Multiple Distinct Targeting Signals in Integral Peroxisomal Membrane Proteins

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Abstract. Peroxisomal proteins are synthesized on free polysomes and then transported from the cytoplasm to peroxisomes. This process is mediated by two short well-defined targeting signals in peroxisomal matrix proteins, but a well-defined targeting signal has not yet been described for peroxisomal membrane proteins (PMPs). One assumption in virtually all prior studies of PMP targeting is that a given protein contains one, and only one, distinct targeting signal. Here, we show that the metabolite transporter PMP34, an integral PMP, contains at least two nonoverlapping sets of targeting information, either of which is sufficient for insertion into the peroxisome membrane. We also show that another integral PMP, the peroxin PEX13, also contains two independent sets of peroxisomal targeting information. These results challenge a major assumption of most PMP targeting studies. In addition, we demonstrate that PEX19, a factor required for peroxisomal membrane biogenesis, interacts with the two minimal targeting regions of PMP34. Together, these results raise the interesting possibility that PMP import may require novel mechanisms to ensure the solubility of integral PMPs before their insertion in the peroxisome membrane, and that PEX19 may play a central role in this process.

Key words: peroxisome • organelle biogenesis • PEX19 • targeting signal • PMP34

Introduction

Proteins that are directed to subcellular compartments contain targeting information that specifies their final location. Often a single class of amino acid sequences directs a multitude of proteins to the same cellular compartment. In the case of the peroxisome, targeting to the organelle matrix occurs posttranslationally and is mediated by two cis-acting sequences, the peroxisome-targeting signal (PTS)[1] 1 (Gould et al., 1987, 1989b) and PTS2 (Swinkels et al., 1991). Proteins containing these signals are directed to the peroxisome upon binding the corresponding signal sequence receptors, PEX5 and PEX7, and are then imported into the peroxisome matrix in an ATP-dependent manner by a common matrix protein import machinery.

Less is known about the import of proteins destined for insertion into the peroxisome membrane. Like matrix proteins, integral peroxisomal membrane proteins (PMPs) are synthesized on free polyribosomes and imported posttranslationally from the cytosol (Fujiki et al., 1984; Suzuki et al., 1987; Diestelkotter and Just, 1993; Imanaka et al., 1996), though their import does not require the hydrolysis of ATP (Diestelkotter and Just, 1993). In addition, integral PMPs lack functional PTS1 or PTS2 signals, and their import is independent of the PTS1 and PTS2 receptors (Chang et al., 1999; Hettema et al., 2000). Integral PMPs are therefore thought to be imported into peroxisomes by a distinct targeting mechanism from that used by peroxisomal matrix proteins.

Multiple studies have attempted to define the targeting information in integral PMPs. These include studies of *Candida boidinii* PMP47 (McCammon et al., 1994; Dyer et al., 1996), PEX3 from *Pichia pastoris*, *Hansenula polymorpha*, and humans (Baerends et al., 1996, 2000; Wiemer et al., 1996; Kammerer et al., 1998; Soukupova et al., 1999), *P. pastoris* PEX2, PEX10, PEX13, PEX17, and PEX22 (Snyder et al., 2000), rat PMP22 (Pause et al., 2000), and human PMP70 and PEX11β (Sacksteder et al., 2000). Of these studies, only the examination of *C. boidinii* PMP47 defined PMP targeting information at the level of functional residues. In that study, Dyer et al. (1996) reported that the PMP47 targeting information lies between transmembrane domains 4 and 5 in a positively charged intraperoxisomal loop. Although these studies have all advanced our understanding of PMP targeting, they have not yet yielded a clear model of what constitutes a PMP targeting signal. In addition, virtually all of the above studies have assumed that PMPs contain one and only one set of targeting information.

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[1]*Abbreviations used in this paper:* HA, hemagglutinin; NLS, nuclear localization signal; PMP, peroxisomal membrane protein; PTS, peroxisome-targeting signal.

Here, we report an examination of the targeting information in PMP34, the human homologue of *C. boidinii* PMP47. We demonstrate that PMP34 contains not one but at least two targeting signals, either of which is sufficient for targeting to peroxisomes. We also show that two nonoverlapping segments of PEX13 target to peroxisomes, indicating that the presence of multiple independent targeting regions within a single PMP is not unique to PMP34. Last, we demonstrate that the minimal targeting regions of PMP34 both bind to PEX19. The implications of these results for PMP recognition and targeting are discussed.

Materials and Methods

Plasmids

The plasmids pcDNA3-PMP34myc and pcDNA3-PEX13myc have been described (Liu et al., 1999; Sacksteder et al., 2000). All PMP34 and PEX13 truncation mutants were generated by amplifying the desired fragment using primers that append the sequence 5′-GGTACCATG-3′ (encoding an Asp718 site and a start codon) at the 5′ end of the fragment, and the sequence 5′-GGATCC-3′ (encoding a BamHI site) at the 3′ end of the fragment, using the published sequences as a guide (Bjorkman et al., 1998; Wylin et al., 1999). PCR products were then digested with Asp718 and BamHI and cloned upstream of, and in frame with, either 3 sequential c-*myc* epitopes in the plasmid pcDNA3/3xmyc (Geisbrecht et al., 1998) or 13 sequential c-*myc* epitopes in the plasmid pcDNA3/13xmyc. The plasmid pNHA is designed to append a 17–amino acid hemagglutinin (HA) epitope tag, NH_3-MAYPYDVPDYAGGSGSS-COOH, to the NH_2 terminus of a protein. The expression vector pNHA-PTE1 was constructed by inserting a BamHI/XbaI fragment of pNmyc-*PTE1* (Jones et al., 1999), containing the *PTE1* ORF, into the BamHI and XbaI sites of pNHA. The plasmid pNHA-*PEX19* was made by excising the *PEX19* ORF from $pT7_{his6}$-*PEX19* (Sacksteder et al., 2000) by digestion with SalI and NotI and inserting it into the XhoI and NotI sites of pNHA. The p3xNLS vector is designed to append three contiguous copies of the viral SV-40 T antigen nuclear localization signal (Adam and Gerace, 1991), in the form of the amino acid sequence NH_3-MACPKKKRKVGDGGCPKKKRK-VGDGGCPKKKRKVGDGS-COOH, to the NH_2 terminus of a protein. To construct the p3xNLS vector, a PCR product was generated that encoded the above amino acid sequence and contained HindIII and BamHI sites at its 5′ and 3′ ends, respectively. The PCR product was then digested with HindIII and BamHI and inserted into the HindIII and BamHI sites of pNmyc-*PTE1* to make p3xNLS-*PTE1*, effectively exchanging the 3xNLS sequence for the c-*myc* epitope tag. The plasmids p3xNLS-*PEX19* and p3xNLS-*HAOX3* were made by excision of the appropriate ORF from $pT7_{his6}$-*PEX19* or $pT7_{his6}$-*HAOX3* by digestion with SalI and NotI and inserting them into the XhoI and NotI sites of p3xNLS-*PTE1*, effectively swapping the *PEX19* or *HAOX3* ORFs for that of *PTE1*. All clones generated by PCR amplification were sequenced to ensure the absence of any unintended mutations.

Indirect Immunofluorescence and Fluorescence Microscopy

Indirect immunofluorescence studies were performed on normal human skin fibroblasts (GM 5756-T) or the *PEX10*-deficient fibroblast cell line, PBD100, which has been described previously (Warren et al., 1998). Cell lines were cultured and transfected as described (Chang et al., 1997). After transfection, cells were transferred to cover glasses and incubated in complete medium for 1 d. The cells were then fixed in 3% formaldehyde, permeabilized with 1% Triton X-100, and processed for indirect immunofluorescence as described (Chang et al., 1997). The anti–c-*myc* mouse monoclonal antibody was derived from tissue culture medium of the mouse hybridoma line 1-9E10 (Roche Molecular Biochemicals). Sheep anti-PMP70 and rabbit anti-PEX19 antibodies (Sacksteder et al., 2000) and rabbit anti-HAOX3 antibodies (Jones et al., 2000) have been described. Fluorescent secondary antibodies were obtained from commercial sources.

Cell Lysates, Na_2CO_3 Extraction, Immunoprecipitation, and Immunoblots

Normal human skin fibroblasts (GM5756-T) or *PEX10*-deficient PBD100 fibroblasts were cultured and transfected with the appropriate expression plasmids as described (Chang et al., 1997). 1 d after transfection, cells were washed twice in PBS (GIBCO BRL) and harvested by gentle scraping. For whole cell lysates, transfected cells were briskly resuspended in PBS plus 1% Triton X-100. Protein content of lysates was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Equal quantities of total protein from each lysate were separated by PAGE, transferred to membranes, and analyzed by immunoblot using standard protocols (Crane et al., 1994).

For membrane extraction experiments, cells were resuspended in hypotonic lysis buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA, Complete™ protease inhibitor cocktail [Boerhinger]) and lysis was achieved by passing cell slurries through a 20-gauge syringe needle five times. Cellular membranes were harvested by centrifugation at 100,000 *g* for 1 h. Supernatant and pellet were separated, pellets were resuspended in 100 mM Na_2CO_3 at a final protein concentration of 0.4 mg/ml, and the suspensions were incubated for 30 min on ice with gentle agitation. Membranes were pelleted by centrifugation at 100,000 *g* for 1 h. Extraction samples were diluted to the initial volume of lysate, and equal volumes of the samples were analyzed by immunoblot using standard techniques (Crane et al., 1994). Detection of the c-*myc* epitope was performed by a commercially available anti–c-*myc* polyclonal antibody (Santa Cruz Biotechnology, Inc.), and detection of catalase was performed by a commercially available sheep anticatalase antibody (The Binding Site). Anti-PEX13 antibodies were raised in rabbit against a synthetic peptide corresponding to the COOH-terminal 14 amino acids of human PEX13 (Gould et al., 1996).

For immunoprecipitation experiments, cells expressing either PMP34aa1-147/13xmyc or PMP34aa244-307/3xmyc in combination with either HA-PEX19 or HA-PTE1 were lysed in 500 μl hypotonic lysis buffer as above. Lysates were then subjected to centrifugation at 25,000 *g* for 15 min to pellet large organelles such as peroxisomes. The resulting supernatant was brought to 1 ml final volume by addition of PBS (Life Technologies) plus Complete™ protease inhibitor cocktail (Boehringer). 20 μl of a 50% slurry of protein A agarose (Sigma-Aldrich) in PBS was added and the mixture, incubated with mixing at 4°C for 30 min. The protein A agarose was pelleted, and the supernatant was removed to a new tube. 15 μl of a 50% slurry of anti-HA monoclonal antibodies coupled to agarose beads (Santa Cruz Biotechnology, Inc.), which had been preincubated in 0.1 mg/ml BSA in PBS for 30 min, was added to each sample, and the mixtures were incubated with mixing at 4°C for 3 h. The anti-HA beads were recovered by centrifugation, washed 4 times with 1 mL PBS, and resuspended in SDS-PAGE sample buffer. Corresponding proportions of lysate and immunoprecipitate samples were analyzed by immunoblot using the anti–c-*myc* polyclonal antibody as above.

Results

Epitope-tagged PMP34 Localizes to Peroxisomes

C. boidinii PMP47 was the subject of the first detailed examination of PMP targeting signals (Dyer et al., 1996). We felt that its human homologue (Wylin et al., 1999) would therefore be a useful model protein for the study of targeting information in mammalian PMPs. We proceeded to search for targeting information in this protein using the same experimental system that was used to identify the peroxisomal matrix protein targeting signals PTS1 and PTS2 (Gould et al., 1989a; Swinkels et al., 1991). Specifically, we designed genes to express a set of mutant PMP34 proteins, expressed those genes in mammalian cells by transient transfection, and assessed the distribution of their products by indirect immunofluorescence.

To distinguish between endogenously expressed PMP34 and PMP34 proteins expressed from plasmids, the plasmid-encoded proteins all contained the c-*myc* epitope at their COOH terminus. The full-length PMP34myc fusion protein was expressed in wild-type human fibroblasts (Fig. 1 a) and was efficiently targeted to peroxisomes (b and c). The Zellweger syndrome fibroblast cell line PBD100 is unable to import peroxisomal matrix proteins but imports PMPs normally (Warren et al., 1998). PMP34myc was ex-

pressed in PBD100 cells (Fig. 1 a) and was targeted to peroxisomes (d and e), demonstrating that PMP34myc is targeted to peroxisomes by a membrane protein targeting pathway, as previously suggested (Wylin et al., 1999).

PMP34 Contains Multiple Peroxisomal Targeting Signals

One strategy for identifying targeting information in a protein is to identify mutations that disrupt its targeting and then deduce the necessary targeting information from the positions of the mutations. However, this method is unreliable since protein import can be disrupted by improper folding, insolubility, or instability of a protein rather than a specific defect in a protein's targeting information. For example, mutations that disrupted the peroxisome targeting of luciferase were very misleading in the search for the PTS1 (Gould et al., 1987). Another strategy is to identify the minimal region(s) of a protein that are sufficient for its proper subcellular localization. This method is less sensitive to artifacts and was, in fact, the approach used to identify both the PTS1 (Gould et al., 1989a) and PTS2 (Swinkels et al., 1991). Therefore, we proceeded to scan PMP34 for regions that were sufficient for peroxisomal targeting (Fig. 2).

We first made a series of deletions starting at the COOH terminus of the protein and found that the NH_2-terminal 147 amino acids were capable of targeting to peroxisomes (Fig. 3, a and b). Truncation mutants shorter than this either failed to express in multiple trials or did not target to peroxisomes. Next, we made a series of truncations beginning from the NH_2 terminus and found that the COOH-terminal 64 amino acids of PMP34 retained excellent targeting to peroxisomes (Fig. 3, c and d). In these studies, PMP34 truncations that targeted to peroxisomes but no other compartments were designated +++, those that targeted to peroxisomes and targeted to other compartments in some cells were designated ++, those that targeted to peroxisomes and targeted to other compartments in all cells were designated +, and those that showed no peroxisomal targeting were designated −. Most other studies of PMP targeting information have assumed that each PMP contains a single targeting signal (Baerends et al., 1996, 2000; Wiemer et al., 1996; Kammerer et al., 1998; Pause et al., 2000; Snyder et al., 2000), including some from our own laboratory (Sacksteder et al., 2000). Thus, the identification of two discrete targeting signals in PMP34 was entirely unexpected.

Next, we made small deletions in the NH_2- and COOH-terminal targeting regions. These attempts failed to identify smaller targeting segments of the NH_2-terminal region. However, we did identify a smaller region at the COOH terminus of PMP34 that retained targeting ability. Specifically, we found that amino acids 255–307 of PMP34 targeted to peroxisomes with good efficiency, whereas a construct containing amino acids 270–307 failed to target. Dyer et al. (1996) and others (Baerends et al., 2000; Pause et al., 2000) have emphasized the importance of basic amino acids in PMP targeting signals, and there was a patch of basic residues, HQRVRR, within the COOH-terminal targeting region of PMP34 (amino acids 255–260). However, we found that a smaller protein (amino acids 260–307) that lacked this patch of basic residues still targeted to peroxisomes, albeit at reduced efficiency. Although small truncations from the COOH terminus of this region (constructs ending at amino acid 303 or 291) re-

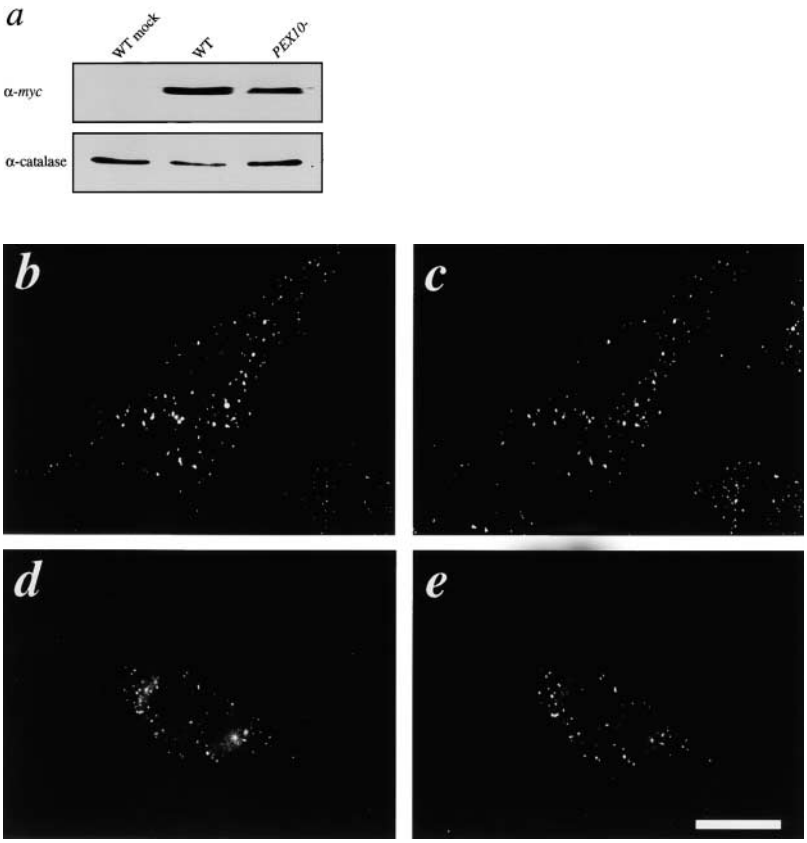

Figure 1. PMP34myc localizes to peroxisomes by a pathway distinct from that used by matrix proteins. (a) Lysates of mock-transfected human fibroblasts, fibroblasts expressing PMP34myc, and *PEX10*-deficient PBD100 fibroblasts expressing PMP34myc were analyzed by immunoblot using anti-*myc* and anticatalase antibodies. (b–e) Human skin fibroblasts expressing PMP34myc were processed for double indirect immunofluorescence by fixing cells and permeabilizing them with 1% Triton X-100. The distribution of PMP34myc was examined using anti-*myc* antibodies (b) and anti-PMP70 antibodies (c). The distribution of PMP34myc was also examined in fibroblasts of the *PEX10*-deficient cell line PBD100, again by double indirect immunofluorescence using anti-*myc* antibodies (d) and anti-PMP70 antibodies (e). Bar, 15 μm.

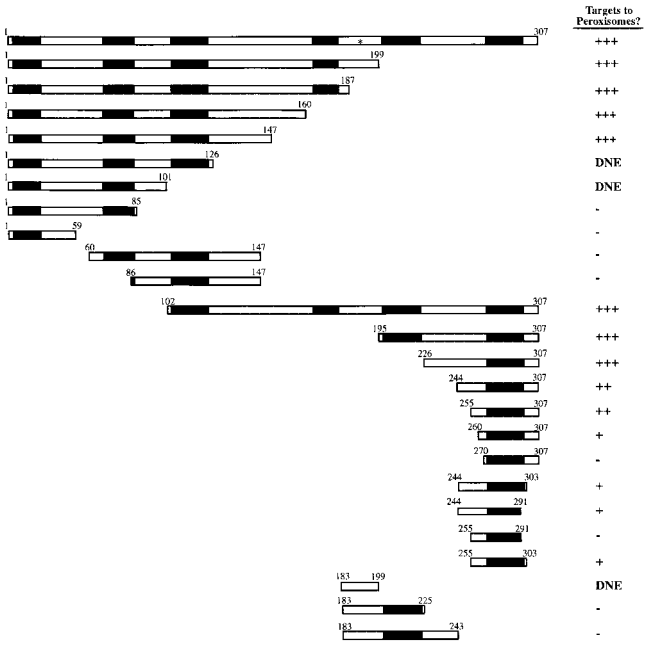

Figure 2. Peroxisomal targeting of PMP34 mutants. Human skin fibroblasts expressing a series of PMP34 mutants were analyzed by double indirect immunofluorescence using anti-*myc* antibodies and antibodies to the peroxisomal marker protein PMP70. Peroxisomal targeting of the mutants was scored as follows: +++, peroxisomal targeting with no targeting to other structures; ++, peroxisomal targeting with some cells showing staining of other compartments; +, peroxisomal targeting with all cells also showing staining of other compartments; −, no peroxisomal targeting. DNE, did not express.

duced the efficiency of targeting, they did not disrupt targeting completely, suggesting that the extreme COOH terminus of PMP34 did not contain essential targeting information. Comparison of the NH_2- and COOH-terminal minimal targeting regions did not identify a common sequence motif but did reveal that both regions contained a putative membrane spanning domain.

Dyer et al. (1996) found that the peroxisomal targeting signal of *C. boidinii* PMP47 resided in the loop between transmembrane spans 4 and 5. To determine whether the corresponding loop in human PMP34 (Fig. 2, *) is also important for targeting to peroxisomes, we assayed the targeting ability of several proteins containing this region. Although the loop alone (amino acids 183–199) failed to express to levels detectable by immunofluorescence, we found that two larger constructs containing this segment (amino acids 183–225 and amino acids 183–243) failed to target to peroxisomes.

The Minimal Targeting Regions in PMP34 Are Sufficient for Membrane Insertion

Peroxisomal localization of the two PMP34 fragments could reflect insertion into the peroxisome membrane. However, it could also have been caused by interaction with a binding partner at the peroxisome surface or by cryptic import into the peroxisome matrix. To address these concerns, fibroblasts expressing the NH_2- and COOH-terminal targeting elements of PMP34 were lysed in hypotonic buffer. Soluble proteins were separated from membranes by centrifugation, the membranes were extracted with alkaline sodium carbonate, and the resulting suspension was separated into a membrane pellet and a soluble supernatant. These fractions were then assayed by immunoblot. Proteins containing either the NH_2-terminal PTS (PMP34aa1-147/3xmyc) or the COOH-terminal PTS (PMP34aa244-307/3xmyc) remained in the membrane even after carbonate extraction, demonstrating that these signals direct proteins into the peroxisome membrane (Fig. 4). As expected, control immunoblots showed that the peroxisomal matrix enzyme catalase was released to the supernatant by hypotonic lysis and that the integral PMP PEX13 was resistant to carbonate extraction.

PEX13 Also Contains Multiple Independent Targeting Signals

The presence of two distinct targeting signals in a single PMP was unexpected and challenges the assumption that only one targeting signal exists in a given PMP. To deter-

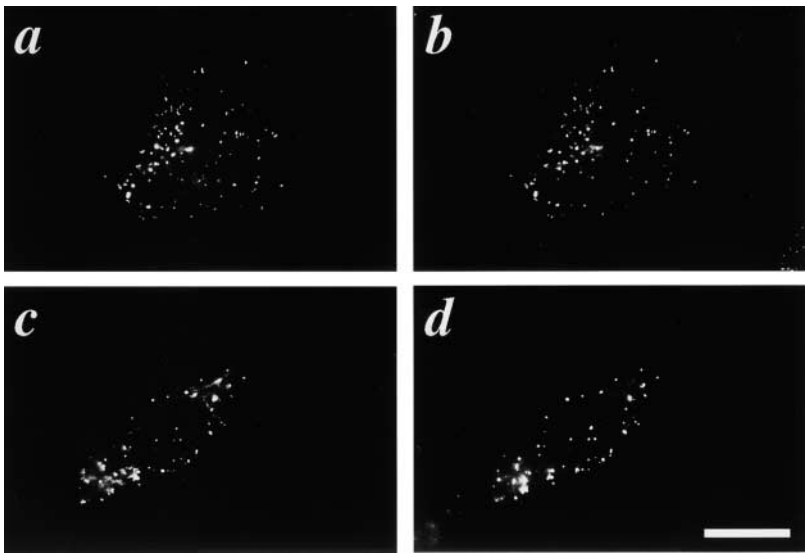

Figure 3. Two distinct regions of PMP34 are sufficient for peroxisomal targeting. Human skin fibroblasts expressing PMP34aa1–147/3xmyc (a and b) or PMP34aa244–307/3xmyc (c and d) were processed for double indirect immunofluorescence by fixing cells and permeabilizing with Triton X-100. The distribution of each PMP34 mutant was examined using anti-*myc* antibodies (a and c) and antibodies to the peroxisomal marker protein PMP70 (b and d). Bar, 15 μm.

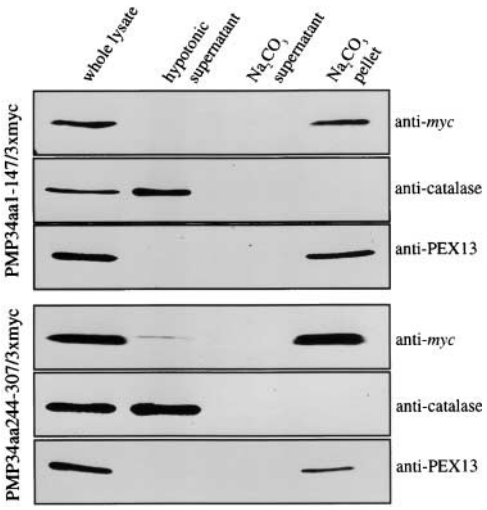

Figure 4. The two minimal targeting regions of PMP34 are inserted into the peroxisomal membrane. Human skin fibroblasts expressing PMP34aa1–147/3xmyc or PMP34aa244–307/3xmyc were lysed by thorough mixing in hypotonic buffer. Membranes were pelleted by centrifugation, separated from the supernatant, and incubated in alkaline sodium phosphate buffer. Membranes were then pelleted again, separated from the supernatant, and resuspended. All fractions were diluted to the initial volume of whole lysate. Equal volumes of each fraction were then assayed by immunoblot using antibodies against the c-*myc* epitope, the soluble peroxisomal matrix enzyme catalase, and the integral PMP PEX13.

mine whether the presence of multiple PTSs was a unique feature of PMP34 or might also occur in other PMPs, we examined the targeting information in another integral PMP, human PEX13. Full-length PEX13 was efficiently targeted to peroxisomes, as expected (Fig. 5, a and b). A fragment of PEX13 containing only amino acids 1–193 targeted to peroxisomes (Fig. 5, c and d), as did a fragment containing only amino acids 194–403 (e and f), although this COOH-terminal fragment of PEX13 was also detected in a perinuclear structure. Thus, PMP34 is not the only integral PMP that contains multiple, nonoverlapping targeting signals.

Interaction of PEX19 with Targeting Signals from PMP34

In humans, PEX19 is required for the import of PMPs and the synthesis of peroxisome membranes (Sacksteder et al., 2000). We previously established that human PEX19 is a predominantly cytoplasmic, partly peroxisomal protein and interacts with 12 different PMPs and the targeting signals of at least two PMPs, PMP70 and PEX11β (Sacksteder et al., 2000). Furthermore, we showed that mislocalization of PEX19 to the nucleus results in the misdirection of newly synthesized PMPs to the nucleoplasm rather than the peroxisome membrane. Based on these and other results, we proposed that PEX19 might function as a PMP-specific chaperone, an import receptor for newly synthesized PMPs, or both.

We used the nuclear mislocalization assay to determine whether PEX19 might also interact with the two targeting signals of PMP34. A mutant version of the *PEX19* cDNA was constructed that encoded three tandem copies of the nuclear localization signal (NLS) from SV-40 large T antigen (Adam and Gerace, 1991) at the 5′ end of the *PEX19* ORF. The two small targeting elements of PMP34, PMP34aa1-147/3xmyc, and PMP34aa244-307/3xmyc were coexpressed with 3xNLS-PEX19 in normal human fibroblasts, and their subcellular distribution was determined by immunofluorescence microscopy. Both fragments of PMP34 were efficiently targeted to the nucleoplasm when coexpressed with 3xNLS-PEX19 (Fig. 6, a–d). In contrast, coexpression of these PMP34 fragments with a control protein, 3xNLS-HAOX3, had no effect on their trafficking to peroxisomes (Fig. 6, e–h; HAOX3 is a peroxisomal oxidase that is not normally expressed in fibroblasts and is not involved in PMP import [Jones et al., 2000]).

Next, we used coimmunoprecipitation as an independent test of whether PEX19 can interact with the smaller of the PMP34 targeting signals. Human fibroblasts were cotransfected with plasmids designed to express (i) PMP34aa1-147/13xmyc or PMP34aa244-307/3xmyc and (ii) either an HA-tagged form of PEX19 or an HA-tagged form of PTE1, a peroxisomal matrix enzyme not involved in PMP import (Jones et al., 1999). Lysates were prepared from the two cell populations and then processed for immunoprecipitation using anti-HA monoclonal antibodies. The resulting immunoprecipitates were separated by SDS-PAGE and blotted with anti-myc antibodies (Fig. 7). Both PMP34aa1-147/13xmyc and PMP34aa244-307/3xmyc were coimmunoprecipitated with HA-PEX19 but not HA-PTE1, supporting the hypothesis that PEX19 interacts with PMP targeting signals.

Discussion

Our data on PMP34 targeting raise three general questions. First, what is the nature of PMP targeting signals? Second, why is it that some PMPs have multiple nonoverlapping PMP targeting signals? Third, does PEX19 interact with the PMP targeting signals identified in PMP34?

Properties of the PMP34 Targeting Signals

The two PTSs we identified correspond to amino acids 1–147 and 244–307 of PMP34. An inspection of these sequences failed to reveal any common sequences with each other or with previously identified PTSs from human PMP70 (Sacksteder et al., 2000), human PEX11β (Sacksteder et al., 2000), human PEX14 (Sacksteder et al., 2000), human PEX3 (Kammerer et al., 1998; Soukupova et al., 1999), rat PMP22 (Pause et al., 2000), or PTSs identified in fungal forms of PMPs (Baerends et al., 1996, 2000; Wiemer et al., 1996; Dyer et al., 1996; Snyder et al., 2000). This was not altogether surprising, given that these previously described PMP targeting signals lack significant sequence similarities. We have also considered the possibility that there may be multiple types of PMP targeting signals, just as there are at least two types of peroxisomal matrix protein targeting signals. However, our efforts to identify subgroups of PMP targeting signals that share conserved sequence motifs have also failed.

Although PMP targeting signals lack recognizable amino acid sequence motifs, they do have some common features. One is that they are relatively long. A targeting signal in hu-

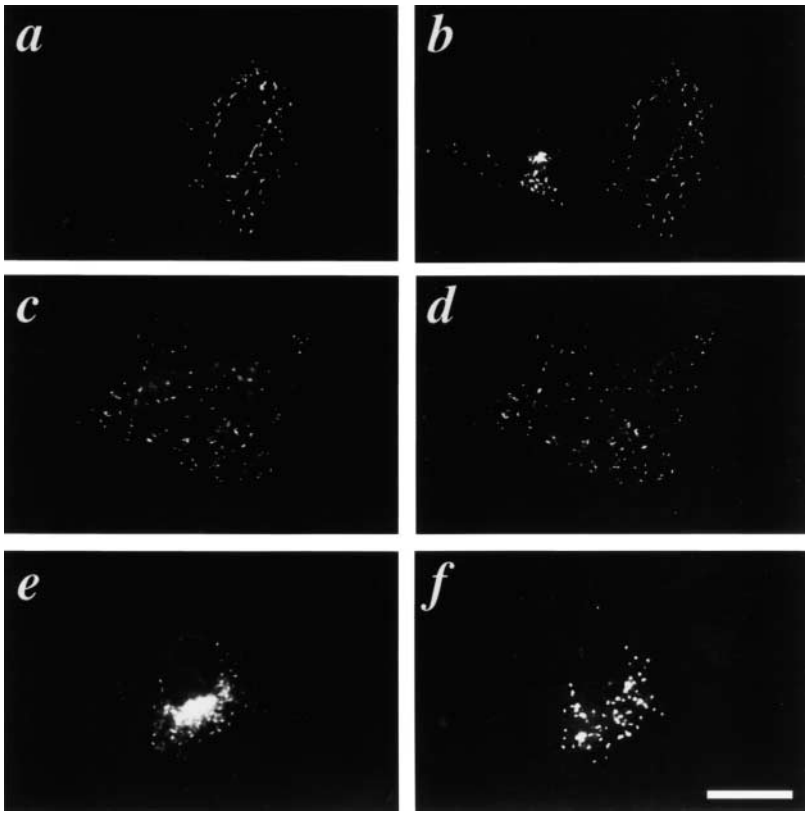

Figure 5. Two nonoverlapping regions of PEX13 are sufficient for peroxisomal targeting. Normal human fibroblasts expressing either PEX13myc (a and b), PEX13aa1–193/3xmyc (c and d), or PEX13aa194–403/3xmyc (e and f) were analyzed by double indirect immunofluorescence using anti-*myc* antibodies (a, c, and e), or antibodies to the peroxisomal marker protein PMP70 (b, d, and f). Bar, 15 μm.

man PMP70 is 61 amino acids long (Sacksteder and Gould, 2000), a targeting signal from PEX11β is 47 amino acids long (Sacksteder and Gould, 2000), a targeting signal in rat PMP22 is 97 amino acids long (Pause et al., 2000), and the targeting signals we identified in human PMP34 are 147 and 64 amino acids long. In fact, the shortest PMP targeting signals yet identified are the first 33 amino acids of PEX3, which functions only inefficiently (Soukupova et al., 1999), and the first 25 amino acids of *P. pastoris* PEX17 (Snyder et al., 2000). The long length of PMP targeting signals contrasts sharply with the small size of the targeting signals for peroxisomal matrix proteins, the PTS1 and PTS2, which are three and nine amino acids long, respectively (Gould et al., 1987, 1989b; Swinkels et al., 1991). This difference in targeting signal size may reflect the nature of protein targeting assays and the fundamental differences between membrane and matrix protein targeting mechanisms. All protein targeting assays measure two processes, the targeting of the protein to its destination and the retention of the protein at its destination. In the case of peroxisomal matrix protein targeting, retention is provided by the passive barrier of the peroxisome membrane and the targeting signals correspond to the minimal sequence necessary for binding the PTS1 and PTS2 receptors, PEX5 and PEX7 (Rehling et al., 1996; Zhang and Lazarow, 1996; Gatto et al., 2000). However, the only retention mechanism for PMPs is insertion into the peroxisomal membrane. PMP insertion likely requires a transmembrane domain on the targeted protein, but additional sequences may also be required for the insertion process. Thus, it is not surprising that the second common property of PMP targeting signals is that they contain at least one membrane-spanning domain.

Another feature of the PMP34 targeting signals that warrants discussion is their relationship to the mPTS of PMP47. PMP47 was the subject of the first PMP targeting signal studies (McCammon et al., 1994; Dyer et al., 1996), which concluded that its targeting signal, the mPTS, resided within a hydrophilic loop between membrane spanning domains 4 and 5 (Dyer et al., 1996). We assumed that the targeting signal of human PMP34 would be similar, particularly since this loop is relatively well conserved between human PMP34 and *C. boidinii* PMP47 (Wylin et al., 1999). This is not the case. The two PTSs we identified in human PMP34 differ in many respects from the mPTS of *C. boidinii* PMP47. The most obvious differences are that the two targeting signals of human PMP34 do not correspond in position or sequence to the mPTS of PMP47. Also, the region of human PMP34 that does correspond to the mPTS of PMP47 does not function as a PMP targeting signal. Another notable difference is that the PMP34 targeting signals both contain at least one putative membrane-spanning domain, whereas the mPTS of PMP47 lacks a membrane-spanning domain. Another difference worth discussing is the role of a basic patch of amino acids in PMP targeting. A basic patch of amino acids was thought to be important for the mPTS of PMP47 (Dyer et al., 1996), and much attention has been given to the importance of basic amino acid patches in targeting of other PMPs (Baerends et al., 2000; Pause et al., 2000). We identified a small patch of basic amino acids (amino acids 255–259) in the COOH-terminal PMP34 targeting signal. However, a smaller fragment of PMP34 lacking these basic residues (PMP34aa260–307/3xmyc) retained peroxisomal targeting, indicating that a basic patch of amino acids may

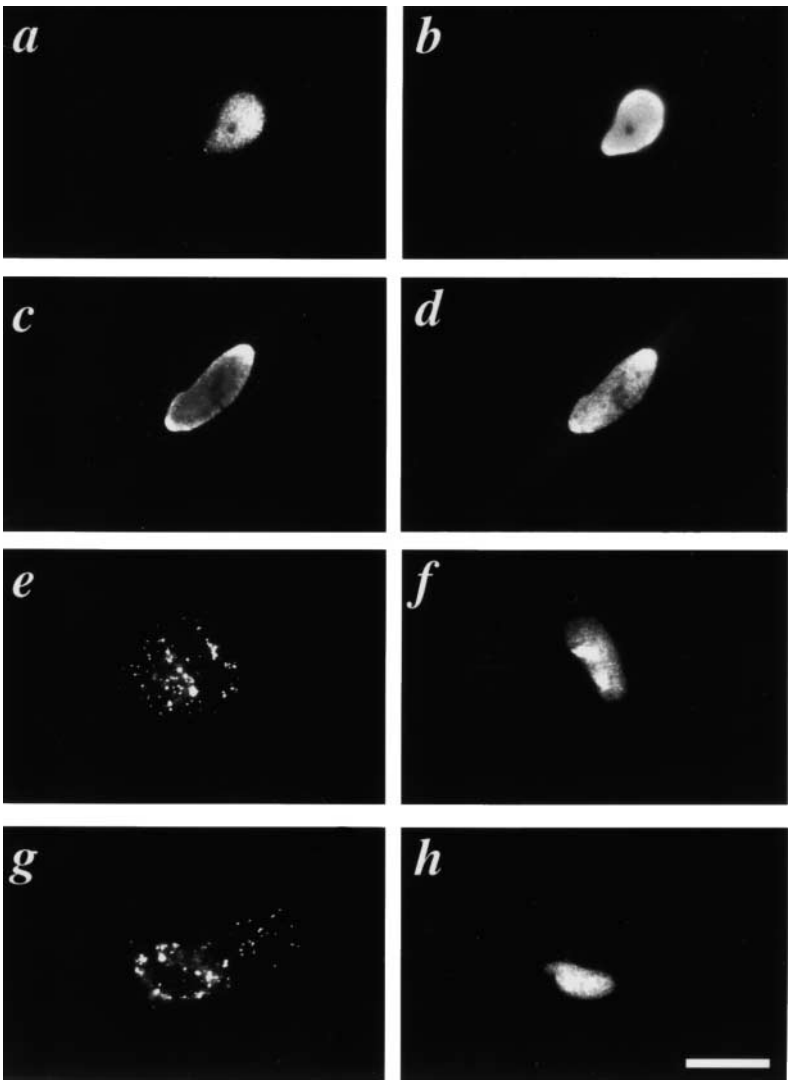

Figure 6. PEX19 controls the subcellular distribution of the PMP34 targeting elements. PMP34aa1–147/3xmyc (a, b, e, and f) or PMP34aa244–307/3xmyc (c, d, g, and h) were transiently expressed in normal human fibroblasts in combination with either 3xNLS-PEX19 (a–d) or 3xNLS-HAOX3 (e–h). The distributions of the PMP34 fragments were determined using antibodies to the c-*myc* epitope (left), and the distributions of 3xNLS-PEX19 and 3xNLS-HAOX3 were determined using antibodies to PEX19 and HAOX3, respectively (right).

not be an essential feature of all PMP targeting signals. While this work was in progress, Wang et al. (2001) revisited the peroxisomal targeting of *C. boidinii* PMP47 and found that PMP47 does require a transmembrane domain for targeting and that multiple fragments containing the mPTS identified by Dyer et al. (1996) did not target to peroxisomes. Furthermore, additional sequences far upstream of the mPTS facilitated peroxisomal targeting of PMP47, though there is as yet no data on whether PMP47 contains more than one functional peroxisomal targeting signal.

While our study was in progress, an independent study of PMP34 targeting information was reported by Honsho and Fujiki (2001). However, their report concluded that at least three transmembrane domains were necessary for targeting, that PMP34 contained a single targeting signal, and that the loop between transmembrane domains 4 and 5 of PMP34 was essential for targeting. Given that our study and the Honsho and Fujiki study (2001) both examined the same protein (human PMP34) using a very similar protein targeting assay in very similar cells, we feel compelled to address the significant differences between the two studies. Although there are some differences in the experimental results, the major difference between the two reports is in the interpretation of the data. Our conclusions are drawn entirely from PMP34 fragments that target to peroxisomes. In contrast, Honsho and Fujiki based many of their conclusions on PMP34 fragments that failed to be targeted. For example, Honsho and Fujiki concluded that the NH_2-terminal half of PMP34 lacks targeting information because a single fusion protein containing amino acids 1–186 of PMP34 did not localize to peroxisomes, whereas we showed that even smaller NH_2-terminal fragments did target to peroxisomes. Their conclusion ignores other reasonable explanations, such as aggregation, insolubility, or the masking of protein targeting signals in the artificial fusion protein. Given the many possible reasons that a mutant form of a protein might fail to target, we feel that there is a logical basis for relying on positive experimental results in the interpretation of these types of experiments, and prior studies of the PTS1 support this view (Gould et al., 1987).

Why Do Some PMPs Have Multiple Targeting Signals?

Previous studies of PMP targeting, including ours, have assumed that there is one and only one targeting signal per PMP (Baerends et al., 1996, 2000; Dyer et al., 1996; Kam-

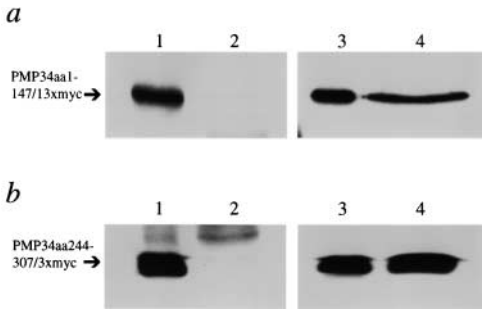

Figure 7. Coimmunoprecipitation of PEX19 and the peroxisomal targeting elements of PMP34. (a) Normal human fibroblasts were transfected with a plasmid designed to express PMP34aa1–147/13xmyc and plasmids designed to express either HA-PEX19 or HA-PTE1. Cells were lysed in hypotonic buffer, and the resulting lysates were cleared by centrifugation and processed for immunprecipitation using antibodies to the HA epitope. Immunoprecipitates and corresponding proportions of each lysate were separated by SDS-PAGE and analyzed by immunoblot using anti-*myc* antibodies. (lane 1) Anti-HA immunprecipitate from lysate of cells expressing PMP34aa1–147/13xmyc and HA-PEX19; (lane 2) anti-HA immunoprecipitate from cells expressing PMP34aa1–147/13xmyc and HA-PTE1; (lane 3) lysate of cells expressing PMP34aa1–147/13xmyc and HA-PEX19; (lane 4) lysate of cells expressing PMP34aa1–147/13xmyc and HA-PTE1. (b) Fibroblasts were transfected with a plasmid designed to express PMP34aa244–307/3xmyc and plasmids designed to express either HA-PEX19 or HA-PTE1. Cell lysis, immunoprecipitation, and immunoblot were performed as in panel a. (lane 1) Anti-HA immunprecipitate from lysate of cells expressing PMP34aa244–307/3xmyc and HA-PEX19; (lane 2) anti-HA immunoprecipitate from lysate of cells expressing PMP34aa244–307/3xmyc and HA-PTE1; (lane 3) lysate of cells expressing PMP34aa244–307/3xmyc and HA-PEX19; (lane 4) lysate of cells expressing PMP34aa244–307/3xmyc and HA-PTE1.

merer et al., 1998; Pause et al., 2000; Sacksteder et al., 2000; Snyder et al., 2000). Our data show that this assumption is flawed. We detected two distinct PTSs in PMP34 and showed that two nonoverlapping fragments of PEX13, another integral PMP, are targeted to peroxisomes. PMP70 also appears to have multiple targeting signals; we have identified a PTS at the protein's NH_2 terminus (amino acids 1–61 [Sacksteder et al., 2000]), and Imanaka et al. (1996) demonstrated that another PTS exists in a COOH-terminal 50-kD fragment of PMP70. Because these targeting signals are sufficient to direct proteins to and into the peroxisome membrane, the identification of multiple targeting signals in several PMPs warrants further discussion.

We can envision two general hypotheses that might explain our observations. One is that the presence of multiple targeting signals in PMP34, PEX13, and PMP70 reflects a random meaningless event, namely the unselected acquisition of a second set of targeting information in these proteins. This hypothesis suggests that all or most other PMPs will be found to have one and only one PMP targeting signal and that our results do not reveal anything significant about the mechanism of PMP import. Although we cannot exclude this possibility, it seems unlikely that multiple proteins would acquire and maintain superfluous targeting information. Thus, we must also consider the alternative hypothesis that the presence of multiple peroxisomal targeting signals in PMP34, PEX13, and PMP70 is meaningful and reflects the properties of the underlying PMP import mechanism. But what mechanism would require multiple targeting signals per PMP?

Current paradigms of protein import systems do not provide an obvious answer to this question. Both co- and posttranslation import systems are known that rely on a single set of targeting information. In addition, import mechanisms that are heavily reliant on maintaining import substrates in the unfolded state, such as translocation through the endoplasmic reticulum and mitochondrial membranes (Deshaies et al., 1988), are mediated by a single set of targeting information, as is peroxisomal matrix protein import, which appears to be less reliant on protein unfolding before import (Subramani, 1993). In fact, there is only one substantive difference we can see between existing paradigms of protein import and PMP import. PMP import involves the posttranslational import of proteins that contain membrane-spanning domains (Lazarow and Fujiki, 1985).

It is generally appreciated that hydrophobic membrane-spanning domains cannot be exposed to the aqueous environment of the cytoplasm without deleterious affects on protein folding and solubility. Thus, it may well be that posttranslational import of PMPs requires special mechanisms to prevent exposure of their transmembrane domains to the cytoplasm. This could be accomplished by a specific protein or protein complex that binds hydrophobic regions of PMPs during and/or after PMP synthesis and before insertion of the PMP into the peroxisome membrane. Furthermore, PMPs with multiple transmembrane domains such as PMP34, PEX13, and PMP70 might be expected to contain multiple sites for interaction with such a PMP binding factor.

A priori, the existence of such a PMP binding factor does not demand the presence of multiple targeting signals in a polytopic PMP. However, we can imagine one circumstance in which it would. If the interaction between a PMP and the PMP binding factor is sufficient to direct the PMP into the peroxisomal import pathway, then we would expect that the sites along the PMP that interact with the binding factor would function as PMP targeting signals. PMPs with multiple membrane-spanning domains would be expected to have multiple interaction sites for the PMP binding factor and would therefore be expected to contain multiple nonoverlapping targeting signals.

Although this speculative model remains to be tested, it is interesting to consider known proteins that might fulfill the role of the proposed PMP binding factor. Molecular chaperones are clear candidates since they are known to mask hydrophobic domains and prevent protein aggregation. In addition to Hsp70 and Hsp40 molecules that have been implicated in peroxisome biogenesis, a recent study has suggested that the multicomponent cytosolic ring chaperone, TriC, may interact with newly synthesized PMPs (Pause et al., 1997). However, it is difficult to imagine how these somewhat generic chaperones, which affect proteins destined for many functions and subcellular compartments, would specify entry into the PMP import pathway. Our hypothesis may make more sense if the PMP binding factor acts in a pathway-specific manner, and one such candidate has been identified. PEX19 is required for

PMP import, interacts with all known human PMPs, and interacts with the PMP targeting signals of PMP70 and PEX11β (Sacksteder et al., 2000). We have shown here that PEX19 also interacts with the two targeting signals of PMP34. Mislocalization of PEX19 to the nucleus results in the nuclear accumulation of proteins containing either PMP34 targeting signal. Also, we detected coimmunoprecipitation of PEX19 and the PMP34 targeting signals. A previous study of PEX19–PMP interactions in the yeast *P. pastoris* concluded that PEX19 does not interact with PMPs before their import and therefore could not act as the proposed PMP binding factor (Snyder et al., 2000). However, in this study, we established that PEX19 does interact with PMPs in the cytosol, since our coimmunoprecipitation experiments were performed with cytosolic lysates that lack peroxisomal membranes. Although our results are consistent with the hypothesis that PEX19 may function as a chaperone and/or import receptor for newly synthesized PMPs, a direct test of this hypothesis remains to be performed.

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