Topogenesis of Peroxisomal Membrane Protein Requires a Short, Positively Charged Intervening-loop Sequence and Flanking Hydrophobic Segments

STUDY USING HUMAN MEMBRANE PROTEIN PMP34*

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Human 34-kDa peroxisomal membrane protein (PMP34) consisting of 307 amino acids was previously identified as an ortholog of, or a similar protein (with 27% identity) to the, 423-amino acid-long PMP47 of the yeast Candida boidinii. We investigated membrane toponogenesis of PMP34 with six putative transmembrane segments, as a model peroxisomal membrane protein. PMP34 was characterized as an integral membrane protein of peroxisomes. Transmembrane topology of PMP34 was determined by differential permeabilization and immunofluorescent staining of HeLa cells ectopically expressing PMP34 as well as of Chinese hamster ovary-K1 expressing epitope-tagged PMP34. As opposed to expressing PMP34 as well as of Chinese hamster ovary-K1 expressing epitope-tagged PMP34. As opposed to PMP47, PMP34 was found to expose its N- and C-terminal parts to the cytosol. Various deletion variants of PMP34 and their fusion proteins with green fluorescent protein were expressed in Chinese hamster ovary-K1 and were verified with respect to intracellular localization. The loop region between transmembrane segments 4 and 5 was required for the peroxisome-targeting activity, in which Ala substitution for basic residues abrogated the activity. Three hydrophobic transmembrane segments linked in a flanking region of the basic loop were essential for integration of PMP34 to peroxisome membranes. Therefore, it is evident that the intervening basic loop plus three transmembrane segments of PMP34 function as a peroxisomal targeting and topogenic signal.

The peroxisome is a model system for addressing protein traffic and membrane biogenesis, especially where it is linked to human neurological and metabolic diseases, called peroxisome biogenesis disorders. Import of matrix proteins into peroxisomes has been investigated to a greater extent at the molecular and cellular levels (1, 2). Peroxisomal matrix proteins, such as 22-kDa PMP (PMP22) (6), PMP70 (7), and Pex2p (8) of rat liver are also synthesized on free polysomes. Post-translational import of PMP22 and PMP70 were shown in vitro (9, 10). It is noteworthy that Pex16p and Pex2p are N-glycosylated in the yeast Yarrowia lipolytica (11). Pts of membrane protein, termed mPTS, was previously suggested for PMP47 with six putative transmembrane segments of the yeast Candida boidinii (12). The loop region between the fourth transmembrane segment (TM4) and TM5, which was enriched in positively charged amino acids was functional as a PTS when fused to cytosolic proteins (12). It is also noteworthy that an internal region, including the predicted TM3, of rat PMP70, a six-TM protein, is essential for the peroxisomal localization (10). The N-terminal amino acid residues at positions 1–40, which are relatively enriched in positively charged residues, were recently shown to be required for translocation of Pex3p, an integral membrane peroxin, to peroxisomes in mammals (13–15) and yeast (16, 17). Accordingly, to delineate mPTS and to establish a general paradigm for topogenesis of PMPs, more information is required.

In the present study, we have chosen PMP34 as a model protein, to address these issues. PMP34 was recently cloned by expressed sequence tag data base search using C. boidinii PMP47 and was shown to be localized to peroxisomes (18). We found that PMP34 is an integral membrane protein of peroxisomes. In contrast to PMP47, PMP34 exposes both N- and C-terminal parts to the cytosol. We also identified the loop region between TM4 and TM5 as a potential mPTS. This loop plus three TM5s was essential for targeting and integration of PMP34. These results provide the first evidence for a functional mPTS for the topogenesis of a membrane protein that spans membranes multiple times in mammalian peroxisomes.

EXPERIMENTAL PROCEDURES

Reagents and Biochemicals—Restriction enzymes and DNA modifying enzymes were purchased from Nippon Gene (Tokyo, Japan), Toyobo acyl-CoA oxidase; FITC, fluorescein isothiocyanate; GFP, enhanced green fluorescent protein; HA, influenza virus hemagglutinin; mPTS, peroxisome targeting signal for membrane protein; PAGE, polyacrylamide gel electrophoresis; PMP47, 47-kDa peroxisomal integral membrane protein; PMP34, 34-kDa PMP; TM, transmembrane segment; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; FNS, postnuclear supernatant.

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(Tokyo, Japan), and Takara (Tokyo, Japan). Fetal calf serum and Ham’s F-12 were from Life Technologies, Inc. Anti-PMP34 antibody was raised in rabbits by immunizing with synthetic peptide comprising the C-terminal, 19-amino acid sequence of human PMP34, supplemented with cysteine at the N terminus that had been linked to keyhole limpet hemocyanin (19). Rabbit antibody against influenza virus hemagglutinin (HA) was likewise raised using synthetic peptide, CYPDVYPDY- YASLRS-NH₂. Guinea pig antibody to human catalase (Sigma) was raised by conventional subcutaneous injection. We also used rabbit antibodies to PTSS peptide (20), acetyl-CoA oxidase (AOx) (21), and C-terminal 19-amino acid residues of rat Pex14p (4). Rabbit antibody to fetal calf serum, under 5% CO₂, 95% air (21).

**Cell Culture**—CHO-K1 and HeLa cells were cultured in Ham’s F-12 medium and RPMI 1640, respectively, both supplemented with 10% fetal calf serum, under 5% CO₂, 95% air (21).

**Isolation and Epitope Tagging of HsPMP34**—Human PMP34 cDNA (HsPMP34) was cloned by PCR-based technique on the human liver cDNA library in pCMVSPORT (Life Technologies, Inc.) (22), using as a set of primers, HsPMP34F and HsPMP34R (Table I). Six independent cDNA clones were sequenced, all showing that nucleotide residue at position 315 in a codon (CTG) for Leu was G instead of CTG reported by Wylin et al. (18). HsPMP34 cDNA was cloned into the NotI-SalI site in pUCD2HysSRα (23). Tagging of epitopes, flag, and tandem HA (HA-HA) to the N and C terminus, respectively, of human PMP34 was conducted as follows. The full length of HsPMP34 was amplified by PCR of primers, HsPMP34F and PMP34R. The PCR product was digested with NotI and NheI, then ligated into the NotI-NheI sites, upstream of a double-HA tag sequence, of pUCD2HysSRα. Then the NotI-SalI fragment of vector pBluescript SK(−) was inserted into the NotI-SalI sites of pBluescript SK(−)–flag-PMP34-HA (24) and pUCD2HysSRα. The SalI fragment of pBluescript SK(−)–flag-PMP34-HA was subcloned into pUCD2HysSRα, termed pUCD2HysSRαflag-HsPMP34-HA. All plasmid constructs were assessed by sequence analysis.

**Construction of PMP34 Variants**—N-terminus truncation mutants of PMP34 were generated by PCR, using as forward primers, ΔN30F, ΔN125F, ΔN186F, or ΔN204F, and a reverse primer PMP34R. C-terminal deletion mutants were likewise constructed by PCR, using reverse primers, ΔN204R, ΔN186R, and a forward primer HsPMP34F. HA tagging at their C terminus was done as described above. To construct enhanced green fluorescent protein (GFP)-fusion proteins, SpeI site was introduced to the initiation codon of GFP-expression plasmid pGFP105-C1, a generous gift from T. Tsuchimoto and T. Osumi (Himeji Institute of Technology, Himeji, Hyogo, Japan) by PCR using primers, GFPSpeF and GFPshortR. The resulting PCR fragment was digested with SpeI and introduced into SpeI-NcoI sites of pBluescript SK(−), termed pBluescript SK(−)–GFP, SpeI-NcoI fragment of pBluescript SK(−)–GFP and NotI and NheI fragment of ΔN125, ΔN186, 204Ter, or 186Ter, cloned in pGEM/T-easy vector were introduced into the NotI-SalI site of pUCD2HysSRα. Chimera constructs for 30/204GFP and 86/204GFP were generated by PCR, using a forward primer ΔN30F or ΔN68F, and a reverse primer 204TerR. Fusion for 86/273GFP and 125/273GFP were likewise constructed, using a forward primer ΔN86F or ΔN125F, and a reverse primer 273TerR. GFP tagging was performed as described above. Expression plasmid for 187/204GFP, the fourth loop domain of PMP34 fused to the N terminus of GFP, was constructed by PCR, using as a template pUCD2HysSRα and as a primer ΔN186F and GFPshortR. For construction of GFP187/204HA, BglII site was introduced by PCR on pUCD2HysSRα using BglII1187F and pUCD2HysSRα reverse (D3R) as forward and reverse primers, respectively. The BglII and SalI fragments of 187/204HA and NotI-BglII fragment of pBluescript SK(−) were GFP fusion were introduced into the NotI-SalI site of pUCD2HysSRα.

**Expression of PMP34 and Its Derivatives in HeLa and CHO-K1 Cells**—DNA transfection was done using LipofectAMINE (Life Technologies, Inc.), as described (23). The cells were cultured for 36 h after transfecting the plasmid into the cells. Cells were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100, and peroxisomes were visualized by indirect immunofluorescence light microscopy, as described (26). Antibody-antibody complexes were detected under a Carl Zeiss Axioskop FL microscope. FITC-labeled sheep anti-mouse antibody (Amersham Pharmacia Biotech, Tokyo, Japan), FITC-labeled sheep anti-rabbit immunoglobulin (Ig) G antibody (Cappel), or Texas Red-labeled goat anti-mouse IgG antibody (Jackson Immunoresearch) were used, respectively. The cells were cultured for 36 h after transfection, fixed with 4% paraformaldehyde at room temperature, permeabilized with 1% Triton X-100, and stained with a mixture of mouse anti-pig catalase antibody and guinea pig anti-human catalase antibody, as described (26). Cells were washed with PBS and permeabilized with 10% guinea pig IgG (Vector Laboratories) and rabbit IgG (Leinco Technologies). GFP was directly observed by fluorescent microscopy with the use of the same filter for FITC after fixation (27). Flag-PMP34-HA was detected using rabbit anti-HA antibody and mouse anti-flag antibody, in cells that has been fixed as above and then permeabilized with either 25 μg/ml digitonin or 1% Triton X-100 (23, 28).

**HeLa Cells** were transfected with pUCD2HysSRα for 204GFP. Cells were stained with rabbit antibody in PMP34 C-terminal peptide antibody and guinea pig anti-human catalase antibody, after permeabilization for 5 min using either 25 μg/ml digitonin or for 2 min with 1% Triton X-100. Protease Sensitivity Assay—HsPMP34-transfected CHO-K1 cells (1 × 10⁵) were homogenized in 0.5 ml of a homogenizing buffer: 0.25 M sucrose, 25 mM ammonium carbonate, pH 7.4, 20 μg/ml each of leupeptin and antipain, and 500 units/ml aprotinin, by 10 strokes of an Elvehjem-Potter homogenizer. A postnuclear supernatant (PNS) fraction was prepared by centrifugation of homogenate at 10,000 x g for 5 min. The PNS from 1 × 10⁶ cells was treated with 2 μg of Staphylococcus aureus V8 protease (Roche Molecular Biochemicals) at 25 °C for 1.5 h in 0.5 ml of the homogenizing buffer, in the absence or presence of 1% Triton X-100. The reaction was terminated by precipitation using trichloroacetic acid, and whole proteins were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot.
Other Methods—Liver peroxisomes were isolated from a normal rat, as described (29). Western blot analysis was done using primary antibodies and a second antibody, donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech). Antigen-antibody complex was visualized with ECL. Western blotting and detection reagent (Amersham Pharmacia Biotech).

RESULTS

Characterization of HsPMP34 Protein—The intracellular localization of PMP34 was determined by ectopic expression of epitope-tagged HsPMP34 and immunofluorescence microscopy. In wild-type CHO-K1 cells expressing PMP34 tagged with HA at its C terminus, PMP34 was detected as a punctate staining pattern using anti-HA antibody. The staining pattern was superimposable with that obtained using anti-Pex14p antibody, thereby suggesting that PMP34-HA was targeted to peroxisomes (Fig. 1A). Similar results were obtained when HsPMP34 was expressed in HeLa cells and stained with anti-PMP34 peptide antibody (see Fig. 1C). The rabbit antibody raised to the C-terminal peptide of human PMP34 specifically reacted with an endogenous, single protein with an apparent molecular mass of ~34 kDa in immunoblot of rat liver peroxisomes (Fig. 1B, left), confirming that PMP34 is a bona fide protein of peroxisomes. By sequence similarity to C. boidinii PMP47 (12, 18), HsPMP34 is likely to be an integral membrane protein with six putative transmembrane segments. The integrity of PMP34 in peroxisomal membranes was verified by extraction with 0.1 m sodium carbonate, pH 11.3 (30). PMP34-HA expressed in CHO-K1 cells was not extracted with sodium carbonate (Fig. 1B, right) and was recovered in membrane fraction at a similar level as in postnuclear supernatant fraction (data not shown). Pex14p, a peroxisomal membrane peroxin, was likewise in membrane pellet, whereas a matrix enzyme AOX was in soluble fraction. These data thereby indicated that PMP34 is integrated into membranes.

Next, we determined transmembrane topology of PMP34 by the differential permeabilization/immunofluorescence microscopy method (28, 31). HeLa cells were transfected with HsPMP34. Upon treatment with 1% Triton X-100, which solubilizes all cellular membranes, a punctate staining pattern was observed using anti-PMP34 C-terminal peptide antibody, in a superimposable manner with catalase presumably representing peroxisomes (Fig. 1C, a and c). Similar morphological pattern, PMP34-staining, was observed after permeabilization with 25 µg/ml digitonin, which selectively permeabilized plasma membranes (28, 31) (Fig. 1C, b), whereas none of the cells were stained with anti-catalase antibody under the same condition (Fig. 1C, d), indicating that peroxisomal matrix proteins are not accessible to antibody. Taken together, C-terminal part of PMP34 is most likely to be exposed to the cytosol. Moreover, the C-terminal part of PMP34 in HsPMP34-overexpressing CHO-K1 cells was likewise detected by the digitonin treatment, although requiring much higher concentration of digitonin under which conditions matrix proteins still remained inaccessible to antibodies (data not shown). We do not know the reason why PMP34 was visible only at higher concentration of digitonin in this particular case, as compared with that normally permeabilizing plasma membranes (4, 23). Membrane topology of PMP34 was also determined using N-terminally flag- and C-terminally HA-tagged PMP34. When flag-HsPMP34-HA-transfected CHO-K1 cells were permeabilized with 25 µg/ml digitonin, a punctate staining pattern was observed using both anti-flag and anti-HA antibodies (Fig. 1D, b and f). In contrast, PTS1 proteins were not detectable under the same condition, indicating that luminal proteins are not accessible to the antibody (Fig. 1D, d). Upon treatment with Triton X-100, PTS1 proteins as well as PMP34 were discernible in numerous particles, peroxisomes (Fig. 1D, a, c, and e). Therefore, it is apparent that N- and C-terminal portions are exposed to the cytosol. Of note, the topology of PMP34 contrasts with that of PMP47, which exposes both terminal parts to matrix of peroxisomes (12).

Furthermore, PMP34 expressed in CHO-K1 cells was resistant to the treatment with exogenously added S. aureus protease V8, as verified using PNS fraction, under which condition
Pex14p was digested (Fig. 1E, left panel). Matrix enzyme Aox was also fully protected from the digestion. Both PMP34 and Aox were no longer discernible after protease treatment in the presence of Triton X-100 (Fig. 1E, lane 3). These results were interpreted to mean that multiple sites, C-terminal side of Gln, for cleavage by V8 protease, locate inside of peroxisomes (Fig. 1E, right panel). It is conceivable that three potential cleavable Gln sites may not be readily attacked by the protease, probably owing to their location close to the transmembrane-spanning segments. The data together support the notion described above that the N- and C-terminal parts of PMP34 are exposed to the cytosol.

The Fourth Loop Domain of PMP34 Contains Peroxisome Membrane Targeting Signal—Although transmembrane topology of PMP34 is opposite to that of CbPMP47, amino acid sequence of the fourth intervening-loop region of PMP34 is similar to the one containing mPTS of PMP47 (12) (see Fig. 3A). To search for peroxisome targeting signal of PMP34, various mutants with deletion either from N or C terminus were constructed (Fig. 2A). Mutants ΔN30HA, ΔN125HA, ΔN186HA and ΔN204HA, lacking the sequence from N terminus to the first, third, and fourth transmembrane segments, and to the fourth loop domain, respectively, were HA-tagged at the C terminus. These truncation mutants were transfected into wild-type CHO-K1 cells and analyzed for intracellular localization by immunofluorescence microscopy using anti-HA antibody (Fig. 2B). In the cells expressing ΔN30HA, punctate immunofluorescence pattern was observed and superimposable to that using anti-Pex14p antibody, thus indicating that ΔN30HA was localized to peroxisomes (Fig. 2B, a and b). ΔN125HA was likewise detected in a superimposable manner to Pex14p, demonstrating peroxisomal localization (Fig. 2B, c and d). In the case of ΔN186HA, punctate pattern was obtained, however, some signal did not correspond to that obtained from anti-Pex14p antibody (Fig. 2B, e and f, arrowheads). To confirm the intracellular localization of these two mutants, we also expressed GFP-tagged ΔN125 and ΔN186 and their peroxisomal localization were analyzed. The full-length PMP34 fused with GFP was localized to peroxisomes, as seen for PMP34-HA, when expressed in CHO-K1 cells (data not shown). Punctate signals were observed in the cells expressing ΔN125GFP, and colocalized with those noted using anti-Pex14p antibody (Fig.
Topogenic Sequence of PMP34

2B, g and h). In the case of \(\Delta N186GFP\), two types of punctate structures were found, where one type was superimposable with those seen using anti-Pex14p antibody and the other was not (Fig. 2B, i and j, arrowheads). \(\Delta N204HA\) was apparently localized to endoplasmic reticulum-like structures in addition to peroxisomes (Fig. 2B, k), thereby inferring a decrease in the topogenic activity. Together, these results suggest that 79-amino acid sequence between the loop domains 3 (L3) and 4 (L4) is required for localization of PMP34 to peroxisomes.

Next, we constructed two other types of PMP34 mutants truncated in the C-terminal region and analyzed their intracellular localization. A deletion mutant, 204HA, truncated from the C terminus to the fifth transmembrane segment was localized to peroxisomes in CHO-K1, as assessed by colocalization with Pex14p (Fig. 2B, m and n). Moreover, the expressed 204HA protein showed the membrane topology exposing the HA-tagged C terminus to the cytosol and was resistant to the sodium carbonate treatment (data not shown), hence indicating that 204HA was properly targeted and integrated to peroxisome membranes. In contrast, cytoplasmically diffused staining was observed in the cells expressing 186HA truncated from C terminus to fourth loop segment (Fig. 2B, l). Essentially the same results were obtained using GFP as a reporter fusion protein; a fusion protein, 204GFP, was found in peroxisomes (Fig. 2B, o and p), while 186GFP was not localized to peroxisomes (data not shown).

We further investigated transmembrane topology of C-terminal portion of AN125GFP and 204GFP, using anti-GFP antibody. Fig. 2C shows the topology of their C-terminal portion. GFP of the both fusion proteins was recognized by anti-GFP antibody at 25 \(\mu g/ml\) digitonin, suggesting that both mutant proteins were integrated into peroxisomal membranes and exposed the C-terminal part to the cytosol, as wild-type PMP34. These results demonstrate that the fourth cytosolically faced hydrophilic loop of 18 amino acids is necessary for targeting of PMP34 to peroxisomes.

**Positively Charged Region of the Fourth Loop Functions as a Peroxisome Targeting Signal**—The fourth loop domain of PMP34 has positively charged amino acids at positions 190, 191, 195–197, and 199 (Fig. 3A). The positively charged amino acid cluster in the fourth loop of ChPMP47 was shown to be sufficient for localizing soluble reporter proteins to peroxisomes in yeast (12). To investigate whether the sequence enriched in basic amino acids in the fourth loop of PMP34 functions as an mPTS, we first replaced all basic amino acids to Ala of this region in 204GFP, termed 6A204GFP (Fig. 3B). In the cells expressing 6A204GFP, GFP fluorescence was diffused to the cytosol, suggesting that positively charged amino acids are required for peroxisome targeting (Fig. 3C, a). To determine which amino acid is important for the peroxisome-targeting function, we subdivided the fourth loop domain into two parts and changed basic amino acids in respective parts to Ala. Mutants 4A204GFP and 3A204GFP were partially localized to peroxisomes as well as in the cytoplasm (Fig. 3C, b and c). In contrast, 2A204GFP and A204GFP was localized to peroxisomes (Fig. 3C, d and e), as efficiently as 204GFP. Four chimera proteins, 4A204GFP as well as 3A204GFP localized to peroxisomes, 2A204GFP, and A204GFP, showed the same membrane topology, exposing C-terminal GFP to the cytoplasm (data not shown). Therefore, it is more likely that KR in the first portion and KKRMK in the second part function as mPTS and that the latter half of the loop is the most important.

**The Fourth Loop Is Not Sufficient for Peroxisomal Localization**—Dyer et al. (12) reported that the last 12 amino acids of the fourth loop domain of ChPMP47 was sufficient for targeting to peroxisomes. So we fused the fourth loop domain of PMP34 to N- or C terminus of GFP and analyzed its sufficiency for peroxisomal localization. To determine whether the fourth loop functions as a sufficient information for peroxisomal targeting, a fusion protein 187/204GFP, the loop domain fused to the N terminus of GFP was expressed in CHO-K1, and found not to be localized to peroxisomes (Figs. 4A and 4B). Contrary to this, another fusion protein, GFP187/204HA, was targeted to mitochondria (Fig. 4B), although the positively charged loop in the fusion construct was located at position distinct from a general, N-terminal mitochondrial targeting sequence. These results demonstrated that the fourth loop domain of PMP34 is necessary for transport of PMP34 to peroxisomes, but not sufficient for integration into peroxisomal membranes.

**Three Transmembrane Segments of PMP34 Are Required for Integration**—204GFP and \(\Delta N125GFP\) which possess four and three transmembrane segments, respectively, were localized to peroxisomes as efficiently as wild-type PMP34, suggesting that the region(s) required for integration to peroxisomal membranes is in both N- and C-terminal domains (see above). \(\Delta N125GFP\) with three TMs containing the fourth TM, was localized to peroxisomes, while \(\Delta N186GFP\) with two TMs, but lacking the fourth TM, was localized to not only peroxisomes but also another organelles. These results suggest two possibilities for integration into peroxisomal membrane. One is that fourth TM is important for integration into peroxisomal membranes. The other is that at least three TMs are required. To

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*Fig. 3. Sorting sequence of PMP34.* A, amino acid alignment of putative peroxisome membrane-targeting signal sequence in PMP34 and mPTS of PMP47. Positively charged amino acids are underlined. B, site mutation of the loop region between TM4 and TM5 of PMP34. C, subcellular localization of PMP34 variants mutated in the loop region. Each construct was expressed in CHO-K1 cells and detected by GFP fluorescence. a, 6A204; b, 4A204; c, 3A204; d, 2A204; e, A204. Bar, 20 \(\mu m\).
address this issue, two mutants, 86/204GFP and 125/273GFP, containing the fourth TM plus one TM, were constructed (Fig. 5A). Their intracellular localization is shown in Fig. 5B. Both 86/204GFP and 125/273GFP were not localized to peroxisomes (Fig. 5B, a and e). It is of interest to note that 86/204GFP was instead transported to mitochondria, as verified by staining using anti-malate dehydrogenase antibody (Fig. 5B, b). These results suggest that at least three TMs, not the fourth TM, are required for integration into peroxisomal membranes.

To ask if three TMs of PMP34 are also required in the N-terminal portion of the fourth loop, we expressed 30/204GFP carrying three TMs, TM2–TM4, and analyzed its intracellular localization. The 30/204GFP was detected mostly in particles (Fig. 5B, c). In the GFP-positive cells, punctate structures were only partly superimposable with those stained using Pex14p antibody (Fig. 5B, c and d). These results suggest that the presence of simply three TMs upstream of the loop is not sufficient for localization, rather implying that TMs from the first to the third or the fourth to the sixth are required for integration to peroxisomal membranes. To confirm this result, a fusion protein 86/273GFP containing the third to fifth TMs of PMP34 was expressed in CHO-K1. Localization of 86/273GFP was not in peroxisomes (Fig. 5B, f). A chimera 86/230GFP, where GFP was fused to immediately downstream of TM5, showed very similar GFP fluorescence pattern as 86/273GFP (data not shown). Taken together, it is most likely that the fourth loop and three TMs, comprising either TM1–TM3 or TM4–TM6, coordinate function as peroxisomal membrane-targeting and integration information.

**DISCUSSION**

To elucidate the molecular mechanisms involved in topogenesis of PMPs, we selected PMP34 as a model PMP in the present study. We first determined the transmembrane topology of PMP34 by expressing the full-length human PMP34 in HeLa cells as well as epitope-tagged PMP34 in CHO-K1 cells. From several lines of morphological assessment using a combination of ectopic expression and the differential permeabilization method, we concluded that PMP34 exposes both N- and C-terminal portions to the cytoplasm. The orientation of PMP34 contradicts that of PMP47, a potential yeast ortholog of PMP34, of which both terminal regions reside in the matrix side of peroxisomes (12). One possible interpretation of these observations would be that PMP34 is not an ortholog of Cb-PMP47, at least with respect to the transmembrane topology; another one is that it is a homolog but the membrane topology is distinct. Otherwise, PMP34 is closely related to PMP47 but a distinct protein. Physiological function of PMP34 has not been elucidated, while PMP47 was recently suggested to be a transporter of ATP required in activating middle-chain fatty acids with CnA (32). We noted that PMP34 contains in the first loop region a highly conserved sequence, PX(D/E)XX(K/R)XX20–30(D/E)XX(K/R), found in the extramembrane loop of mitochondrial carrier proteins (33, 34). PMP4 may also be a transporter like others, such as a Ca2+-dependent peroxisomal transporter exposing both terminal regions to the cytoplasm (35).

We demonstrated that the hydrophilic loop between TM4 and TM5 of PMP34, similar to that of PMP47, is required for transport to peroxisomes but is not sufficient for integration. Instead, this basic loop plus at least three TMs, such as TM1–TM3, are likely to be responsible for the proper localization of PMP34. In the assay system we used, it was very difficult to discriminate between the activities of targeting and integration. We concluded, however, that the TM4-TM5 loop domain functions as mPTS, based on the following observations. 1) A PMP34 mutant 186HA, including TMs 1–4 but devoid of the
fourth loop domain, was not transported to peroxisomes; 2) a
mutant PMP34-GFP fusion, 6AGFP, in which all of the six
basic amino acids were substituted by Ala, was completely
mislocalized to the cytosol; 3) the fourth loop domain faces
to the cytosol; but it is unlikely that this loop can translocate
through the membrane into the matrix side and finally re-
translocate back to the cytoplasmic face. Together, these find-
ings strongly suggest that the fourth loop domain functions as
an mPTS.

The fourth loop region of PMP47 is enriched in positively
charged amino acids and functions as an mPTS (12). The region
responsible for peroxisomal targeting of other membrane pro-
teins, including Pex3p and PMP70, has been searched for.
N-terminal region, residues at positions 1–40, of the mem-
brane protein Pex3p was shown to be necessary and sufficient
for peroxisomal targeting in mammals (13–15) and yeast,
*Pichia pastoris* (16) and *Hansenula polymorpha* (17). The
highly conserved residues at 9–15, LKRHKK, of human and
*H. polymorpha* Pex3p was recently suggested as an mPTS (14,
17). This sequence is very similar to that of the basic loop in
C6PMP47 (12), thereby suggesting that the mechanisms of
targeting Pex3p and PMP47 may resemble. Imanaka et al. (10)
demonstrated that the N-terminal sequence encompassing one
third of the full-length rat PMP70 was necessary for an *in vitro*
targeting and integration. In this regard, Sacksteder et al. (36)
very recently showed the N-terminal residues at 1–124 of hu-
man PMP70 are targeted to peroxisomes *in vivo*. It is also
noteworthy that positively charged amino acid residues are
noted immediately downstream of the TM1 (residues at 28–42)
as well as upstream of the TM3 (resides at 117–124).

Between PMP34, PMP47, PMP70, and Pex3p, the cluster
enriched in relatively positively charged amino acids which
located flanking region of hydrophobic segment is a common
feature. However, no conserved amino acid sequence is ob-
served. In our Ala-scanning analysis of the basic loop of
PMP34, the peroxisomal targeting activity decreased as the
number of mutated residues in the basic amino acids increased.
A mutant PMP34-GFP fusion, 4AGFP, in which four of the six
basic amino acids were substituted by Ala, was partially local-
ized to peroxisomes. Another mutant, 204Rev-GFP, where five
basic amino acids were substituted by Ala, was localized
specifically to peroxisomes as efficiently as was seen in the case
of 204GFP and ΔN125GFP. Thus, the fourth basic loop domain is
most likely to function as mPTS, despite there being apparently
no need to specify the positively charged amino acids. Whether such basic amino acids are prerequisite for targeting of other PMPs remains to be determined. Simi-
larly, a conformational requirement is postulated for the mPTS
of PMP47 (12). In contrast to PMP47, the fourth loop region of
PMP34 was predicted to form an α-helix structure (data not
shown). Moreover, Ala is not a helix breaker. Therefore, it is
most likely that the positively charged loop works as mPTS.

We conclude from the following observations that three TMs
1–3 or 4–6 are essential for integration of PMP34 to peroxi-
somes. 1) PMP34 mutants with only two TMs were not local-
ized to peroxisomes; 2) fusion proteins, 204GFP and
ΔN125GFP, were specifically localized to peroxisomes as effi-
ciently as the full-length PMP34-GFP, although 86/273GFP
and 86/230GFP, both despite with three TMs 3–5, were not
localized to peroxisomes; 3) 80/240GFP with deletion of TM1 of
TMs 1–4, or ΔN186GFP likewise deleted in TM4 of TMs 4–6
was significantly reduced in the level of peroxisomal localiza-
tion. Thus, we infer that three TMs are necessary to integrate
into peroxisome membrane. This conclusion was confirmed by
Ala substitution mutants. Since Ala substitution mutants were
integrated into peroxisomal membrane in the same manner as
wild-type PMP34, although their targeting activity is weak, the
implication is that integration of PMP34 is dependent on its
transmembrane segment but does not depend on the fourth
loop domain. It is of interest to note that the fourth basic loop
domain fused to GFP was transported to mitochondria, pre-
sumably recognized as a mitochondrial targeting signal. Hence,
three TMs are more likely to play a role in allowing the basic
loop to be readily recognized by a putative mPTS recognition
factor (see Fig. 6, X) and not by a mitochondrial signal receptor,
as was seen in the case of 204GFP and ΔN125GFP specifically
localized to peroxisomes.

Several peroxisomal proteins have been suggested to be gly-
cosylated, including N-glycosylated Pex2p and Pex16p of *Y.
lipolytica* (11). We investigated this issue extensively using
mamalian cells. Mutation of two potential N-glycosylation
sites, including N*167*GT to DGT or N*241*RT to DRT did not alter
the mobility of PMP34 in SDS-PAGE (data not shown), sug-
gest that PMP34 is not N-glycosylated. Moreover, we ob-
served no staining of endoplasmic reticulum, using anti-
PMP34 antibody, in CHO-K1 cell overexpressing PMP34 (data
not shown). These results imply that endoplasmic reticulum is
less likely to be involved in biogenesis of PMP34.

Given the findings described here, we propose a hypothetical
model of the topogenesis of PMP34 (Fig. 6). After the synthesis
on cytoplasmic free polyribosomes, PMP34 is recognized by a soluble
factor such as putative “mPTS receptor” (designated by X) at the loop
region between TM4 and TM5, transported to the surface of peroxisome
membrane, imported by a “membrane protein import receptor,” then
finally localized to the membrane by vectorial translocation. At least
three TMs are also responsible for the targeting and/or integration.
recently reported to weakly interact with PMP34 (36). The region containing the basic loop of PMP34 may mediate binding to Pex19p. Other cytosolic factors may also be involved in the transport of PMP34.

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