH(35) (Fig. 4C). As shown in Fig. 4B, rGFP was accumulated in the nucleus after a 30-min incubation at 37°C. In marked contrast, rGFPALDH(35) showed a reticular staining pattern that surrounded the nucleus and extended throughout the cytoplasm in the cells fixed after a 30-min incubation at 37°C (Fig. 4E). Indirect immunofluorescence microscopy using anti-PDI antibodies (Fig. 4F) showed co-localization of the two proteins in the ER. Thus, localization of rGFP and rGFPALDH(35) in COS-7 cells fixed after incubation at 37°C is consistent with that (Fig. 2) of GFP and GFPALDH(35) expressed from cDNAs, respectively.

We next investigated intracellular localization of rGFPALDH(35) by immunoelectron microscopy. When cells were fixed just after injection, gold particles for the recombinant protein were detected in the cytoplasm (Fig. 5A). On the contrary, gold particles selectively labeled the cytoplasmic face of the ER in the cells fixed after a 1-h incubation at 37°C (Fig. 5B). These results indicate that cytoplasmically injected rGFPALDH(35) is correctly localized to the ER. Taken together, we conclude that the tail-anchored protein is post-translationally targeted to the ER in COS-7 cells.

Localization of GFPALDH(18) and rGFPALDH(18) in COS Cells—To investigate the role of the ER-targeting sequences, we determined subcellular localization of GFPALDH(18) and rGFPALDH(18), which contain the transmembrane domain of msALDH but lack the ER-targeting sequences (Fig. 6A). When expressed from cDNA, GFPALDH(18) was distributed in the cytoplasm and the nucleoplasm of COS-7 cells (Fig. 6B, panel a), and it did not co-localize with PDI (Fig. 6B, panel b). Immunoelectron microscopy showed that gold particles were detected in the cytoplasm (Fig. 6C). However, immunogold staining in the nucleus was weak compared with fluorescent intensity, probably because of low accessibility of IgG-gold complexes to the nucleus.

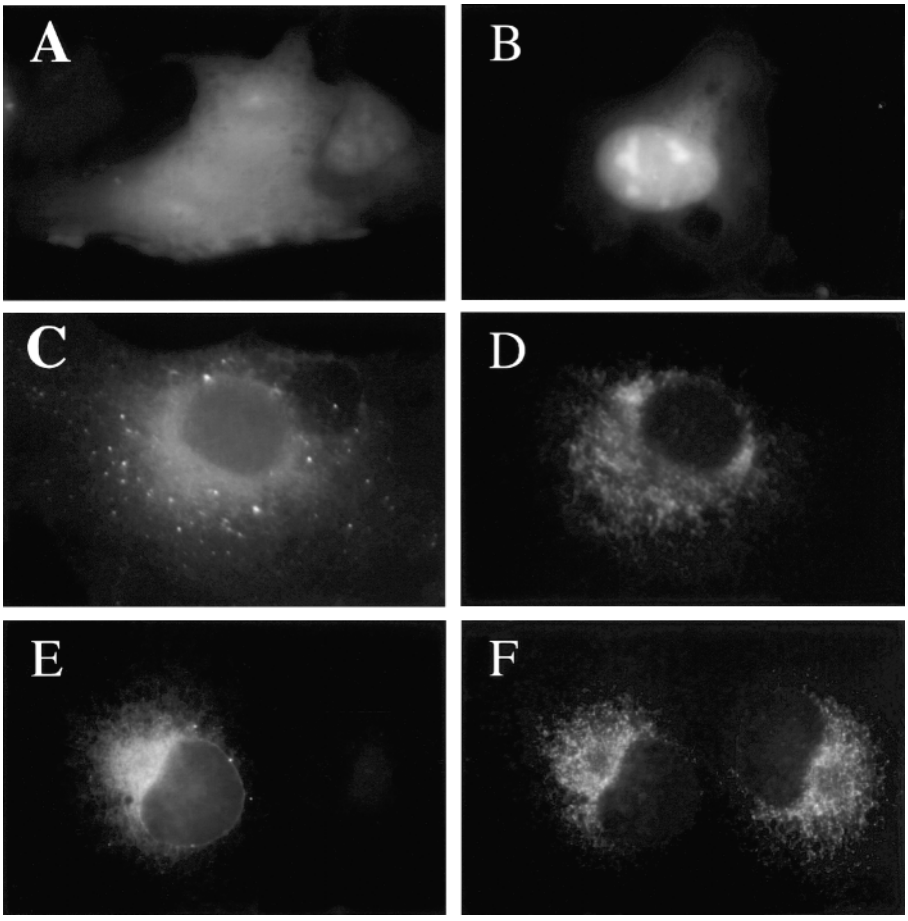

Fig. 4. **Localization of rGFP and rGFPALDH(35) injected into COS cells.** COS-7 cells were grown on CEL-Locate coverslips at 37°C. rGFP (A and B) or rGFPALDH(35) (C and E) was injected into the cytoplasm of COS-7 cells. The cells were fixed just after injection (A and C) or after incubation at 37°C for 30 min (B and E). For detection of endogenous PDI (D and F), the cells were permeabilized and stained with rabbit anti-rat PDI antibodies and a fluorolink Cy3-labeled anti-rabbit IgG.

We then prepared rGFPALDH(18), which exhibited almost identical mobility to rGFP (31 kDa) on SDS-PAGE (data not shown). In sedimentation of rGFPALDH(18) (Fig. 7A), fast sedimenting components were observed at earlier sedimenting times in addition to a slowly sedimenting major component, though the sedimenting boundary of the former could not be clearly identified as in the case of rGFPALDH(35). The sedimentation coefficient of the slowly sedimenting component was determined to be 5.8S, and the average value for the fast sedimenting components was estimated to be 50S. This results suggests that rGFPALDH(18) forms molecular assemblies different from those of rGFPALDH(35).

The recombinant protein was injected into the cytoplasm of COS-7 cells, and intracellular localization was examined by fluorescence microscopy. When the cells were fixed just after injection, rGFPALDH(18) was distributed throughout the cytoplasm (data not shown). In contrast to rGFPALDH(35), punctate signals were not observed. After a 1-h incubation at 37°C, the recombinant protein was detected in the cytoplasm and nucleoplasm (Fig. 7B). A prolonged incubation at 37°C (8 h) did not change localization of rGFPALDH(18) (data not shown). Immunoelectron microscopic observation confirmed the cytoplasmic distribution of rGFPALDH(18) (Fig. 7C). Thus, intracellular localization of the recombinant protein was consistent with that of GFP-ALDH(18). These findings confirm that the ER-targeting sequences of msALDH in addition to the transmembrane domain are necessary for correct localization of GFP to the ER.

Temperature- and ATP-Dependent ER Targeting—To examine the effect of temperature on the ER-targeting reaction of rGFPALDH(35), COS-7 cells were incubated at 4°C for 1 h after injection. As shown in Fig. 8A (panel a), intracellular localization of rGFPALDH(35) was similar to that just after injection (Fig. 4C), indicating that the recombinant protein remained in the cytoplasm under the cold condition. However, the protein was localized to the ER after subsequent incubation at 37°C for 1 h (Fig. 8A, panel b). Thus, the reaction is temperature-dependent.

Next, we conducted ATP-depletion experiments to investigate the role of ATP. Since COS-7 cells became fragile upon ATP depletion and were easily disrupted by microinjection, they were subjected to ATP depletion after injection. Injected COS-7 cells were cultured at 4°C for 1 h in DMEM without glucose and FBS containing 2-deoxyglucose and antimycin A as described by Podbilewicz and Mellman (*27*). By treating cells at 4°C, the ER-targeting reaction of the recombinant protein was presumably inhibited. As shown in Fig. 8B, these conditions reduced the ATP level to about 10% of the initial value. Upon warming to 37°C, intracellular ATP was further reduced to about 2% after 30 min and to about 1% after 1.5 h. On the other hand, the ATP level in control cells

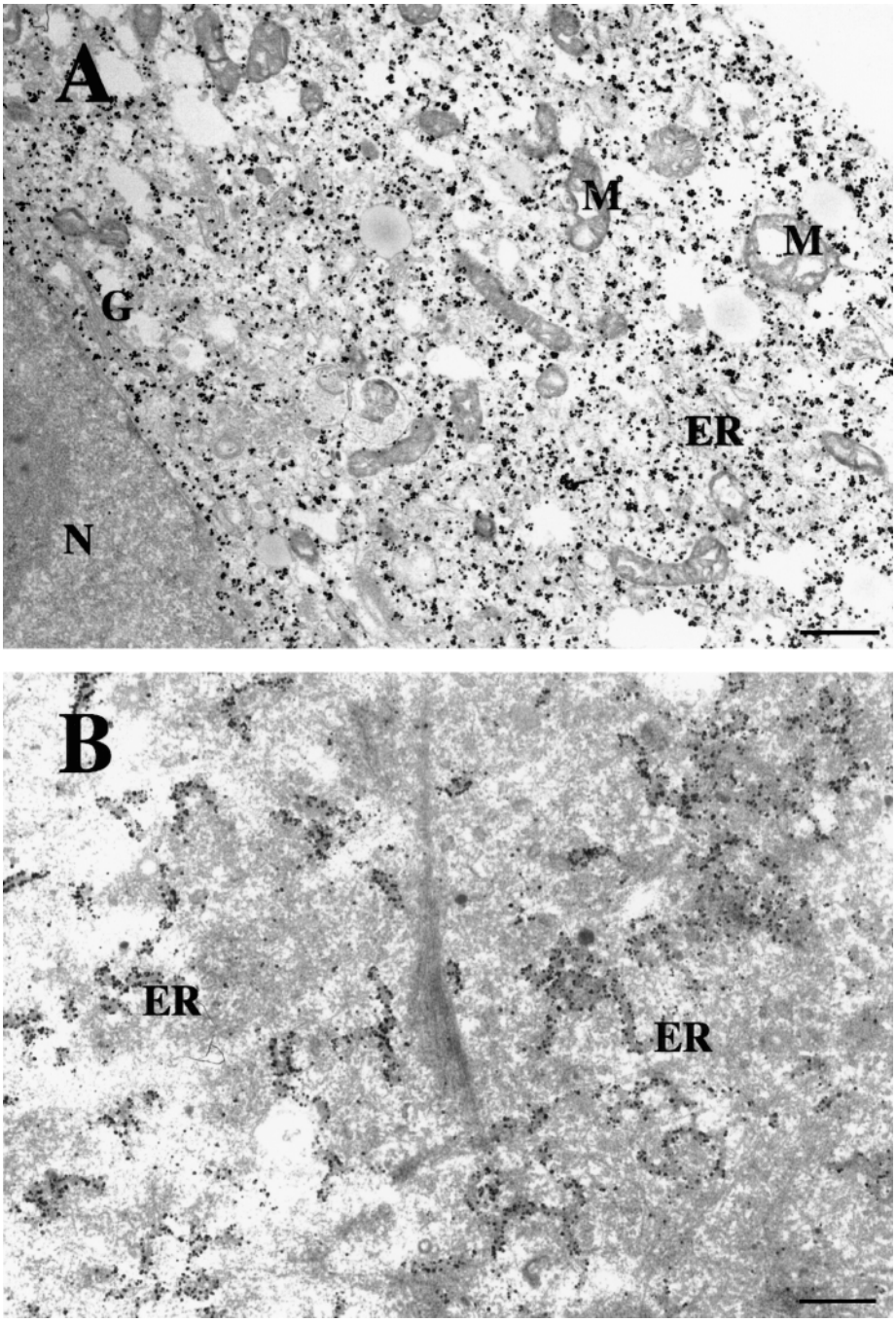

Fig. 5. **Immunogold localization of rGFPALDH(35) injected into COS cells.** COS-7 cells were grown on CELLocate coverslips at 37°C. rGFPALDH(35) was injected into the cytoplasm of COS-7 cells. The cells were fixed just after injection (A) or after incubation at 37°C for 1 h (B). They were permeabilized and reacted with anti-GFP antibodies, then with immunogold particles. ER, endoplasmic reticulum; G, Golgi complex; N, nucleus; M, mitochondria; bar, 500 nm.

without the metabolic inhibitors was maintained about 60% of the initial value after 1.5 h of incubation at 37°C. The reduction of internal ATP pool in control cells was probably due to elimination of glucose from the medium. As expected, the recombinant protein was distributed in the cytoplasm after a 1-h incubation at 4°C in the absence or presence of the metabolic inhibitors (Fig. 8C, panels a and c). However, rGFPALDH(35) in control cells was localized to the ER after subsequent incubation at 37°C for 1.5 h (Fig. 8C, panel b). When COS-7 cells were subjected to ATP depletion, the protein remained in the cytoplasm even after 1.5 h at 37°C (Fig. 8C, panel d). Thus, our data indicate that the ER-targeting reaction of rGFPALDH(35) is dependent on both temperature and ATP.

Application to Different Cell Types—We then examined whether GFPALDH(35) and rGFPALDH(35) are localized to the ER in different cell types and species. First, the expression plasmid, pEGFPALDH(35), was transiently transfected into NRK cells (rat kidney cell line), A431 cells (human epidermoid carcinoma) or CHO-K1 cells (hamster ovary cell line). Although expression efficiencies in these cells were lower than that in COS-7 cells, the expressed protein showed the reticular staining pattern including the nuclear membrane (Fig. 9, A, C, and E). These reticular compartments were confirmed to be the ER by co-localization with PDI (data not shown). Next, rGFPALDH(35) was injected into the cytoplasm of these cells. As shown in Fig. 9, B, D, and E, the injected

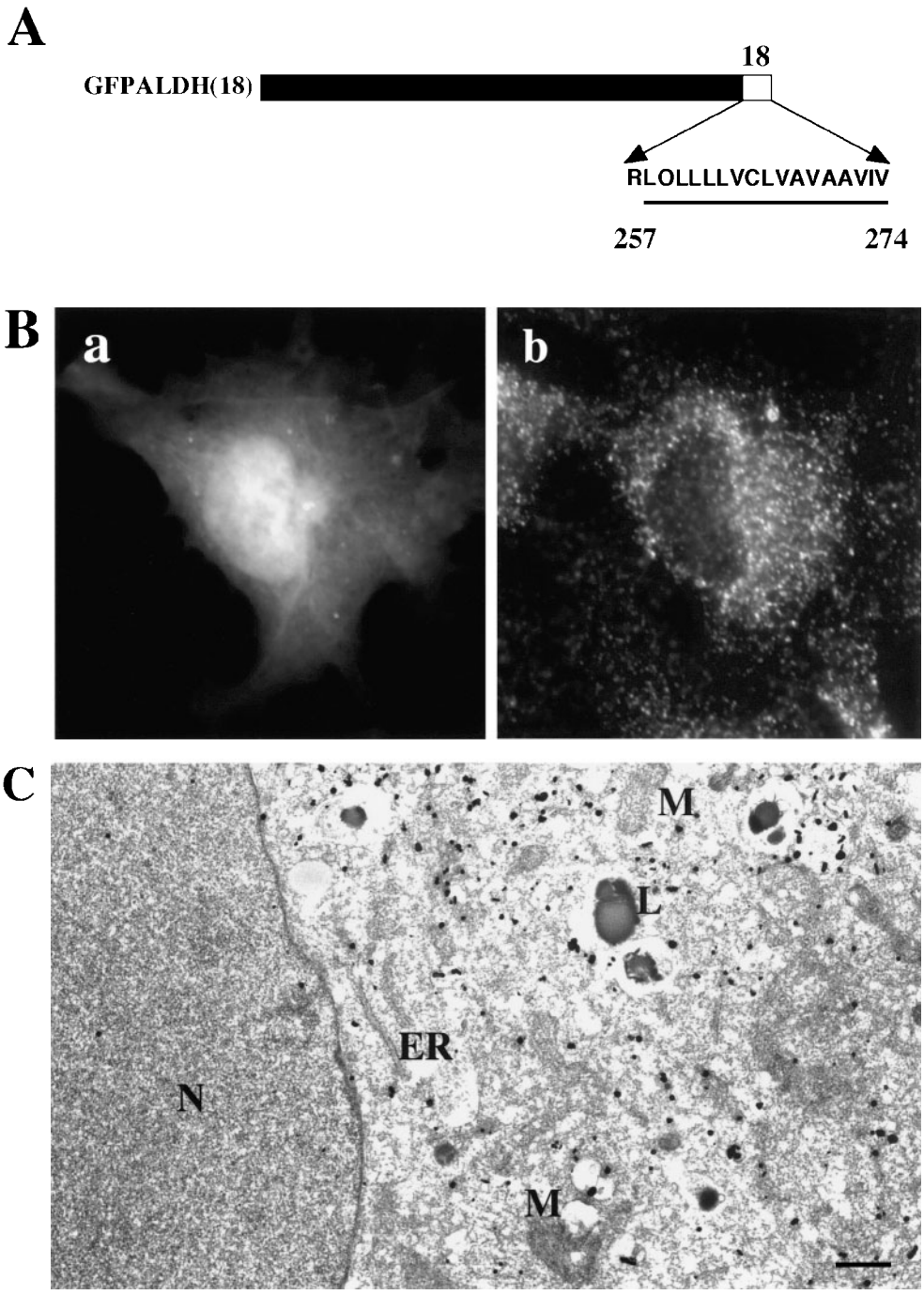

Fig. 6. **Localization of GFPALDH(18) expressed in COS cells.** (A) A schematic diagram of GFPALDH(18) is shown. The solid bar shows amino acids 1–256 of GFP. The single amino acid code is used to represent amino acids 463–480 of msALDH. The membrane-binding domain of msALDH is underlined. (B) COS-7 cells were grown on glass coverslips at 37°C and transfected with pEGFPALDH(18) (a). The cells were fixed 44 h after transfection, permeabilized, then stained with rabbit anti-rat PDI antibodies and a fluorolink Cy3-labeled anti-rabbit IgG (b). (C) For immunogold localization, COS-7 cells were grown on Celldesk coverslips at 37°C, transfected with pEGFPALDH(18), and fixed 44 h after transfection. The cells were permeabilized and reacted with rabbit anti-GFP antibodies and immunogold particles. ER, endoplasmic reticulum; L, lysosome; M, mitochondria; N, nucleus; bar, 500 nm.

protein was localized to the ER in these cells after a 30-min incubation at 37°C. These results suggest a common mechanism for ER targeting of the tail-anchored protein among mammalian cells.

DISCCUSION

In this report we describe an *in vivo* system to study ER targeting of msALDH using GFP as a reporter protein. We first investigated localization of GFPALDH(35) in transfected COS-7 cells and found that the fusion protein is correctly localized to the ER (Figs. 1 and 2). These results confirm our previous conclusion (*21*) that the carboxyl-terminal 35 amino acids of msALDH contain both the ER-targeting sequences and the membrane-binding sequence. Additionally, our recent investigation has suggested that GFPALDH(35) is retained in the ER by a static retention mechanism (Yamamoto, A. *et al.*, manuscript in preparation), similar to msALDH (*22*). Thus, these results allow us to use the fusion protein to study mechanisms by which ER localization of msALDH occurs.

Since GFPALDH(35) is localized to the ER *via* the last 35 amino acids of msALDH, it is most likely that the fusion protein is targeted to the ER by a mechanism different from an SRP-dependent pathway for proteins with

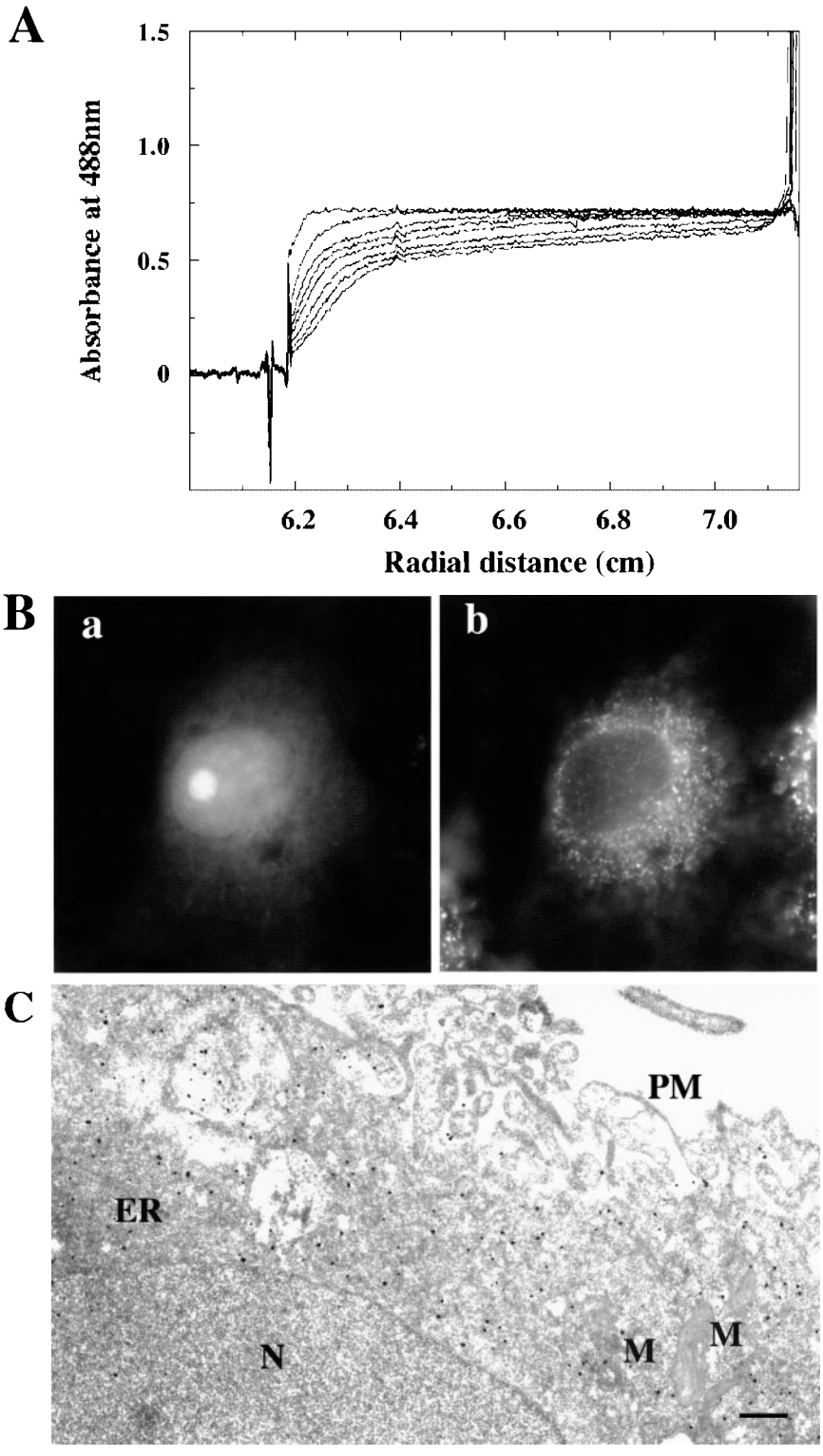

Fig. 7. **Localization of rGFPALDH(18) injected into COS cells.** (A) Sedimentation velocity run for rGFPALDH(18) was performed at 30,000 rpm and 20°C. The sample was scanned at a wavelength of 488 nm. Recorded times were 267, 487, 784, 1,087, 1,679, 2,285, 2,880, and 3,484 s from left to right, respectively. (B) COS-7 cells were grown on CELLocate coverslips at 37°C. rGFPALDH(18) was injected into the cytoplasm of COS-7 cells. The cells were fixed after incubation at 37°C for 1 h (a). For detection of endogenous PDI (b), the cells were permeabilized and stained with rabbit anti-rat PDI antibodies and a fluorolink Cy3-labeled anti-rabbit IgG. (C) For immunogold localization, COS-7 cells were fixed after incubation at 37°C for 1 h. They were permeabilized and reacted with anti-GFP antibodies, then with immunogold particles. ER, endoplasmic reticulum; N, nucleus; M, mitochondria; PM, plasma membrane; bar, 500 nm.

an amino-terminal signal sequence. To prove this idea *in vivo*, we microinjected rGFPALDH(35) into the cytoplasm of COS-7 cells. We demonstrated by fluorescence microscopy that cytoplasmically injected rGFPALDH(35) is localized to the ER (Fig. 4). In addition, immunoelectron microscopic data showed that the protein is selectively localized to the cytoplasmic face of the ER (Fig. 5B), similar to GFPALDH(35) expressed from cDNA (Fig. 2E). Therefore, it is concluded that the fusion protein is post-translationally targeted to the ER and that the ER-targeting reaction is highly selective in living cells. Although microinjection experiments have previously been conducted using soluble proteins or antibodies against intracellular proteins, we show here that the system works for the ER-resident membrane protein. To the best of our knowledge, ours is the first *in vivo* evidence

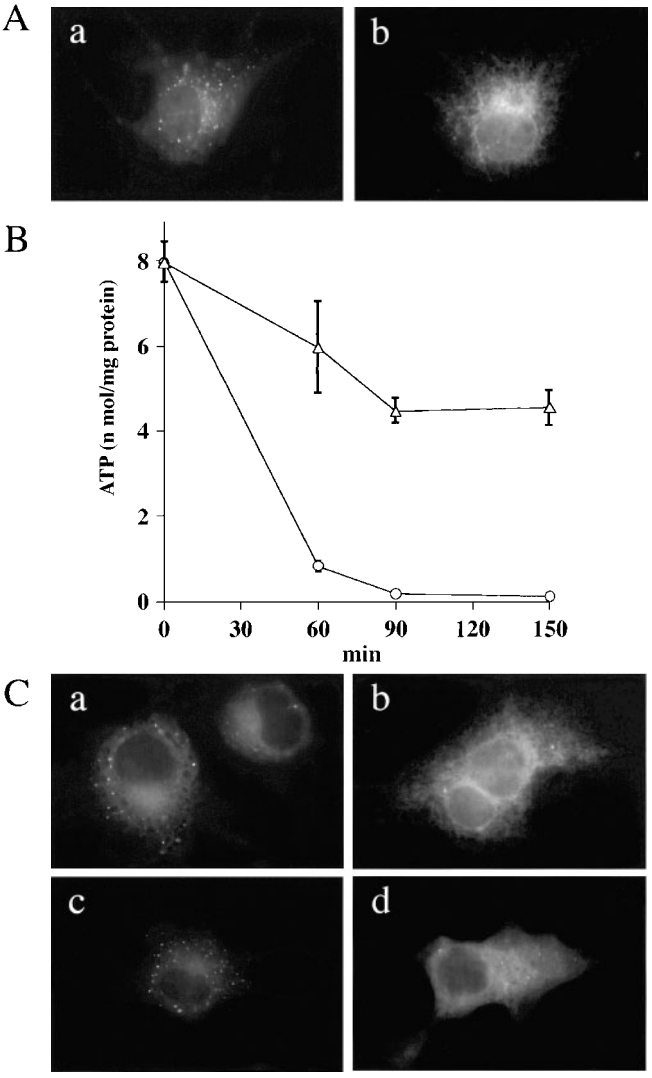

Fig. 8. **Temperature- and ATP-dependent ER targeting of rGFPALDH(35).** (A) COS-7 cells were grown on CELLocate coverslips at 37°C. rGFPALDH(35) was injected into the cytoplasm of COS-7 cells. The cells were incubated at 4°C for 1 h (a), then at 37°C for 1 h (b) before fixation. (B) ATP depletion (circles) was achieved by treating COS-7 cells at 4°C for 1 h in DMEM without glucose and FBS containing 2-deoxyglucose and antimycin A. The cells were then warmed to 37°C. Control cells (triangles) were incubated under the same conditions except for elimination of the metabolic inhibitors. ATP concentrations were determined as described in "MATERIALS AND METHODS." The bars show the mean ± SD (n = 3). (C) COS-7 cells were grown on CELLocate coversplips at 37°C. rGFPALDH(35) was injected into the cytoplasm of COS-7 cells. After injection, the cells were cultured as in (B) in the absence of (a and b) or presence of the metabolic inhibitors (c and d). The cells were incubated at 4°C for 1 h (a and c), then at 37°C for 1.5 h (b and d) before fixation.

for post-translational targeting of a tail-anchored protein to the ER. This is consistent with our recent result from an *in vitro* transcription and translation system that msALDH is post-translationally integrated into microsomal membranes from canine pancreas (Masaki, R. *et al.*, manuscript in preparation). Thus, two different approaches support post-translational ER targeting of msALDH.

Neither GFPALDH(18) nor rGFPALDH(18) was targeted to the ER (Figs. 6 and 7), confirming that the ER-targeting sequences in addition to the transmembrane domain of msALDH are necessary for correct intracellular localization of GFP to the ER. These results also demonstrate that the ER-targeting reaction of rGFPALDH(35) reflects biological processes occurring in living cells. rGFPALDH(35) remains in the cytoplasm under conditions of low temperature (Fig. 8A) or ATP depletion (Fig. 8C), indicating that the ER-targeting reaction is temperature- and ATP- dependent. Therefore, it is likely that ATP hydrolysis is necessary for the reaction. ATP requirement together with the selectivity of the targeting membrane strongly suggests a novel mechanism for ER targeting. One possible model is that a soluble factor(s) and a membrane-bound receptor(s) on the ER are involved to help efficient and correct ER localization of the tail-anchored protein, although the identities of these factors are not known.

In sedimentation velocity measurements, the sedimentation coefficient of rGFP was estimated to be 5.9S, corresponding to a molecular mass of about 75–85 kDa determined from a correlation between sedimentation coefficients and molecular masses of globular proteins (*28*). This result is consistent with a recent report (*29*) that GFP is present as a stable dimeric form in aqueous solution. rGFPALDH(35) was found to form higher order molecular assemblies with the sedimentation coefficients of 19S, 35S and 49S, which correspond to molecular masses of about 550, 1,400, and 2,300 kDa, respectively. In sedimentation of rGFPALDH(18), fast sedimenting components with an average sedimentation coefficient of 50S were observed in addition to a slowly sedimenting component with a sedimentation coefficient of 5.8S (Fig. 7A). However, the molecular assemblies of rGFPALDH(18) seemed to be unstable upon dilution with PBS and dissociated to the 5.8S component during centrifugation. These results suggest that rGFPALDH(18) is different from rGFPALDH(35) in forming higher order molecular assemblies. It seems that interactions of the last 35 amino acids of msALDH in addition to the GFP domain result in formation of stable, large assemblies of rGFPALDH(35). Therefore, the punctate signals detected in the cytoplasm just after injection of rGFPALDH(35) would reflect the large assemblies of the recombinant protein. However, the fusion protein should be in a monomeric form to be localized to the ER. Therefore, we speculate that a soluble factor(s) may interact with the ER-targeting sequences and the hydrophobic domain of the fusion protein to depolymerize molecular assemblies to a monomeric form. Thereafter, the fusion protein would be transferred to a membrane-bound receptor(s) for integration into the ER membrane. Since the fusion protein is localized to the ER in various cell types (Fig. 9) as well as COS-7 cells, it seems that common machineries for ER localization of the protein exist among mammalian cells. Further studies on protein factors and the role of ATP are required to understand the mechanisms by which ER targeting and integration of the tail-anchored protein occur.

We here provide *in vivo* evidence for post-translational ER targeting of GFPALDH(35). This pathway is consistent with a current idea that tail-anchored proteins reach their targeting membranes post-translationally,

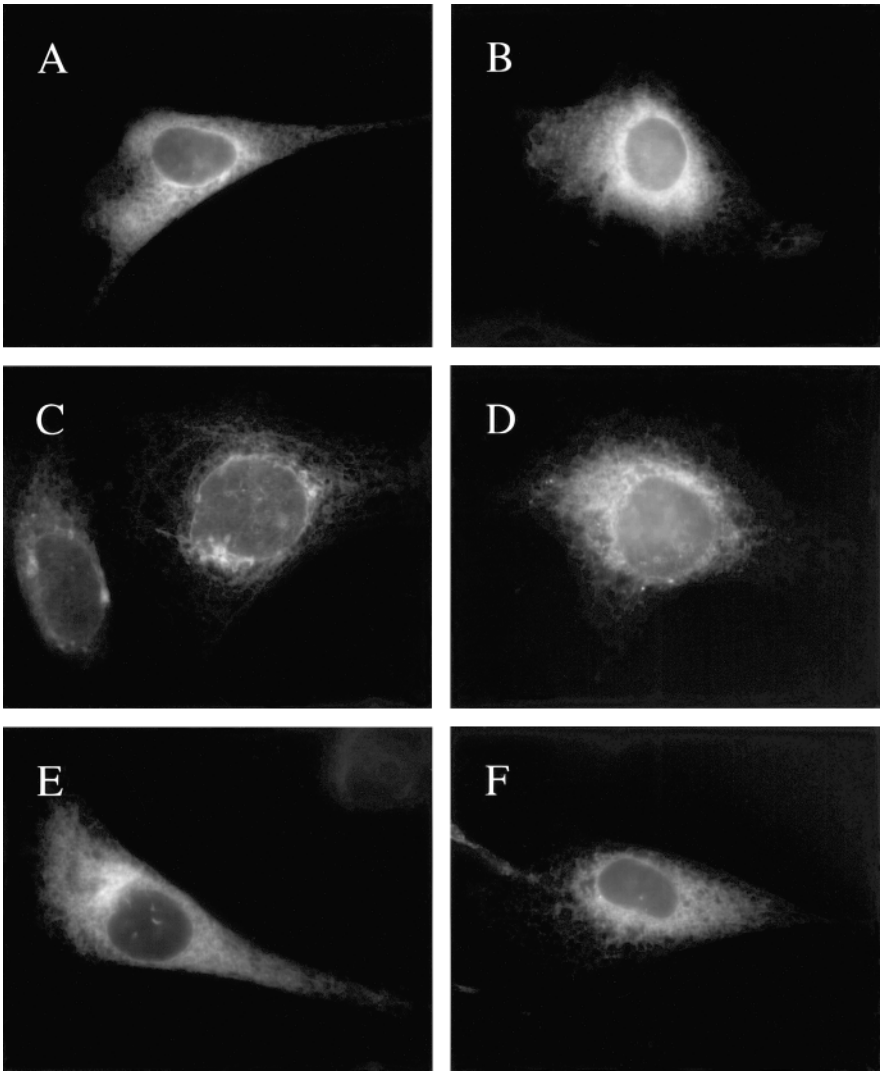

Fig. 9. **ER localization of GFPALDH(35) and rGFPALDH(35) in different cell types.** NRK (A), A431 (C) and CHO-K1 (E) cells were grown on glass coversplips at 37°C, transfected with pEGFPALDH(35), and fixed 44 h after transfection. For microinjection, NRK (B), A431 (D) and CHO-K1 (F) cells were grown on CELLocate coverslips at 37°C. rGFPALDH(35) was injected into the cytoplasm of each cell. The cells were fixed after a 30-min incubation at 37°C.

although most available information has come from *in vitro* analyses. It has been shown using a reticulocyte lysate cell-free system that tail-anchored proteins such as microsomal cytochrome *b*5 (*30*, *31*), vesicle-associated membrane proteins (Vamps) (*17*, *31*) and Jaw1 (*32*) are post-translationally integrated into canine pancreatic microsomes. Of these proteins Vamps have been well studied. These proteins require ATP and a trypsin-sensitive membrane component(s) to be integrated into microsomal membranes and do not bind to other membranes such as mitochondria and lysosomes. As for an insertion machinery in the ER membrane, Kutay *et al*. have shown that Sec61p is not involved in membrane integration of Vamp-1A/synaptobrevin (*17*). So far as ATP requirement and the selectivity of targeting membrane are concerned, it seems that the ER-targeting pathway of Vamps is similar to that of GFPALDH(35) in spite of the difference in experimental approaches. Unlike Vamps, microsomal cytochrome b_5 binds post-translationally to a variety of different membranes including lysosomes and liposomes (*31*, *33*). In addition, microsomal cytochrome b_5 does not require either ATP or a trypsin-sensitive membrane component(s) for integration into microsomes (*31*). Based on *in vitro* evidence, a spontaneous insertion model of microsomal cytochrome b_5 has been proposed. However, it has been shown that microsomal cytochrome b_5 is exclusively localized to the ER, when expressed from cDNA in mammalian cells (*12*, *34*). Further investigation is required to elucidate the difference in membrane selectivity of microsomal cytochrome *b*5 between *in vitro* and *in vivo* systems. In addition, it remains to be confirmed that a common mechanism exists to target these tail-anchored proteins to the ER.

In conclusion, our microinjection experiments have demonstrated that GFPALDH(35) is post-translationally targeted to the ER and that the reaction is highly selective and ATP-dependent in living cells. The strategy used here should also be useful for investigating intracellular localization of tail-anchored proteins found not only in the ER but also in other membranes.

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