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Multiple organelle-targeting signals in the N-terminal portion of peroxisomal membrane protein PMP70

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Most membrane proteins are recognized by a signal recognition particle and are cotranslationally targeted to the endoplasmic reticulum (ER) membrane, whereas almost all peroxisomal membrane proteins are post-translationally targeted to the destination. Here we examined organelle-targeting properties of the N-terminal portions of the peroxisomal isoform of the ABC transporter PMP70 (ABCD3) using enhanced green fluorescent protein (EGFP) fusion. When the N-terminal 80 amino acid residue (N80)-segment preceding transmembrane segment (TM) 1 was deleted and the TM1–TM2 region was fused to EGFP, the TM1 segment induced ER-targeting and integration in COS cells. When the N80-segment was fused to EGFP, the fusion protein was targeted to the outer mitochondrial membrane. When both the N80-segment and the following TM1–TM2 region were present, the fusion located exclusively to the peroxisome. The full-length PMP70 molecule was clearly located in the ER in the absence of the N80-segment, even when multiple peroxisome-targeting signals were retained. We concluded that the TM1 segment possesses a sufficient ER-targeting function and that the N80-segment is critical for suppressing the ER-targeting function to allow the TM1–TM2 region to localize to the peroxisome. Cooperation of the organelle-targeting signals enables PMP70 to correctly target to peroxisomal membranes.

Keywords: topogenesis/membrane protein/signal sequence/peroxisome/ABC transporter.

Abbreviations: CNX, calnexin; EGFP, enhanced green fluorescent protein; EndoH, endoglycosidase H; ER, endoplasmic reticulum; HA, hemagglutinin; H-segment, hydrophobic segment; N80, N-terminal 80 amino acid residues of PMP70; PBS, phosphate-buffered saline; PMP70, 70-kDa peroxisomal membrane protein; PMP, peroxisomal membrane protein; ProK, proteinase K; RM, rough microsomal membrane; TM, transmembrane.

Signal sequences destined for the endoplasmic reticulum (ER) are primarily determined by hydrophobic segments (H-segments) of nascent polypeptide chains. An H-segment consisting of only seven hydrophobic residues can exert the signal function (*1*). The H-segment on the polypeptide chain emerging from the ribosome is scanned and recognized by the signal recognition particle at the exit of the ribosome and then the ribosome-nascent chain complex is targeted to the ER translocon. At this stage, the amino acid sequence downstream of the signal sequence is not released from the ribosome nor synthesized. ER-targeting of H-segment can be modulated only by the flanking and upstream regions. On the ER membrane, the H-segment is released from the signal recognition particle, recognized by the Sec61 complex, and inserted into the translocon pore. At the following insertion step on the ER, the orientation of the H-segment is affected by the flanking charged residues [*e.g.* references (*2*, *3*)]. The ribosomes cooperate with the translocon to integrate membrane proteins into the membrane (*4*, *5*). The translocon scans the nascent chain emerging from the ribosome, recognizes the hydrophobic transmembrane (TM) segment, and releases it into the lipid phase (*6*, *7*).

On the other hand, almost all membrane proteins in the peroxisomes and mitochondria are targeted to their destinations after protein synthesis. Because the cotranslational mechanism for the ER is triggered by recognition of the short H-segment, membrane proteins with a hydrophobic TM segment in the peroxisomes and mitochondria must include somewhat different information by which they escape ER-targeting before being targeted to the ER translocon. A simple hypothesis is that the cotranslational ER-targeting of the hydrophobic TM segment is suppressed by its upstream sequence.

The ATP binding cassette (ABC) transporter family members are ideal for studying organelle-targeting of multispanning hydrophobic membrane proteins. Specific isoforms are localized to specific destinations, even though all of them possess H-segments that appear to be sufficient as ER-targeting signals. Typical ABC transporter isoforms possess 12 TM segments, including two sets of six TM segments and two sets of ATP-binding cassettes (*8*). Half-type isoforms

possess six TM segments and a single ATP binding cassette. Some subfamily isoforms possess an extra TM segments upstream of the conserved TM segments. Isoforms localized in the exocytic and endocytic pathways are synthesized by membrane-bound ribosomes, integrated into the ER membrane, and then sorted to their final destinations via vesicle transport. Mitochondrial inner membrane isoforms possess long presequences that suppress ER-targeting: e.g. the ABCB10 isoform possesses a presequence of 105 residues (*9*, *10*). When the presequence is deleted, the membrane domain is integrated into the ER membrane and TM1 shows the same topology as the TM1 of MDR1 (*11*). The long presequence suppresses cotranslational ER-targeting and the targeting mechanism is switched to posttranslational mitochondrial import (*11*).

Peroxisomal membrane proteins (PMPs), including the ABC transporter isoforms, also possess hydrophobic TM segments and escape ER-targeting. Multispanning PMPs possess multiple peroxisome-targeting signals in a single polypeptide chain (*12*, *13*). Pex19p, a peroxisome-assembly factor, binds multiple sites of PMPs and maintains them in a soluble form before they are imported into the peroxisomal membrane (*14*–*17*). A half-type ABC-transporter, PMP70 (ABCD3 isoform), is one of the major membrane proteins of the peroxisome (*18*). It possesses multiple peroxisome-targeting sequences and multiple Pex19p binding sites (*19*, *20*). In the absence of Pex19, the PMP70 molecule is not imported into the peroxisome, but is instead rapidly degraded in the cytosol (*21*).

Here, as a first step to address the ER-targeting suppression of PMPs, we examined organelle-targeting properties of the N-terminal region of PMP70. We demonstrated that the TM1 sequence possesses an intrinsic ER signal-anchor function, which is integrated into the ER membrane with an N_{cyt}/C_{lumen} topology. The ER-targeting is suppressed by the N-terminal 80 amino acid residue (N80)-segment that is upstream of the TM1 segment. Interestingly, the N80-segment alone, however, is integrated into the outer membrane of mitochondria. A combination of the N80-segment and the TM1–TM2 region achieves peroxisome targeting. We propose that the N80-segment with a mitochondria-targeting function modulates the ER-targeting function of TM1, allowing for correct targeting of the TM1–TM2 region to the peroxisomal membrane.

Materials and Methods

Materials

The rabbit anti-calnexin C-terminal segment antibodies (SPA-860; Stressgen, San Diego, CA), mouse anti-HA monoclonal antibody (MMS-101R; Covance, Gaithersburg, MD), rabbit anti-Pex14 antibodies (*22*), rabbit anti- enhanced green fluorescent protein (EGFP) antibodies (*23*) and rabbit anti-human Tom20 antibodies (sc-11415; Santa Cruz Biotechnology, Santa Cruz, CA) were described. Rabbit anti-AIF antibodies (*24*) were kindly provided by Mr Ohsakaya and Dr Mihara (Kyushu University, Fukuoka). Human PMP70 cDNA was previously described (*25*). Mouse PMP70 cDNA was amplified from mouse cDNA library that was kindly provided by Dr Yamazaki (Kyushu University, Fukuoka) and subcloned in the XbaI site of HA-fusion vector, pRcCMV-HA (*26*), to obtain pmPMP70-HA. Endoglycosidase H (EndoHf; New England BioLabs, Ipswich, MA), FuGene6 transfection reagent (Roche, Madison, WI), digitonin (Wako Chemicals, Tokyo), proteinase K (Merck, Whitehouse Station, NJ), MG132 (Sigma Chemical, St. Louis, MO) and Mitotracker Orange (Invitrogen) were purchased from the indicated companies.

Construction of model proteins

In the following procedures, the desired DNA fragments were obtained by PCR with oligonucleotide primers containing the appropriate restriction enzyme sites, which are indicated by parentheses. The DNA fragments were digested with restriction enzymes and ligated with plasmid vectors that had been digested with the indicated restriction enzymes. At each junction, six bases of the restriction enzyme site encoded two codons. For EGFP fusion proteins (Fig. 1), the DNA fragments encoding the N-terminal portions of human PMP70 were amplified by PCR using either forward primer CGAAAAGCTTCCACCATGGCGGCCTTCAGC for the 5′-end of cDNA (the HindIII site, Kozak sequence, and initiation codon are underlined) or ATCGAAGCTTCCACCATGAAAGAGACAG GTTAC for the N80-segment deleted construct, and the appropriate reverse primers containing the XbaI site. The obtained each DNA fragment (HindIII/XbaI) and EGFP cDNA (XbaI /ApaI) were ligated with pRcCMV vector (HindIII/ ApaI). For the N-terminal deletion of full-length mouse PMP70, the N-terminal segment (2–80) was deleted by the Quickchange method using pmPMP70-HA. Tom20-EGFP fusion protein, in which full-length rat Tom20 was fused to EGFP, was previously described (*27*). Fusion protein of the ABCB10 presequence and EGFP was described previously (*11*).

Cell culture, transfection, extraction and immunoblotting

Transfections using FuGene 6 reagent were performed as described previously (*23*). To minimize artificial effects as much as possible, minimal amounts of plasmids were used for transfection and the expression period was minimized to <24 h. Where indicated, the proteasome inhibitor MG132, which was dissolved in dimethyl sulfoxide, was added to the culture medium (final concentration 20 μM) 12 h after transfection and the cell were further incubated for 12 h. For immuno-blotting analysis, one-fifth of the cells on the 3.5-cm dish were solubilized with SDS–PAGE sample buffer. Where indicated, samples were subjected to EndoH treatment. Reaction buffer supplied by the company and 2000 U EndoHf were added to the samples in SDS–PAGE sample buffer and incubated for 1 h at 37°C.

For ProK treatment of organelle membranes, cytosolic proteins were removed by permeabilization. Cells on a 10-cm culture dish were washed twice with 5 ml phosphate-buffered saline (PBS) and treated with 5 ml permeabilizing buffer [20 mM HEPES–KOH (pH 7.5), 0.25 M sucrose, 2.5 mM magnesium acetate, 25 mM KCl, 2.5 mM EGTA and 1 μM Taxol] containing 25 μg/ml digitonin at room temperature for 5 min, washed twice with 5 ml permeabilizing buffer, scraped in 2 ml of permeabilizing buffer, and then suspended by pipetting. Aliquot (0.67 ml) of the cell suspension was mixed with equal volume of ProK solution (120 μg/ml in the permeabilizing buffer) and incubated in the presence or absence of 1% Triton-X100 at 4°C for 60 min. The enzyme reactions were terminated with 10% trichloroacetic acid. The proteins were precipitated, washed with 80% acetone at −20°C, and then subjected to immuno-blotting.

For extraction of the organelle membranes, the transfected cells were homogenized and post-nuclear supernatant was obtained as previously described (*28*). The post-nuclear supernatant was subjected to extraction under various conditions and analyzed by immuno-blotting as described (*28*).

Immunofluorescence microscopy

For mitochondrial staining, MitoTracker orange solution (1 mM) dissolved in dimethyl sulfoxide was added to the medium (final concentration 50 nM), incubated for 20 min, washed twice with DMEM, and fixed with paraformaldehyde (4%) in DMEM. For immuno-staining with anti-Pex14 antibodies, cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed twice with PBS, and permeabilized with 0.2% Triton-X100 in PBS for 15 min. For immuno-staining with anti-calnexin antibodies, cells were fixed with mixture of methanol and acetone (1:1) for 2 min at room temperature. The fixed cells were incubated with antibodies as

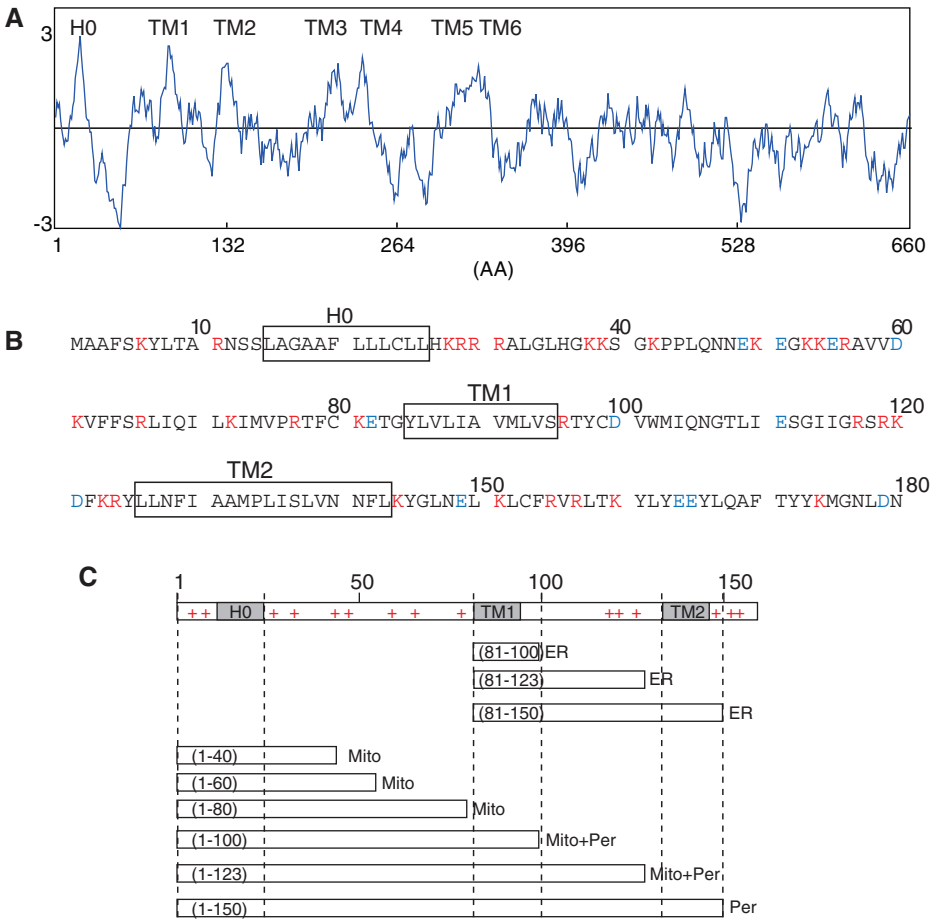

Fig. 1 N-terminal sequence of PMP70 and fusion proteins used in this study. (A) Hydropathy profile of PMP70 was calculated according to the method of Kite and Doolittle (window set at 15 residues). Hydrophobic peaks predicted to be transmembrane (TM) segments are numbered. (B) Amino-acid sequence of the N-terminal region of human PMP70. Hydrophobic segment (H0) and TM segments are boxed. Positively- and negatively-charged residues are indicated with red and blue letters, respectively. (C) Indicated N-terminal portions were fused to the N-terminus of EGFP: the regions included are indicated within the parentheses of their names. Location in COS cells assessed in this study are summarized. Two fusion proteins showed dual localization in mitochondria and peroxisome (Mito+Per).

previously described (*23*). After washing, the cells were mounted with ProLong Antifade (Invitrogen).

Results

TM1 possesses a signal function for ER-targeting and membrane integration

PMP70 possesses six hydrophobic TM segments that correspond to the consensus TM segments of the ABC transporter family (Fig. 1). The N80-segment contains a positive-charge rich sequence and a hydrophobic segment (H0). To assess the functions of the intracellular localization and membrane integration of the N-terminal portions, we constructed EGFP-fusion proteins (Fig. 1C). One series of fusion proteins included N-terminal portions initiated at the authentic Met[1]. The other series lacked the N80-segment. The fusion proteins were named according to the included sequences.

Initially, we assessed the TM1–TM2 region using N-terminally deleted fusions. The (81–100)-EGFP and (81–123)-EGFP fusions contained the TM1 segment and the (81–150)-EGFP fusion contained the TM1–TM2 region. The constructs were transiently expressed in COS cells and the extracts were probed by immuno-blotting (Fig. 2A). To avoid overexpression as much as possible, minimum amount of expression plasmid was used and expression was carried out for 24 h. The N-terminally deleted constructs were observed only in the presence of the proteasome inhibitor MG132, whereas the fusion including the N80-segment, (1–150)-EGFP, was observed irrespective of the presence of MG132 (compare lanes 7 and 10). When treated with EndoH, (81–123)-EGFP and (81–150)-EGFP were quantitatively shifted down by 2 kDa (Fig. 2A, lanes 6 and 9), indicating that they possessed an Asn-linked sugar chain. In contrast, the (81–100)-EGFP was not affected by the EndoH treatment, indicating that the sugar chain was added between 101 and 123. In the region, there is one potential glycosylation site, Asn^{106}–Gly–Thr.

Immunofluorescence observations revealed that (1–150)-EGFP was located exclusively in the punctuated structure, which was co-stained with Pex14p, peroxisomal marker (*22*) (Fig. 2B). On the other hand, the fusion lacking the N80-segment, (81–150)-EGFP, was located in the reticular structure but not in the punctate pattern of peroxisomes. The reticular structure was co-stained with the ER marker calnexin.

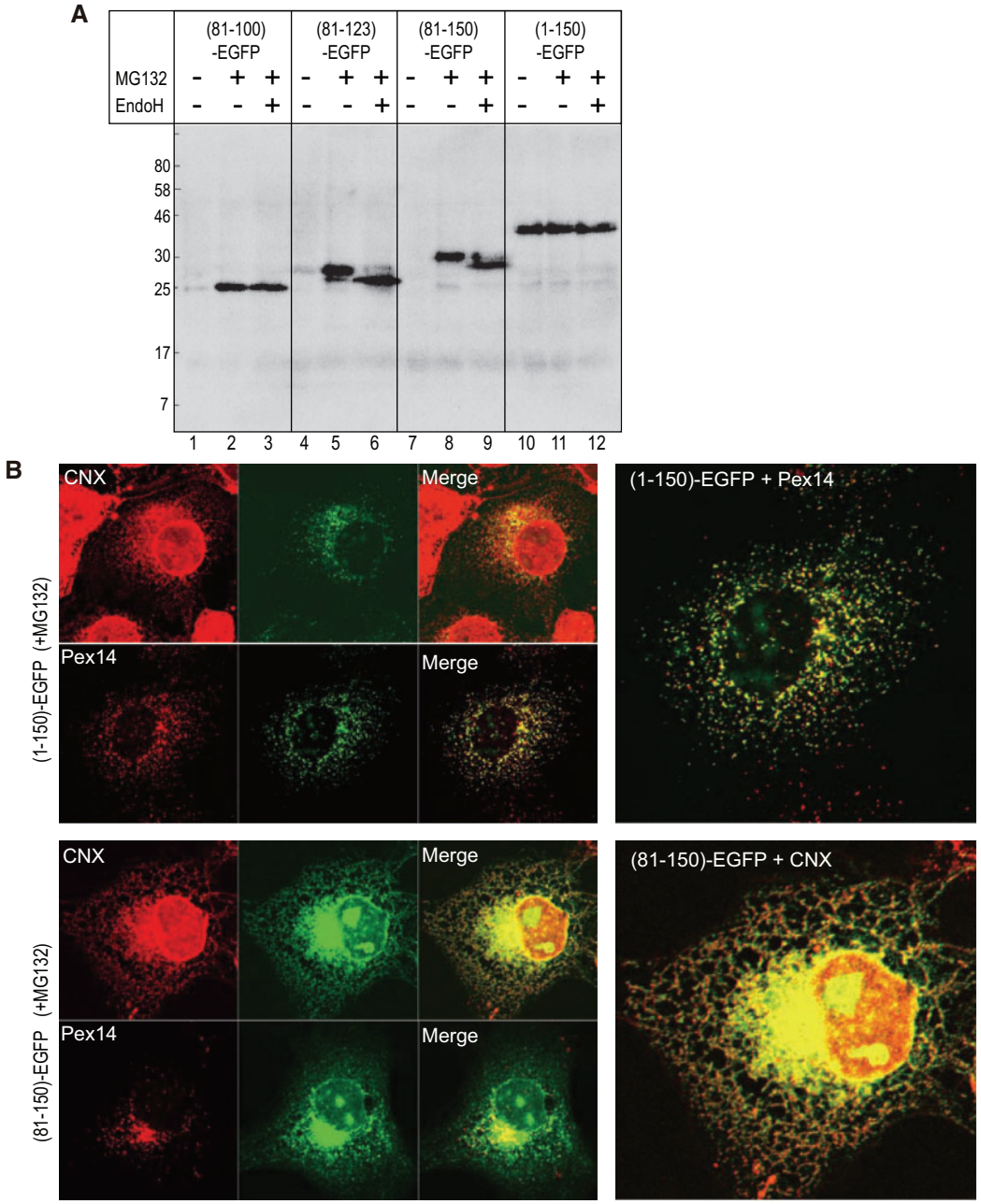

Fig. 2 TM1 is targeted to and integrated into the ER membrane. (A) The fusion proteins were transiently expressed in COS cells in the presence or absence of MG132. Total cell lysates were subjected to an immuno-blotting analysis using anti-EGFP antibodies. Aliquots were treated with EndoH. (B) The fusions were expressed in the COS cells in the presence of MG132 and localizations were analyzed. The ER marker (calnexin, CNX) and peroxisomal marker (Pex14p) were stained with antibodies.

The TM1 segment possesses features by itself sufficient for targeting to and integration into the ER. The ER-targeting function intrinsic to the TM1–TM2 region was suppressed by the N80-segment.

N-terminal segment upstream of TM1 possesses mitochondria-targeting activity

We next examined the targeting functions of the N-terminal region (Fig. 3). The (1–12)-EGFP fusion protein was dispersed in the whole cell, including both the cytosol and the nucleus. Unexpectedly, the (1–40)-EGFP, (1–60)-EGFP and (1–80)-EGFP fusion proteins were localized exclusively in the mitochondria that were stained with Mitotracker. They were not observed in the peroxisomes at all. The N-terminal region upstream of TM1 contains mitochondria-targeting information rather than peroxisome-targeting information. On the other hand, fusions including the TM1 segment, (1–100)-EGFP and (1–123)-EGFP, localized both in the mitochondria and in the peroxisome. The dual localization was observed even when the expression level was decreased to a minimum level for immuno-blotting detection (data not shown). Fusion protein including TM1–TM2 region, (1–150)-EGFP, was localized exclusively to peroxisomes. Expression level analysis

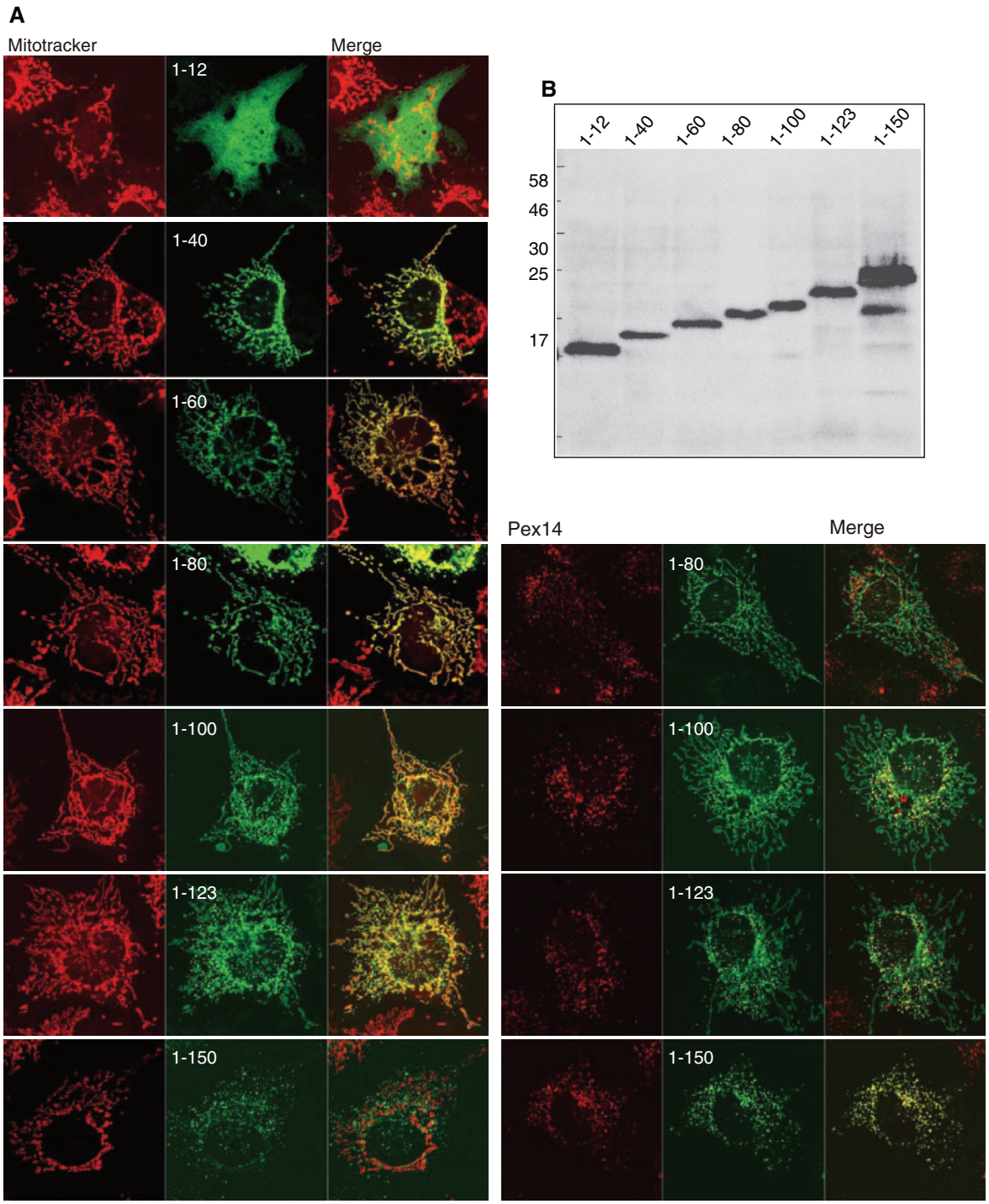

Fig. 3 Location of N-terminal fusion proteins in COS cells. (A) Each EGFP fusion construct was transfected to COS cells and observed by fluorescent microscopy. Mitochondria were visualized with Mitotracker Orange. Peroxisomes were stained with anti-Pex14 antibodies. (B) Whole extract of the transfected COS cells was analyzed by immuno-blotting using anti-EGFP antibodies.

by immuno-blotting indicated that the longest model protein, (1–150)-EGFP, was expressed at a higher level than the others (Fig. 3B). Despite having the highest expression level, the longest one was exclusively localized in the peroxisome (Fig. 3A), indicating that the peroxisomal-targeting mechanism was not saturated under the expression level. Thus, the dual localization of (1–100)-EGFP and (1–123)-EGFP was not caused by overexpression. Overall, we concluded that the N80-segment of PMP70 possesses a mitochondria-targeting function, rather than a peroxisomal targeting function, and is essential for suppressing the ER-targeting induced by the TM1 segment.

N80-segment is inserted in the mitochondrial outer membrane like Tom20

To examine the membrane topology of the (1–80)-EGFP fusion in mitochondria, organelle membranes

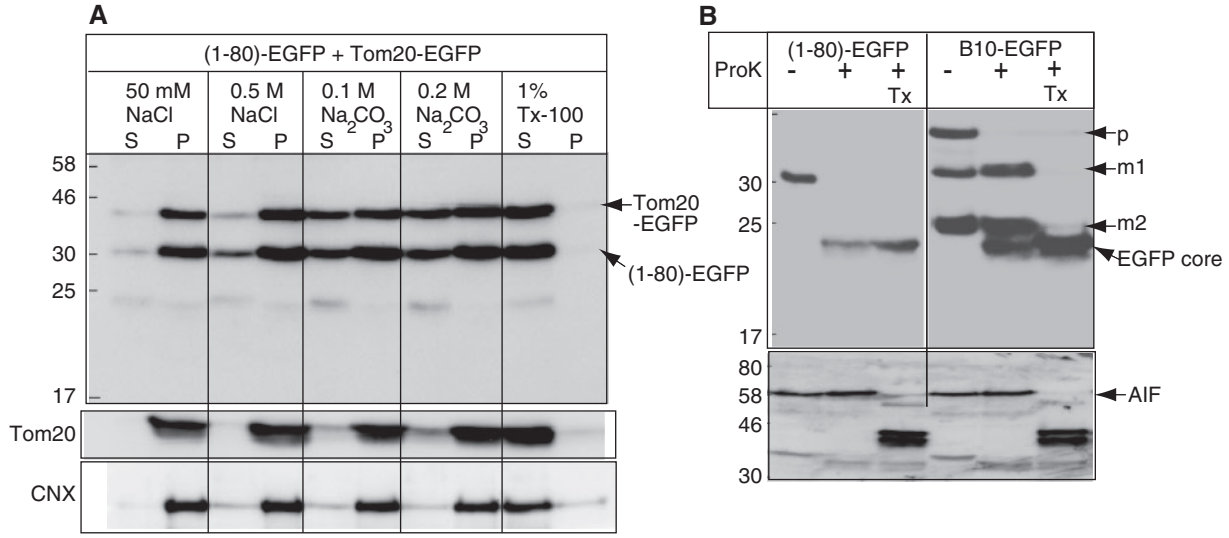

Fig. 4 (1–80)-EGFP is anchored to mitochondrial outer membrane as Tom20 is. (A) (1–80)-EGFP and Tom20-EGFP were cotransfected into COS cells. Post-nuclear supernatant of the transfected cells were extracted under high salt and various alkaline conditions and separated to membrane precipitates (P) and soluble fraction (S) by ultracentrifugation. Fractions were subjected to immuno-blotting analysis using anti-EGFP antibodies. Endogenous Tom20 and calnexin (CNX) were also visualized using their antibodies. (B) The transfected cells were permeabilized and treated with ProK in the absence and presence of detergent (Triton-X100, Tx). The ABCB10-EGFP fusion (N135-H1-EGFP) and intermembrane space protein (AIF) were also probed as controls.

were extracted under various conditions. The (1–80)-EGFP fusion protein was co-expressed in COS cells with a Tom20-EGFP fusion protein, which is integrated into the mitochondrial outer membrane (*27*). Under low salt (50 mM NaCl) and high salt (500 mM NaCl) conditions, (1–80)-EGFP was recovered in membrane precipitates as was Tom20-EGFP. Under alkaline conditions that extract peripheral and lumenal soluble proteins (*29*), more than half of (1–80)-EGFP and Tom20-EGFP were similarly recovered in the membrane precipitates (Fig. 4A). The endogenous Tom20 of the mitochondrial outer membrane and calnexin in the ER membrane were recovered almost completely in membrane fractions, even under the alkaline conditions. It is highly likely that some of these externally expressed EGFP-fusion proteins were not stably inserted into the membrane and remained in alkaline-soluble states. All the proteins examined were completely solubilized in the supernatant by a mild non-ionic detergent, Triton-X100, indicating that they were not insoluble aggregates. Thus, more than half of the transiently expressed EGFP fusion proteins were integrated into the membrane in an alkaline-resistant manner.

Membrane topology was examined using proteinase K (ProK) treatment. When the post nuclear supernatant was treated directly with ProK, even the mitochondria matrix proteins, such as hsp60, were degraded. We assumed that the ProK generated peptide fragments of cytoplasmic proteins that might permeabilize the organelle membrane during ProK-treatment, as previously reported (*30*). Therefore, the cells were permeabilized with a low concentration of digitonin and the soluble cytoplasmic proteins were removed from the cytoplasm. Upon ProK treatment, the apoptosis-inducing factor (AIF), which exists in the intermembrane space of mitochondria, is resistant to ProK (Fig. 4B), indicating that the pretreatment kept the organelle membranes intact during the ProK treatment. The (1–80)-EGFP was degraded and only the EGFP core domain was observed. The ProK treatment in the presence of mild detergent (Triton-X100) gave essentially the same result. As a control for the mitochondrial matrix protein, the N-terminal 135-residue segment of the ABCB10 isoform, which contains a matrix-targeting pre-sequence, was fused to EGFP and expressed in the cells. It produced three bands. Upon ProK treatment the precursor form (P) was degraded but the other two processed mature forms (m1 and m2) were resistant, as previously reported (*11*). In the presence of detergent (Tx), the fusion proteins were degraded and only the EGFP core domain remained. The (1–80)-EGFP fusion protein was integrated into the mitochondrial outer membrane in an alkaline-resistant manner and the EGFP domain remained on the cytoplasmic side.

N80-segment is essential for correct location of PMP70

Peroxisome-targeting signal has been reported to exist in multiple portions of PMP70. To examine the importance of the N80-segment in the context of full-length PMP70, the N80-segment was deleted from the full-length PMP70. The wild-type and mutant PMP70 were expressed in COS cells. The mutant was unstable and was detected only in the presence of the proteasome inhibitor MG132 (Fig. 5A). The wild-type PMP70 molecule was similarly observed, irrespective of the presence of MG132. Upon immunofluorescent observation, the PMP70 was exclusively localized in the peroxisomal dots (Fig. 5B). In contrast, the N80-segment deleted PMP70 was hardly observed in

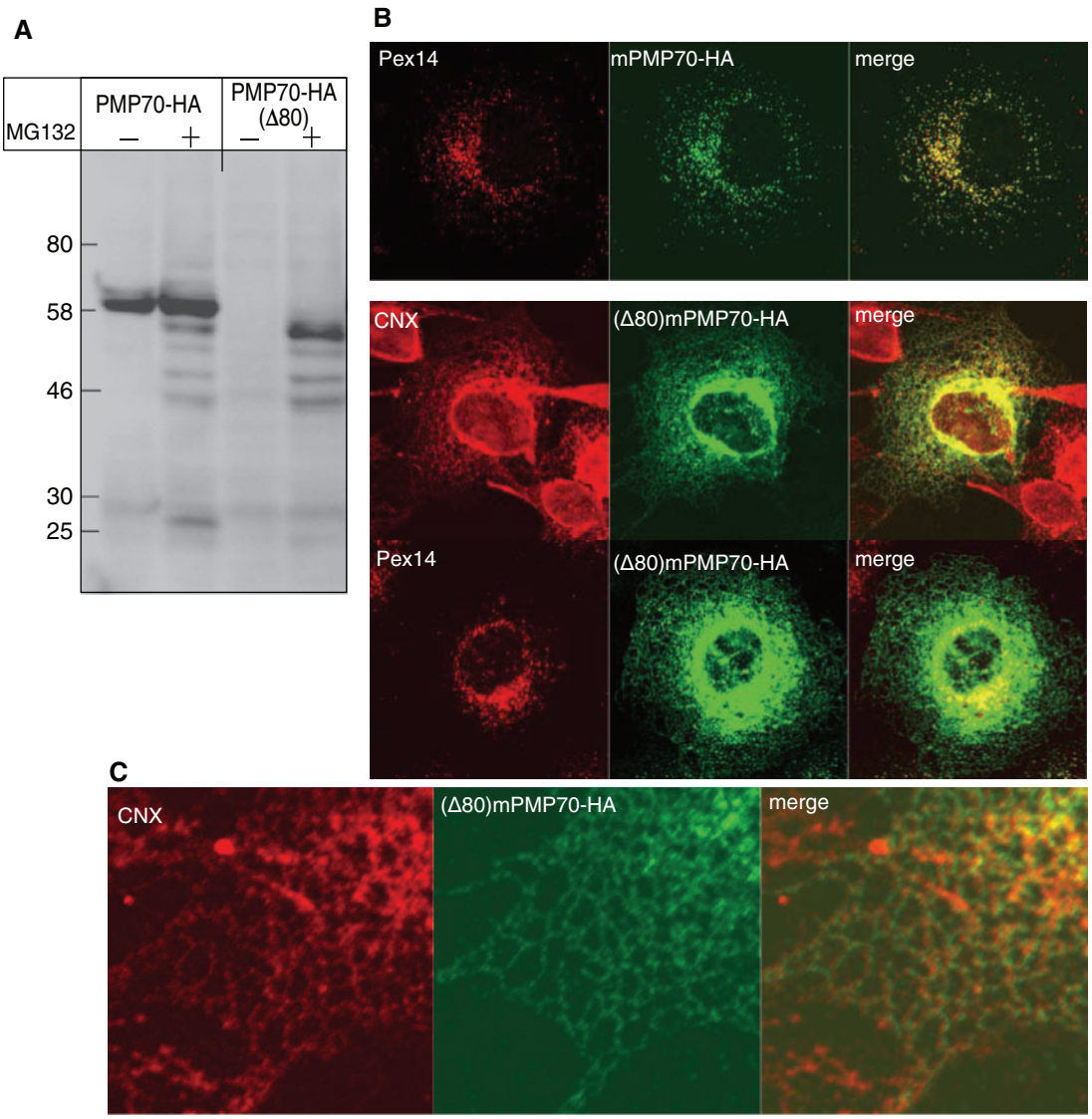

Fig. 5 N80-segment is essential for correct localization of PMP70. (A) Hemagglutinin (HA)-tagged full-length mouse PMP70 and the N80-segment deleted mutant were expressed in COS cells in the presence or absence of MG132. Total cell extracts were probed by immuno-blotting using anti-HA monoclonal antibody. (B) Localization of the constructs was probed by fluorescent microscopy using anti-HA monoclonal antibody. Peroxisomes were visualized by anti-Pex14 antibodies, and ER was by anti-calnexin (CNX) antibodies. (C) High magnification revealed the reticular structure of the N80-deleted PMP70.

peroxisomes, and the majority was observed in the reticular structure. Superimposition of the structure over the ER pattern stained with calnexin indicated that it was located in the ER. The expression level of the N80-deleted mutant was similar to that of the wild-type PMP70 molecule, indicating that the ER-location was not caused by overexpression. Thus, the N80-segment was essential for correct localization of the full-length PMP70, even though the multiple peroxisome-targeting sequence and Pex19p-binding sites were retained in the N80-segment deleted mutant.

Discussion

In this work, we demonstrated that multiple organelle-targeting and insertion signals exist in the N-terminal portion of PMP70. TM1 has intrinsic signal activity to be inserted into the ER membrane with an N_{cyto}/C_{lumen} topology. The N80-segment suppresses the ER-targeting activity of TM1 and allows the TM1–TM2 region to target and insert into the peroxisomal membranes. In addition, the whole PMP70 molecule lacking the N80-segment was localized to the ER, even though multiple peroxisome-targeting signals and Pex19 binding site were still retained. In contrast to this critical function, the N80-segment itself did not show peroxisomal-targeting activity and was inserted into the mitochondrial outer-membranes. Overall, through the cooperation of these multiple organelle-targeting activities, the N-terminal portion of 150 residues achieves posttranslational peroxisome-targeting.

During the ER-targeting process from the signal recognition in the cytosol to the insertion into the translocon, the short unit of the H-segment in the nascent polypeptide chain exerts its signal functions. The intrinsic ER-targeting activity of the TM1–TM2

region should thus be suppressed directly and/or indirectly by other segments within the nascent chain. Our findings demonstrated that the N80-segment possesses this function. The sequence might directly perturb the ER-targeting function by forming a tertiary structure. Alternatively, some factors might recognize the N80-segment to inhibit the ER-targeting and induce peroxisome-targeting. The hydrophobic residues in the N80-segment of PMP70 are suggested to include the peroxisome-targeting signal as well as the binding site of Pex19p (*20*). Studies to determine the structure and function relationship of the N80-segment and analysis of binding factors are now in progress.

The N80-segment is integrated into the mitochondrial outer membrane, highly likely via the H0-segment (Fig. 1B). Its hydrophobicity and the following positive charges are quite similar to the mitochondrial outer-membrane targeting signal of Tom20; weakly hydrophobic segment and the following positive charge cluster are characteristic of the signal (*27*). When the N80-segment of full-length PMP70 was replaced with the mitochondrial outer-membrane targeting signal of Tom20, the chimeric protein localized to the ER (data not shown). A specific combination of the N80-segment and the TM1–TM2 region in PMP70 should be critical for the correct localization.

Even though the TM1–TM2 region shows peroxisome-targeting activity with the N80-segment, TM1 itself is integrated into the ER membrane in the absence of the N80-segment. Peroxisome-targeting signals exist at multiple sites in redundant fashion in the PMP70 molecule; those are the hydrophobic regions adjacent to TM1 and TM5 (*20*). The signal adjacent to TM1 cannot exert the full function even in the presence of the N80-segment, and requires the TM2 for efficient peroxisomal targeting. In the PMP70 molecule, Pex19p binding sites have been identified in the N-terminal 60 residue portion and around the TM6 segment of PMP70 (*19*); the fusion protein of dihydrofolate reductase and the N-terminal 61-residues segment of human PMP70 interacted with Pex19p and the segment 263–347, including TM5-6, is critical for Pex19p binding. These peroxisome-targeting signals and Pex19p-binding motifs are functional only after ER-targeting suppression by the N80-segment.

We demonstrated the importance of the N-terminal region of PMP70 that suppresses ER-targeting of TM1. It is highly likely that the ABC transporters in mitochondria and peroxisomes generally possess an N-terminal sequence that suppresses the ER-targeting of the downstream TM segments. The ER-targeting of the ABCB10 isoform is suppressed by its long N-terminal import presequence. The B7 and B9 isoforms also possess an orthologous pre-sequence in the N-terminus. When the N80-segment of PMP70 was replaced with the B10 presequence, the chimeric PMP70 was imported into the mitochondria (data not shown). The pre-sequence of the B10 isoform is so strong that it dominates not only the ER-targeting signal but also any peroxisome-targeting signals. We assume that the N80-segment, which is destined to the mitochondrial outer membrane, is a moderate mitochondrial signal that suppresses ER-targeting and can cooperate with peroxisome-targeting signals. We also observed that ABCD1 and ABCD2 isoforms are located at the peroxisome depending on the upstream region of the TM1 segment. In contrast to these cases, the ABC isoforms that possess no ER-targeting suppressor in the N-terminal region are cotranslationally targeted to the ER as noted with the following examples. Different locations have been proposed for the ABCB6 isoform: mitochondria (*31*) and secretory organelles (*31–33*). The existence of five highly hydrophobic segments at the N-terminus strongly suggests that it is sorted to the ER via a cotranslational mechanism. The ABCD4 isoform, which was proposed to localize in peroxisome, has recently been demonstrated to localize in the secretory pathway (*34*). Cells and/or tissue-specific switching of organelle localization might be an interesting hypothesis and should be addressed. Similarly, another multispanning membrane protein, NHE6, which was reported to be in mitochondria has also been demonstrated to be targeted to the ER and sorted to the exocytic endocytic pathway (*23*, *35*, *36*). We propose that the sequence elements of the ER-targeting suppressor play a key role in correct organelle-targeting of hydrophobic multispan membrane proteins.

We propose the following model for initial stage of PMP70 biogenesis: (i) The N-terminal region emerging from the ribosome suppresses cotranslational ER-targeting of the following TM segments. The nascent chain remains in the cytosol. The upstream region might be recognized by unknown trans-factor(s) or directly suppresses the ER-targeting function of TM1. (ii) Pex19p and other chaperones bind nascent chain and maintain its import-competent state. In this state, the N80-segment and TM2 are critical for peroxisome-targeting. (iii) After polypeptide chain elongation is completed, the nascent chain is targeted to the peroxisome, as previously proposed (*20*).

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Conflict of interest

None declared.

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