Two Different Targeting Signals Direct Human Peroxisomal Membrane Protein 22 to Peroxisomes*

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The 22-kDa peroxisomal membrane protein (PMP22) is a major component of peroxisomal membranes in mammals. Although its precise role in peroxisome function is poorly understood, it seems to be involved in pore forming activity and may contribute to the unspecific permeability of the organelle membrane. PMP22 is synthesized on free cytosolic ribosomes and then directed to the peroxisome membrane by specific targeting information. Previous studies in rats revealed that PMP22 contains one distinct peroxisomal membrane targeting signal in the amino-terminal cytoplasmic tail. We cloned and characterized the targeting signal of human PMP22 and compared it with the already described characteristics of the corresponding rat protein. Amino acid sequence alignment of rat and human protein revealed 77% identity including a high conservation of several protein motifs. We expressed various deletion constructs of PMP22 in fusion with the green fluorescent protein in COS-7 cells and determined their intracellular localization. In contrast to previous studies on rat PMP22 and most other peroxisomal membrane proteins, we showed that human as well as rat PMP22 contains two distinct and nonoverlapping peroxisomal membrane targeting signals, one in the amino-terminal and the other in the carboxyl-terminal protein region. They consist of two transmembrane domains and adjacent protein loops with almost identical basic clusters. Both of these peroxisomal targeting regions interact with PEX19, a factor required for peroxisome membrane synthesis. In addition, we observed that fusing the green fluorescent protein immediately adjacent to the targeting region completely abolishes targeting function and mislocalizes PMP22 to the cytosol.

Peroxisomes are spheroid organelles present in virtually all eukaryotic cells (1). The peroxisomal matrix and the single organelle membrane contain a wealth of organelle-specific proteins. All peroxisomal matrix and membrane proteins are encoded by nuclear genes, synthesized on free cytosolic polyribosomes, and post-translationally imported into peroxisomes (1–5). Over the past years, a clear picture has emerged of how these proteins are targeted to peroxisomes. The majority of these proteins have a carboxyl-terminal peroxisomal targeting signal (PTS1) with the consensus sequence -S-K-L-COOH or a conservative variant (6–8). A few matrix proteins have a targeting signal at the amino terminus (PTS2) with the consensus sequence (R/K)(L/I/V)X(A/T/Y) (9). PTS1 and PTS2 are recognized by specific cytosolic receptors, PEX5 and PEX7, respectively. The subsequent import process involves receptor and translocation complexes on the peroxisome surface and in the peroxisomal membrane (1, 10–14). Peroxisomal membrane proteins (PMPs) are directed to the organelle membrane by mechanisms that are separate from and less well understood than those used by matrix proteins. Previous studies on peroxisomal membrane protein targeting of PMP22, PMP47, PMP47, PMP70, PEX3, PEX11β, PEX14, and PEX22 in different species, including yeast and mammals, have not yet yielded a consistent targeting hypothesis (15–22). They all describe a single targeting signal that is either contained in internal protein regions or at the amino terminus of these membrane proteins. Two recent studies report the existence of multiple distinct targeting signals for yeast PMP47, human PMP34, and human PEX13 (23, 24).

The insertion mechanisms of peroxisomal membrane proteins are also poorly understood. Cytosolic factors including organelle-specific chaperones may promote post-translationally import by presenting the membrane protein in a conformation suitable for insertion into the peroxisome membrane. Studies of PEX19 indicate that this protein may act as such a chaperone for newly synthesized peroxisomal membrane proteins (20, 24). PEX19 is involved in peroxisome membrane synthesis, and mislocalization of PEX19 to the nucleus led to the accumulation of newly synthesized peroxisomal membrane proteins in the nucleoplasm. In addition, PEX19 binds to the targeting regions of various peroxisomal membrane proteins.

To better understand the molecular mechanisms of peroxisome membrane synthesis and especially import of human peroxisomal membrane proteins, we cloned the human PMP22 and analyzed the molecular and biochemical properties of different PMP22 regions. We describe that either discrete amino-terminal or carboxyl-terminal targeting elements are necessary and sufficient to direct the protein to the peroxisome membrane. We also report on the interaction of the peroxisomal targeting regions with PEX19 and the influence of the green fluorescent protein on targeting characteristics.

**EXPERIMENTAL PROCEDURES**

**Materials**—We obtained reagents and biochemicals from the following sources: synthetic oligonucleotides from MWG Biotech Ebersberg; 1 The abbreviations used are: PTS, peroxisomal targeting signal; AA, amino acid(s); GFP, green fluorescent protein; OAT, ornithine–α-amino transferase; PEX, peroxin; PMP, peroxisomal membrane protein(s); PMP22, 22-kDa peroxisomal membrane protein; PCR, polymerase chain reaction; mPTS, peroxisomal membrane targeting signal; TMD, transmembrane domain; TRITC, tetramethylrhodamine isothiocyanate; bp, base pair(s); HsPMP22, human PMP22; RnPMP22, rat PMP22; HsPEX, human PEX.
Two Peroxisomal Targeting Signals in PMP22

TABLE I

Sequences of oligonucleotides used for the construction of the GFP-PMP22 fusion proteins cloned in pEGFP-C1 (top), pEGFP-N1 (middle), and pCMV-Tag3B (bottom)

| Fusion protein | Oligonucleotides for pEGFP-N1 constructs |
|----------------|------------------------------------------|
| GFP-HsPMP22-(A56–74) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A38–94) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A38–61) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A62–94) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A1–94) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-RnPMP22-(A1–93) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A75–195) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-RnPMP22-(A74–194) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A1–195) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A95–195) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-RnPMP22-(A94–194) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A75–174) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A95–174) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A115–195) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |
| GFP-HsPMP22-(A75–132) | 5’-CTTCGAATTTCGAGAAGGAGCCAGGCAA-3’ |

| Fusion protein | Oligonucleotides for pCMV-Tag3B constructs |
|----------------|------------------------------------------|
| Myc-HsPEX2(A1–306) | 5’-GGCGGATCCATGGAGTTTGGTCAGG-3’ |
| Myc-HsPMP70(A1–180) | 5’-GGCGGATCCATGGAGTTTGGTCAGG-3’ |

human retina αgt10 cDNA library from David Valle (Johns Hopkins University, Baltimore, MD); pBlueScript II KS vector from Stratagene Heidelberg; pEGFP-C1 and pEGFP-N1 vectors from CLONTECH; pCMV-Tag3A, pCMV-Tag3B, and pcDNA1Neo vectors from Invitrogen; ornithine-δ-amino transferase (OAT) plasmid DNA from David Valle; PEX19-pcDNA expression plasmid DNA from Gabriele Dodt (Ruhr University, Bochum, Germany); and Ania Muntau (Ludwig Maximilian University, Munich, Germany); Vent DNA polymerase and restriction enzymes from New England Biolabs; BigDye Terminator Cycle Sequencing Kit from PerkinElmer Life Sciences; COS-7 cells from DSMZ; Superfect reagent from Qiagen; TNT Coupled Reticulocyte Lysate System from Promega; rabbit anti-human calreticulin antibody from Affinity BioReagents; mouse anti-human Golgi antibody from Chemicon International; rabbit anti-human catalsate antibody and TRITC-conjugated goat anti-mouse antibody from Eurogentec (Cambridge, United Kingdom); TRITC-conjugated goat anti-rabbit antibody from Dianova (Hamburg, Germany); LysoTracker Red and MitoTracker Red from Molecular Probes, Inc. (Leiden, The Netherlands); mouse anti-Myc antibody from Invitrogen; anti-mouse IgG Dynabeads from Dynal (Hamburg, Germany); and [35S]methionine from Amersham Pharmacia Biotech. All other reagents were obtained from Roche Molecular Biochemicals or Sigma.

cDNA Cloning and Sequencing—Using the published sequence for the rat PMP22 cDNA (25), we selected PCR primers to amplify bp 1–582. Pooled recombinant phage DNA from two rat liver cDNA libraries was taken as template for the amplification (26). The amplified fragment was used to probe a human retina αgt10 cDNA library. From a series of screens we isolated three positive clones. All phage clones were excised, and the inserts were ligated into pBluescript II KS by standard techniques. The entire cDNA melt was sequenced in both directions by the dyeode method of Sanger et al. (27).

Construction of PMP22 Expression Plasmids for Subcellular Localization—Different human or rat cDNA fragments were amplified using a full-length human or rat PMP22 cDNA clone as template. PCR-generated fragments with either EcoRI and BamHI or EcoRI and SalI restriction sites were subcloned in frame either into the pEGFP-C1 or into the pEGFP-N1 expression vector. The identity of all subclones was confirmed by semiautomated sequencing on an ABI Prism 377 DNA sequencer following the manufacturer’s instructions (PerkinElmer Life Sciences). The oligonucleotide primers used are listed in Table I. Construction of PMP22, PEX2, PMP70, OAT, and PEX19 Expression Plasmids for Immunoprecipitation—Full-length PMP22 and PEX2...
cDNA as well as truncated PMP22 and PMP70 cDNA fragments were excised from their plasmids and subsequently subcloned in frame into the pCMV-Tag3A or pCMV-Tag3B vector. The OAT cDNA was subcloned into the pcDNA1Neo vector. N-terminally Myc-tagged PEX2 cDNA with BamHI/EcoRI restriction sites and truncated PMP70-(1–180) with BamHI/XhoI restriction sites were generated by PCR using PEX2 and PMP70 plasmid DNA as template, respectively. The oligonucleotide primers used are listed in Table I.

Culturing Conditions and Transient Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5% CO2. 24 h before transfection, 5 × 105 cells were seeded on sterile cover slides. All transfections were performed using Superfect reagent according to the manufacturer’s instructions. 48 h after transfection, the cells were washed twice with phosphate-buffered saline and fixed for 25 min in 3% formaldehyde in phosphate-buffered saline for indirect immunofluorescence.

Indirect Immunofluorescence—The fixed cells were permeabilized in 1% (v/v) Triton X-100 in phosphate-buffered saline for 5 min, washed twice, and incubated with the primary antibody for 1 h at room temperature. Primary antibodies used in this study include rabbit anti-human calreticulin (1:300 dilution), mouse anti-human mitochondria (1:50 dilution), and rabbit anti-human catalase (1:250 dilution). Anti-gene antibody complexes were detected with either TRITC-conjugated goat anti-rabbit IgG (1:50 dilution) or TRITC-conjugated goat anti-mouse IgG (1:50 dilution). We also used the LysoTracker Red and the MitoTracker Red according to the manufacturer’s instructions.

Immunoprecipitation—Immunoprecipitation was essentially performed as described previously (18). Using the TNT Coupled Reticulocyte Lysate System, N-Myc-tagged PMP22, PMP22 fragments, PMP70 fragments, PEX2, and PEX19 as well as untagged PMP22, OAT, and PEX19 were transcribed and translated in vitro for 90 min at 30 °C. The proteins were used either labeled with [35S]methionine or unlabeled. The proteins of interest were incubated with an equal amount of N-Myc-tagged or untagged PEX19 translation reaction. The Myc-tagged proteins were precipitated by anti-mouse IgG Dynabeads saturated with mouse anti-Myc antibodies and further isolated by use of a magnetic device. The collected precipitates were resuspended in SDS sample buffer, denatured at 95 °C for 5 min, separated by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography.

RESULTS

Human PMP22 cDNA—The rat PMP22 cDNA was used to clone the corresponding human PMP22 cDNA. The resulting human PMP22 cDNA meld extends from bp 1 to the apparent poly(A) addition site at bp 884 (GenBank™/EMBL accession number AF044439). Over the 585-bp open reading frame, the nucleotide sequence of the human PMP22 cDNA is 81% identical with that of rat and is 3 bp longer. The amino acid sequence of the human PMP22, as shown in Fig. 1, is 77%
identical with the rat protein, 76% with the mouse protein, and 22% with the Arabidopsis protein. The protein has four putative transmembrane domains; the amino terminus and the carboxyl terminus are facing the cytosol.

**The Basic Cluster in the First Peroxisomal Matrix Loop of PMP22 Is Not Essential for Targeting**—We first performed a database search for conserved protein regions of human PMP22 and other peroxisomal membrane proteins using the BLAST algorithm (28). The alignment detected blocks of conserved amino acid sequences in the first peroxisomal matrix loop of PMP22 (Fig. 2). Interestingly, these conserved amino acids, including the basic cluster KIKKR, had previously been identified as the peroxisomal targeting motif of the 47-kDa peroxisomal membrane protein of *Candida boidinii* (16). Since similar basic motifs are also present in PMP22 and in other peroxisomal membrane proteins, we assumed that the targeting information of human PMP22 is located within the first peroxisomal matrix loop. Amino acids 56–74 of human PMP22 were fused to the green fluorescent protein. However, the resulting GFP-HsPMP22-(56–74) fusion protein did not reveal any peroxisomal localization (Fig. 3, A and B). Because peroxisomal membrane proteins have to be inserted into the peroxisomal membrane, a more complex targeting signal including membrane anchoring domains may be required. Therefore, we added transmembrane regions adjacent to the first peroxisomal loop to allow membrane integration of the fusion proteins.

**FIG. 3.** The first peroxisomal loop of PMP22 is not essential for peroxisomal targeting. A, constructs of human PMP22 deletion mutants. The ellipse shows the position of the GFP. The three blocks (Block I, II, and III) in the first peroxisomal matrix loop are highlighted by different patterns. The four TMDs are indicated in black, and the cytosolic and peroxisomal loops are indicated by cyto and perox, respectively. B, intracellular localization of PMP22 fusion proteins expressed in COS-7 cells. The cellular distribution of the fusion proteins is compared with the localization of the peroxisomal marker enzyme catalase detected by immunofluorescence staining. GFP-HsPMP22-(75–195) co-localizes with catalase. Original magnification was ×850.
Nevertheless, all of these fusion proteins, namely GFP-HsPMP22-(56–74), GFP-HsPMP22-(38–94), GFP-HsPMP22-(62–94), and GFP-HsPMP22-(1–94), were found in the cytoplasm and did not target to peroxisomes (Fig. 3, A and B). Furthermore, GFP-HsPMP22-(75–195), which lacks the first peroxisomal matrix loop region, did target to peroxisomes at an efficiency almost comparable with the full-length protein and yielded a perfect co-localization pattern with the peroxisomal marker enzyme catalase (Fig. 3B). Our data suggest that the first peroxisomal matrix loop does not contain important peroxisomal membrane targeting information for human PMP22.

Peroxisomal Targeting Elements at the Carboxyl Terminus of PMP22—To identify candidate regions responsible for targeting human PMP22 to the peroxisome membrane, the polypeptide was first divided into two nonoverlapping halves and subsequently fused to the carboxyl terminus of the green fluorescent protein. While the amino-terminal half, GFP-HsPMP22-(1–94), did not localize to peroxisomes, the carboxyl-terminal 101 amino acids, GFP-HsPMP22-(95–195), were capable of targeting to peroxisomes (Fig. 4, A and B). To identify the minimal regions within the carboxyl-terminal half of PMP22 that are sufficient for its proper peroxisomal localization, we made a series of deletion constructs. The truncated fusion proteins GFP-HsPMP22-(75–174) and GFP-HsPMP22-(95–195) retained targeting to peroxisomes, whereas all shorter fusion protein constructs failed to target (Fig. 4, A and B). Thus, the cytosolic and peroxisomal loop in the carboxyl-terminal half of PMP22 plus two transmembrane segments function as the peroxisomal targeting signal. These results were inconsistent with those by Pause et al. (19). Their work on rat PMP22 had shown that the amino-terminal cytoplasmic tail is important for peroxisomal targeting.

Peroxisomal Targeting Elements at the Amino Terminus of

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**FIG. 4.** The carboxyl-terminal peroxisomal membrane targeting signals of PMP22. A, constructs of human PMP22 deletion mutants. The ellipse shows the position of the GFP, and the black boxes represent the position of the four TMDs. The cytosolic and peroxisomal loops are indicated by cyto and perox, respectively. B, intracellular localization of PMP22 fusion proteins expressed in COS-7 cells. The cellular distribution of the fusion proteins is compared with the localization of the peroxisomal marker enzyme catalase detected by immunofluorescence staining. GFP-HsPMP22-(95–195) co-localizes with catalase. Original magnification was × 850.
Fig. 5. Two distinct peroxisomal membrane targeting signals in human and rat PMP22. A, constructs of human and rat PMP22 deletion mutants. The ellipse shows the position of the GFP, and the black boxes represent the position of the four TMDs. The cytosolic and peroxisomal loops are indicated by cyto and perox, respectively. B, intracellular localization of PMP22 fusion proteins expressed in COS-7 cells. The cellular distribution of the fusion proteins is compared with the localization of the peroxisomal marker enzyme catalase detected by immunofluorescence staining. GFP-HsPMP22-(95–195) and HsPMP22-(1–94)-GFP co-localize with catalase. Original magnification was × 850.
PMP22—The presence of two entirely different and nonoverlapping targeting regions in the rat and human protein was unexpected and could reflect interspecies differences or the existence of multiple targeting signals in PMP22. To distinguish between these possibilities, we divided the rat polypeptide into two nonoverlapping halves and subsequently fused the two fragments to the carboxyl terminus of the green fluorescent protein. In accordance with our finding of a carboxylterminal targeting signal for human PMP22, we showed that the amino-terminal half of the rat protein, GFP-RnPMP22-(1–93), was also mislocalized in the cytosol and that the carboxylterminal fragments, GFP-RnPMP22-(74–194) and GFP-RnPMP22-(94–194), targeted to peroxisomes (Fig. 5A). Next, we used the experimental conditions previously described by Pause et al. (19) and fused the amino- and carboxyl-terminal halves of human and rat PMP22 to the amino terminus of the green fluorescent protein. These experiments revealed the second and distinct peroxisomal targeting signal for human PMP22 at the amino terminus (Fig. 5, A and B). HsPMP22-(1–94)-GP and RnPMP22-(1–93)-GP were sorted to peroxisomes. In contrast, HsPMP22-(75–195)-GP, HsPMP22-(95–195)-GP, and RnPMP22-(94–194)-GP were not localized in peroxisomes. Comparison of the amino- and carboxyl-terminal protein targeting regions did reveal that both regions consist of two transmembrane domains and an adjacent basic cluster of five amino acids. The amino acid composition of these clusters is almost identical, RRALA or KRALA for the amino-terminal signal and RRLLL or KRLLL for the carboxyl-terminal one (Table II). Taken together, these results indicate that there are two distinct peroxisomal targeting signals in human as well as in rat PMP22. Furthermore, the targeting characteristics of the PMP22 fusion proteins are influenced by the position of the green fluorescent protein. Fusing the green fluorescent protein to the carboxyl terminus of PMP22 masks its carboxyl-terminal peroxisomal targeting signal, while adding this protein to the amino terminus of PMP22 masks its amino-terminal targeting signal.

Role of Transmembrane Domains in Targeting Efficiency—While Dyer et al. (16) found that a hydrophilic 20-amino acid loop in PMP47 is both necessary and sufficient for peroxisomal targeting, further investigations on various peroxisomal membrane proteins revealed that targeting also depends on the number and position of the transmembrane domains (17, 18, 20, 21). To elucidate the effect of the two transmembrane domains in PMP22 on peroxisomal targeting, we made different amino-terminal deletion constructs and determined their efficiency for peroxisomal localization. We found that at least two transmembrane domains were necessary and sufficient for peroxisomal targeting. GFP-HsPMP22-(75–174), including TMD2 and TMD3, was targeted with an efficiency of 85% of all transfected cells, and GFP-HsPMP22-(95–195), including TMD3 and TMD4, was targeted with an efficiency of 74%. Adding an additional transmembrane domain could further increase the targeting efficiency. GFP-HsPMP22-(75–195), including TMD2, TMD3, and TMD4, was directed to peroxisomes at an efficiency indistinguishable from the full-length protein.

Mislocalization of PMP22 Fusion Proteins to Other Organelles—By scanning PMP22 for regions that were sufficient for peroxisomal targeting, we also found PMP22 truncations that specifically targeted to other cellular organelles (Fig. 6, A and B). The fusion proteins GFP-HsPMP22-(95–132) and GFP-HsPMP22-(95–175) targeted to the endoplasmic reticulum or mitochondria, respectively, while GFP-HsPMP22-(115–195) targeted to both of these organelles. In addition, none of our PMP22 truncations targeted to lysosomes. Thus, mitochondrial targeting of PMP22 seems to require the following elements: the second peroxisomal matrix loop and the third transmembrane domain. These regions may contain a conservative variant of a mitochondrial sorting signal.

PEX19 Interactions with Peroxisomal Targeting Elements of PMP22—Previous studies established a role of PEX19 as a chaperone-like protein or a receptor protein for the import of peroxisomal membrane proteins and the synthesis of peroxisomal membranes (20, 29–32). To determine if PEX19 functions as a targeting receptor for PMP22 by binding to the two distinct peroxisomal targeting regions, we analyzed these interactions by co-immunoprecipitation experiments. We co-incubated N-Myc-tagged PMP22 truncations and full-length PMP22 with labeled and unlabeled PEX19. N-Myc-tagged PEX2 and PMP70 truncations as well as untagged ornithine-δ-aminotransferase were used as controls. The results are shown in Fig. 7. The Myc-HsPMP22-(94–194) and Myc-HsPMP22-(75–174) fusion proteins including the carboxyl-terminal peroxisomal targeting elements as well as the Myc-RnPMP22-(1–93) fusion protein including the amino-terminal targeting elements interacted with PEX19. As expected, PEX19 also interacted with the two other peroxisomal membrane proteins, PEX2 and PMP70. In addition, the mitochondrial enzyme OAT did not interact with peroxisomal proteins. The interaction of both the amino-terminal and the carboxyl-terminal peroxisomal targeting signals of PMP22 with PEX19 provides evidence that there are at least two independent PEX19 binding sites.

**DISCUSSION**

Although the translocation apparatus for peroxisomal membrane proteins is still poorly understood, the targeting and subsequent insertion processes seem to be mediated by internal peroxisomal membrane protein targeting signals. In order to obtain further information on these processes, we cloned the human PMP22 and investigated its targeting characteristics. PMP22 consists of four putative transmembrane regions and four protein loops. Loops 1 and 3 are exposed on the cytosolic side, and loops 2 and 4 are exposed on the matrix side of the peroxisomal membrane. We showed that PMP22 includes two distinct targeting signals, one in the amino-terminal and the other in the carboxyl-terminal part of the protein. These signals consist of two transmembrane domains and adjacent protein loops with almost identical basic clusters. We further demonstrated that both of these targeting regions are capable of interacting with PEX19, a mainly cytosolic factor involved in peroxisomal membrane synthesis.

Two Independent Targeting Signals in Peroxisomal Membrane Proteins—We analyzed the targeting characteristics of human and rat PMP22 by determining the intracellular localization of a series of truncated proteins in fusion with the green fluorescent protein. We identified two distinct nonoverlapping targeting signals. While Pause et al. (19) had already described only one targeting signal in the amino-terminal part for rat
PMP22, we identified the second one in the carboxyl-terminal part of human PMP22 as well as in the corresponding rat protein. Within the last few years, a series of targeting signals for peroxisomal membrane proteins have been identified. Most of these previous studies describe only one targeting signal per membrane protein. These single targeting signals were found either in the amino-terminal part, as for PEX3, PEX14, PEX22, and PMP70 (17–21, 33); in the middle part, as for PMP47 and PMP94 (16, 22); or in the carboxyl-terminal part, as for PEX11β and PEX15 (20, 34). Recent studies contradict these results of a single set of peroxisomal targeting information and proclaim the presence of multiple targeting signals per membrane protein. They describe two nonoverlapping targeting regions in PMP47 of C. boidinii, its human ortholog PMP34, and human PEX13 (23, 24). Our finding of a third peroxisomal membrane protein with two distinct targeting signals confirms and extends the observation of multiple targeting signals. Furthermore, the peroxisomal membrane proteins with only one peroxisomal targeting signal might have additional, so far unidentified, targeting signals. The single peroxisomal targeting signals of PEX11β, PEX14, PEX15, and PMP70 have been found by investigating exclusively only one-half of these proteins, namely by subcellular localization of either carboxyl-terminal or amino-terminal truncated fusion proteins (20, 21, 34). Therefore, all of these peroxisomal membrane proteins might contain further targeting signals in the other, as yet unanalyzed, protein half. The presence of multiple peroxisomal targeting signals seems to be meaningful and may reflect the characteristics of the translocation and insertion apparatus for peroxisomal membrane proteins.

**Influence of the Green Fluorescent Protein on Peroxisomal Targeting Characteristics**—We found in a series of truncated PMP22 fusion constructs that the targeting characteristics are strictly dependent on the position of the GFP. In A, constructs of human deletion mutants. The ellipse shows the position of the GFP, and the black boxes represent the position of the four TMDs. The cytosolic and peroxisomal loops are indicated by cyto and perox, respectively. Localization of fusion proteins to peroxisomes (perox), mitochondria (mito), endoplasmic reticulum (ER), and lysosomes (lyso) had been determined. B, intracellular localization of PMP22 truncations expressed in COS-7 cells. The cellular distribution of the fusion proteins is compared with the localization of organelle-specific markers detected by immunofluorescence staining, catalase for peroxisomes, MitoTracker® for mitochondria, and calreticulin for the endoplasmatic reticulum. GFP-HsPMP22-(115–195) targeted to mitochondria and the endoplasmatic reticulum. GFP-HsPMP22-(95–132) targeted to the endoplasmic reticulum, and GFP-HsPMP22-(95–175) targeted to mitochondria. Original magnification was ×850.
protein. Fusing the green fluorescent protein to the amino terminus of PMP22 masks its amino-terminal peroxisomal targeting signal. In this case, the last 101 amino acids but not the first 94 amino acids of PMP22 are able to localize the fusion protein to peroxisomes. In contrast, adding the green fluorescent protein to the carboxyl terminus of PMP22 masks its carboxyl-terminal targeting signal, and only the first 94 amino acids direct the fusion protein to peroxisomes. We conclude from this observation that other peroxisomal membrane proteins for which a single peroxisomal targeting signal has been described might also have multiple signals. Examples are PEX3 and PEX22. Fusing the green fluorescent protein to the carboxyl terminus of these proteins revealed their amino-terminal targeting signal (17, 18). Having the green fluorescent protein at the carboxyl terminus of PEX3 and PEX22 might mask the existence of further targeting signals in carboxyl-terminal protein regions. The huge green fluorescent protein with a molecular size of 240 or 266 amino acids, depending on the vector protein, might alter the protein structure and interfere with the translocation machinery for peroxisomal membrane proteins by inhibiting their binding to cytosolic docking proteins or their membrane insertion.

Role of Transmembrane Domains and Basic Clusters for Peroxisomal Targeting—We and others have described how transmembrane domains within or close to peroxisomal targeting signals are crucial for targeting function (16, 18, 21, 23, 35–37). These transmembrane domains differ in size and number between various peroxisomal membrane proteins. In PEX3, PEX11β, PEX14, and PEX22, one single transmembrane domain is directly integrated in the targeting signal (17, 18, 20). Two transmembrane domains are required for targeting of PMP22, and three are required for PMP34 (23). Besides the hydrophobic transmembrane domains of peroxisomal membrane proteins, basic clusters also influence correct organelle targeting. These clusters are stretches of at least five amino acids consisting of at least two basic amino acids and mainly aliphatic residues (21–23). Substituting two or more basic amino acids with alanines or glycines strongly decreases peroxisomal targeting efficiency (16, 17, 22, 23). Furthermore, substituting all of the amino acids of the carboxyl-terminal basic cluster of PMP47 with alanines leads to partial targeting (23). The residual peroxisomal targeting efficiency of the mutated PMP47 protein might be due to the targeting information maintained in the second basic cluster in the amino terminus of this protein. We further observed that the amino acid compositions of the two basic clusters, one in the amino-terminal and the other in the carboxyl-terminal targeting signal, of PMP22 and the other peroxisomal membrane proteins with two peroxisomal targeting signals are very similar (Table II). Two of the basic amino acids are always identical in side chain structure and position. In contrast, homologous residues can substitute for the other amino acids of the cluster. For example, changing the position of isoleucine within analogous residues can substitute for the other amino acids of the targeting signal, of PMP22 and the other peroxisomal membrane proteins.

Interactions of Peroxisomal Membrane Proteins with PEX19—PEX19 is a farnesylated protein essential for peroxisome biogenesis and most likely involved in the synthesis of the peroxisome membrane (20, 29, 30). Although its precise function is still unknown, it seems to act as a chaperone-like protein for peroxisomal targeting and insertion of various peroxisomal membrane proteins including PMP22 (20, 30–32, 39). To elucidate whether PEX19 binds to the identified targeting ele-
ments of human and rat PMP22, we analyzed its interactions by co-immunoprecipitation with Myc-tagged PMP22 fragments. PEX19 interacts with the amino-terminal half as well as with the carboxyl-terminal half of PMP22, revealing the existence of at least two PEX19 binding sites in PMP22. Furthermore, PEX19 is able to precipitate the minimal carboxyl-terminal targeting element of PMP22. These results suggest that PEX19 binds directly to both peroxisomal targeting regions of PMP22. Our observations are consistent with those by Sacksteder et al. (20) and Jones and co-workers (24), who found that the targeting regions of PMP70, PMP34, PEX11β, and PEX14 are also recognized by PEX19. In contrast, previous studies on various peroxisomal membrane proteins and especially those with single targeting signals revealed that the region of the peroxisomal targeting signal and the PEX19 interaction site do not overlap (18, 21, 32). However, these proteins might have further and so far not identified peroxisomal targeting signals including the putative binding sites for PEX19. Another possibility is that there exists more than one import mechanism for peroxisomal membrane proteins, since there are at least two different import possibilities for peroxisomal matrix proteins. Considering these results together, PEX19 is a strong candidate for a docking protein or a peroxisomal receptor. Its function seems to be specific for peroxisomal proteins, since the mitochondrial protein ornithine–δ-amminotransferase did not show any PEX19 interactions. PMP22 is not essential for early peroxisome membrane synthesis, and the interactions of PEX19 and PMP22 cannot be attributed to a general role of PEX19 in peroxisomal membrane synthesis. Therefore, PEX19 interactions may be connected with a transport function or may facilitate the insertion of proteins into the peroxisomal membrane.

A Hypothetical Model for Targeting of Proteins to the Peroxisomal Membrane—Based on our results, we propose a new hypothetical model for the translocation apparatus of peroxisomal membrane proteins summarized in Fig. 8. After synthesis in the cytosol, PMP22 and other peroxisomal membrane proteins are recognized and bound at their positively charged basic clusters to a receptor protein located in the peroxisome membrane followed by integration. B, after synthesis in the cytosol, PMP22 or other peroxisomal membrane proteins are recognized and bound to a cytosolic docking protein at the two regions with positively charged basic clusters. The complex is transported to the surface of the peroxisome membrane, and the peroxisomal membrane protein is directly integrated. Another possibility is that a receptor protein in the peroxisomal membrane mediates integration.

**Fig. 8.** A hypothetical model for the translocation apparatus of peroxisomal membrane proteins. **A,** after synthesis in the cytosol, PMP22 or other peroxisomal membrane proteins are recognized and bound at their positively charged basic clusters to a receptor protein located in the peroxisome membrane followed by integration. **B,** after synthesis in the cytosol, PMP22 or other peroxisomal membrane proteins are recognized and bound to a cytosolic docking protein at the two regions with positively charged basic clusters.
translocation apparatus might be even more complex and involve further cytosolic factors.

The nature and characteristics of the docking proteins are still unclear. Previous studies on PEX19 show that it is a good candidate protein for such a chaperone-like protein or receptor protein (29–31, 40). PEX19 has been found either in the cytosol or associated to the peroxisome membrane. Human PEX19 consists of 300 amino acids (GenBank™/EMBL accession number AB018541). While only 22 of these amino acids are basic residues, namely arginine and lysine, there are 59 negatively charged amino acids, namely aspartic acid and glutamic acid. The numerous negatively charged amino acids might constitute specific recognition sites for the two positively charged basic clusters within the targeting signals of peroxisomal membrane proteins. Transmembrane domains adjacent to the basic clusters might be necessary to present the membrane proteins in a suitable conformation for binding. It will be interesting to acquire more information on this translocation model and especially on the physiological interactions and functions of the proteins involved.

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REFERENCES

1. Gould, S. J., Raymond, G. V., and Valle, D. (2001) in The Metabolic and Molecular Bases of Inherited Disease (Scherer, C. R., Beaudet, A. L., Valle, D., and Sly, W. S., eds) pp. 3181–3217, McGraw-Hill Inc., New York.
2. Fujiy, Y., Rachubinski, R. A., and Lazarow, P. B. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 7127–7131.
3. Lazarow, P. B., and Fujiy, Y. (1985) Annu. Rev. Cell Biol. 1, 489–530.
4. Suzuki, Y., Orii, T., Takiguchi, M., Mori, M., Hijikata, M., and Hashimoto, T. (1987) J. Biochem. (Tokyo) 101, 491–496.
5. Diestelkötter, P., and Just, W. W. (1989) J. Cell Biol. 123, 1717–1725.
6. Gould, S. J., Keller, G. A., and Subramani, S. (1988) J. Cell Biol. 107, 897–905.
7. Gould, S. J., Keller, G. A., Hsokena, N., Wilkinson, J., and Subramani, S. (1989) J. Cell Biol. 106, 1657–1664.
8. Subramani, S. (1990) Annu. Rev. Cell Biol. 9, 445–454.
9. Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A., and Subramani, S. (1991) EMBO J. 10, 3255–3262.
10. McCollum, D., Monosov, E., and Subramani, S. (1993) J. Cell Biol. 121, 761–774.
11. Marzich, M., Erdmann, R., Veenhuis, M., and Kunau, W. H. (1994) EMBO J. 13, 4988–4998.
12. Dodg, G., Braverman, N., Wong, C., Moser, A., Moser, H. W., Watkins, P., Vallee, D., and Gould, S. J. (1995) Nat. Genet. 9, 115–125.
13. Purdu, P. E., Zhang, J. W., Skoneczny, M., and Lazarow, P. B. (1996) Nat. Genet. 15, 381–384.
14. Braverman, N., Steel, G., Obie, C., Moser, A., Moser, H., Gould, S. J., and Vallee, D. (1997) Nat. Genet. 15, 369–376.
15. McCanmon, M. T., McNew, J. A., Willy, P. J., and Goodman, J. M. (1994) J. Cell Biol. 124, 915–925.
16. Dour, J. M., McNew, J. A., and Goodman, J. M. (1996) J. Cell Biol. 133, 269–280.
17. Koller, A., Snyder, W. B., Faber, N. K., Wenzel, T. J., Rangell, L., Keller, G. A., and Subramani, S. (1999) J. Cell Biol. 146, 99–112.
18. Soukupova, M., Sprenger, C., Gergas, K., Kunau, W.-H., and Dott, G. (1999) Eur. J. Cell Biol. 78, 357–374.
19. Pause, B., Saffrich, R., Hunziker, A., Ansorge, W., and Just, W. W. (2000) FEBS Lett. 471, 23–28.
20. Sacksteder, K. A., Jones, J. M., South, S. T., Li, X., Liu, Y., and Gould, S. J. (2000) J. Cell Biol. 148, 931–944.
21. Biermanns, M., and Gartner, J. (2001) Biochem. Biophys. Res. Commun. 283, 649–655.
22. Honsho, M., and Fukiji, Y. (2001) J. Biol. Chem. 276, 9375–9382.
23. Wang, X., Uruh, M. J., and Goodman, J. M. (2001) J. Biol. Chem. 276, 10987–10995.
24. Jones, J. M., Morrell, J. C., and Gould, S. J. (2001) J. Cell Biol. 153, 1141–1149.
25. Kaldi, K., Diestelkötter, P., Stebbeck, G., Auerbach, S., Jakle, U., Magert, H. J., Wieland, F. T., and Just, W. W. (1993) FEBS Lett. 315, 217–222.
26. Gartner, J., Moser, H. W., and Valle, D. (1992) Nat. Genet. 1, 16–23.
27. Sanger, F., Nickler, S., and Coulsen, A. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.
28. Altschul, S. F., Boguski, M. S., Gish, W., and Wootton, J. C. (1997) Nat. Genet. 15, 79–1129.
29. Gottke, K., Girzalsky, W., Linkert, M., Baumgart, E., Kammerer, S., Kunau, W. H., and Erdmann, R. (1998) Mol. Cell. Biol. 18, 616–628.
30. Snyder, W. B., Faber, N. K., Wenzel, T. J., Keller, A., Luer, G. H., Rangell, L., Keller, G. A., and Subramani, S. (1999) Mol. Cell. Biol. 10, 1745–1761.
31. Snyder, W. B., Keller, A., Choy, A. J., Johnson, M. A., Cregg, J. M., Rangell, L., Keller, G. A., and Subramani, S. (1999) Mol. Biol. Cell 10, 4005–4019.
32. Snyder, W. B., Keller, A., Choy, A. J., and Subramani, S. (2000) J. Cell Biol. 149, 1171–1177.
33. Kammerer, S., Holzinger, A., Welsch, U., and Roseher, A. A. (1998) FEBS Lett. 429, 53–60.
34. Elgersma, Y., Krast, L., van den Berg, M., Snyder, W. B., Distel, B., Distel, S., and Tabak, H. F. (1997) EMBO J. 16, 7236–7241.
35. Hofsfield, J., Veenhuis, M., and Kunau, W. H. (1991) J. Cell Biol. 114, 1167–1175.
36. Baerends, R. J. S., Rasmussen, S. W., Hilbrands, R. E., van der Heide, M., Kaldi, K., Diestelkötter, P., Stebbeck, G., Auerbach, S., Jakle, U., Magert, H. J., Wieland, F. T., and Just, W. W. (1993) FEBS Lett. 315, 217–222.
37. Baerends, R. J. S., Rasmussen, S. W., Hilbrands, R. E., van der Heide, M., Kaldi, K., Diestelkötter, P., Stebbeck, G., Auerbach, S., Jakle, U., Magert, H. J., Wieland, F. T., and Just, W. W. (1993) FEBS Lett. 315, 217–222.
38. Baerends, R. J. S., Rasmussen, S. W., Hilbrands, R. E., van der Heide, M., Kaldi, K., Diestelkötter, P., Stebbeck, G., Auerbach, S., Jakle, U., Magert, H. J., Wieland, F. T., and Just, W. W. (1993) FEBS Lett. 315, 217–222.
39. Baerends, R. J. S., Rasmussen, S. W., Hilbrands, R. E., van der Heide, M., Kaldi, K., Diestelkötter, P., Stebbeck, G., Auerbach, S., Jakle, U., Magert, H. J., Wieland, F. T., and Just, W. W. (1993) FEBS Lett. 315, 217–222.
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