Hydrophobic Regions Adjacent to Transmembrane Domains 1 and 5 Are Important for the Targeting of the 70-kDa Peroxisomal Membrane Protein

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The 70-kDa peroxisomal membrane protein (PMP70) is a major component of peroxisomal membranes. Human PMP70 consists of 659 amino acid residues and has six putative transmembrane domains (TMDs). PMP70 is synthesized on cytoplasmic ribosomes and targeted posttranslationally to peroxisomes by an unidentified peroxisomal membrane protein targeting signal (mPTS). In this study, to examine the mPTS within PMP70 precisely, we expressed various COOH-terminally or NH2-terminally (mPTS). In this study, to examine the mPTS within PMP70 precisely, we expressed various COOH-terminally or NH2-terminally deleted constructs of PMP70 fused with green fluorescent protein (GFP) in Chinese hamster ovary cells and determined their intracellular localization by immunofluorescence. In the COOH-terminally truncated PMP70, PMP70(AA.1–144)-GFP, including TMD1 and TMD2 of PMP70, was still localized to peroxisomes. However, by further removal of TMD2, PMP70(AA.1–124)-GFP lost the targeting ability, and PMP70(TMD2)-GFP did not target to peroxisomes by itself. The substitution of TMD2 in PMP70(AA.1–144)-GFP for TMD4 or TMD6 did not affect the peroxisomal localization, suggesting that PMP70(AA.1–124) contains the mPTS and an additional TMD is required for the insertion into the peroxisomal membrane. In the NH2-terminal 124-amino acid region, PMP70 possesses hydrophobic segments in the region adjacent to TMD1. By the disruption of these hydrophobic motifs by the mutation of L21Q/L22Q/L23Q or I70N/L71Q, PMP70(AA.1–144)-GFP lost targeting efficiency. The NH2-terminally truncated PMP70, GFP-PMP70(AA.263–375), including TMD5 and TMD6, exhibited the peroxisomal localization. PMP70(AA.263–375) also possesses hydrophobic residues (Ile307/Leu308) in the region adjacent to TMD5, which were important for targeting. These results suggest that PMP70 possesses two distinct targeting signals, and hydrophobic regions adjacent to the first TMD of each region are important for targeting.

Peroxisomes are organelles surrounded by a single membrane, and they are found in almost all eukaryotic cells. Peroxisomes have many important metabolic functions, including \( \beta \)-oxidation of fatty acids, especially very long-chain fatty acids, and synthesis of ether phospholipids (1). Therefore, a defect in the biogenesis and function of the organelle causes severe metabolic disease, such as Zellweger syndrome (2–4).

Peroxisomes are thought to be formed by the division of preexisting peroxisomes after the import of newly synthesized proteins (5). Peroxisomal proteins are encoded by nuclear genes, synthesized on cytosolic polyosomes, and posttranslationally targeted to the peroxisomes (5). The import of peroxisomal matrix proteins is gradually becoming well understood. Most matrix proteins are targeted to the peroxisomes by one of two types of a peroxisome targeting signal (PTS). PTS1 is a COOH-terminal tripeptide composed of SKL or a conserved sequence and is found in the majority of matrix proteins (6). PTS2 is a cleavable NH2-terminal nonapeptide with the consensus motif (R/K)(L/V/I)X_2(H/Q)(L/A) and is found in a few matrix proteins (7, 8). PTS1 and PTS2 are recognized by the specific receptors, Pex5p and Pex7p, respectively (9, 10). These receptors target the cargo proteins from the cytosol to the docking complex on the peroxisomal membrane (11–14). However, the pathway for proteins to target to the peroxisomal membrane is less well defined. Peroxisomal membrane proteins (PMPs) do not contain a recognizable PTS1 or PTS2 sequence, and they are still imported even in the mutants that are defective for the import of matrix proteins, indicating that the targeting process of PMPs is not dependent on the components of the pathway for matrix protein targeting (15–17). Recently, Pex3p, Pex16p, and Pex19p have been proposed to be essential for the proper localization and stability of PMPs (18–21).

Pex19p is localized mostly in the cytosol and binds a broad spectrum of PMPs (22–27). These observations led us to the hypothesis that Pex19p functions as a recycling targeting signal receptor for PMPs. Indeed, Pex19p-binding regions of multiple PMPs were found to be an integral part of their targeting elements (22, 25, 28–32). However, other reports have claimed that Pex19p-binding sites of some PMPs were spatially or functionally separated from their peroxisomal sorting regions (23, 24, 27, 33). These results argue against the role of Pex19p as an import receptor for PMPs and assign an alternative, chaperone-like function for Pex19p.

The abbreviations used are: PTS, peroxisome targeting signal; ABC, ATP-binding cassette; GFP, green fluorescence protein; mPTS, peroxisomal membrane protein targeting signal; PMP, peroxisomal membrane protein; PMP70, 70-kDa peroxisomal membrane protein; TMD, transmembrane domain; PCC, Pearson’s correlation coefficient; CHO, Chinese hamster ovary.

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TABLE 1
Oligonucleotide primer sequences used for the generation of PMP70 deletion mutants

| Construct name | Oligonucleotide primer (5′ → 3′) |
|----------------|----------------------------------|
| PMP70(AA.1–375)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.1–324)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.1–276)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.1–228)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.1–124)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.113–228)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.224–259)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.314–347)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.113–375)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.176–375)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.224–375)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.263–375)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.314–375)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |
| PMP70(AA.113–228)-GFP | CTGAGAACCAGCAATTGCAGGTTTGAG |

Restriction sites used for directional cloning are underlined (XhoI or PstI in forward primers; BamHI for reverse primers).

EXPERIMENTAL PROCEDURES

Materials—pEGFP-N1 and pEGFP-C3 were purchased from Clontech (Palo Alto, CA), pQE30 and pEU3-NII were from Qiagen (Valencia, CA) and TOYOBO (Osaka, Japan), respectively. PRO-MIX™L-[35S] in vitro cell labeling mix (70% L-[35S]methionine and 30% L-[35S]cysteine, >37 TBq/mmol) was purchased from Amersham Biosciences. PROTEOS™, a wheat germ cell-free protein synthesis core kit, was obtained from TOYOBO (Osaka, Japan). Nucleotides, such as ATP, CTP, UTP, and GTP, for mRNA synthesis were obtained from Promega (Madison, WI). The protein G-agarose was from Sigma. Rabbit anti-Living Colors A. v. peptide antibody was from Invitrogen. Preparation of the antibody against the COOH-terminal 15 amino acids of rat PMP70 is described in Ref. 45. The anti-rat liver catalase antibodies were raised in a rabbit (46).

Construction of PMP70 Expression Plasmids for Subcellular Localization and Immunoprecipitation—pEU3-NII/PMP70(AA.1–659) and expression constructs encoding PMP70(AA.1–659)-GFP and PMP70(AA.1–144)-GFP were prepared as described in Ref. 27. Different NH2- or COOH-terminal truncation mutants of PMP70 were generated by PCR using a full-length human PMP70 cDNA (47) as a template. The oligonucleotide primers used are listed in Table 1. PCR-generated fragments with XhoI or PstI and BamHI restriction sites were subcloned in frame into a pEGFP-N1 expression vector. To construct NH2-terminal GFP fusion proteins, NH2- or COOH-terminally truncated cDNA fragments of PMP70 were amplified from each PMP70-GFP expression vector encoding the corresponding truncated cDNA fragment of PMP70 using the forward primer.
5'-AAATGGGCAGTGGCGGTGAAAACAGG-3', which annealed to a site upstream of the unique PstI site in pEGFP-N1, or 5'-CTCGAGATGCGGCGCTCTACAG-3', which introduced an XhoI site at the amino terminus, and the reverse primer 5'-CGCTGAACCTGAGCCGCTCTACAG-3', which annealed to a site downstream of the unique BamHI site in pEGFP-N1. PCR-generated fragments with PstI and XhoI and BamHI restriction sites were subcloned in frame into a pEGFP-C3 expression vector. To construct PMP70(AA.1–124/TMD4)-GFP and PMP70(AA.1–124/TMD6)-GFP, in which TMD4 or TMD6 of PMP70(AA.1–659) were confirmed by semiautomated sequencing on an ABI 310 DNA sequencer using the forward primer 5'-CTCGAGATGCGGCGCTCTACAG-3', which introduced an XhoI site at the amino terminus, and the reverse primer 5'-CGCTGAACCTGAGCCGCTCTACAG-3', which annealed to a site downstream of the unique BamHI site in pEGFP-N1. PCR-generated fragments with PstI or XhoI and BamHI restriction sites were subcloned in frame into a pEGFP-C3 expression vector. Two sets of the oligonucleotide primers used were designed on the basis of their sequences (Table 2). The mutations in the constructions were confirmed by semiautomated sequencing on an ABI 310 DNA sequencer (PerkinElmer Life Sciences).

### Table 2

| Construct name | Forward primer (5’ → 3’)
|----------------|------------------------------------------------|
| K28A/R29A      | GCTTGCCCTGCTCAGGCGGGCGGCAGGCGCCCTCAGG |
| R30A/R31A      | CTCGCCTGAGGCGGCGGCAGGCGCCCTCAGG |
| K38A/K39A      | CTCGCCTGAGGCGGCGGCAGGCGCCCTCAGG |
| P76A/R77A      | CAGATCGTGGTACGATTTTGAGGGGAGCGAGGCGAGG |
| K72A           | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| R66A           | CAGAGGCGTCTTATCGGAGGCGAGGCGAGG |
| K61A           | CAGATCGTGGTACGATTTTGAGGGGAGCGAGGCGAGG |
| K53A/K54A/R56A | CAATGAGGAGAGGGGCGAGGCGAGGAGCGAGGAGCGAGG |
| K123A/R124A    | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| R119A/K120A    | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| R117A          | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| L21A/L22A/L23A | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| L21Q/L22Q/L23Q | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| I70A/I71A      | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| I70N/I71N      | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |
| I307N/I308Q    | GCTGCATACGAGATTGCTGAGCTGAGCTGAG |

Site-directed Mutagenesis of Conserved Amino Acids and Hydrophobic Amino Acids in the Targeting Elements of PMP70—Site-directed mutagenesis was performed on pEGFP-N1/PMP70(AA.1–659), pEGFP-N1/PMP70(AA.1–144), pEGFP-C3/PMP70(AA.263–375), and pE33U-NII/PMP70(AA.1–659) using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Two sets of the oligonucleotide primers used were designed on the basis of their sequences (Table 2). The mutations in the constructions were confirmed by semiautomated sequencing on an ABI 310 DNA sequencer (PerkinElmer Life Sciences).

### Culturing Conditions and Transient Transfection—CHO-K1 cells were cultured in F12K medium (ICN, Aurora, OH) with 10% fetal bovine serum at 37 °C and 5% CO2. 48 h before transfection, the cells were washed three times with phosphate-buffered saline and fixed for 10 min in 5% paraformaldehyde in phosphate-buffered saline for indirect immunofluorescence.

Indirect Immunofluorescence—Immunostaining was performed by essentially the same procedure as described in Ref. 7. The fixed cells were permeabilized in 0.1% (w/v) Triton X-100 in phosphate-buffered saline for 10 min, washed twice with phosphate-buffered saline, and incubated with the primary antibodies for 1 h at room temperature. The primary antibodies used in this study were a rabbit antibody against the COOH-terminal 15 amino acids of rat PMP70 (1:200) and a rabbit antibody against rat catalase (1:200). Cy3-conjugated goat anti-rabbit IgG antibody (Amersham Biosciences) was used to label the first antibody. The cells were mounted in 90% glycerol in 100 mM Tris-HCl (pH 8.0), and the samples were examined by confocal microscopy (LSM510; Carl Zeiss, Jena, Germany). To analyze the efficiency of peroxisomal localization, samples were examined by TCS-SP5 software (Leica, Wetzlar, Germany). The per pixel scatter diagrams were generated using the built in software of the Leica TCS-SP5. Pearson’s correlation coefficient (PCC) and the peroxisome colocalization rate were employed to evaluate colocalization. PCC is one of the standard measures to assess the relationship between fluorescence intensities (48). Its value ranges between –1.0 and 1.0, where –1.0 represents no overlap and 1.0 represents complete colocalization. The peroxisome colocalization rate was expressed as a ratio of colocalization area showing certain red pixel intensity of peroxisomal marker and certain green pixel intensity of each GFP fusion protein against the area foreground. In a typical experiment, 20 randomly chosen areas containing some of the cells expressing GFP fusion protein were examined for each culture, and each experiment was repeated at least three times.

Purification of His-Pex19p—Purification of the NH2-terminal His6-tagged human Pex19p (His-Pex19p) was performed by essentially the same procedure as described in Ref. 27. M15 pREP4 Escherichia coli cells (Qiagen, Valencia, CA) harboring pQE30/Pex19 were grown at 37 °C in LB medium containing 0.1 mg/ml ampicillin. At a cell density of 0.5 (A600), protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h at 37 °C. The cells were harvested by centrifugation at 4,000 × g for 20 min, resuspended in 35 ml of the lysis buffer (50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 5 mM imidazole, 0.1 mM phenylmethylsulfonyl fluoride), and disrupted 20 times for 20 s in an ice bath by an Astrason XL-2020 ultrasonic processor (Misonix Inc., Farmingdale, NY). The lysate was cen-
trifuged at 20,000 × g for 30 min and the His-Pex19p in the supernatant was immediately applied to 10 ml of TALON Metal affinity resin (Clontech) equilibrated with the lysis buffer. After extensive washing, the His-Pex19p was eluted with the lysis buffer containing 250 mM imidazole. The eluted fractions containing His-Pex19p were dialyzed against 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10 mM dithiothreitol and stored at −80 °C.

In Vitro Transcription and Translation—The plasmids encoding wild type and mutant PMP70s were transcribed in vitro using T7 RNA polymerase, and the synthesized mRNAs were isolated by a MicroSpin G-25 column (Amersham Biosciences). Using the purified mRNA, cell-free translation was performed according to the bilayer method using PROTEIOS™, a wheat germ cell-free protein synthesis core kit, according to the manufacturer’s procedure. In a typical experiment, the synthesized mRNAs were translated for 24 h at 26 °C in a 300-µl wheat germ cell-free protein synthesis system containing 50 µCi of [35S]methionine in the presence of 100 µg of His-Pex19p. After translation, the reaction mixture was centrifuged for 20 min at 17,000 × g, and the supernatant was used for co-immunoprecipitation.

Co-immunoprecipitation—Translation products (50 µl) were pre-cleaned with an appropriate amount of protein G-agarose in 200 µl of the binding buffer (20 mM Hepes-KOH, pH 7.5, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EDTA, 0.2% Triton X-100, 10 mM dithiothreitol). After this step, the supernatant was removed and incubated with protein G-agarose beads saturated with anti-His G antibody. After incubation of the suspensions for 2 h at 4 °C, the beads were collected by centrifugation and washed five times with 250 µl of the binding buffer. Immunoprecipitated proteins were analyzed on a 7–15% SDS-polyacrylamide gradient gel. The gels were dried, and the radioactivity of the band corresponding to PMP70 was quantified by a Fuji BAS 5000 imaging analyzer (Fuji Film, Tokyo, Japan).

RESULTS

Subcellular Localization of COOH-terminal Truncated PMP70—To determine the mPTS of PMP70, we first expressed various COOH-terminal deletion constructs of PMP70 fused with GFP in CHO cells and examined their intracellular localization.

FIGURE 1. Subcellular localization of COOH-terminal truncated PMP70 in fusion with the COOH terminus of GFP. A, constructs of the PMP70 truncation mutants and their peroxisomal localization. The ellipse shows the position of GFP, and black boxes represent the position of the six putative TMDs. B, GFP-PMP70 fusion proteins were expressed in CHO cells. The subcellular distribution of the fusion proteins was compared with the localization of the endogenous PMP70 detected by immunofluorescence staining with anti-PMP70 COOH-terminal antibody. The peroxisomal staining pattern is shown on the left, EGFP fluorescence in the center, and a superimposition of both stains on the right.
ization by immunofluorescence. We have recently shown that PMP70(AA.1–659)-GFP and PMP70(AA.1–375)-GFP, which possess whole NH2-terminal transmembrane segments, were localized to peroxisomes and that PMP70(AA.1–144)-GFP was still targeted to peroxisomes, indicating that PMP70 possesses an mPTS in the NH2-terminal 144-amino acid region (27).

It is suggested that the targeting characteristics are influenced by the position of GFP. Therefore, we expressed various COOH-terminal deletion constructs of PMP70 in fusion with the COOH terminus of GFP (Fig. 1A). As shown in Fig. 1B, GFP-PMP70(AA.1–375) exhibited a punctated immunofluorescent pattern that coincided with that of peroxisomes. GFP-PMP70(AA.1–324) was still localized to peroxisomes, and further COOH-terminal deletion constructs, including GFP-PMP70(AA.1–276), GFP-PMP70(AA.1–228), and GFP-PMP70(AA.1–144), were still directed to peroxisomes. These results also indicate that, even in the case of NH2-terminal GFP fusion, the mPTS of PMP70 could still exist in the NH2-terminal 144-amino acid region.

Role of TMD2 in the Targeting of PMP70—To identify the minimal region in the NH2-terminal region of PMP70 that is sufficient for its proper peroxisomal localization, we made small deletions in the NH2-terminal region and examined the subsequent intracellular localization (Fig. 2A). As shown in Fig. 2B, PMP70(AA.1–144)-GFP was localized to peroxisomes. However, further deletion of TMD2 led to mislocalization of the PMP70(AA.1–124)-GFP fusion protein. Quantitative colocalization assessment of the relationship between fluorescence intensities confirmed the high extent of colocalization of PMP70(AA.1–144)-GFP with peroxisomes (PCC of 0.86 ± 0.02), and ~80% of the fluorescence coincided with that of peroxisomes. On the other hand, PCC of PMP70(AA.1–124)-GFP decreased to 0.39 ± 0.01, and only 15% of the fluorescence of PMP70(AA.1–124)-GFP coincided with that of peroxisomes. These values were almost the same as in the case of GFP alone (PCC of 0.28 ± 0.03, and 10% of the fluorescence coincided with that of peroxisomes). Furthermore, subcellular fractionation showed that the distribution of PMP70(AA.1–124)-GFP was different from that of endogenous PMP70 and was almost the same as that of calnexin, a marker protein of endoplasmic reticulum (data not shown). PMP70(TMD2)-GFP did not target to peroxisomes by itself either. PMP70(TMD2)-GFP was mainly recovered in the nuclear fraction and some was in the cytosol fraction (data not shown). To elucidate the role of the second TMD in the peroxisomal targeting of PMP70, TMD2 in PMP70(AA.1–144)-GFP was swapped for TMD4 or TMD6 of PMP70, respectively. PMP70(TMD4)-GFP and PMP70(TMD6)-GFP did not target to peroxisomes by themselves. However, PMP70(AA.1–124/TMD4)-GFP and PMP70(AA.1–124/TMD6)-GFP displayed the peroxisomal localization. These results suggest that the important targeting information of PMP70 is contained within an NH2-terminal 124-amino acid region and that at least two TMDs are required for integration into the peroxisomal membrane.

Basic Amino Acid Clusters in the NH2-terminal Region of PMP70 Are Not Essential for the Peroxisomal Targeting of PMP70—In the NH2-terminal 124-amino acid region, there are three clusters of basic amino acids (the first cluster is at amino acid positions 28, 29, 30, 31, 38, and 39; the second is at positions 53, 54, 56, 61, 66, 72, and 77; the third is at positions 117, 119, 120, 123, and 124) (Fig. 3A). The positively charged amino acid cluster is suggested to be the peroxisomal targeting motif of other PMPs. To examine whether these basic clusters function as an mPTS of PMP70, we replaced these basic amino acids in the respective parts of PMP70(AA.1–144)-GFP with Ala. PMP70(AA.1–144)-GFP was localized to peroxisomes (Fig. 2B). As shown in Fig. 3B, PMP70(AA.1–144 K28A/R29A/R30A/R31A/K38A/K39A)-GFP, PMP70(AA.1–144 K53A/K54A/R56A/K61A/R66A/K72A/R77A)-GFP, and PMP70(AA.1–144 R117A/R119A/K120A/K123A/R124A)-GFP were localized to peroxisomes, and the targeting efficiency of each mutant protein was almost the same as that of PMP70(AA.1–144)-GFP. Under the same condition, PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP were not localized to peroxisomes as negative controls (see Fig. 4). These data suggest that these basic clusters are not essential for the targeting of PMP70, and the mPTS of PMP70 is located in another part of the molecule.

In addition to the basic clusters, there are highly conserved amino acid sequences among peroxisomal ABC proteins (PMP70, adrenoleukodystrophy protein, and ALDRP) in the NH2-terminal 124-amino acid region (Fig. 3A). We replaced these amino acids with Ala or Val in PMP70(AA.1–144)-GFP as follows: A18V/A19V/F20V, V62A/F63A, L67A/I68A, E82A/T83A, G84A/Y85A/L86A/L88A, S95A/R96A, and G113V/I114A, respectively. However, all mutant proteins we examined were localized to peroxisomes (data not shown).

Hydropathic Motifs Adjacent to the NH2-terminal Side of TMD1 Are Important for the Stability and the Peroxisomal Targeting of PMP70—Based on the hydropathy profiling, peroxisomal ABC proteins possess two hydrophobic segments adjacent to the NH2-terminal side of TMD1 (Fig. 3A). To examine whether these hydrophobic motifs are important for the targeting of PMP70, we disrupted these hydrophobic properties by the mutation of L21Q/L22Q/L23Q or I70N/L71Q and examined the subcellular localization. When these amino acid residues were substituted for alanines considered to retain the minimum hydrophobic property of these sequences in PMP70(AA.1–144)-GFP, PMP70(AA.1–144 L21A/L22A/L23A)-GFP and PMP70(AA.1–144 I70A/L71A)-GFP were both localized to peroxisomes (Fig. 4A). On the other hand, we could not detect the fluorescence of PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP or PMP70(AA.1–144 I70N/L71Q)-GFP. In addition, PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP were not detected by immunoblot analysis under the conditions in which PMP70(AA.1–144)-GFP was detected (Fig. 4B, left). However, in the presence of MG132, a proteasome inhibitor, degradations of these mutant proteins were inhibited, and both PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP exhibited almost the same expression levels as that of PMP70(AA.1–144)-GFP (Fig. 4B, right). In this condition, PMP70(AA.1–144)-GFP was still localized to peroxisomes, but PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP did not show any peroxisomal localization (Fig. 4C). Furthermore, full-length PMP70 with the same mutations (PMP70(AA.1–659 L21Q/
FIGURE 2. An NH₂-terminal 124-amino acid region containing the targeting information and an additional TMD are required for the sufficient peroxisomal localization of PMP70. A, constructs of the PMP70 fragments and the TMD2 substitution mutants of PMP70(AA.1–144) and their peroxisomal localization. The ellipse shows the position of GFP, and black boxes represent the position of the six putative TMDs. B, the subcellular distribution of GFP fusion proteins of the indicated PMP70 fragments and TMD2 substitution mutants of PMP70(AA.1–144)-GFP was compared with the localization of the endogenous PMP70 detected by immunofluorescence staining with anti-PMP70 COOH-terminal antibody. The peroxisomal staining pattern is shown on the left, EGFP fluorescence in the center, and a superimposition of both stains on the right.
Amino acids identical in at least two of the aligned proteins are boxed in black. +, three positively charged amino acid clusters in PMP70.

**Basic amino acid residues in the NH$_2$-terminal region of PMP70 are not necessary for the targeting of PMP70.**

Interaction between Mutant PMP70 and Pex19p—We recently found that Pex19p, a protein required for peroxisomal membrane biogenesis, interacts with the NH$_2$-terminal region of PMP70 to maintain it in soluble and proper conformation in the cytosol (27). Therefore, we investigated the interaction between Pex19p and mutant PMP70s in which the NH$_2$-terminal hydrophobic motifs were disrupted. As shown in Fig. 5A, wild type and mutant PMP70s were translated in a wheat germ *in vitro* translation system in the presence or absence of purified His-Pex19p. Wild type PMP70 was solubilized by the addition of purified His-Pex19p, and about 60% of wild type PMP70 synthesized in the cell-free system was recovered in the supernatant fraction after centrifugation at 17,000 × g for 20 min (Fig. 5, A and B). The solubilized PMP70 was co-immunoprecipitated with His-Pex19p (Fig. 5, C and D). On the other hand, PMP70(AA.1–659 L21Q/L22Q/L23Q) was not well retained in a soluble form, and only 25% of PMP70(AA.1–659 L21Q/L22Q/L23Q) was recovered in the soluble fraction even in the presence of His-Pex19p. Furthermore, the efficiency of the interaction between the soluble mutant PMP70 and His-Pex19p decreased to 40% compared with that of the interaction between wild type PMP70 and His-Pex19p. PMP70(AA.1–659 L21Q/L22Q/L23Q) existed in a soluble form in the presence of His-Pex19p (Fig. 5, A and B). The solubilized PMP70 was co-immunoprecipitated with His-Pex19p (Fig. 5, C and D). On the other hand, PMP70(AA.1–659 L21Q/L22Q/L23Q) was not well retained in a soluble form, and only 25% of PMP70(AA.1–659 L21Q/L22Q/L23Q) was recovered in the soluble fraction even in the presence of His-Pex19p. Furthermore, the efficiency of the interaction between the soluble mutant PMP70 and His-Pex19p decreased to 40% compared with that of the interaction between wild type PMP70 and His-Pex19p. PMP70(AA.1–659 L21Q/L22Q/L23Q) existed in a soluble form in the presence of His-Pex19p and still interacted with His-Pex19p at almost the same level as wild type PMP70 did, but the mutant PMP70 was mislocalized to endoplasmic reticulum-like structures, as
Peroxisome Targeting Signal of PMP70

(A) PMP70(AA.1–144 L21A/L22A/L23A)-GFP
PMP70(AA.1–144 L70A/L71A)-GFP
PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP
PMP70(AA.1–144 I70N/L71Q)-GFP

(B) None
10 µM MG132

(C) PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP
PMP70(AA.1–144 I70N/L71Q)-GFP
PMP70(AA.1–659 L21Q/L22Q/L23Q)-GFP
PMP70(AA.1–659 I70N/L71Q)-GFP

FIGURE 4. The importance of the NH₂-terminal hydrophobic motifs for the stability and targeting of PMP70. A, PMP70(AA.1–144 L21A/L22A/L23A)-GFP, PMP70(AA.1–144 L70A/L71A)-GFP, PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP, and PMP70(AA.1–144 I70N/L71Q)-GFP were expressed in CHO cells. The subcellular distribution of the fusion proteins was compared with the localization of the endogenous PMP70 detected by immunofluorescence staining with anti-PMP70 COOH-terminal antibody. The peroxisomal staining pattern is shown on the left, EGFP fluorescence in the center, and a superimposition of both stains on the right. B, CHO cells transiently expressing the indicated PMP70(AA.1–144)-GFP mutants were cultured in the absence or presence of 10 µM MG132 for 10 h. Cell lysates were subjected to immunoblot analysis using anti-GFP antibody. The arrows indicate the position of the PMP70(AA.1–144)-GFP mutants and GFP, respectively. C, PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP, PMP70(AA.1–144 I70N/L71Q)-GFP, PMP70(AA.1–659 L21Q/L22Q/L23Q)-GFP, and PMP70(AA.1–659 I70N/L71Q)-GFP were expressed in CHO cells. After incubation with 10 µM MG132 for 10 h, the subcellular distribution of PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP was compared with the localization of the endogenous PMP70 detected by immunofluorescence staining with anti-PMP70 COOH-terminal antibody, and the subcellular distribution of PMP70(AA.1–659 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–659 I70N/L71Q)-GFP was compared with the localization of catalase. The peroxisomal staining pattern is shown on the left, EGFP fluorescence in the center, and a superimposition of both stains on the right.

We also examined the subcellular localization of NH₂-terminal truncated PMP70 in fusion with the NH₂ terminus of GFP (Fig. 6). GFP-PMP70(AA.1–375) was localized to peroxisomes as described above. GFP-PMP70(AA.113–375), which lacks the NH₂-terminal hydrophobic region required for the targeting of PMP70, still retained the ability to target to peroxisomes (PCC of 0.81 ± 0.03). GFP-PMP70(AA.176–375) was still partially localized to peroxisomes (PCC of 0.74 ± 0.04). By the further removal of TMD3 from PMP70, GFP-PMP70(AA.224–375) lost the targeting ability. Image quantification implied the random localization of GFP-PMP70(AA.224–375) (PCC of 0.51 ± 0.01), and GFP-PMP70(AA.224–375) did not exhibit any punctated pattern like peroxisomes. However, GFP-PMP70(AA.263–375), which comprises TMD5 and TMD6 of PMP70, restored the peroxisomal localization (PCC of 0.82 ± 0.04). The localization was diminished by the removal of TMD5, and GFP-PMP70(AA.314–375) was diffused in the cytosol and partially localized to endoplasmic reticulum-like structures. These data suggest that, in addition to the NH₂-terminal hydrophobic motif, PMP70 possesses a second and distinct mPTS in the region of PMP70(AA.263–375).

PMP70 Possesses a Second and Distinct mPTS in the Region of TMD5–TMD6—Furthermore, we examined the subcellular localization of NH₂-terminal truncated PMP70 in fusion with the COOH terminus of GFP (Fig. 6). However, with progressive NH₂-terminal shortening of PMP70, GFP-PMP70(AA.176–375)-GFP, GFP-PMP70(AA.224–375)-GFP, GFP-PMP70(AA.263–375)-GFP, and GFP-PMP70(AA.314–375)-GFP lost the targeting ability. Among these deletion constructs, GFP-PMP70(AA.224–375)-GFP was localized to a subcellular compartment. However, the colocalization analysis resulted in no positive relation between the fluorescence intensities of the GFP fusion protein and peroxisomes (PCC of 0.28 ± 0.03). Furthermore, only 15% of the fluorescence of GFP-PMP70(AA.224–375)-GFP coincided with that of peroxisomes, which was comparable...
FIGURE 5. Co-immunoprecipitation of PMP70 and the mutants with Pex19p. A, PMP70 and the mutants were translated and labeled with \[^{35}S\]methionine \textit{in vitro} in the absence or presence of Pex19p. These translation products (T) were separated into the soluble (S) and pellet (P) fractions by centrifugation at 17,000 \( \times g \) for 20 min at 4 °C. Equivalent portions of these fractions were analyzed by SDS-PAGE followed by autoradiography. The arrowhead indicates the position of PMP70 and the mutants. The amounts of soluble and insoluble PMP70 in A were quantified with a Fuji BAS 5000 imaging analyzer, and the relative ratio of soluble and insoluble PMP70 was calculated. The open bars show the soluble percentage of PMP70 in the absence of Pex19p, and the closed bars show the soluble percentage of PMP70 in the presence of Pex19p. B, PMP70 and the mutant proteins were translated and labeled with \[^{35}S\]methionine in the presence of His-Pex19p. After centrifugation at 17,000 \( \times g \), the soluble and insoluble PMP70 was calculated. The relative ratio of soluble and insoluble PMP70 was determined as a soluble protein in the presence of purified His-Pex19p, and PMP70(AA.1–659 I307N/L308Q)-GFP and PMP70(AA.1–659 L21Q/L22Q/L23Q)-GFP were not (see Fig. 4C). These data suggest that the hydrophobic motif located adjacent to the NH\(_2\)-terminal side of TMD5 is also important for the peroxisomal targeting of PMP70.

To characterize the hydrophobic motif in the targeting process of PMP70 more precisely, we examined the interaction between PMP70(AA.1–659 I307N/L308Q) and Pex19p. As shown in Fig. 9, PMP70(AA.1–659 I307N/L308Q) translated in the wheat germ \textit{in vitro} translation system was almost recovered as a soluble protein in the presence of purified His-Pex19p, and PMP70(AA.1–659 I307N/L308Q) interacted with Pex19p at almost the same efficiency as PMP70(AA.1–659 L21Q/L22Q/L23Q)-GFP. These data suggest that the hydrophobic region constituted by Ile\(^{307}\) and Leu\(^{308}\) could function as an mPTS rather than a region that is required to stabilize PMP70 in the targeting process through interaction with Pex19p.

Subcellular Localization of TMD3-TMD4 of PMP70—Both the NH\(_2\)-terminal and COOH-terminal minimal targeting regions possess two putative TMDs and one peroxisomal luminal loop. The finding suggests the possibility that PMP70 possesses discrete mPTSs at each region that comprises two TMDs and one peroxisomal luminal loop. However, PMP70(AA.176–276)-GFP as well as GFP-PMP70(AA.176–276), which corresponded to TMD3-TMD4, did not show any peroxisomal localization, excluding the possibility that only two TMDs and a

with that of GFP alone. These values excluded the peroxisomal localization of the fusion protein. These data were not consistent with those of the NH\(_2\)-terminal GFP-fused protein. However, PMP70(AA.113–228)-GFP, the deletion of TMD5-TMD6 of PMP70(AA.113–375)-GFP, was not located to peroxisomes. In addition, PMP70(AA.113–183)-GFP was not sorted to peroxisomes (data not shown). These data suggest that, in addition to the NH\(_2\)-terminal hydrophobic motif, PMP70 possesses the second and distinct mPTS on the region of PMP70(AA.263–375), and the ability of the region as a targeting signal could be influenced by the position of the fusion protein, and it might be disturbed by the misfolding of the polypeptide if TMD1 and TMD2 were absent.

Characterization of the Second Peroxisome Targeting Signal at the TMD5-TMD6 Region of PMP70—Comparison of the amino acid sequences of the NH\(_2\)-terminal and COOH-terminal minimal targeting regions did not show any apparent sequence similarity, but PMP70(AA.263–375) also possesses a hydrophobic motif at the region adjacent to the NH\(_2\)-terminal side of the first TMD. To examine the importance of the hydrophobicity for the peroxisomal targeting of PMP70, we substituted Ile\(^{307}\) and Leu\(^{308}\) which comprise the hydrophobic segment adjacent to TMD5, to Asn and Gln, respectively, and determined the subcellular localization of the mutant protein. GFP-PMP70(AA.263–375 1307N/L308Q) was detected only in the presence of MG132. GFP-PMP70(AA.263–375) was localized to peroxisomes even in the presence of the proteasome inhibitor. However, under the same conditions, GFP-PMP70(AA.263–375 1307N/L308Q) did not show any peroxisomal localization (Fig. 8). The mutation also affected the targeting of full-length PMP70, so PMP70(AA.1–659 I307N/L308Q)-GFP was not localized to peroxisomes as PMP70(AA.1–659 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–659 L70N/L71Q)-GFP were not (see Fig. 8C). These data suggest that the hydrophobic motif located adjacent to the NH\(_2\)-terminal side of TMD5 is also important for the peroxisomal targeting of PMP70.
Peroxisome Targeting Signal of PMP70

At first, it was found that a targeting element of PMP70 existed within the NH₂-terminal 124 amino acids, and at least two TMDs were required for the integration into peroxisomal membrane by the following observations. 1) PMP70(AA.1–144)-GFP was localized to peroxisomes. 2) PMP70(AA.1–124)-GFP, which was deleted of the region of TMD2(AA.125–144), lost the ability to localize to peroxisomes, but substitutions of TMD2 in PMP70(AA.1–144)-GFP with TMD4 or TMD6 of PMP70 did not affect the peroxisomal localization (Fig. 2B). 3) The finding that TMD2, TMD4, or TMD6 did not locate to peroxisomes excludes the possibility that these regions possess sufficient information for peroxisomal localization (Fig. 2B). As for the mPTS in the NH₂-terminal region of PMP70, Sacksteder et al. (22) reported that the COOH-terminally Myc-tagged PMP70(AA.1–124) was able to target to peroxisomes, and the efficiency of targeting was much decreased in Myc-tagged PMP70(AA.1–61). Biermanns and Gärntner (49) determined that a region of 20 amino acids (positions 61–80) contained important targeting information from their observations that GFP-PMP70(AA.61–180) targeted to peroxisomes and that GFP-PMP70(AA.81–160) did not display any peroxisomal localization. Thus, our data were partly consistent with these observations.

On the other hand, we found an additional novel mPTS of PMP70 in the region of amino acids 263–375, including TMD5 and TMD6 (Fig. 6). We have previously shown that the PMP70 produced by the in vitro translation system was inserted into rat liver peroxisomes, and the truncated PMP70 that was deleted of the NH₂-terminal 20-kDa region adjacent to TMD3 was also associated with peroxisomes (45). These data imply the existence of another mPTS, which is outside of the NH₂-terminal region. Indeed, in some PMPs, it is suggested that there are multiple targeting signals. As for the targeting of PMP47, Dyer et al. (35) first reported that the targeting information was contained in the basic cluster within matrix loop 2, which connects TMD4 and TMD5 of PMP47. In addition to this observation, Wang et al. (41) showed that TMD2 and an adjacent region of

DISCUSSION

Most of the PMPs are synthesized on free cytosolic polyribosomes and posttranslationally targeted to peroxisomes. However, the precise targeting process of PMPs is still unknown, and furthermore the common peroxisome targeting signal of the PMPs is not yet identified. In this study, we examined the characteristics of the mPTS of PMP70.

FIGURE 6. Subcellular localization of NH₂-terminal truncated PMP70 in fusion with the COOH terminus of GFP. A, constructs of the PMP70 truncation mutants and their peroxisomal localization. The ellipse shows the position of GFP, and black boxes represent the position of the six putative TMDs. B, GFP-PMP70 fusion proteins were expressed in CHO cells. The subcellular distribution of the fusion proteins was compared with the localization of the endogenous PMP70 detected by immunofluorescence staining with anti-PMP70 COOH-terminal antibody. The peroxisomal staining pattern is shown on the left, EGFP fluorescence in the center, and a superimposition of both stains on the right.
cytosolic loop 1 were also crucial for the targeting of PMP47. Jones et al. (40) reported that PMP34 contained multiple distinct targeting signals, and Brosius et al. (38) showed that PMP22 contained two distinct and nonoverlapping peroxisomal membrane targeting signals. The meaning of the presence of multiple mPTTs in these PMPs is not well understood yet, but Wang et al. (50) suggest that two mPTTs of PMP47 function differently among the peroxisome populations in Saccharomyces cerevisiae. These data give impetus to the possibility that PMP70 possesses another mPTS in a region distinct from the NH2-terminal region, and PMP70 adopts the mPTTs depending on the condition of the peroxisomes. Indeed, PMP70 has the property to be highly enriched in the rodent liver peroxisomal membrane under the condition in which peroxisomes are induced by the administration of hypolipidemic agents, such as clofibrate (51, 52).

Our results also showed that the NH2-terminal basic clusters did not function as the mPTS of PMP70, but the hydrophobic motifs just adjacent to the first TMD were important for the targeting of PMP70, as suggested by the following observations. 1) Disruption of basic amino acid clusters that exist in the NH2-terminal region did not affect the peroxisomal targeting efficiency of PMP70(AA.1–144)-GFP (Fig. 3B). 2) PMP70(AA.1–144 L21A/L22A/L23A)-GFP and PMP70(AA.1–144 I70A/L71A)-GFP, which possess a hydrophobic property similar to that of PMP70(AA.1–144)-GFP, were localized to peroxisomes. On the other hand, both PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP were degraded by proteasomes and did not show any peroxisomal localization even in the presence of proteasome inhibitor (Fig. 4). Similar characteristics were observed in the second mPTS. In the region of PMP70(AA.263–375), there is no positively charged amino acid clusters, and the hydrophobic amino acid clusters did not function as the mPTS of PMP70, but the hydrophobic motifs just adjacent to the first TMD were important for the targeting of PMP70, as suggested by the following observations. 1) Disruption of basic amino acid clusters that exist in the NH2-terminal region did not affect the peroxisomal targeting efficiency of PMP70(AA.1–144)-GFP (Fig. 3B). 2) PMP70(AA.1–144 L21A/L22A/L23A)-GFP and PMP70(AA.1–144 I70A/L71A)-GFP, which possess a hydrophobic property similar to that of PMP70(AA.1–144)-GFP, were localized to peroxisomes. On the other hand, both PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP were degraded by proteasomes and did not show any peroxisomal localization even in the presence of proteasome inhibitor (Fig. 4). Similar characteristics were observed in the second mPTS. In the region of PMP70(AA.263–375), there is no positively charged amino acid clusters, and the hydrophobic amino acid clusters did not function as the mPTS of PMP70, but the hydrophobic motifs just adjacent to the first TMD were important for the targeting of PMP70, as suggested by the following observations. 1) Disruption of basic amino acid clusters that exist in the NH2-terminal region did not affect the peroxisomal targeting efficiency of PMP70(AA.1–144)-GFP (Fig. 3B). 2) PMP70(AA.1–144 L21A/L22A/L23A)-GFP and PMP70(AA.1–144 I70A/L71A)-GFP, which possess a hydrophobic property similar to that of PMP70(AA.1–144)-GFP, were localized to peroxisomes. On the other hand, both PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP were degraded by proteasomes and did not show any peroxisomal localization even in the presence of proteasome inhibitor (Fig. 4). Similar characteristics were observed in the second mPTS. In the region of PMP70(AA.263–375), there is no positively charged amino acid clusters, and the hydrophobic amino acid clusters did not function as the mPTS of PMP70, but the hydrophobic motifs just adjacent to the first TMD were important for the targeting of PMP70, as suggested by the following observations. 1) Disruption of basic amino acid clusters that exist in the NH2-terminal region did not affect the peroxisomal targeting efficiency of PMP70(AA.1–144)-GFP (Fig. 3B). 2) PMP70(AA.1–144 L21A/L22A/L23A)-GFP and PMP70(AA.1–144 I70A/L71A)-GFP, which possess a hydrophobic property similar to that of PMP70(AA.1–144)-GFP, were localized to peroxisomes. On the other hand, both PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP were degraded by proteasomes and did not show any peroxisomal localization even in the presence of proteasome inhibitor (Fig. 4). Similar characteristics were observed in the second mPTS. In the region of PMP70(AA.263–375), there is no positively charged amino acid clusters, and the hydrophobic amino acid clusters did not function as the mPTS of PMP70, but the hydrophobic motifs just adjacent to the first TMD were important for the targeting of PMP70, as suggested by the following observations. 1) Disruption of basic amino acid clusters that exist in the NH2-terminal region did not affect the peroxisomal targeting efficiency of PMP70(AA.1–144)-GFP (Fig. 3B). 2) PMP70(AA.1–144 L21A/L22A/L23A)-GFP and PMP70(AA.1–144 I70A/L71A)-GFP, which possess a hydrophobic property similar to that of PMP70(AA.1–144)-GFP, were localized to peroxisomes. On the other hand, both PMP70(AA.1–144 L21Q/L22Q/L23Q)-GFP and PMP70(AA.1–144 I70N/L71Q)-GFP were degraded by proteasomes and did not show any peroxisomal localization even in the presence of proteasome inhibitor (Fig. 4). Similar characteristics were observed in the second mPTS. In the region of PMP70(AA.263–375), there is no positively charged amino acid clusters, and the hydrophobic amino
acid pair existing just adjacent to TMD5 was important for the targeting to peroxisomes; GFP-PMP70(AA.263–375 I307N/L308Q) was directed to peroxisomes, but GFP-PMP70(AA.263–375 I307A/L308A) was not (Fig. 8). In a previous paper (27), we proposed that the mPTS of PMP70 was located near the TMD2, including a positively charged cluster of basic amino acids, in a study using COOH-terminal truncated PMP70-GFP. However, the present study suggests that the region is essential for the insertion of PMP70 into the peroxisomal membranes, but a positively charged cluster is not involved in the process.

Concerning the targeting element of PMPs, positively charged amino acid clusters in the matrix loop are suggested to be essential. Dyer et al. (35) first defined a basic cluster of amino acids of the sequence KIKKR existing on the second intraperoxisomal loop as a targeting motif of PMP47. Baerends et al. (36) also reported that RHKKK at the NH2 terminus of Hansenula polymorpha Pex3p was important for sorting. In addition, a matrix-oriented positively charged amino acid cluster was seen in many PMP fragments that can target to peroxisomes, such as PMP22, Mpv17-like protein, Pex16p, and ascorbate peroxidase (39, 41, 51–56). Our data are inconsistent with these observations. However, Pause et al. (37) reported that YYX₂LX₃PX₃(K/Q/N), a conserved motif among PMP22 orthologues, comprised the core of the mPTS of PMP22, and the basic cluster in the first peroxisomal matrix loop was not essential for targeting. They also found that the hydrophobic sequence was necessary for the targeting and/or insertion of PMP22. Recently, Landgraf et al. (57) identified a 14-amino acid motif (F(F/L)X(R/Q/K)(L/F)(L/I/K)XLLKIL(F/I/V)P/) as an mPTS of adrenoleukodystrophy protein, one of the peroxisomal ABC proteins, and found that the substitution or deletion of these hydrophobic residues significantly reduced the targeting efficiency. In particular, the deletion of three amino acids (Leu78-Leu79-Arg80) lost peroxisomal targeting of adrenoleukodystrophy protein. The region corresponds to Ile 70-Leu71-Lys72 in PMP70, which we found to be an mPTS of PMP70. Taking these observations into consideration, we suggest that in a group of PMPs, the mPTS is composed of hydrophobic regions but not basic amino acid clusters.
Another point we addressed in this study is the function of Pex19p in the targeting process of PMP70. Pex19p is a farnesylated protein essential for the early steps of peroxisome biogenesis and most likely is involved in peroxisomal membrane synthesis. Pex19p is mainly located in the cytosol and is known to bind multiple PMPs. From these findings, Pex19p has been proposed to function either as a receptor for the mPTS of PMPs or as a chaperone that stabilizes PMPs in the cytosol. Sacksteder et al. (22) found that the targeting regions of multiple PMPs were also recognized by Pex19p. Jones et al. (28) reported that the attachment of a nuclear localization signal to Pex19p lead to accumulations of mPTS regions of PMP34, PEX11β, PEX16, PMP22, and PMP70 in the nucleus. Furthermore, Rottensteiner et al. (29) deduced the amino acid sequence, X4(C/F/I/L/T/V/W)X3(A/C/F/I/L/Q/V/W/Y)(C/I/L/Y)X3(A/C/F/I/L/V/W/Y)(I/L/Q/R/V)X3 as the common Pex19p-binding motif in PMPs. They also reported that the Pex19p-binding site in conjugation with one or more adjacent TMDs of Pex13p possessed peroxisome targeting ability, and a mutation within the Pex19p-binding site that disrupted the Pex19p binding impaired the peroxisomal targeting of Pex13p (29). A similar Pex19p-binding site-dependent targeting was observed in Pex17p (31). These findings strongly support the function of Pex19p as a PMP import receptor, which recognizes the mPTS of peroxisomal membrane proteins and delivers them to peroxisomes. Recently, we found that an NH2-terminal 61-amino acid region and TMD5-TMD6 of PMP70 interacted with Pex19p in in vitro binding experiments (27). These regions partly overlap with the mPTSs which we found in this study. However, we also found that PMP70(AA.1–659 I307N/L308Q) lost the targeting activity, although these mutant proteins still interacted with Pex19p and were solubilized by Pex19p, suggesting that the targeting element and Pex19p-binding site of PMP70 are functionally separated. This finding seems to claim the function of Pex19p as a mPTS receptor. Snyder et al. (23) reported that the domains of Pex3p, Pex10p, Pex13p, and Pex22p, which interact with Pex19p, did not function as an mPTS. Fransen et al. (24) also found that the Pex19p-binding sites of Pex3p and Pex12p were separated from their mPTS regions. Furthermore, they found that the mutant Pex13p did localize to peroxisomes but did not show binding affinity for Pex19p (58). Recently, Vizeacoumar et al. (33) showed that both Pex30p and Pex32p of S. cerevisiae interacted with Pex19p in regions that did not overlap with their mPTSs. These data suggest that Pex19p functions as a chaperone for PMPs in the targeting process rather than acting as an mPTS receptor. In this study, some of the deletion constructs of PMP70 still possessed peroxisomal targeting ability. These deletion constructs might have different conformation with respect to native PMP70. However, similar results were obtained for various PMPs (34–41, 49, 54, 57). These data suggest that these PMP fragments possess the ability to form a proper conformation by themselves. In this process, Pex19p is supposed to play an important role. Consistent with the hypothesis, the fragments of various PMPs, including Pex16p, PMP22, and PMP34, which can target to peroxisomes, were shown to be able to interact with Pex19p (28). As for the PMP70, our recent study showed that various COOH-terminal or NH2-terminal deletion constructs still interacted with Pex19p, although the NH2-terminal 61-amino acid region and TMD5-TMD6 of PMP70 are required for efficient binding (27). These interactions might prevent entire conformational changes of deletion constructs and might keep them in a proper conformation for the peroxisomal targeting.

In summary, based on our results, we propose a hypothetical model for the targeting of PMP70 (Fig. 10). After being synthesized in the cytosol, PMP70 is recognized and bound to Pex19p at the NH2-terminal hydrophobic motif constituted by Leu21–Leu22–Leu23 and the region of TMD5-TMD6. The PMP70-Pex19p complex is transported to peroxisomes by the mPTSs located in the NH2-terminal 124-amino acid region and the region of PMP70(AA.263–375) (the hydrophobicities of Ile307–Leu308 might be essential). Finally, PMP70 is inserted into peroxisomal membranes through the unidentified proteinaceous components on the peroxisomal membranes. In this process, at least two TMDs are required for correct insertion.

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