Multiple Targeting Modules on Peroxisomal Proteins Are Not Redundant: Discrete Functions of Targeting Signals within Pmp47 and Pex8p

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Several peroxisomal proteins have two nonoverlapping targeting signals. These signals have been termed “redundant” because targeting can still occur with only one signal. We now report that separate targeting motifs within both Pmp47 and Pex8 provide complementary function. Pmp47 is an ATP translocator that contains six transmembrane domains (TMDs). We had previously shown that the TMD2 region (termed TMD2R, consisting of TMD2 and a short adjacent segment of cytosolic loop) was required for targeting to proliferated peroxisomes in *Saccharomyces cerevisiae*. We now report that the analogous TMD4R, which cannot target to proliferated peroxisomes, targets at least as well, or much better (depending on strain and growth conditions) in cells containing only basal (i.e., nonproliferated) peroxisomes. These data suggest differences in the targeting pathway among peroxisome populations. Pex8p, a peripheral protein facing the matrix, contains a typical carboxy terminal targeting sequence (PTS1) that has been shown to be nonessential for targeting, indicating the existence of a second targeting domain (not yet defined in *S. cerevisiae*); thus, its function was unknown. We show that targeting to basal peroxisomes, but not to proliferated peroxisomes, is more efficient with the PTS1 than without it. Our results indicate that multiple targeting signals within peroxisomal proteins extend coverage among heterogeneous populations of peroxisomes and increase efficiency of targeting in some metabolic states.

INTRODUCTION

All peroxisomal proteins begin their life in the cytoplasm and must be recognized by receptors to be successfully imported into the organelle. The peroxisome has two principal compartments, the matrix, which itself can comprise several subcompartments, and a unit membrane surrounding it (Masters and Crane, 1995; Usuda *et al.*, 1995). The targeting of matrix proteins from cytoplasm to peroxisomes is becoming well understood (Subramani *et al.*, 2000; Holroyd and Erdmann, 2001; Purdue and Lazarow, 2001; Gould and Collins, 2002): most matrix proteins contain a short targeting signal at their carboxy terminus (termed PTS1) that recruits a shuttling cytosolic receptor, and the resulting complex then binds to a docking element on the peroxisomal surface. Translocation of cargo and receptor recycling then ensues. Several players in these processes have been identified, although the precise mechanism of cargo translocation and receptor recycling remains obscure.

The trafficking of membrane proteins to peroxisomes is less well understood. Although there is ample documentation for a cytosolic to peroxisome pathway, a subset of peroxisomal membrane proteins (PMPs) probably arrive at

peroxisomes from the exocytic pathway (Titorenko *et al.*, 2000; Geuze *et al.*, 2003). Because they do not have secretion signal sequences are not glycosylated (with two exceptions in the yeast *Yarrowia lipolytica* [Titorenko and Rachubinski, 1998]), and do not require Sec61p for integration (South *et al.*, 2001), they seem not to traverse the membrane of the rough endoplasmic reticulum (ER), or at least not in a classical way. The recent evidence for continuities between membranes of the ER and peroxisomal tubules (reminiscent of micrographs published decades ago [Novkoff and Novikoff, 1982] offers strong support for the ER as the source of the peroxisomal membrane [Geuze *et al.*, 2003].

Many studies have been directed toward identifying targeting signals on PMPs (Snyder *et al.*, 2000; Jones *et al.*, 2001; Purdue and Lazarow, 2001). Although no conservation in primary sequence among them has been found, a few general principles that govern most peroxisomal membrane targeting signals (mPTSs) have emerged. Nearly all PMP fragments that can target contain at least one transmembrane domain (TMD) as well as a matrix-oriented segment that contains a cluster of basic amino acids, or at least has a net basic charge, close to the membrane span. The TMD itself probably contains targeting information, although low-efficiency targeting can occur with a hydrophobic span of random sequence (Mullen and Trelease, 2000) or with no span at all (Dyer *et al.*, 1996). The TMD and matrix basic cluster may be all that is required for type I (i.e., amino terminus facing the matrix) single spanning PMPs; for type II (amino terminus out) proteins or those that span the membrane multiple times, cytoplasmically oriented fragments have been found to

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Abbreviations used: GFP, green fluorescent protein; PMP, peroxisomal membrane protein; PTS, peroxisomal targeting signal; TMD, transmembrane domain.

also be important for targeting (Pause *et al.*, 2000; Jones *et al.*, 2001; Wang *et al.*, 2001).

Interestingly, several peroxisomal proteins have been shown to have two discrete targeting signals. One of these is human Pmp34. Pmp34, as well as Pmp47, its homolog in the yeast *Candida boidinii*, are members of the solute carrier family and translocate adenine nucleotides. This class of transporters contains six TMDs and is thought to have originated from an ancient gene triplication (Jank *et al.*, 1993). We have studied the localization of Pmp47 in some detail (McCammon *et al.*, 1990a, 1994; Goodman *et al.*, 1992; Dyer *et al.*, 1996). Although a basic cluster within a matrix loop was very important for targeting (Dyer *et al.*, 1996), efficient localization required TMD2, a fragment of the adjacent cytoplasmic loop, and the matrix-oriented basic cluster (Wang *et al.*, 2001). Although we found that these sequences were essential for targeting in our system (*S. cerevisiae* cells expressing Pmp47-GFP fusions and grown in oleic acid to proliferate their peroxisomes), Jones *et al.* (2001) reported that Pmp34 contained distinct redundant signals.

Another protein with two targeting signals is Pex8p, a matrix-oriented peripheral membrane protein that is essential for protein import perhaps by bridging two protein complexes that serve different roles in protein translocation (Agne *et al.*, 2003). Pex8p has a carboxy terminal PTS1 that can be removed without affecting targeting. The remaining targeting signal is either another identifiable matrix targeting motif, PTS2 (Waterham *et al.*, 1994), or is as yet undefined (Smith *et al.*, 1997; Rehling *et al.*, 2000).

We now report that Pmp47 has a second and distinct targeting signal, consistent with its human orthologue Pmp34. Moreover, we demonstrate that the two signals within Pmp47 and Pex8 provide additional targeting function for the host proteins. For Pmp47, the two targeting signals show preference in cells containing basal organelles or proliferated organelles, implying that these two populations of peroxisomes differ in chaperone or targeting elements. For Pex8p, the PTS1 signal enhances the targeting to basal peroxisomes but does not affect targeting to proliferated organelles. Considering the great plasticity of peroxisomes (Goodman *et al.*, 1990; Masters and Crane, 1995), our results suggest that multiple targeting signals on peroxisomal proteins are not redundant, as has been supposed, but ensure efficient targeting to peroxisomes under different metabolic conditions.

MATERIALS AND METHODS

Strains and Culturing Conditions

S. cerevisiae strain MMYO11 α (McCammon *et al.*, 1990a) was used as the parental strain for all experiments. Pmp47-related proteins were expressed on plasmids (details are described below). Strains expressing hemagglutinin (HA)-tagged Pex8p and Pex14p contain gene replacements in which the DNA encoding the sequence FYPYDVPDYAGYPYDVPDY (two copies of the HA epitope) is fused immediately before the stop codon, leaving all upstream sequences intact. The ρ^0 strain was generated from MMYO11 α by incubation in ethidium bromide (Fox *et al.*, 1991).

Several media were used for yeast liquid culture: Glucose medium (SD) contains 2% glucose and 0.67% yeast nitrogen base without amino acids (YNB-WO) (Difco, Detroit, MI); glycerol medium (SGd) contains 3% glycerol, 0.1% glucose, and 0.67% YNB-WO; oleate medium (HMYOT) is semisynthetic medium (van Dijken *et al.*, 1976) supplemented with 0.1% (vol/vol) oleic acid and 0.2% (vol/vol) Tween 40; and raffinose medium contains 2% (wt/vol) raffinose and 0.67% YNB-WO. YPD contains 10% yeast extract, 20% peptone, and 20% dextrose. All media were supplemented with the appropriate amino acids and bases to complement auxotrophic markers. Induction of peroxisomal proliferation by oleate was performed as described previously (McCammon *et al.*, 1990a), except that 0.2% (vol/vol) Tween 40 was added to the oleate medium for the Pmp47 studies (Elgersma *et al.*, 1997). Adenine at 0.8 mg/ml was added into all cultures to reduce vacuolar autofluorescence (Dorfman, 1969; Roeder and Shaw, 1996).

Recombinant DNA Procedures and Reagents

The construction of all Pmp47-containing plasmids and the DsRed-AKL plasmid were described previously (Wang *et al.*, 2001). They are low copy number plasmids and expression is driven by the PGK promoter. Yeast strains were transformed by the lithium acetate method (Ito *et al.*, 1983).

For the Pex-HA knock-in strains, a cassette was assembled within Bluescript pKS⁻ (Stratagene, La Jolla, CA) consisting of an HAX2-stop sequence (TTTATCCATATGATGTTCCAGATTATGCTGGTTACCTTACGATGTTCTGATTACGACAGTTAA) inserted between the *SacII* and *NotI* sites of the plasmid, the 3'-untranslated region of the PGK gene between the *BamHI* and *HindIII* sites, and *HIS3* between *SalI* and *ApaI*. The cassette (HA-term-*HIS3*) was amplified by polymerase chain reaction (PCR) by using oligonucleotides that ended with 50 bases either upstream or downstream of the PEX stop codon, so that the cassette sequences would be inserted at the end of the PEX open reading frames. The PCR products were introduced into yeast, histidine prototrophs were selected, and they were screened for correct insertion by PCR of genomic DNAs. On average, one in six his⁺ colonies contained the proper insertion.

Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). Isolation of DNA fragments and plasmids was carried out using the QIAEXII gel extraction kit and QIAprep spin miniprep kit from QIAGEN (Valencia, CA). PCR was performed with a PTC-100 programmable thermal controller from MJ Research (Watertown, MA) by using Pfu or *Taq* DNA polymerase. Standard recombinant DNA techniques were used (Sambrook *et al.*, 1989).

Fluorescent and Confocal Microscopy

Fluorescence in most living cells was visualized with an Axiovert 100M fluorescent microscope (Carl Zeiss, Thornwood, NY) with Openlab imaging software (Improvision, Lexington, MA). Green fluorescent protein (GFP) was visualized with fluorescein isothiocyanate filter set, and DsRed (BD Biosciences Clontech, Palo Alto, CA) and MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) were observed with the Texas Red filter set. Some living cells and all fixed cells were observed with an Axioplan fluorescent microscope (Carl Zeiss) with a MRC 600 confocal imaging system (Bio-Rad, Hercules, CA). For confocal imaging, GFP and DsRed were excited at 488 and 568 nm, respectively. The fixed cells were converted to spheroplasts and permeabilized (Pringle *et al.*, 1991) before observation to minimize background autofluorescence.

Quantification of Peroxisomal Localization

The extent of punctate fluorescence was determined as described previously (Wang *et al.*, 2001). Briefly, living cells were harvested and spread in a single layer on microscope slides and observed by fluorescent or confocal microscopy. Both fluorescent and transmitted light images were recorded at the equatorial plane of the cells. Five randomly chosen $24 \times 33\text{-}\mu\text{m}$ areas were examined for each culture. For each area, the percentage of the cells showing punctate fluorescence was determined. A mean and SE were determined for each culture from a typical experiment.

Cell Fractionation and Biochemical Analyses

Peroxisome localization was confirmed for Pmp47-GFP fusion proteins by organelle fractionation by differential centrifugation and Nycodenz gradient separation as described previously (McNew and Goodman, 1994). For localization of Pex8p-related proteins, this procedure was modified in that the supernatant from the $25,000 \times g$ (13.5K) spin was centrifuged at $277,000 \times g$ (55,000 rpm for 90 min at 2°C in a Beckman 70.1Ti rotor) to generate a high speed (55K) supernatant and pellet. Proteins in the supernatant were concentrated by precipitation with cold 10% trichloroacetic acid. Equal percentages of pellets and supernatants were subjected to SDS-PAGE (Laemmli, 1970) and immunoblotting (Towbin *et al.*, 1979). The immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Biosciences UK, Buckinghamshire, Little Chalfont, England). The relative amounts of proteins in different gradient fractions were quantified by densitometry analysis using NIH Image (version 1.60).

Sources for Antibodies

Antibodies used in this study were mouse anti-HA antibodies from Roche Diagnostics (Indianapolis, IN); anti-GFP monoclonal antibody from Chemicon International (Temecula, CA); anti-glucose 6-phosphate dehydrogenase from Sigma-Aldrich (St. Louis, MO); anti-Pex11p polyclonal antibodies (Marshall *et al.*, 1995); and goat anti-mouse Ig (G+L)-horseradish peroxidase and goat anti-rabbit Ig-horseradish peroxidase from Biosource International (Camarillo, CA).

Figure 1. Diagrams of Pmp47- and Pex8p-derived proteins used in this study. (A) Pmp47 and Pmp47 deletion-GFP fusion proteins. White rectangles show TMDs with their limiting residues. The topology of Pmp47 is shown with C denoting cytosolic side and M, matrix side. The indicated topology of the deletion-fusion proteins is inferred from full-length Pmp47. (B) Tagged Pex8p shown as a peripheral membrane protein. Two copies of the HA epitope tag are indicated by a single rectangle. Asterisks denote the native PTS1, masked by the HA tag.

RESULTS

A Pmp47 Fragment Containing TMD4 Targets to Unproliferated Peroxisomes but Not to Oleate-induced Organelles

The topology for wild-type Pmp47 and diagrams of the GFP fusion proteins used in this study are shown in Figure 1A. The “minimal construct” used in our previous study is termed 70-267(Δ); it is comprised of three fused segments: residues 70–110, consisting of the distal segment of the first cytoplasmic loop and TMD2 (in combination termed the TMD2 region, or TMD2R); residues 225–267, corresponding to matrix loop 2, which contains a basic cluster important for targeting, and adjacent TMD5; and GFP (Wang *et al.*, 2001). Although the first matrix loop, also basic, could substitute for the second matrix loop, the TMD2R was essential for peroxisomal targeting. TMD5 could be deleted without eliminating the targeting of GFP fusions, but its presence substantially increased targeting efficiency. It is not clear whether its principal effect is in inserting or stabilizing TMD2 in the membrane (TMD2 is not very hydrophobic), providing additional targeting information, or simply making it unnecessary to translocate GFP across the membrane.

In *S. cerevisiae* and several other fungi, growth in medium containing fatty acids as the sole carbon source causes a major proliferation of peroxisomes, increasing peroxisomal size and number, to accommodate the β -oxidation pathway (Veenhuis *et al.*, 1987). For this reason, oleate-cultured yeast

is a standard system for peroxisomal studies, and the one that we previously used to elucidate the mPTS of Pmp47. In this system, the peroxisomal marker GFP-AKL, 1–267 (near full-length Pmp47 fused to GFP), and 70-267(Δ) yielded punctate patterns (Figure 2A, first column of paired images); the Pmp47-derived GFP fusions colocalize completely with peroxisomal markers (Wang *et al.*, 2001). While testing the importance of the TMD2 region, we swapped it for the analogous TMD4 and its upstream loop fragment (the TMD4 region, TMD4R), generating the GFP fusion 176–267. Unlike 70-267(Δ), 176–267 yielded diffuse staining at a low level, indicating that it was unable to target to peroxisomes in this system (Wang *et al.*, 2001).

A few tiny peroxisomes exist in cells grown in glucose medium. The organelles are somewhat larger and more abundant in glycerol medium, under which condition the absence of glucose causes several peroxisomal matrix enzymes to be derepressed (Veenhuis *et al.*, 1987; McCammon *et al.*, 1990b). However, Pex11p, a key peroxin that causes peroxisomal proliferation, remains at low levels in glucose and glycerol and thus a large increase in peroxisomal number is avoided (Marshall *et al.*, 1995). In the course of pre-culturing cells for peroxisome experiments, we inspected the targeting of the Pmp47 fusion proteins to peroxisomes in cells grown in glucose and glycerol medium (Figure 2A, second and third columns of paired images). Both 1–267 and 70-267(Δ) partially targeted to punctate organelles in these

Figure 2. Distinct targeting of 70-267(Δ) and 176-267 to peroxisomes induced to proliferate with oleic acid and basal organelles from glucose and glycerol cultures. Pairs of panels in this and subsequent figures represent fluorescent and transmitted light micrographs through the medial plane. For comparison, all cells were imaged at the same intensity in all panels, except for GFP-AKL, where the intensity setting was lowered to prevent oversaturation. (A) Fields of cells. The carbon source of the cultures is indicated at top. The large puncta for 1-267 represent aggregated peroxisomes, as shown previously (Wang *et al.*, 2001). Arrowheads indicate examples of mistargeting to nonpunctate organelles. Bar, 10 μ m. (B) Colocalization of Pmp47-GFP fusion products with the peroxisome marker DsRed-AKL in single cells. Bar, 2 μ m. In some images peroxisomes seem artifactually large due to bright peroxisomal fluorescence and oversaturation of the image. (C) Quantification of the percentage of the cells displaying a punctate pattern. Averages and SEs from five randomly selected fields of at least 50 cells each are shown.

cells (these were identified as peroxisomes, as shown by colocalization with DsRed-AKL, which targets to peroxisomes by virtue of its PTS1, in Figure 2B, and quantitation in Figure 2C), although some mistargeting was also seen in the glucose culture (Figure 2A, arrowheads). Mistargeting to mitochondria was common in these cultures (our unpublished data), probably the result of overexpression. Less mistargeting of 70-267(Δ) was evident in glycerol-cultured cells compared with cells growing in glucose, suggesting a limitation of a critical element in the targeting machinery in glucose-grown cells.

Surprisingly, peroxisomal targeting of 176-267 (containing TMD4R), which did not occur in oleate cultures, was clearly seen in both glucose and glycerol-grown cells (Figure 2, A and B). The targeting efficiency of 176-267 to basal peroxisomes (i.e., those from glucose and glycerol-cultured cells) was at least as good as that of 70-267(Δ), measured by counting cells with punctate patterns (Figure 2C).

The percentage of cells with punctate patterns in Figure 2C is somewhat underrepresented because a medial plane of optical sectioning was used; peroxisomes at both ends of the cells were not visible and therefore not counted.

These data indicate that the Pmp47 fragments containing the TMD2 and TMD4 regions target with different efficiencies to peroxisomes in cells repressed or induced for perox-

isomal proliferation. TMD4R targets well to basal peroxisomes but poorly to oleate-induced peroxisomes. In contrast, TMD2R targets best in cells grown in oleate medium.

Differential Targeting of TMD2R and TMD4R in Peroxisomes of ρ^0 Cells

Although our data suggest that TMD2R and TMD4R interact differently with the targeting machinery for basal and induced peroxisomes, cells growing in oleic acid undergo a major metabolic shift and change their intracellular morphology to accommodate the large flux of fatty acids. Several large lipid droplets that contain esterified fatty acids in flux with peroxisomes dominate the cytoplasm (Veenhuis *et al.*, 1987; Sorger and Daum, 2003). These large intracellular changes may alter the cytoplasmic milieu through which the Pmp47 fragments travel en route to peroxisomes. For example, the high concentration of fatty acids may alter the stability or conformation of the Pmp47 deletion proteins, thus altering targeting efficiency.

Cells in which mitochondrial DNA is eliminated (the ρ^0 state) have been shown to proliferate their peroxisomes when grown in raffinose, a result of intensified signaling between mitochondria and nuclei, known as the RTG response (Epstein *et al.*, 2001). This is beneficial because mito-

Figure 3. Distinct targeting of 70-267(Δ) and 176-267 to peroxisomal populations in ρ^0 cells. (A) Representative pairs of fluorescent and transmitted light images are shown. To visualize targeting in the two different cultures at equal intensity settings, fluorescent images of the raffinose are oversaturated; puncta are larger than the corresponding organelles in this case. (B) Single cells are shown, illustrating colocalization with DsRed-AKL. (C) Quantification of cells containing punctate fluorescent organelles.

chondria and peroxisomes share enzyme activities required for metabolism of nonfermentable carbon sources.

To test whether the different targeting behaviors of TMD2R and TMD4R were specific to effects of fatty acid flux or were dependent on the state of peroxisomal proliferation, we compared the targeting of the two minimal deletion proteins, 70-267(Δ) and 176-267, in ρ^0 cells growing in glucose or raffinose. The 1-267 construct, which contains both TMD2R and TMD4R, targeted to peroxisomes in both glucose and raffinose cultures, although the micrographs (Figure 3A) and quantitation results (Figure 3C) indicated more efficient targeting to the induced organelles. The TMD2R construct, 70-267(Δ), yielded clear targeting to the induced organelles but only weak targeting to the basal peroxisomes in glucose-grown cells. (The weaker targeting of 70-267(Δ) in glucose cultures of ρ^0 versus wild-type cells may reflect slower growth of the mutant cells, and thus slower targeting of peroxisomal constructs, leading to degradation of those with weaker signals. It may also reflect possible differences in peroxisomal gene expression, i.e., those for receptors, between the two strains in the basal state.) The converse was seen with the TMD4R construct, 176-267: significant targeting was clear in glucose cultures, but targeting to induced organelles was virtually absent in raffinose cultures. In all cases, the puncta seen represented peroxisomal targeting, as shown by colocalization with DsRed-AKL (Figure 3B).

Thus, comparing the two Pmp47 TMD targeting regions, TMD2R targets at least as well, or much better, to proliferated peroxisomes (depending on inducing pathway), whereas TMD4R targets principally to basal peroxisomes.

The Cytoplasmically Oriented Sequence before TMD4 Is Essential for Its Targeting

We have previously shown that the cytoplasmically oriented region upstream of TMD2 (residues 70-95) was essential for

targeting to peroxisomes (Wang *et al.*, 2001). To determine whether the analogous region upstream of TMD4 is essential for targeting to basal peroxisomes, we constructed 199-267, in which most of this region is eliminated. Similar to the results with TMD2-adjacent sequences, the region before TMD4 is essential for targeting to basal peroxisomes (Figure 4). Localization of 199-267 to organelles is apparent on all carbon sources (Figure 4A), but the pattern completely overlaps that of the mitochondrial marker MitoTracker in all media (Figure 4B). Thus, the regions upstream of TMD2 and TMD4 are essential parts of the induced and basal targeting elements, respectively. Switching these upstream regions between TMD2 and TMD4 within the minimal fusion proteins resulted in a loss of peroxisomal targeting, suggesting that these segments of proximal loops and TMDs comprise a unit targeting domain (Wang *et al.*, 2001; our unpublished data).

125-267 Yields Punctate Pattern in All Cultures but Only Targets to Basal Peroxisomes

The TMD4-containing construct 176-267 is difficult to discern on micrographs in cells induced for peroxisomal proliferation. Western blots and organelle fractionation revealed that the protein is present but widely distributed in the cell (our unpublished data). We found that a larger fragment of Pmp47 (termed 125-267), containing TMDs 3-5, conferred high stability of the molecule, and it was easily visible by GFP fluorescence on all carbon sources. 125-267 yielded a clear punctate pattern regardless of growth conditions (Figure 5). In cells growing in glucose, virtually all the puncta were peroxisomes (as expected because the construct contains TMD4R), whereas in glycerol there was some mistargeting, although the brightest spots colocalized with the peroxisome marker (Figure 5A). In contrast, colocalization with peroxisomes was very rare in oleate medium (Fig-

Figure 4. Cytoplasmic fragment preceding TMD4 is important for targeting. (A) Micrographs of cells expressing 176–267 (containing the fragment) and 199–267 (missing the fragment) in glucose, glycerol, and oleate media are shown. (B) Colocalization of 199–267, but not 176–267, with MitoTracker Red is shown.

ure 5B). This was also true for ρ^0 cells growing on raffinose (our unpublished data). The identity of the organelles to which 125–267 mistargets is not known. The behavior of 125–267 is consistent with the behavior of 176–267: TMD4R allows targeting to basal peroxisomes and the lack of TMD2R prevents targeting to induced organelles.

The PTS1 of Pex8p Improves Targeting to Basal Peroxisomes

The function of the PTS1 at the carboxy terminus of Pex8p, if any, is unknown because it can be removed while preserving localization and function in the import complex (Waterham *et al.*, 1994; Smith *et al.*, 1997). To confirm and extend this finding, we HA-epitope tagged the chromosomal copy of PEX8 so that two copies of the tag were inserted at the carboxy terminus of Pex8p. Addition of HA masks the PTS1, because there must be a free carboxy terminus to allow recognition by and binding to Pex5p, the PTS1 receptor (Gatto *et al.*, 2000). Cells with the gene replacement grew normally on oleic acid medium, indicating the protein was functioning normally (our unpublished data).

In cells growing on oleic acid, Pex8-HA was found almost exclusively in an organellar pellet (Figure 6A), and comigrated with peroxisomes on Nycodenz gradients (our unpublished data), consistent with the known behavior of PTS1-less Pex8p. The same was true for Pex14-HA, a control

that was generated similarly. In cells growing in rich glucose medium, however, only 56% (the average of several experiments) of Pex8-HA was found in this organellar pellet, whereas the distribution of Pex14-HA was unchanged. Much of the remaining protein pelleted at high speed, suggesting it has localized to small vesicles. The result indicates that Pex8p, without a functional PTS1, targets inefficiently to basal peroxisomes but completely to induced organelles.

To confirm that the Pex8p PTS1 was important for targeting to basal peroxisomes, we compared the localization of Pex8-HA with Pex8-HA-SKL (again replacing the normal gene). This addition improved the targeting of Pex8p-HA to peroxisomes in every experiment performed, an average of 20%. Because the context for PTS1 can greatly affect, and even destroy, the efficacy of the targeting motif (Kragler *et al.*, 1993; Neuberger *et al.*, 2003), the PTS1 may function much better in the context of the wild-type protein than fused to the HA sequence. Nevertheless, we can conclude that the PTS1 within Pex8p assists in the targeting of the protein to basal peroxisomes.

DISCUSSION

In this report, we have demonstrated that two peroxisomal targeting modules within Pmp47 target differently in cells containing basal versus proliferated peroxisomes and that the

Figure 5. 125–267, which contains TMD4R but lacks TMD2R, only localizes to peroxisomes in uninduced cells. Colocalization of Pmp47-GFP fusion proteins and DsRed-AKL is shown. (A) Representative single cells from glucose and glycerol cultures. (B) Representative cells from oleate cultures.

Figure 6. PTS1 of Pex8p increases targeting efficiency to basal peroxisomes. Strains in which the chromosomal copy of *PEX8* or *PEX14* was replaced with one containing sequences for HA or HA-SKL (as indicated) were cultured in oleate medium or YPD and then subjected to organelle fractionation. (A) Immunoblots using anti-HA, or G6PDH, against an equal percentage of fractions are shown. The distribution of glucose-6-phosphate dehydrogenase among fractions was similar in all strains; only one is shown. Asterisks denote a comigrating band present in equal intensity in the 55K supernatants of all strains, such that the contribution of Pex8p-HA proteins to this band is minimal. (B) Percentage of Pex8-HA or Pex8-HA-SKL in the 13.5K peroxisomal fraction (compared with the sum in 13.5 and 55K pellets) is shown, based on densitometry. The data represent five separate experiments with means + SE shown. In every experiment, the addition of SKL increased targeting efficiency.

PTS1 of Pex8p increases the efficiency of targeting to basal, but not proliferated peroxisomes. Both are cases where two targeting signals function in a nonredundant way.

Pmp47: Signals for Different Subpopulations of Peroxisomes?

Gould and coworkers reported the presence of two independent targeting signals within human Pmp34 (Jones *et al.*, 2001), and we have now confirmed this finding for the *C. boidinii* homologue Pmp47. The two fragments within Pmp34 that target well contain TMDs1–3 or TMD6, consistent with a targeting role for the even-numbered TMDs. Because these TMDs are homologous (as a result of the presumed ancient gene triplication; Jank *et al.*, 1993), we might expect that TMDs 2, 4, and 6 all contain targeting activities. Such activity within TMD4 of Pmp34, or TMD6 of Pmp47, has not yet been tested. The targeting fragments of Pmp34 also contain cytoplasmic loop sequence and matrix-oriented sequence with net positive charge, similar to Pmp47. Also, similar to our results for TMD2R and TMD4R (Wang *et al.*, 2001; our unpublished data), targeting is gradually lost as more of the cytoplasmic loop sequence preceded-

ing TMD6 of Pmp34 is deleted. These cytoplasmic loop fragments have amphipathic character and function with specific TMDs (at least for our Pmp47 fusion proteins), suggesting that the loop-TMD is a unit that is recognized in targeting or integration.

Jones *et al.* (2001) suggested that integral membrane proteins contain multiple targeting sequences because they represent domains of interaction with Pex19p, a good candidate for a shuttling mPTS receptor. According to this model, Pex19p shields hydrophobic sequences from solvent, thus protecting them from aggregation. Because Pex19p may be the receptor, such Pex19-binding motifs would function as targeting elements. This is a reasonable idea, but it cannot explain the behavior of the Pmp47-targeting modules that preferentially interact with the peroxisomal targeting machinery of basal versus proliferated organelles. Our data are also inconsistent with the notion that the two identified Pmp47 TMDR modules represent a weak and strong targeting signal and that competition occurs between them, because each is preferred under different conditions.

Rather, we hypothesize that there are differences in targeting or membrane integration factors within cells containing basal or proliferated peroxisomes. One possibility is that the difference exists in the cytosol of these cells, that Pmp47 folds differently in the cytosol under varying metabolic states, exposing alternative mPTS motives. This might occur if the protein binds to different critical chaperones or co-chaperones during its folding pathway depending on growth substrate, creating different metastable folding states. In this regard, data indicate that among members of the cytosolic Hsp70 family, the transcription of *Ssa1p* is up-regulated by oleate, whereas *Ssa3p* is repressed by this lipid (Kal *et al.*, 1999). However, these changes were not seen in conversion of cells to ρ^0 , suggesting that they are not responsible for the Pmp47 TMD2R/TMD4R preference, which we see in both peroxisomal induction systems (Epstein *et al.*, 2001). Similarly, Pex19p, which could bind to cytosolic Pmp47 influencing its folding, is clearly induced by oleic acid (Gotte *et al.*, 1998), but it is lower in raffinose-grown than uninduced ρ^0 cells (Epstein *et al.*, 2001). Although we could find no candidate chaperones to differentially regulate folding of Pmp47 in a way consistent with our results, the existence of such factors remains a possibility.

A second way in which redundant signals may function differentially evokes differences in the targeting or assembly machinery on the surface of subpopulations of peroxisomes, or differences in the lipid composition of the peroxisomal membrane. We expect that the lipid composition would be much different in membranes of peroxisomes whose major activity is the metabolism of fatty acids than in organelles from cells growing on nonlipid substrates. There may be specific interactions of the amphipathic loop sequences with these bilayers, although the composition of the peroxisomal membrane bilayer may be very different in oleate- and raffinose-cultured cells, both of which prefer the TMD2R-targeting motif.

We think it likely, however, that the efficacy of peroxisomal targeting motifs is influenced by proteinaceous factors specific for subpopulations of organelles. Peroxisomes in yeast are highly plastic, varying their protein content greatly depending on growth substrate. In *C. boidinii*, for example, growth on glucose, oleate, methanol, and D-alanine as sole carbon sources leads to peroxisomes with completely different constellations of matrix proteins (Goodman *et al.*, 1990). Although the most important peroxins that catalyze matrix protein import have probably been identified, much less is known about the targeting and assembly of peroxisomal

membrane proteins. The lack of consensus thus far in what constitutes an mPTS targeting sequence may reflect heterogeneity in those sequences and in the corresponding receptors or membrane assembly factors yet-to-be-discovered.

Peroxisomal heterogeneity is found not only in yeast. In plants, the glyoxysomes in seeds, which are essential for conversion of storage lipid into carbohydrate, develop into peroxisomes, organelles that are functionally very distinct from glyoxysomes, during germination (Hayashi *et al.*, 2000). Although regulation of transcription of peroxisomal matrix proteins may well explain the heterogeneity in yeast or plant peroxisomes in cells under different metabolic or developmental programs, animal cells have been shown to simultaneously contain peroxisomal subpopulations with different matrix components. A subset of peroxisomes in animal cells lack catalase (Klucis *et al.*, 1991) or D-amino acid oxidase (Angermüller and Fahimi, 1988). It is not known how differential targeting of proteins to these separate populations of organelles could occur; subcompartments cannot easily be explained by regulation of transcription. It seems reasonable that there may be at least quantitative differences in peroxin complexes among populations of peroxisomes that vary in the capacity to import or assemble specific cargo. Therefore, we expect that there are peroxisomal membrane proteins that preferentially target to basal or proliferated peroxisomes.

Pex8: Signal Reinforcement

As previously reported, Pex8p can target without its PTS1, and we find virtually all of the protein that has its PTS1 masked by the epitope tag is in peroxisomes in oleate-cultured cells. However, the targeting efficiency drops to 56% in cells cultured in rich glucose-based medium.

The limiting factor for Pex8p-HA targeting under these conditions is not clear. Oleic acid does not increase the level of Pex5p, the receptor for both PTS1 and the uncharacterized Pex8p internal signal (Rehling *et al.*, 2000; Rottensteiner *et al.*, 2003). Thus, both signals cannot function in combination by reinforcing the binding to this receptor. However, both Pex13p and Pex14p, components of the docking complex, are higher in oleate than in glucose medium (Rottensteiner *et al.*, 2003). Thus, the limitation may be in docking of Pex5-cargo complexes to the membrane. According to the preimport hypothesis (Gould and Collins, 2002), large complexes of receptors and cargo are formed on the surface of peroxisomes before import. Perhaps the two signals within Pex8p aid in the cross-linking of Pex5p, attracting more docking units, which are more scarce on basal peroxisomes, leading to more efficient import.

Discrete targeting signals within peroxisomal proteins may function both in peroxisomal discrimination (as shown here for Pmp47) as well as signal reinforcement (as for Pex8p). In fact, the signals within Pmp47 seem to reinforce each other because large fragments containing TMDs 1–5 target to peroxisomes in oleate medium more efficiently than smaller ones (McCammon *et al.*, 1994; Wang *et al.*, 2001).

Other Peroxisomal Proteins with Two Known Targeting Signals

Besides Pmp47 and Pex8, five other proteins also have discrete peroxisomal targeting signals. Yeast catalase also contains two targeting signals: a carboxy-terminal noncanonical PTS1 sequence (SSNSKF), which is sufficient for targeting but dispensable, and a poorly defined signal contained in the first third of the protein (Kragler *et al.*, 1993). In contrast, human catalase contains only one signal, a C-terminal PTS1, KANL (Purdue and Lazarow, 1996). Yeast carnitine acetyl-

transferase also contains a C-terminal PTS1 that is dispensable for peroxisomal targeting (Elgersma *et al.*, 1995). Similar to these examples, Eci1p (enoyl-CoA isomerase) contains a PTS1 sequence that is expendable (Gurvitz *et al.*, 2001). In this case, however, the protein can enter as a complex with Dci1, by using its own targeting sequence, so it is likely that the redundant signal on Eci1p is actually a Dci1p binding domain (Yang *et al.*, 2001).

Among integral membrane proteins, human Pex13p has also been found to contain two discrete targeting signals (Fransen *et al.*, 2001; Jones *et al.*, 2001). Also, different sufficient sequences within Pmp70 identified in two laboratories suggest discrete signals for targeting (Imanaka *et al.*, 1996; Sacksteder *et al.*, 2000).

Many of these proteins are from mammalian sources. There are classes of peroxisomal-proliferating agents for rodent peroxisomes (Lake, 1995), and it is not clear whether the effects of such treatments are analogous to proliferation of yeast peroxisomes with respect to mPTS targeting. However, because the composition of peroxisomes are heterogeneous in different tissues and even within the same cells, such systems seem to be good candidates in which to explore variations in targeting pathways similar to those we have seen in yeast.

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