An Internal Region of the Peroxisomal Membrane Protein PMP47 Is Essential for Sorting to Peroxisomes

Mark T. McCammon, James A. McNew, Patricia J. Willy, and Joel M. Goodman

Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041

Abstract. Targeting sequences on peroxisomal membrane proteins have not yet been identified. We have attempted to find such a sequence within PMP47, a protein of the methylotrophic yeast, *Candida boidinii*. This protein of 423 amino acids shows sequence similarity with proteins in the family of mitochondrial carrier proteins. As such, it is predicted to have six membrane-spanning domains. Protease susceptibility experiments are consistent with a six-membrane-spanning model for PMP47, although the topology for the peroxisomal protein is inverted compared with the mitochondrial carrier proteins. PMP47 contains two potential peroxisomal targeting sequences (PTS1), an internal SKL (residues 320–322) and a carboxy terminal AKE (residues 421–423). Using a heterologous in vivo sorting system, we show that efficient sorting occurs in the absence of both sequences. Analysis of PMP47-dihydrofolate reductase (DHFR) fusion proteins revealed that amino acids 1–199 of PMP47, which contain the first three putative membrane spans, do not contain the necessary targeting information, whereas a fusion with amino acids 1–267, which contains five spans, is fully competent for sorting to peroxisomes. Similarly, a DHFR fusion construct containing residues 268–423 did not target to peroxisomes while residues 203–420 appeared to sort to that organelle, albeit at lower efficiency than the 1–267 construct. However, DHFR constructs containing only amino acids 185–267 or 203–267 of PMP47 were not found to be associated with peroxisomes. We conclude that amino acids 199–267 are necessary for peroxisomal targeting, although additional sequences may be required for efficient sorting to, or retention by, the organelles.

PEROXISOMES are found in virtually all eukaryotic organisms. These organelles, composed of a matrix and surrounding unit membrane, perform reactions in diverse metabolic pathways including the oxidative degradation of purines and fatty acids and the synthesis of bile acids and ether lipids (Tolbert, 1981; van den Bosch et al., 1992). The organelles are highly specialized in certain tissues and organisms. The metabolic importance of peroxisomes in humans has been better appreciated in recent months with the cloning of the gene, encoding a new member of the P-glycoprotein family, that is responsible for X-linked adrenoleukodystrophy (Mosser et al., 1993), a catastrophic peroxisomal disease. Another serious disease, familial amyotrophic lateral sclerosis, is now linked to a defect in Cu/Zn superoxide dismutase (Rosen et al., 1993), an enzyme that has been localized to peroxisomes (Keller et al., 1991). The role of peroxisomes in Zellweger syndrome has been appreciated for several years (Moser et al., 1989). Since the most severe peroxisomal disorders are caused by improper assembly of the organelle, mechanisms of protein targeting and assembly of this organelle have been the subject of increasingly intense research.

Peroxisomal matrix proteins are synthesized in the cytoplasm and are posttranslationally translocated directly into peroxisomes (Lazarow and Fujiki, 1985). Two distinct targeting sequences on different matrix proteins have been identified. Peroxisomal targeting sequence 1 (PTS1)[1] consists of the sequence SKL at the extreme carboxy terminus (Gould et al., 1988), although several substitutions are allowed (Gould et al., 1989; Swinkels et al., 1992; Hansen et al., 1992), and there are variations that are specific to species (Aitchison et al., 1991; Hansen et al., 1992). PTS2, a sequence of at least 11 amino acids, was identified on 3-ketoacyl-CoA thiolase and is contained on a cleavable amino-terminal extension (Swinkels et al., 1991). PTS2-like sequences have been noted for a few other peroxisomal proteins, including watermelon malate dehydrogenase and amine oxidase of *Hansenula polymorpha* (Faber et al., 1993; Gietl, 1990). There is also evidence for important targeting information at internal sites that may be distinct from PTS1

Address all correspondence to J. M. Goodman, Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235-9041.

M. T. McCammon's present address is Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205.

1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; PMP, peroxisomal membrane protein; PTS, peroxisomal targeting sequence.

and PTS2 (Kragler et al., 1993; Small et al., 1988). Receptors for these PTS elements are assumed to exist; the PTS1 receptor may be the product of the *PAS8* gene in the yeast *Pichia pastoris* (McCollum et al., 1993).

Much less information is available concerning the targeting of peroxisomal membrane proteins. This class of proteins is synthesized on nonmembrane-bound polysomes (Fujiki et al., 1984; Koester et al., 1986; Suzuki et al., 1987) and thus assumed to sort directly from the cytoplasm, similar to matrix proteins. To date the sequences of only a few peroxisomal membrane protein (PMPs) have been described, all from analysis of the isolated genes. Mammalian PMPs include PMP70 from rat (Kamijo et al., 1990) and human (Gärtner et al., 1992; Kamijo et al., 1992), PAF-1 from rat (Tsukamoto et al., 1991) and human (Shimozawa et al., 1992), and PMP22 from rat (Kaldi et al., 1993). The X-linked adrenoleukodystrophy gene may also encode a PMP (Mosser et al., 1993). Yeast PMPs include PAS3 from *Saccharomyces cerevisiae* (Höhfeld et al., 1991) and PMP47 from the methylotrophic yeast *Candida boidinii* (McCammon et al., 1990*a*).

We have been studying the structure and function of peroxisomal membranes in yeast. PMP47 is a protein in *C. boidinii* that is regulated by diverse peroxisomal proliferators (Sulter et al., 1990; Veenhuis and Goodman, 1990). It has significant homology to proteins in the mitochondrial carrier family of transporters (Kuan and Saier, 1993; Jank et al., 1993). The protein consists of 423 amino acids and contains two strong candidates for membrane spanning domains and several weaker candidates as well (McCammon et al., 1990*a*). Its homology with mitochondrial transporters strongly suggests that it crosses the membrane six times. PMP47 contains the peroxisomal targeting sequence (PTS) tripeptide -SKL- at amino acids 320–322; its carboxy terminal sequences is -AKE, which is close to the accepted PTS1 signal of -AKL (Gould et al., 1989). We have previously shown that PMP47 sorts normally when expressed in *S. cerevisiae* under conditions of peroxisomal induction (McCammon et al., 1990*a*). We now report an attempt to define a peroxisomal membrane targeting signal. We find that a fusion protein containing amino acids 1–267 of PMP47 targets to peroxisomes, whereas one with amino acids 1–199 does not and remains in the cytoplasm or missorts to the nucleus. A fusion containing amino acids 202–420 also appears to sort at low efficiency. However, amino acids 199–267 may not be sufficient; surrounding sequences may be important for sorting or retention of PMP47.

Materials and Methods

Cell Strains and Culture Conditions

C. boidinii strain ATCC 32195 was cultured as previously described (Goodman et al., 1990). *S. cerevisiae* strain MMYO11 (*MATα* ade2-1 his3-11,15 leu2-3,112 trp1-1 can1-100 Ole$^+$; McCammon et al., 1990*b*) was used throughout and cultured as previously described (McCammon et al., 1990*a*), except as noted. Transformants (Ito et al., 1983) were selected and maintained on 0.7% Yeast Nitrogen Base (GIBCO BRL, Gaithersburg, MD) containing 2% glucose and supplemented with amino acids and bases as needed (Sherman 1991). Plasmid shuttle vectors were derivatives of pRS314(CEN4 ARSH6 TRP1) or pRS316 (CEN4 ARSH4 URA3) as described (Sikorski and Hieter, 1989). *E. coli* strains used were TG1 (supE hsdΔ5 thi Δ(lac-proAB) F′ [traD36 proAB$^+$ lacI^q lacZΔM154]) and CJ236 (dutl ungl thi-1 relA1/pCJ105(cam^{r}F′).

Expression and Sorting

To measure expression of PMP47 mutants, PMP47-DHFR, and DHFR-PMP47 hybrids, transformants were precultured 1.5 d at 30°C in 0.7% Yeast Nitrogen Base, 3% glycerol, 0.1% glucose, and the appropriate amino acid supplements, and then "boosted" with 10X YP (10% yeast extract, 20% peptone) to a 1× concentration for 4 h. Cells were harvested by centrifugation and resuspended to an OD_{600} of 1.0 in a semisynthetic medium containing 0.1% oleic acid (Aldrich Chemical Co., Milwaukee, WI). Cultures were harvested after 22–26 h. When present, galactose was added to a concentration of 0.1-0.2% for the final 4 h before harvesting.

Peroxisomes were prepared from *S. cerevisiae* essentially as described (Goodman et al., 1986; McCammon et al., 1990*a*) except for the data shown in Fig. 7. Cells from a two-liter culture grown in semisynthetic oleate (~8,000–10,000 OD_{600} units) were converted to spheroplasts and osmotically lysed (McCammon et al., 1990*b*). A 22,000 g_{max} pellet consisting primarily of mitochondria and peroxisomes was isolated and resuspended at a concentration of 5–10 mg/ml protein in 1 M sorbitol, 5 mM 4-morpholinoethane sulfonic acid-NaOH, pH 5.5, 1 mM phenylmethylsulfonyl fluoride. For fractionation on sucrose gradients, the resuspended pellet (2.5 ml) was layered on top of a 32-ml discontinuous gradient (3.0 ml 35% sucrose, 6.5 ml of 40, 43, 46, and 50% sucrose, and 3.5 ml of 60% sucrose, all wt/wt) and centrifuged at 4°C for 6 h at 27,000 rpm in a Beckman SW28 rotor (100,000 g_{ave}) (Beckman Instruments, Inc., Fullerton, CA). The gradient was fractionated by hand from the top such that each sucrose interface was within one fraction. Proteins were precipitated from aliquots (0.5–2% of pellet and gradient fractions) by the addition of trichloroacetic acid to a final concentration of 10%. Precipitates were washed twice with cold acetone and resuspended in 1% SDS, 0.1 M NaOH before adding an equal volume of 2× Laemmli sample buffer (Laemmli, 1970). For Nycodenz fractionation (see Fig. 7), 200 μl of a resuspended 25,000 *g* pellet was applied to a 15–45% continuous Nycodenz gradient containing 0.25 M sorbitol and 5 mM 4-morpholinoethane sulfonic acid-NaOH, pH 5.5, and centrifuged for 55 min at 108,000 g_{ave} in a Beckman VTi65 rotor. Gradients were fractionated from the bottom into eleven 425-μl fractions.

Proteins were separated by electrophoresis on 9% polyacrylamide gels. After electrophoresis the gels were stained with Coomassie brilliant blue or transferred to nitrocellulose and subjected to immunoblotting (Towbin et al., 1979). Antibodies used were a monoclonal antibody against PMP47 (IVA7; Goodman et al., 1986), or rabbit antisera against DHFR (Rb 3519; Dr. F. Huennekens, Scripps Research Institute, La Jolla, CA), acyl CoA oxidase (McNew et al., 1993), or porin (G. Schatz, Biozentrum, Basil). Immunodetection was performed via commercial luminescence procedures (ECL; Amersham Corp., Arlington Heights, IL) or via 4-chloro-1-napthol oxidation (Fig. 2 *B* only).

Mutagenesis

Oligonucleotide-directed mutagenesis and mutant enrichment were performed by the method of Kunkel (Kunkel et al., 1987). Mutagenesis was performed on two M13mp18 or M13mp19 phage constructs containing fragments of the *C. boidinii PMP47* gene: EcoRI to HindIII, and XbaI to PstI (Fig. 1 *B*; McCammon et al., 1990*a*). Single stranded templates were prepared from strain CJ236 as described (Kunkel et al., 1987). After mutagenesis several plaques were purified for analysis. Most of the mutations resulted in the insertion of SalI restriction sites at specific locations within the *PMP47* and dihydrofolate reductase (*DHFR*) genes, and plaques were screened by restriction digests of the replicative form of the phage for those sites. Mutations were confirmed by sequencing the single-stranded template. One strand of the entire fragment to be reconstructed back into a yeast–*E. coli* shuttle plasmid was sequenced. Once the correct sequence was confirmed, the mutation was reconstructed from replicative form DNA into the mutant and/or fusion construct. Deletions of restriction fragments, Klenow fill-ins, etc. were performed on phage replicative forms or plasmid DNA using standard procedures (Sambrook et al., 1989). These constructs were confirmed by single-stranded or double stranded DNA sequencing, respectively.

Constructions

Table I lists the amino acid sequences at gene fusion junctions and deletion sites.

Expression Vector for Dihydrofolate Reductase. An NheI restriction site was inserted at codons 2–3 of *PMP47* by mutagenesis. This was ligated to the XbaI site upstream of the *DHFR* gene from plasmid pMT2PC (gift

Table I. Mutations and Fusion Constructs

Construct	*Amino acid sequence*$_{codon}$
267DHFR	...TLRS$_{267}$SR$_3$PLN...
231DHFR	...KSFI$_{231}$GR$_3$PLN...
199DHFR	KNEG$_{199}$CR$_3$PLN...
88DHFR	...KLQG$_{88}$R$_3$PLN...
DHFR268	...VYEK$_{185}$SIR$_{268}$MHV...
DHFR376	...VYEK$_{185}$STG$_{376}$IIKS...
DHFR	M$_1$LEAIM$_1$VRPL...
47Δ200-267	...KNEG$_{199}$CPR$_{268}$MHV...
47Δ232-267	...KSFI$_{231}$R$_{268}$MHV...
235-267DHFR	MS$_2$RLK$_{235}$KRN...TLRS$_{267}$SR$_3$PLN...
Δ234DHFR	MS$_2$RLK$_{235}$KRN...VASS$_{420}$R$_3$PLN...
203-267DHFR	MS$_2$RQL$_{203}$FT...TLRS$_{267}$SR$_3$PLN...
Δ202DHFR	MS$_2$RQL$_{203}$FT...VASS$_{420}$R$_3$PLN...
185-267DHFR	MS$_2$RL$_{185}$NTI...TLRS$_{267}$SR$_3$PLN...
Δ184DHFR	MS$_2$RL$_{185}$NTI...VASS$_{420}$R$_3$PLN...
DHFR203Δ267-403	...VYEK$_{185}$QL$_{203}$FTG...SR$_{268}$A$_{404}$LSM...A$_{421}$KE
DHFR203	...VYEK$_{185}$QL$_{203}$FTG...

Bold text, PMP47 sequence; underlined text, residues inserted at fusion junction; normal text, DHFR residues; subscript, residue number at fusion junctions.

of R. Bassel-Duby, Univ. of Texas Southwestern Medical Center). This resulted in an expression construct consisting of the 5′ nontranslated DNA and start codon of PMP47, a four-codon linker region, and the start codon and remaining sequence of mouse DHFR (see DHFR construct in Table I).

Four intermediates (A–D) were generated in the preparation of further constructs. 420DHFR (Construct A): a SalI site, created at codons 419–420 of *PMP47* was ligated to a SalI site at codons 2–3 of *DHFR*, resulting in an in-frame fusion. DHFR47 (Construct B): a SalI site, created at codons 185–186 of *DHFR*, was ligated to a SalI site created at codons 1–3 of *PMP47*, resulting in an in-frame fusion. PMP47/200 (Construct C): a SalI site was created at codons 200–202 of *PMP47*, resulting in a three amino acid substitution (CRQ).

Introduction of SalI mutations. SalI restriction sites were inserted into *PMP47* at the following locations: codons 231–232 (for 231DHFR), 183–185 (construct D), 199–200 (for 199DHFR and construct C), 184–185 (for Δ184DHFR and construct D), 87–88 (for 88DHFR). Where noted these SalI sites were used to create in-frame gene fusions with *PMP47* at the 5′ end and *DHFR* at the 3′ end. The SalI mutation at codons 419–420 created a frame-shift that resulted in the A421 Tail mutation of Fig. 2.

Constructs Created through Ligations at SalI Sites. SalI restriction sites were inserted into *PMP47* at the following locations to create in-frame gene fusions with *DHFR* at the 5′ end and *PMP47* at the 3′ end: codons 2–3 (construct B) and codons 375–376 (DHFR376).

Constructs Created by AccI–AccI deletions. Deletions of DNA between the natural AccI site at codons 1–3 of *PMP47* and the AccI site within the SalI mutations were performed by sequential AccI digestion, Klenow-mediated fill-in, and blunt-end ligation. Construct C was used to create Δ202DHFR and 203-267DHFR; and construct D was used to create Δ184DHFR and 185–267DHFR; the construct 231DHFR was used to create Δ234DHFR and 235–267DHFR.

Constructs Created by XbaI–SalI Deletions. Deletion of codons 268–420 of *PMP47* within construct A to create 267DHFR was accomplished by digestion of construct A with XbaI and SalI, filling in the overhangs with Klenow, and blunt-end ligation; this deletion regenerated a SalI restriction site at the fusion junction. This deletion strategy was also used to generate construct 203–267DHFR from Δ202DHFR, 185–267DHFR from Δ184DHFR, and 235–267DHFR from Δ234DHFR. DHFR268 was generated by SalI and XbaI digestion of construct C followed by fill-in with Klenow and blunt-end ligation.

Constructions by HincII–XbaI Site Deletions. Deletion of codons 200–267 in the construct 199DHFR was accomplished by digestion with XbaI and HincII (the HincII site is within the SalI site created at codons 199–200), Klenow fill-in, and blunt-end ligation to create 47Δ200–267. The identical reactions in construct 231DHFR were performed to generate 47Δ232–267.

Construction of DHFR203. The SalI site at the 3′ end of DHFR (construct B) was ligated to the SalI site created at codons 199–200 of construct C, resulting in an out-of-frame gene fusion. These genes were made in-frame by digesting with SalI, deletion of single-strand overhangs with mung bean nuclease, and religation.

Construction of DHFR203Δ267–403. The XbaI–HindIII fragment from DHFR203 was deleted, the ends were filled in by Klenow and ligated.

GAL1 constructs were similar to those previously reported (McCammon et al., 1990*a*), the promoter was inserted at the upstream EcoRI or BclI restriction site of *PMP47*.

Immunofluorescence

Immunofluorescence was performed on transformants essentially as described in Azpiroz and Butow (Azpiroz and Butow, 1993). Briefly, transformants were precultured and cultured in semisynthetic oleate, as above. For constructs other than those in Fig. 2, galactose was added to 0.05–0.1% final concentration for the last 4–12 h as necessary. Cells were fixed by adding formaldehyde directly to the culture flask to a final concentration of 4% and incubated for 1 h at 30°C. The formaldehyde was then washed out of the culture with three washes of 40 mM potassium phosphate, pH 6.5, containing 0.5 mM $MgCl_2$, and once with the same buffer with 1.2 M sorbitol added (PS buffer). These cells were either used immediately or stored at 4°C for up to 2 d before further processing. Fixed cells were converted to spheroplasts using 40–50 μg of Zymolyase 20T (ICN Biomedicals, Inc., Costa Mesa, CA) per 1 OD_{600} unit of cells. Spheroplasts were washed two times in PS buffer and resuspended in the same buffer at a final concentration of 20–30 OD/ml. Aliquots (10 μl) were applied to polylysine-coated multiwell slides and permeabilized with 0.1% NP-40 in PS buffer for 15 min in a moist chamber. Wells were thoroughly washed with PS buffer and twice with PBS (0.8% NaCl, 0.02% KCl, 0.114% Na_2HPO_4, 0.02% KH_2PO_4, pH 7.3) containing 0.1% fatty acid free bovine serum albumin (PBS-BSA). Wells were incubated with freshly diluted antibodies in PBS-BSA for 1–1.5 h. The antibodies had been incubated overnight with 200 μg yeast cell lysate on nitrocellulose filters to remove antibodies against cell wall and other cross-reacting antigens. Before use all antibody solutions were microcentrifuged for 15 min at 4°C. Primary antibodies (IVA7 or anti-thiolase; J. Rothblatt, Dartmouth University) were thoroughly washed from the wells with multiple drops of PBS-BSA before addition of second antibodies conjugated to fluorescent dyes (fluorescein isothiocyanate for anti-rabbit sera, Texas red for anti-mouse sera). Second antibodies were from either Boehringer Mannheim Corp. (Indianapolis, IN), or Jackson ImmunoResearch Laboratories (West Grove, PA), and were similarly incubated overnight with cell lysate filters and spun as previously described. Wells were washed with PBS-BSA and PBS before staining with DAPI (1 μg/ml H_2O) for 5 min. Wells then were thoroughly washed with PBS and dried. Slides were mounted with *p*-phenylenediamine antioxidant as described (Pringle et al., 1991) and viewed with a Zeiss Axiomat fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY).

Protease Mapping

Yeast spheroplasts derived from a 300-ml oleate culture of *C. boidinii* were osmotically lysed as previously described (Goodman et al., 1986). Unlysed cells and debris were subsequently removed by centrifugation at 500 *g* to yield a lysate in 1 M sorbitol, 5 mM 4-morpholineethane sulfonic acid–NaOH, pH 5.5, at a protein concentration of ~1.5–2.0 mg/ml. For incubation with proteinase K, reactions consisted of 50 μl cell lysate (~100 μg protein), 2.65 μl 10% Triton X-100, and 2.5 μl proteinase K to yield the concentrations indicated in the legend of Fig. 1. Samples were incubated for 30 min at 0°C. Proteolysis was terminated by the addition of 2.5 μl 100 mM [4-(2-aminoethyl)-benzenesulfonylfluoride, HCl] (Calbiochem Corp., La Jolla, CA) and one volume of 2× Laemmli sample buffer immediately prior to boiling. Clostripain (Sigma Chemical Co., St. Louis, MO) was preactivated with dithiotheitol according to the manufacturer's instructions, and 2 μl of activated enzyme were incubated with 50 μl lysate and 2 μl 10% Triton X-100. The final concentration of DTT in these reactions was 1 mM to ensure continued enzyme activity. Final Triton X-100 concentrations in reactions were 0.4–0.5%. Reactions were stopped with 2× sample buffer as above. 10 microliters of all samples were subjected to SDS gel electrophoresis and immunoblotting with IVA7 monoclonal antibody. Detection was by ECL as described above.

Results

Topology of PMP47

PMP47 is an integral membrane protein. It is very tightly bound to the peroxisomal membrane (Goodman et al., 1986, 1990; McCammon et al., 1990*a*) and partitions in the detergent phase after extraction of membranes with Triton X-114 (Goodman et al., 1992). While clearly membrane bound, the topology of this protein has not been addressed experimentally. Recently it has been reported that PMP47 is a member of the mitochondrial carrier family (Kuan and Saier, 1993); currently it is the only member of this family that is a peroxisomal protein. These proteins have a conserved structure with six membrane spans, although many of these transmembrane domains are not readily predicted from hydropathy plots. We have adopted a six-membrane span structure for PMP47 and initiated studies to test this model. An osmotic lysate of spheroplasts from *C. boidinii* grown on oleic acid was used as a source of peroxisomes since the organelles from this preparation are of the highest structural integrity (Goodman et al., 1990). These samples were treated with proteinase K at various concentrations in the absence or presence of Triton X-100. Lysates were then subjected to SDS gel electrophoresis and immunoblotting with anti-PMP47 monoclonal antibody. This antibody binds to an epitope within the first 88 amino acids of the protein (see below). There was little digestion in the absence of exogenous protease under these conditions (Fig. 1 *A*, lanes *1* and *5*). In the presence of protease, PMP47 was degraded to two immunodetectable species with molecular masses of ~30 and 32 kD; at the highest concentration of protease the 30-kD species predominates (lanes *2–4*). Since the epitope is located at the amino terminus of PMP47, this indicates that cleavage was occurring closer to the carboxy than the amino terminus, probably in the large loop between putative membrane spans 5 and 6 (Fig. 1 *B*; Kuan and Saier, 1993). Similar digestion products were seen in the presence of detergent. However, there was a dramatic loss of antigenic signal at 10 μg/ml proteinase K (Fig. 1 *A*, lane *8*), indicating that the detergent is allowing access to sites within the first 88 amino acids of the molecule.

We found similar results with clostripain, a protease that cleaves at the carboxyl linkage of arginine residues. In the absence of detergent there was little digestion of PMP47 until 10 μg/ml, when a digestion product of ~32 kD is seen (Fig. 1 *A* land *12*). This may correspond to a product cleaved after the arginine at residue 285 (McCammon, et al., 1990*a*). In the presence of Triton X-100, a slightly lower band could be seen, perhaps as a result of cleavage at residues 266 or 268, sites immediately following span 5. All of these sites are in the same loop as that which was sensitive to proteinase K. In the presence of detergent with 10 μg/ml clostripain, only a small fraction of the antigen was detected (lane *16*). Since the only arginine within the epitope (i.e., the first 88 amino acids) is at position 4, this amino acid is probably part of it, and it is degraded by clostripain in the presence but not the absence of detergent. The disappearance of the epitope cannot be a result of cleavage beyond the extreme amino termi-

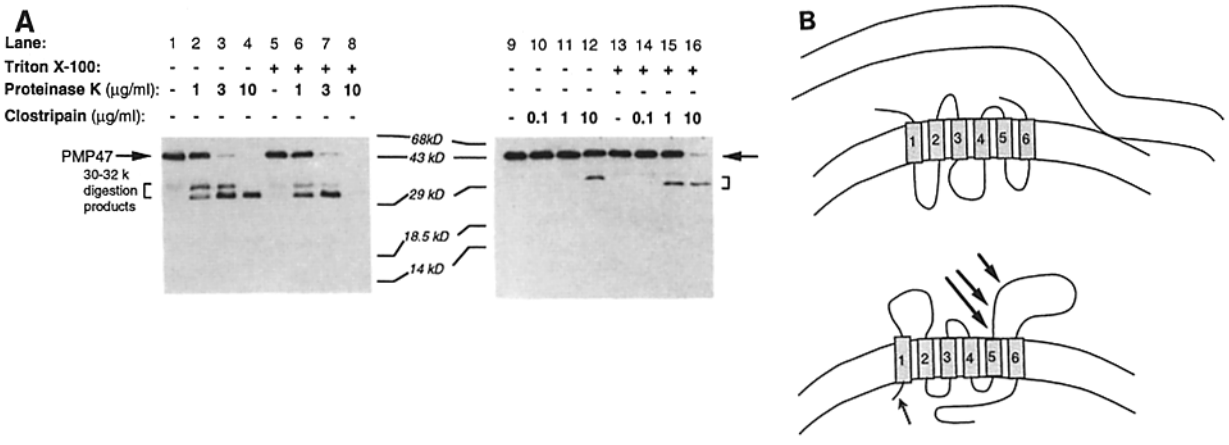


Figure 1. Evidence for the transmembrane orientation of PMP47. (*A*) Yeast lysates were exposed to proteinase K, clostripain, and Triton X-100 as indicated. They were boiled, subjected to SDS electrophoresis and immunoblotting with IVA7. (*B*) The generally accepted topology of the ADP/ATP carrier and a model for the topology of PMP47 that is consistent with the protease digests. Hatched rectangles represent the proposed membrane spanning domains. (*Solid arrows*) Protease-sensitive sites that are independent of detergent; (*dashed arrow*) sensitive site in the presence of detergent.

nus, since the next arginine in the sequence is at position 123, which would generate a 13.5-kD fragment, and such a fragment was not seen. In toto, these experiments suggest that the extreme amino terminus is within the peroxisomes while amino acids in the 270–300 region of the sequence are exposed to cytoplasm. Fig. 1 *B* presents a model of PMP47 that is consistent with both the protease data and the relative positions of the six membrane spanning domains predicted from homology with other members of the mitochondrial carrier family (Kuan and Saier, 1993). Surprisingly, in comparison with the most well defined member of this family, the ADP/ATP carrier (Nelson et al., 1993), it can be seen that these proteins are inverted with respect to each other such that the amino and carboxy termini of the ADP/ATP carrier extend into the mitochondrial intermembrane space while the ends of PMP47 face into the peroxisomal matrix. Corresponding hydrophilic loops between the membrane spans are also predicted to be in the opposite orientation relative to the matrix of the respective organelles (see especially the region between spans 5 and 6). Further experiments to probe other parts of PMP47 will be required to confirm this unexpected result.

PMP47 Sorting and PTS1-like Sequences

To address the mechanism by which PMP47 targets to peroxisomes, we first chose to ask whether either of the two PTS1-type signals is necessary for this process. For these experiments we used a system of constitutive expression in *S. cerevisiae* in which PMP47 assembled into the peroxisomal membranes (McCammon et al., 1990*a*). We previously reported that altering either or both lysine residues in the internal-SKL-(residues 320–322) or the C-terminal-AKE (residues 3421–423) did not measurably affect peroxisomal sorting of PMP47 (Goodman et al., 1992). To determine whether these tripeptidyl sequences were necessary at all, we deleted them singly or together (Fig. 2 *A*). Similar to the wild type protein, the major fraction of all of these mutant constructs were observed in a crude 25,000 *g* organellar pellet consisting mainly of peroxisomes and mitochondria (not shown). These organelle pellets were further fractionated on discontinuous sucrose gradients to separate peroxisomes from mitochondria. In this system mitochondria are observed in fractions 1–3 while peroxisomes migrate to fractions 4 and 5, based on marker enzyme analysis (Goodman et al., 1992). Fig. 2 *C* shows an SDS gel of these fractions, illustrating the distinctive pattern of peroxisomal proteins which peak in fractions 4 and 5 and are separated from mitochondria in fractions 1–3. The localization of the PMP47 constructs was determined after immunoblotting these fractions and detection with the anti-PMP47 monoclonal antibody (Fig. 2 *B*). Neither a truncation mutant (A421U) in which the terminal -AKE was deleted nor an internal deletion of residues 320–322 (ΔSKL) affected the sorting of PMP47 to peroxisomes, as determined by fractionation. Similarly, the double mutant (ΔSKL + A421U) was also localized to peroxisomes. Another construct was tested in which a frame shift mutation caused the insertion of the random peptide sequence -TIRINLKII at the carboxy terminus in place of the -AKE tripeptidyl sequence (A421 Tail); this mutation also had no noticeable effect on targeting.

These results were confirmed by indirect immunofluorescence (data not shown). The wild-type construct, the A421 Tail mutant, and the ΔSKL + A421U double mutant yielded a punctate pattern of fluorescence characteristic of peroxisomes from *S. cerevisiae* (Thieringer et al., 1991). Although colocalization of these constructs with a peroxisomal marker was not performed in these experiments, a construct in which the 156 COOH-terminal residues were deleted (267 DHFR) colocalized with the peroxisomal marker thiolase and confirms these results (Fig. 4, see below). Taken together, the fractionation and immunofluorescence data indi-

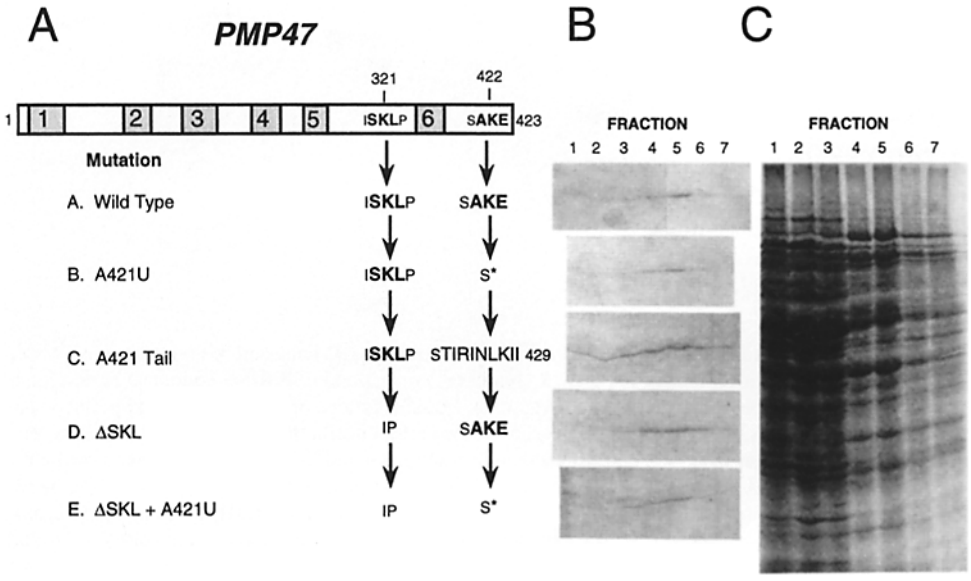


Figure 2. PTS-1-like sequences are not necessary for sorting of PMP47: cell fractionation. (*A*) Diagram of constructions. Hatched boxes are putative membrane spans. Letters in bold are PTS-like sequences; those in plain text are adjacent amino acid residues. Numbers are positions in the sequence. (*B*) Gradient fractions to localize the constructs. Mitochondria peak in fractions 1–3, peroxisomes in fractions in 4–6. Shown are immunoblots with the anti-PMP47 monoclonal antibody. (*C*) Fractions from construct E from the same experiment are shown stained with Coomassie blue, showing the distinct mitochondrial and peroxisomal protein profiles. Gels from the other constructs throughout this report are virtually identical.

cate that neither the extreme carboxy terminus nor the internal SKL sequence are necessary singly or in combination for the sorting of PMP47 to peroxisomes in *S. cerevisiae*. Hence, PMP47 does not sort via a PTS1-mediated pathway.

PMP47-DHFR Fusions to Define Sorting Signals

To define the regions of PMP47 that are necessary for sorting, deletions were constructed through a series of gene fusions of *PMP47* to mouse *DHFR* (Fig. 3 *A*). The dihydrofolate reductase protein was added to the carboxy termini of PMP47 sequences to improve stability of the hybrid proteins and as an antigenic tag. DHFR fused to intact PMP47 sorted to peroxisomes, as determined by immunofluorescence assays (not shown). However, the amount of hybrid protein detected by immunoblotting was lower than when intact PMP47 was expressed. To increase the amount of antigenic signal the *PMP47-DHFR* constructs were placed behind the yeast *GAL* promoter (McCammon et al., 1990*a*). We added a low concentration of galactose (0.1 or 0.2%) to minimize possible negative effects of catabolite repression on peroxisomal assembly (Simon et al., 1991) and to avoid other problems associated with the fractionation and separation of mitochondria and peroxisomes following growth on galactose (McCammon et al., 1990*a*). PMP47, when expressed in *S. cerevisiae*, is not extractable with sodium carbonate, suggesting that it has properly assembled into the peroxisomal membrane (McCammon et al., 1990*a*; Goodman et al., 1992). Up to 156 amino acids could be eliminated from the carboxy terminus of PMP47 without noticeably affecting sorting, as demonstrated by the 1-267DHFR construct, containing the first 267 residues of PMP47 (Fig. 3). This construct, in which DHFR was fused at the end of span 5 in the proposed model of PMP47, was found in the peroxisomal fractions of sucrose gradients (Fig. 3 *B*), and colocalized with the peroxisomal marker thiolase by indirect immunofluorescence (Fig. 4). DHFR fusions containing only the last 155 (containing span 6) or the last 47 amino acids of PMP47 (constructs DHFR268 and DHFR376, respectively), were found almost exclusively in the cytosolic fraction, as was DHFR alone (Fig. 3).

A hybrid containing the first 231 amino acids of PMP47, eliminating the putative membrane spanning domain 5 but still containing the more proximal hydrophobic sequence between amino acids 203–224 (span 4), yielded a weak signal on immunoblots (Fig. 3 *B*) and could not be easily detected by immunofluorescence, indicating that this protein was poorly expressed and/or unstable. We also tried to detect this and other fusions with available polyclonal antibodies to PMP47 or antibodies against DHFR, but they were not useful for yeast immunofluorescence, even after affinity purification, due to nonspecific fluorescence and weak signals. The protein was found both in cytosolic and crude organellar fractions, and it was seen both in mitochondrial and peroxisomal fractions. It is difficult to conclude whether the fraction comigrating with peroxisomes represents real targeting (at low efficiency) to this organelle coupled with mistargeting to mitochondria, or the nonspecific binding of this hydrophobic molecule to accessible organellar membranes. Thus, while the targeting of the fusion containing amino acids 1–267 was excellent, that with amino acids 1–231 was marginal at best.

While a hybrid containing the first five spans of PMP47 (267DHFR) was clearly peroxisomal, shorter hybrids with either the first three spans (199DHFR), or with only one span (88DHFR) were not targeted to peroxisomes. Both constructs were observed almost exclusively in the 25,000 *g* supernatant; none of the hybrid protein in the 25,000 *g* pellet colocalized with peroxisomes during subsequent fractionation (Fig. 3 *B*). Our ability to detect the 88DHFR construct

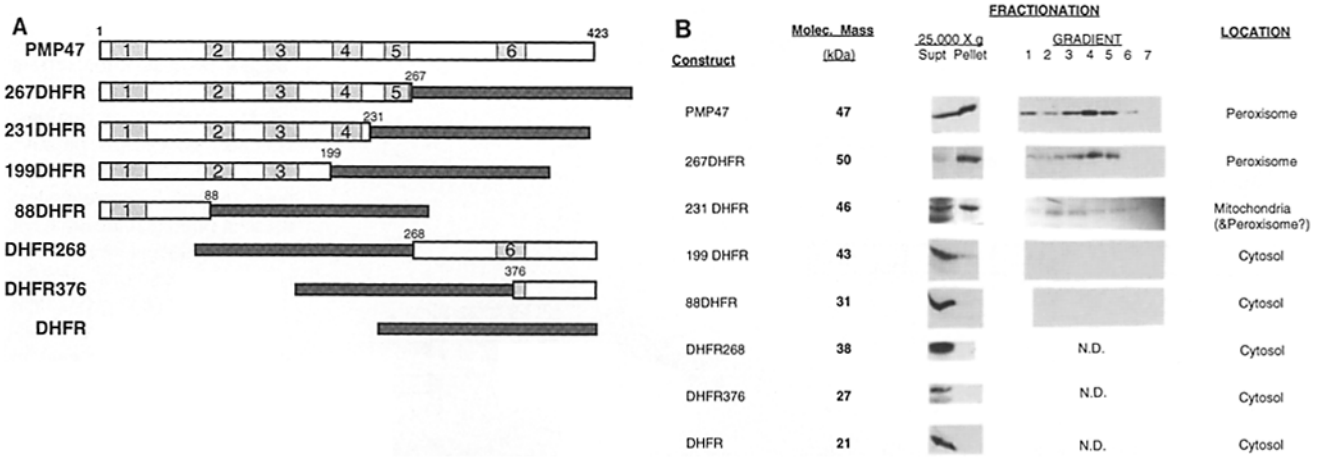


Figure 3. Amino acids 199–267 contain information important for targeting: organelle fractionation. (*A*) Diagram of constructions. Wider rectangles represent PMP47 sequences, narrower gray rectangles represent DHFR. Numbers correspond to PMP47 codons at fusion junctions (see Table I for exact fusion sequences). (*B*) Immunoblots from organellar fractions. Equal fractions of supernatants and pellets from cells expressing the indicated proteins were loaded onto gels, and equal aliquots of the sucrose gradient fractions were loaded. In all experiments, mitochondrial activity peaked in fractions 1–3, and peroxisomal activity peaked in fractions 4 and 5. Anti-PMP47 monoclonal antibody was used in all of the immunoblots except the lowest three constructs, where anti-DHFR antiserum was used. Cross-reacting bands in cytosolic fractions were seen with poorly expressed proteins (such as 231DHFR), and the endogenous yeast DHFR was a visible cross-reacting band with the DHFR antiserum (DHFR376). Reactivity in fraction 1 of gradients probably corresponds to unbroken cells, and was not reproducible.

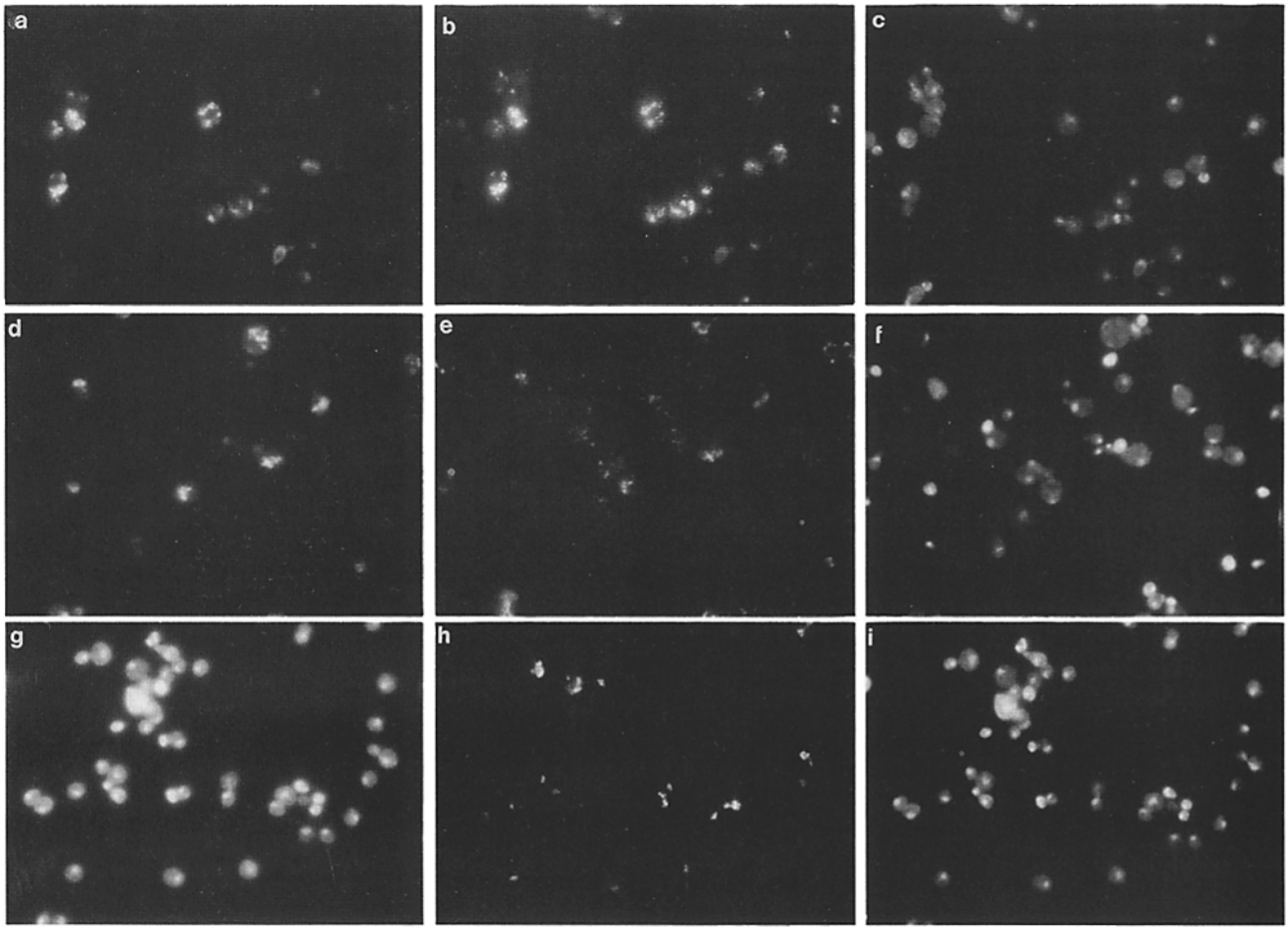
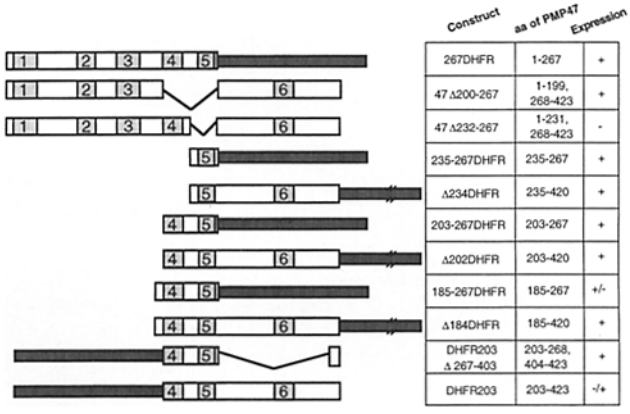


Figure 4. Amino acids 199–267 contain information important for targeting: indirect immunofluorescence. Cells expressing the indicated proteins were prepared for immunofluorescence and probed with anti-PMP47 monoclonal antibody (*left column*), with peroxisomal thiolase antibody (*middle column*), or with DAPI (*right column*). (*a*, *b*, and *c*) PMP47; (*d*, *e*, and *f*) 267DHFR; (*g*, *h*, and *i*) 199DHFR.

was instructive since it allowed us to determine that the epitope recognized by the monoclonal antibody was localized within the first 88 amino acids of PMP47, as stated above. The cytosolic location of the 1–199 fusion was also observed by immunofluorescence (Fig. 4). Diffuse cytoplasmic staining can be seen as expected, although there was also significant staining of the nucleus (Fig. 4, compare *g* and *i*). PMP47 contains many clusters of basic residues, such as KKLK at positions 41–44 and KKILK at positions 81–85, which may be responsible for the nuclear mislocalization. We believe that the apparent discrepancy between the fractionation and immunofluorescence data is caused either by leakage of nuclear material during fractionation or the fact that the 500 *g* pellet (containing nuclei) is normally discarded in our fractionation protocol.

Our data indicated that amino acids 200–267 contain critical information for the sorting of PMP47 to peroxisomes. This is supported by a construct in which only this region of the protein was deleted (47Δ200–266, Figs. 5 and 6). This protein was found principally in the cytoplasm and also bound slightly to mitochondria. A shorter deletion that eliminated only amino acids 232–267 (construct 47Δ232–267) was not expressed sufficiently to ascertain its localization.

We attempted to determine whether the 199–267 sequence was sufficient for sorting. Fusions were constructed in which half of this region (amino acids 235–267), most of it (203–267) or all of it (185–267) were fused to DHFR (Figs. 5 and 6). Hybrids that also contained the carboxy-terminal



Construct	aa of PMP47	Expression
267DHFR	1-267	+
47Δ200-267	1-199, 268-423	+
47Δ232-267	1-231, 268-423	-
235-267DHFR	235-267	+
Δ234DHFR	235-420	+
203-267DHFR	203-267	+
Δ202DHFR	203-420	+
185-267DHFR	185-267	+/-
Δ184DHFR	185-420	+
DHFR203 Δ267-403	203-268, 404-423	+
DHFR203	203-423	-/+

Figure 5. Summary of sorting experiments to probe the importance of the middle region of PMP47. Constructs are diagrammed at the left; see Fig. 3 *A* for further explanation of symbols. Expression ranges from easily detectable, +, to not detectable, −.

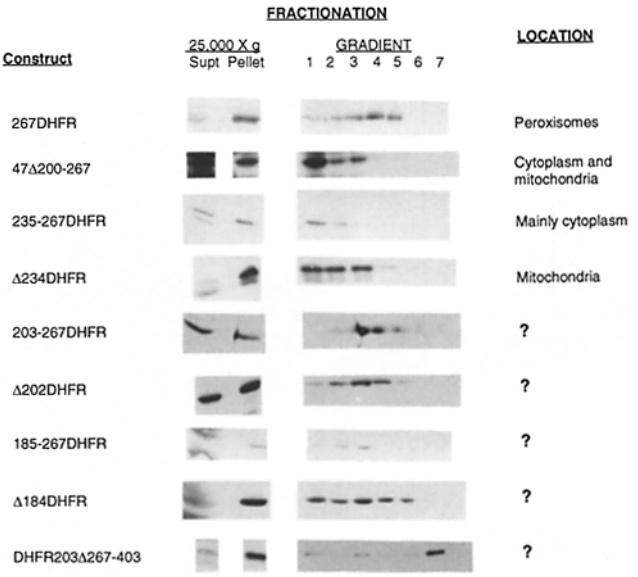


Figure 6. Results of sorting experiments. Fractions were prepared from cells expressing the indicated constructs as in Fig. 3 *B*. "?" indicates ambiguous sorting in the sucrose-gradient system.

downstream region of PMP47 (residues 268–420) were intermediates in these constructions and were also investigated for sorting in case this region added to stability or targeting efficiency. Many of these constructs gave ambiguous results upon organellar fractionation on sucrose gradients. Neither of the first pair (235–267DHFR and Δ234DHFR) sorted to peroxisomes, although construct Δ234DHFR was clearly found in mitochondrial fractions (Fig. 6). Activity in fraction 1 is attributable to contaminating unbroken spheroplasts that do not enter these gradients and are visible by light microscopy; the amount of hybrid proteins in this fraction varied between experiments. Addition of sequence starting at amino acid 203 (constructs 203–267DHFR and Δ202DHFR) did not restore clear peroxisomal targeting; however, these proteins were usually found midway between mitochondrial and peroxisomal markers, in fractions 3 and 4. Addition of amino acids from 185–201 resulted in the appearance of the hybrid protein in both mitochondrial and peroxisomal fractions in one of the constructs (Δ184DHFR); the other chimera of the pair, 185–267DHFR, was weakly expressed in this experiment and only mitochondrial localization could be detected. A unique behavior was reproducibly seen with one of our constructs, DHFR203Δ267–403, which provides PMP47 sequence from the middle of the protein (amino acids 203–267) and twenty amino acids at the extreme carboxy terminus (Fig. 5). Unlike the previous chimeras, DHFR was placed in front of this PMP47 sequence. This protein did not associate to a large degree with mitochondria but was found instead mainly in fraction 6 of the sucrose gradients (Fig. 6), one to two fractions removed from the peak of peroxisomal mass. The organelle to which this construct associated is not clear at present. Finally, expression of the construct DHFR203 was barely detectable and did not allow assessment of its sorting behavior by any criteria (data not shown).

Four of the constructs whose sorting was ambiguous from these experiments were analyzed by separation of organelles on Nycodenz gradients. A protocol was developed in which mitochondria and peroxisomes were at opposite ends of a continuous gradient (Fig. 7 *A*). The positive control in this experiment, 267DHFR, was found mainly in peroxisomes by this analysis, as expected (Fig. 7 *B*). It is clear, however, that neither 203–267DHFR nor 185–267DHFR was associated with peroxisomes; both were found exclusively with mitochondria. Interestingly, the addition of downstream residues (in the constructs Δ202DHFR and Δ184DHFR) which contain membrane span 6, led to significant peroxi-

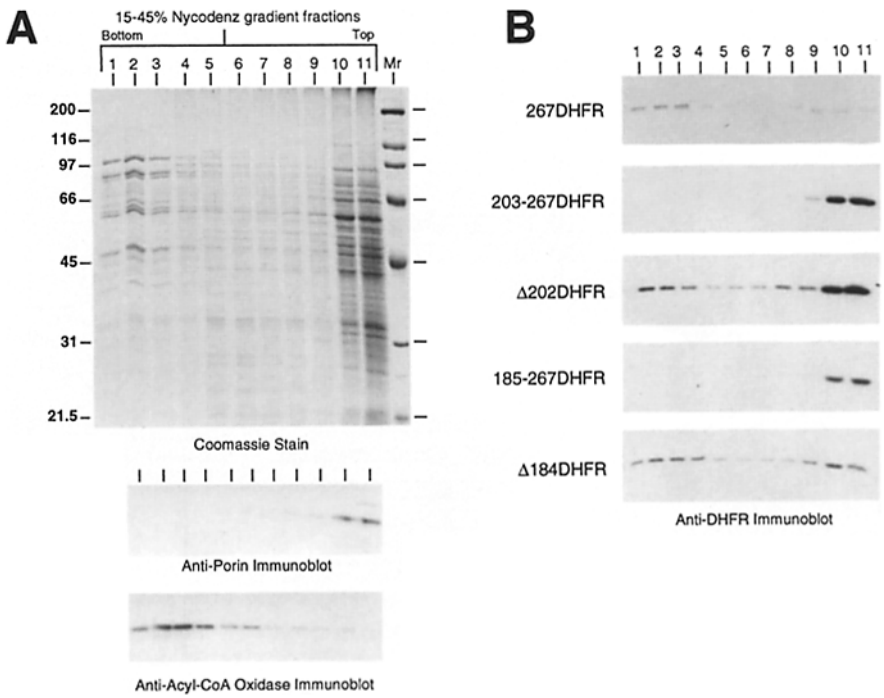


Figure 7. Fractionation of organelles containing chimeric constructs on Nycodenz gradients. Cells expressing various chimeric constructs were lysed and subjected to Nycodenz gradients as described in Materials and Methods. Proteins from gradient fractions were electrophoresed through SDS gels and stained with Coomassie brilliant blue or transferred to nitrocellulose and immunoblotted. (*A*) A representative stained gel and immunoblots (from construct Δ202DHFR) are shown, utilizing antibodies against mitochondrial porin or peroxisomal acyl-CoA oxidase. (*B*) Immunoblots with anti-DHFR antibody to visualize the chimeras are shown.

somal association, although the major fraction of both proteins was observed with mitochondria.

In summary, amino acids 199–267 of PMP47 are clearly important for peroxisomal targeting. These amino acids do not appear to be sufficient, however, for targeting to (or perhaps retention in) the organelles, at least within the context of our DHFR-containing chimeras. Efficient peroxisomal association requires upstream sequences (within amino acids 1–199) as well, although downstream sequences (within amino acids 268–420) may substitute to allow sorting to some extent. Thus, only amino acids 203–267 appear absolutely necessary for peroxisomal targeting.

Discussion

In this report we have begun to identify a targeting signal on the integral peroxisomal membrane protein, PMP47. We have identified a region of 67 amino acids that is critical for correct targeting, although it may not be sufficient, at least in the context of DHFR as the reporter molecule.

The essential region corresponds to transmembrane segments 4 and 5 and the short intervening hydrophilic loop, using the model structure of PMP47 as a member of the mitochondrial carrier family (Fig. 1 *B*; Kuan and Saier, 1993; Nelson et al., 1993). Of the ~40 genes of this family isolated from many different eukaryotic species, PMP47 and a maize amyloplast protein, brittle1, are the only members that do not normally reside in mitochondria (Jank et al., 1993). The other members of this family catalyze metabolite transport across the mitochondrial inner membrane through a mechanism of exchange transport. Among these transporters are the ADP/ATP carriers, phosphate carriers, tricarboxylic acid carriers, oxoglutarate/malate carriers, and the uncoupling protein of brown adipose tissue. These proteins function as homodimers or tetramers. PMP47 is among the most distantly related of the family. It is most closely related to the maize solute carrier, the solute carrier protein and the mitochondrial splicing suppressors of *S. cerevisiae*. Membership in this family suggests that PMP47 may be a carrier molecule for the transport of metabolites, cofactors, and/or import of precursor proteins across the peroxisomal membrane. This is not surprising since PMP47 is induced very early in the pathway of peroxisomal proliferation, before induction of the matrix proteins (Veenhuis and Goodman, 1990), and is positively regulated by all peroxisomal proliferators of *C. boidinii* (Goodman et al., 1990).

Our initial topology mapping experiments suggest that PMP47 is oriented oppositely across the membrane compared to the mitochondrial carriers (Fig. 1 *B*). Thus, while the amino termini of the mitochondrial proteins face the intermediate space, the amino terminus of PMP47 is protected from both proteinase K and clostripain except in the presence of nonionic detergent, suggesting that it faces the peroxisomal matrix. We observed protease-sensitive sites downstream of span 5 that were not detergent enhanced, indicating that they face the cytoplasm. Most models have the corresponding region in mitochondrial carriers facing the matrix, although there is some recent disagreement about this orientation (Marty et al., 1992). Further experiments are planned to confirm the orientation and topology of PMP47.

It is interesting to consider the implications of a reversed topology of PMP47 compared with the mitochondrial carrier proteins. The ADP/ATP carrier first binds to a receptor on the outer membrane (Steger et al., 1990) and then inserts into the inner membrane; the initial step of insertion is dependent on the proton-motive force across the inner membrane (Pfanner and Neupert, 1985, 1987). The protein does not appear to translocate into the matrix before assembling into the inner membrane because assembly is not dependent on mitochondrial hsp60 (Mahlke et al., 1990) nor on mitochondrial ATP (Wachter et al., 1992). These factors are known to be required for assembly of other proteins which may first fully translocate to the matrix before sorting to other mitochondrial compartments. There is evidence for a proton-motive force across the yeast peroxisomal membrane (Bellion and Goodman, 1987; Waterham et al., 1990) but it is in an opposite orientation compared with that across the mitochondrial inner membrane. Perhaps different spanning segments of PMP47 and the mitochondrial carrier are initially inserted into their respective membranes, dictated by the direction of the proton motive force, which ultimately leads to opposite orientations of the two proteins. Alternatively, it may be the number of membranes with which the mitochondrial or peroxisomal precursor interacts during import that is critical for the ultimate topology of the assembled protein.

Analysis of three successive deletions at the carboxy terminus of PMP47 may offer hints concerning the mechanism of assembly of this protein. The construct 199DHFR is cytosolic and nuclear and is not found to be associated with either mitochondria or peroxisomes. Its behavior indicates that it is able to fold into a stable structure that is protected from cytoplasmic proteases and does not undergo noticeable aggregation, even though it is predicted to contain three membrane spanning regions. The construct 231DHFR, containing the predicted membrane span 4 behaves very differently. It is proteolytically unstable (assuming a normal transcriptional rate) and associates with both mitochondria and to a smaller extent, peroxisomes. One interpretation from these data is that amino acids 199–231 confer a more open structure to the molecule that exposes both protease sites, allowing degradation, and cryptic mitochondrial targeting sites, or simply permits nonspecific aggregation to membranes. It also is possible that these amino acids contain sorting information that allows specific but inefficient binding to peroxisomes in the absence of downstream sequences. Perhaps the presence of DHFR in this construct prevents insertion of span 4 across the membrane. The further addition of 36 amino acids (containing the predicted span 5) in the 267 DHFR construct permits efficient localization. According to the topologic model of PMP47 (Fig. 1 *B*), span 5 is directed outward such that DHFR would not be required to cross the peroxisomal membrane during assembly, assuming that the entire precursor does not have to cross the membrane into the matrix during import. Whether specific targeting information exists in these 36 amino acids or only that they allow the stable insertion of span 4 is unclear. Insertion of a random hydrophobic sequence in place of span 5 may provide an answer to this question.

Deletion of amino acids 200–267 in an otherwise wild-type PMP47 (47Δ200–267) completely abolished our ability to detect the protein in any peroxisomal fractions, indicating that this region was necessary for sorting. Several constructs were tested to determine whether amino acids 185–267 were

sufficient for targeting DHFR to peroxisomes. There was no peroxisomal association of constructs containing residues 235–267 (constructs 245–267DHFR and Δ235DHFR, Figs. 5 and 6). Initially, proteins containing amino acids 203–267 were observed in fractions of intermediate density between mitochondria and peroxisomes. However, when these constructs were analyzed on a different gradient separation system, the constructs that contained only this region, 203–267DHFR and 185–267DHFR, were observed exclusively in mitochondria (Fig. 7). Addition of residues 268–420 permitted some peroxisomal association, although most of the protein was observed in the mitochondrial fractions. Hence, the localization of constructs in intermediate densities probably represented the sum of the association of the chimeras with peroxisomes and mitochondria, rather than association with a subset of peroxisomes (Veenhuis et al., 1989; Heinemann and Just, 1992; Lüers et al., 1993).

Considering its membership in a family that is almost exclusively mitochondrial, it is not surprising that PMP47 might contain cryptic or ancestral signals for sorting to mitochondria. When peroxisomal proliferation is not induced, or when PMP47 is overexpressed, the protein is detected in mitochondria (Veenhuis, M., M. T. McCammon, and J. M. Goodman, unpublished results). It is also found in mitochondria when it is overexpressed in another yeast, *H. polymorpha* (Sulter et al., 1993). Since the mitochondrial missorting observed with several of our constructs occurred whether the PMP47 sequences were limited to the amino terminal half of the molecule (in 231DHFR) or to the nonoverlapping carboxy terminal half (in Δ234DHFR), the missorting cannot be caused by a single cryptic mitochondrial targeting sequence within PMP47. DHFR contains an amphipathic helix that can cause mitochondrial sorting under certain circumstances (Hurt and Schatz, 1987), and perhaps some of our constructs cause this region to become exposed. Further constructs will be necessary using a carrier other than DHFR to determine if these amino acids are sufficient to direct a protein to peroxisomes.

While the region within PMP47 delimited by spans 4 and 5 contains essential targeting information, we cannot rule out other upstream or downstream regions that are also important. Multiple targeting domains exist on the ADP/ATP translocator, although these areas have been only crudely defined. The first 111–115 amino acids were required for binding of DHFR or β-galactosidase fusion proteins to mitochondria (Adrian et al., 1986; Smagula and Douglas, 1988*a*;*b*). It was found, however, that the first third of the protein (102 amino acids) could be deleted without abolishing in vitro import or assembly into the inner membrane (Pfanner et al., 1987). The authors postulated that a stretch of 20 amino acid residues at the carboxy-terminal half of each of the three repeated domains of the molecule may contain targeting information. These small regions show similarity to normal mitochondria presequences; they contain no negative charge and are predicted to form α helices. Unfortunately, these putative mitochondrial targeting signals have not been formally tested. Hydrophobic transmembrane domains have also been postulated to be important for the efficiency of import and assembly of this molecule (Pfanner et al., 1987; Smagula and Douglas, 1988*a*). Whether the regions of PMP47 upstream of amino acid 199 or downstream from amino acid 267, both of which contain predicted transmembrane regions, have targeting information or only serve to anchor the protein once targeted, is a question for future research.

We thank Drs. F. Huennekans (Scripps Research Institute) for anti-DHFR antiserum, J. Rothblatt (Dartmouth University) and H. L. Chiang (University of California, Berkeley) for anti-thiolase antiserum, and G. Schatz for anti-porin antiserum. We also thank Kim Campbell for excellent technical assistance and Mary Jane Gething for the use of her fluorescent microscope.

This work was supported by National Institutes of Health Institute grants for General Medical Sciences and the Welch Foundation.

Received for publication 16 September 1993 and in revised form 10 December 1993.

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