A *Saccharomyces cerevisiae* homolog of the human adrenoleukodystrophy transporter is a heterodimer of two half ATP-binding cassette transporters

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ABSTRACT The adrenoleukodystrophy protein (ALDP) and the 70-kDa peroxisomal membrane protein (PMP70) are half ATP-binding cassette (ABC) transporters in the human peroxisome membrane. ALDP and PMP70 share sequence homology and both are implicated in genetic diseases. *PXA1* and *YKL741* are *Saccharomyces cerevisiae* genes that encode homologs of ALDP and PMP70. Pxa1p, a putative ortholog of ALDP, is involved in peroxisomal β -oxidation of fatty acids while *YKL741* is an open reading frame found by the yeast genome sequencing project. Here we designate *YKL741* as *PXA2* and show that its protein product, Pxa2p, like Pxa1p, is associated with peroxisomes but not required for their assembly. Yeast strains carrying gene disruptions of *PXA1*, *PXA2*, or both have similar and, in the case of the latter, nonadditive phenotypes. We also find that the stability of Pxa1p, but not Pxa2p, is markedly reduced in the absence of the other. Finally, we find that Pxa1p and Pxa2p coimmunoprecipitate. These genetic and physical data suggest that Pxa1p and Pxa2p heterodimerize to form a complete peroxisomal ABC transporter involved in fatty acid β -oxidation. This result predicts the presence of similar heterodimeric ABC transporters in the mammalian peroxisome membrane.

ATP-binding cassette (ABC) transporters are members of a superfamily of membrane proteins involved in the transport of a variety of molecules across biological membranes (1, 2). ABC transporters are comprised of two homologous halves, each containing two parts: a membrane spanning domain with multiple transmembrane segments; and a nucleotide binding domain (NBD) with Walker A and B consensus motifs. Mammalian ABC transporters are found either as complete transporters [e.g., the multidrug resistance (MDR1) and the cystic fibrosis transmembrane conductance regulator proteins] or as half transporters which require dimerization to become functional (1). Although half ABC transporters have been identified in many organisms, the structure of the functional transporter formed by dimerization of these molecules is known for only a few. Genetic evidence suggests that the bacterial HlyB half ABC transporter forms a homodimer (3) while the *Drosophila white*, *brown*, and *scarlet* proteins form heterodimeric transporters (4). In as far as we know, the only dimeric ABC transporter for which the subunit makeup has been physically established is the transporter of antigenic peptides or TAP, a heterodimer of the homologous TAP1 and TAP2 proteins (39% overall amino acid identity) (5).

At least three half ABC transporters have been identified in the human peroxisome membrane: the adrenoleukodystrophy (ALD) protein (ALDP) (6), the 70-kDa peroxisomal membrane protein (PMP70) (7), and the recently described ALDP-related protein (8). Mutations in the ALD gene cause

X-linked ALD, an inborn error of peroxisomal β -oxidation of very long chain fatty acids (9–13). Mutations in the PMP70 gene have been identified in two patients with Zellweger syndrome (14), an inborn error of peroxisome biogenesis. The role PMP70 plays in this disease, however, is unclear (15). *PXA1* and *YKL741* are *Saccharomyces cerevisiae* genes that encode homologs of ALDP and PMP70. Pxa1p, the protein product of the *PXA1* gene, is involved in peroxisomal β -oxidation of fatty acids and is a putative ortholog of ALDP (16). *YKL741* is an open reading frame (ORF) found by the yeast genome sequencing project (17). Although we have shown that yeast carrying a disruption of the *YKL741* gene exhibit impaired growth on oleic acid medium (16), the subcellular localization, function and physical interactions of its protein product are not known.

Based on the results presented in this paper, we designate *YKL741* as *PXA2*. We show that Pxa2p is a peroxisomal half ABC transporter similar in function to Pxa1p. We also demonstrate that Pxa1p and Pxa2p heterodimerize to form a yeast peroxisomal ABC transporter involved in β -oxidation of fatty acids.

MATERIALS AND METHODS

Strains. Gene disruptions of *PXA1* and *PXA2* (*pxa1::LEU2*, *ykl741::URA3*, and the *pxa1::LEU2* + *ykl741::URA3* double mutant) in *S. cerevisiae* CH1305 were described (16). All plasmid cloning was in *Escherichia coli* DH5 α (recA, gsdR, supE, endA, gyrA96, thi-1, relA1, and lacZ).

Yeast Culture Conditions. All yeast media were as described (16, 18), except for the pulse media, which contains 1% yeast nitrogen base (without amino acids); 2% galactose; all auxotrophic requirements (40 μ g/ml adenine/20 μ g/ml uracil/20 μ g/ml histidine/60 μ g/ml leucine/30 μ g/ml lysine); 0.2% oleic acid and 0.5% Tween 40. For growth on oleic acid medium we cultured yeast to logarithmic phase in yeast extract/peptone/dextrose (YPD), then pelleted and washed the cells in sterile water. Tenfold dilutions were then plated on fresh oleic acid medium containing 0.1% oleic acid and 0.5% Tween 40.

Plasmids. We used *S. cerevisiae* genomic DNA as a template to PCR amplify a DNA fragment containing the *PXA2* gene (from –310 to +2590 bp where +1 is the A of the ATG translation initiation codon). This fragment was cloned into pCRII vector (Invitrogen) and subcloned into the *Bam*HI site of the low copy number (CEN) vector pRS415 (19), producing pRS415–*PXA2*. To construct epitope tagged Pxa2p, we used PCR to fuse three copies of the hemagglutinin (HA) epitope (–YPYDVPDYAGS–) to the C terminus of Pxa2p. A DNA sequence coding three tandem HA sequences (20) was am-

plified by the sense primer 5'-AAT AAA AGC TTG AAC AAA AAA GTT AAA ACA AAA AAG GAA GAA GGA AAG GAG AGG ATC TTT TAC CCA TAC GAT-3' (which combines DNA sequence encoding the C terminus of Pxa2p with sequence encoding the 5' end of the HA repeat sequence) and the antisense primer 5'-CCG CGG AAG CTT GCG GCC GCT CTA GAT TAG CAC TGA GCA GCG TAA TC-3' (coding for the 3' end of the HA sequence, a stop codon and a *NotI* restriction site). The amplified fragment was cloned into pCRII vector and a 1710-bp *HindIII* fragment was excised and substituted for a *HindIII* DNA fragment (+2510 to +2611 bp) in pRS415-PXA2, producing the construct pRS415-PXA2HA. The manipulated region of the reconstructed plasmid was sequenced to confirm correct reconstruction of the ORF. The DNA fragment containing the PXA2 promoter and the Pxa2p-HA ORF was excised with *NotI* and subcloned into the *NotI* site of pRS317, pRS425, and pRS426 (19, 21) to produce pRS317-PXA2HA, pRS425-PXA2HA, and pRS426-PXA2HA, respectively.

Northern Blot Analysis of RNA Transcripts. After overnight growth in YPD, yeast were transferred to induction media and grown for an additional 4 h at 30°C. Total RNA was isolated and analyzed on a Northern blot (5 µg per lane) (22). We probed duplicate membranes with either an internal *EcoRI* fragment (+179 to +1779) of the *PXA1* gene or a PCR amplified DNA fragment (-33 to +2597) of the *PXA2* gene. As a control for the quality and quantity of RNA, we stripped the membranes in 0.1× standard saline citrate (0.15 M sodium chloride/0.015 M sodium citrate, pH 7) with 1% SDS at 100°C for 20 min and reprobed them with a 1100-bp *BamHI-HindIII* DNA fragment of the yeast actin gene (*ACT1*) (23).

Cellular Fractionation, Enzyme Assays, and Immunoblots. High-density organelles were isolated from oleic acid induced yeast by differential centrifugation and fractionated on continuous 15–35% Nycodense gradients as described (24, 25). The activities of catalase and succinate dehydrogenase were measured by standard techniques (24). Several antibodies were used for immunoblot analysis (enhanced chemiluminescence, Amersham). (i) A hybrid protein composed of the maltose-binding protein (MBP; New England Biolabs) fused to amino acids 626 to 839 of Pxa2p (MBP-Pxa2p) was expressed in bacteria (BL21) and purified on an amylose column (New England Biolabs). A 1/4000 dilution of rabbit antisera (HRP, Denver, PA) raised against this protein was used to detect Pxa2p. (ii) A 1/100 dilution of a 0.4 mg/ml solution of mAb recognizing the HA epitope (Boehringer Mannheim) was used to detect Pxa2p-HA. (iii) A 1/4000 dilution of antisera against MBP-Pxa1p (16) was used to detect Pxa1p. (iv) Based on the MBP-Pxa1p hybrid (16), we constructed a second hybrid protein comprised of gene 10 of T7 (pGEM-Express; Promega) fused to the same C-terminal 163 amino acids of Pxa1p (pGEMEX-Pxa1p). pGEMEX-Pxa1p was expressed in bacteria (BL21) and obtained from SDS/PAGE of total bacterial proteins. Antisera raised against this hybrid protein (HRP) was affinity purified (ImmunoPure; Pierce) using MBP-Pxa1p and was used to detect Pxa1p in Fig. 7b.

β-Oxidation of ¹⁴C-Radiolabeled Oleic Acid. After overnight growth in YPD, yeast were cultured for 24 h in induction medium and then transferred to fresh induction medium containing 2.5 µCi/ml (1 Ci = 37 GBq) [1-¹⁴C]oleic acid (New England Nuclear). Following 20 h incubation at 30°C, the amount of the radiolabeled fatty acid β-oxidized to water-soluble products was measured with the partition method of Folch *et al.* (26).

Pulse-Chase Labeling and Immunoprecipitation. After overnight growth in YPD, yeast were transferred to pulse medium and incubated for 2 h at 30°C. For *in vivo* protein labeling, cells were pelleted and resuspended (5 OD₆₀₀/0.5 ml) in pulse medium containing ³⁵S-labeled methionine and cysteine (150 µCi/0.5 ml Trans-Label; ICN) and cultured for

additional 30 min. Protein labeling was stopped by the addition of unlabeled methionine and cysteine to a final concentration of 20 mM, and the labeled cells were diluted 1:5 in induction medium. The cells were incubated at 30°C and samples of 2.5 ml were taken at the indicated times. The amount of labeled Pxa1p or Pxa2p-HA was analyzed by immunoprecipitation (27) using 10 µl of antiserum against Pxa1p (16) or 10 µl of ascites fluid containing the mAb 12CA5 against the HA epitope (28), followed by SDS/PAGE and fluorography (22).

For coimmunoprecipitation experiments, we isolated high-density organelles from oleic acid induced yeast. Organelles from 150 OD₆₀₀ of yeast were incubated for 15 min on ice in 1 ml solubilization buffer (1% Triton X-100/150 mM NaCl/1 mM EDTA/10 mM Tris-HCl, pH 7.5/1 mM phenylmethylsulfonyl fluoride/25 µg/ml aprotinin/25 µg/ml leupeptin). We then sedimented unsolubilized material by centrifugation at 13,000 × *g* for 15 min and used the supernatant for overnight incubation at 4°C with 10 µl of antiserum against Pxa1p (16) or 20 µg affinity purified 12CA5 mAbs (kindly provided by Sami Malek, Johns Hopkins, Baltimore). After an additional 5-min centrifugation the supernatant was incubated with 40 µl of protein A Sepharose beads (Pharmacia; 1:3 ratio suspension in solubilization buffer) for 2 h at 4°C. We then washed the beads twice with 1 ml of buffer A (0.1% Triton X-100/0.02% SDS/150 mM NaCl/5 mM EDTA/50 mM Tris-HCl, pH 7.5/1 mM phenylmethylsulfonyl fluoride/25 µg/ml aprotinin/25 µg/ml leupeptin) and once with 1 ml of the final buffer (150 mM NaCl/5 mM EDTA/50 mM Tris-HCl, pH 7.5). Proteins bound to the Sepharose beads were then analyzed by SDS/PAGE and immunoblotting.

RESULTS

The ORF YKL741 (PXA2) Is Expressed in Yeast. Previously we showed that yeast with a targeted disruption of *YKL741* have impaired growth on oleic acid medium identical to the phenotype of *pxa1* yeast (16). To confirm expression of *YKL741* we analyzed total RNA isolated from oleic acid induced yeast. Fig. 1 shows a Northern blot probed with labeled DNA fragments of either *PXA1*, *YKL741* (*PXA2*), or the yeast actin gene, *ACT1*. A *PXA1* transcript is present in wild-type and *pxa2* (*pxa2::URA3*) yeast but not in the *pxa1* (*pxa1::LEU2*) strain. The *PXA2* probe detected a single transcript in both wild-type and *pxa1* yeast but not in the *pxa2* strain (Fig. 1). Both the *PXA1* and the *PXA2* transcripts are ≈2.8 kb.

FIG. 1. Expression of *PXA1* and *PXA2*. Northern blots of total RNA (5 µg/lane) extracted from the indicated strains of oleic acid-induced yeast and hybridized to the probes shown on the left.

Complementing the *pxa2* Mutant Phenotype. Examination of the *PXA2* genomic sequence reveals two possible oleate response elements (consensus: CCG NNN T N A (29) located at -215 bp (CGGCAATGA) and -272 bp (CGGCGTTAA) relative to the A of the ATG initiation codon. We amplified a genomic DNA fragment containing these two elements and the *PXA2* ORF (Fig. 2a) and cloned it into the low copy number (CEN) plasmid pRS415. To complement the growth phenotype of *pxa2*, we transformed various *PXA2* expression constructs into *pxa2::URA3* cells and examined growth on plates containing oleic acid as a sole carbon source. Compared with wild type, *pxa2* cells grow poorly on oleic acid medium. Expression of wild-type Pxa2p from a low copy (CEN) vector, or an epitope-tagged Pxa2p (Pxa2p-HA) from either a low (CEN) or a high (2 μ) copy number vectors, restores wild-type growth (Fig. 2b). Thus, lack of Pxa2p is responsible for the growth phenotype and addition of three copies of the HA epitope to the C terminus of Pxa2p does not appreciably alter its function.

Pxa2p, Like Pxa1p, Is Peroxisome-Associated But Not Required for Peroxisome Assembly. To determine the subcellular localization of Pxa2p, we examined its distribution on a Nycodense gradient separation of high density organelles. The distribution of both Pxa2p and Pxa1p corresponds to that of the peroxisomal marker catalase (Fig. 3). This result indicates that Pxa2p, like Pxa1p, is peroxisome associated. Migration of Pxa2p (predicted molecular mass, ≈ 97 kDa) on SDS/PAGE is slightly slower than Pxa1p (predicted molecular mass ≈ 87 kDa).

In contrast to peroxisome assembly (*pas*) mutants (30), peroxisomes are morphologically intact in the *pxa1* yeast (16). To determine the consequences of the *pxa2::URA3* mutation on peroxisome integrity, we examined the partition of catalase by differential centrifugation and Nycodense gradient fractionation. As with wild-type and *pxa1* mutants, most of the

FIG. 2. Complementation of *pxa2::URA3* gene disruption. (a) *PXA2* constructs with and without the HA tag. The gray rectangle represents the *PXA2* ORF and the black box represents an additional segment coding for three copies of the HA epitope. The HA epitope is not drawn to scale. The positions of the ends of the DNA fragment relative to the ATG start codon are indicated. The asterisks represent the location of putative oleate response elements. (b) Ten-fold dilutions (top to bottom) on oleic acid medium of the wild-type strain CH1305 (lane 1), the *pxa2::URA3* mutant (lane 2), and the *pxa2::URA3* mutant transformed with pRS415 (lane 3), pRS415-PXA2 (lane 4), or pRS425-PXA2HA (lane 5).

FIG. 3. Localization of Pxa2p to peroxisomes. The organelle pellet of oleic acid-induced wild-type cells was fractionated on a Nycodense density gradient. (Upper) Gradient fractions were assayed for activity of catalase and succinate dehydrogenase. (Lower) Equal volumes of each fraction were analyzed by immunoblotting with rabbit anti-Pxa1p or anti-Pxa2p antiserum. Note that a somewhat reduced volume was loaded in lane 4 of the immunoblot.

catalase activity in *pxa2::URA3* cells is particulate (61% compared with 69% in wild-type cells) and is distributed in the dense fractions of the gradient containing intact peroxisomes (not shown). Thus, Pxa2p, like Pxa1p, is not required for peroxisomal integrity.

Pxa2p Is Involved in Fatty Acid β -Oxidation. To investigate the metabolic phenotype of *pxa2* cells in oleic acid medium, we assayed the ability of yeast carrying *pxa2::URA3* to β -oxidize 14 C-oleic acid (C18:1). As with *pxa1* yeast, we found that β -oxidation of oleic acid by *pxa2* cells is reduced by $\approx 50\%$. Moreover, the reduction of β -oxidation in the double mutant (*pxa1* plus *pxa2*) is similar to either single mutant (Fig. 4). Thus, the metabolic phenotypes of *pxa1* and *pxa2* are similar and nonadditive.

The Stability of Pxa1p, But Not Pxa2p, Is Affected by Absence of Its Counterpart. High-density organelle pellets

FIG. 4. β -Oxidation of oleic acid by various mutant yeast strains. β -Oxidation of 14 C-oleic acid was measured in the indicated strains and compared with that of the wild-type strain CH1305 assayed simultaneously and set as 100%.

from yeast contain mainly peroxisomes and mitochondria (24). Immunoblots of organelle pellets isolated from the different mutants induced by growth in oleic acid revealed a surprising reduction in the quantity of Pxa1p in *pxa2* cells. The amount of Pxa1p in these cells ranged from 0% to approximately 40% of wild type (Fig. 5). To investigate whether this reduction was due to a decrease in Pxa1p stability, we performed pulse-chase experiments followed by immunoprecipitation. We found that Pxa1p has relatively long half-life in wild-type cells (>9 h), but in *pxa2* yeast the Pxa1p half-life is reduced to ≈ 3 h (Fig. 6a). To determine if the converse is true, we expressed Pxa2p-HA from a CEN plasmid in the presence (*pxa2*) or absence (*pxa1* plus *pxa2*) of Pxa1p. Surprisingly, the stability of Pxa2p-HA was not influenced by the absence of Pxa1p (Fig. 6b).

Pxa1p and Pxa2p Coimmunoprecipitate. The common subcellular localization, nonadditive phenotypes, and destabilization of Pxa1p in the absence of Pxa2p all provide genetic evidence that these two half ABC transporters heterodimerize. To investigate this possibility directly we expressed Pxa2p-HA in cells with or without a wild-type *PXA1* gene. We isolated high-density organelles and solubilized the membranes in solubilization buffer containing 1% Triton X-100. Similar amounts of Pxa2p-HA were present in extracts of yeast with either a wild-type (Fig. 7a, lane 3) or a disrupted (Fig. 7a, lane 4) *PXA1* gene. However when equal volumes of these extracts were precipitated with anti-Pxa1p antiserum, Pxa2p-HA was present only in the immunoprecipitate of cells containing Pxa1p (Fig. 7a, compare lanes 1 and 2). The absence of Pxa2p-HA from immunoprecipitate of cells lacking Pxa1p (Fig. 7a, lane 2) indicates that the presence of Pxa2p-HA in lane 1 is not explained by cross reactivity of the anti-Pxa1p antibodies. We obtained similar results when we performed the converse experiment (Fig. 7b). Pxa1p is detectable in organelles of both wild-type yeast and *pxa2* yeast expressing Pxa2p-HA (Fig. 7b, lanes 3 and 4). When we incubated organelle extracts from these cells with an anti-HA mAb, Pxa1p was detectable only in the immunoprecipitate of cells expressing Pxa2p-HA (Fig. 7b, compare lanes 1 and 2). Thus, immunoprecipitation with antibodies against either Pxa1p or Pxa2p-HA results in coimmunoprecipitation of the other half transporter. These results provide physical evidence that Pxa1p and Pxa2p are part of the same protein complex.

DISCUSSION

ABC transporters are present in all cellular organisms and mediate membrane transport of a variety of substrates including ions (cystic fibrosis transmembrane conductance regula-

FIG. 5. Immunoblot analysis of Pxa1p in wild-type and mutant strains. The organelle pellet (50 μ g protein per lane) of oleic acid-induced yeast was analyzed by immunoblotting using a rabbit antiserum to Pxa1p. The yeast cells analyzed are the wild-type CH1305 strain (lane 1) and the mutant strains *pxa1::LEU2* (lane 2), *pxa2::URA3* (lane 3), and *pxa1::LEU2* plus *pxa2::URA3* (lane 4).

FIG. 6. Stability of Pxa1p and Pxa2p. Pulse-chase analysis of oleic acid-induced yeast. Cells were labeled metabolically for 30 min and chased for the indicated time. Extracts were analyzed by immunoprecipitation and SDS/PAGE. (a) Immunoprecipitation of Pxa1p from wild-type CH1305 yeast and the mutant *pxa2::URA3*. (b) Immunoprecipitation of Pxa2p-HA with monoclonal anti-HA antibodies from *pxa2::URA3* cells transformed with pRS415-PXA2HA or *pxa1::LEU2* plus *pxa2::URA3* cells transformed with pRS317-PXA2HA.

tor) (31), small hydrophobic molecules (MDR1) (32), and peptides (TAP) (33). ABC transporters have a characteristic modular organization consisting of two transmembrane spanning domains, each comprised of multiple transmembrane segments and two hydrophilic NBDs with Walker A and B motifs (1). The structure of the complete transporter may be achieved by assembly of separate polypeptides, each contributing one or more domains, or by a single multidomain protein (2). Half ABC transporters contain a transmembrane domain and an ATP binding domain and are thought to dimerize to form functional transporters.

Previously we showed that the *S. cerevisiae* *PXA1* gene encodes a peroxisomal half ABC transporter that is a putative ortholog of human ALDP (16). Recently, a second group cloned the same gene and referred to it as PAL1 (34). We found that mutations in *PXA1* corresponding to mutations identified in the ALD gene of patients with ALD result in impaired growth on oleic acid (35). These observations suggest that yeast peroxisomes have an ABC transporter system similar to that of mammalian cells. We also suggested that the protein product of the *S. cerevisiae* *YKL741* ORF assembles with Pxa1p to form a complete peroxisomal ABC transporter (16). Here we show that *YKL741* is expressed (Fig. 1) and encodes a second peroxisomal ABC transporter. On this basis we suggest that *YKL741* be designated *PXA2*. Pxa2p shares location (Fig. 3) and weak sequence homology (22.7% overall amino acid identity; 35.5% identity in the NBD) with Pxa1p. Moreover, like Pxa1p, it is required for normal oxidation of fatty acids (Figs. 2 and 4) but not for peroxisome assembly. The phenotypes produced by disruption of either *PXA1* or *PXA2* are identical: impaired growth on oleic acid medium (16) (Fig. 2) and reduced β -oxidation of radiolabeled oleate (Fig. 4). The phenotype of the double mutant is not additive; the growth and oleate oxidation of *pxa1* plus *pxa2* yeast are indistinguishable from those of either of the single mutants (16) (Fig. 4). In contrast to yeast with a complete lack of peroxisomes (16), neither the growth phenotype nor the defect in β -oxidation of the *PXA* mutants are complete, indicating there is some *PXA*-independent peroxisomal fatty acid oxidation.

FIG. 7. Coimmunoprecipitation of Pxa1p and Pxa2p-HA. Analysis of high density organelles isolated from oleic acid induced yeast. (a) Organelles from cells expressing Pxa2p-HA and Pxa1p (*pxa2::URA3* transformed with pRS425-PXA2HA) (lanes 1 and 3), or Pxa2p-HA but no Pxa1p (*pxa1::LEU2* transformed with pRS426-PXA2HA) (lanes 2 and 4) were analyzed by immunoblotting using mAbs for the HA epitope tag. Samples were analyzed either before (lanes 3 and 4) or after (lanes 1 and 2) immunoprecipitation with antiserum to Pxa1p. (b) organelles from cells expressing Pxa1p and Pxa2p-HA (*pxa2::URA3* transformed with pRS425-PXA2HA) (lanes 1 and 3) or Pxa1p and Pxa2p (wild-type yeast) were analyzed by immunoblotting using antibodies for Pxa1p. Samples were analyzed either before (lanes 3 and 4) or after immunoprecipitation (lanes 1 and 2) with an anti-HA mAb.

Disruption of *PXA2* does not influence the expression of the *PXA1* transcript (Fig. 1) but does have a profound effect on Pxa1p stability (Figs. 5 and 6a). This result suggests physical interaction between Pxa1p and Pxa2p. In contrast, Pxa2p stability appears unaltered by the lack of Pxa1p (Fig. 6b), suggesting that Pxa2p is either inherently more stable as a monomer or that it is stabilized by interactions with proteins other than Pxa1p. Consistent with this genetic evidence for heterodimerization of Pxa1p and Pxa2p, we show that these molecules coimmunoprecipitate (Fig. 7). Thus, our results strongly suggest that Pxa1p and Pxa2p heterodimerize to form a complete ABC transporter in the yeast peroxisomal membrane. We refer to this *S. cerevisiae* peroxisomal transporter as the PXA transporter.

In eukaryotes several lines of evidence suggest that the two halves of ABC transporters have separate functions. First, we are unaware of any well-documented evidence for homodimerization of half ABC transporters. By contrast, half ABC transporters have been shown to heterodimerize [e.g., the mammalian TAP1 and TAP2 proteins (5); the *white*, *brown*, and *scarlet* proteins in *Drosophila* (4) and Pxa1p and Pxa2p in this work]. Second, functional analysis of mutant cystic fibrosis transmembrane conductance regulator proteins showed that the two NBDs have different roles in transporter function: ATP hydrolysis by NBD1 starts channel activity while hydrolysis by NBD2 terminates it (36). Third, it has been shown for

several ABC transporters that function is not reconstituted by expression of one of the halves in the absence of the other [shown for Ste6 (37), MDR1 (38), cystic fibrosis transmembrane conductance regulator (39), and TAP (40)]. Taken together these observations suggest that heterodimerization is the rule for half ABC transporters.

Furthermore, genetic studies with the guanine/tryptophan transporters of *Drosophila* pigment cells suggest that different combinations of the half ABC transporters *white*, *brown*, and *scarlet* proteins form transporters with different substrate specificity. The *white* protein interacts with *brown* to form a guanine transporter and with *scarlet* to form a tryptophan transporter (4). A similar possibility was predicted for mammalian peroxisomal half ABC transporters by ourselves (41) and others (8). At least three different half ABC transporters have been identified in the mammalian peroxisome, PMP70, ALDP and the recently identified ALDP-related protein, which is highly homologous to ALDP (8). They have different, yet overlapping, expression patterns (8, 42). Additionally, we have identified a fourth human putative peroxisomal half ABC transporter, most similar to PMP70 (PMP70R) (N.S., G. Steel, M. Dean, and D.V., unpublished results). Based on the precedent from *Drosophila* and the number of available partners, we suggest that in mammalian cells various combinations of peroxisomal half ABC transporters assemble into functional transporters with different properties. This would provide a mechanism for cells and/or tissues to adjust the metabolic capabilities of their peroxisomes. A survey of the recently released complete *S. cerevisiae* genome with ALDP, PMP70, and Pxa1p sequences failed to detect additional homologs with smallest sum probability scores $<10^{-12}$ (for reference, these searches detect Pxa2p with scores $<10^{-45}$). Thus, in contrast to mammalian cells, *S. cerevisiae* appears to have only two peroxisomal half ABC transporters.

The first (or more N terminal) and the second NBD in ABC transporters are less similar to each other than to the corresponding NBDs in other members of a given subgroup of ABC transporters (43). This pattern predicts that the two NBDs of the PXA transporter will be more closely related to their human orthologs than to each other. A comparison of the sequence of the NBDs of the peroxisomal ABC half transporters shows that Pxa1p is more closely related to ALDP than to PMP70 (47.2% amino acid identity, compared with 43.5%, respectively). By contrast, the sequence of the Pxa2p NBD is slightly more similar to that of PMP70 than to that of ALDP (47.5% identity compared with 46.6%, respectively). As expected, the similarity between Pxa1p and Pxa2p NBDs is less (35.5% identity) than with either ALDP or PMP70. These sequence comparisons suggest that Pxa2p may be the yeast ortholog of PMP70. This reasoning together with our demonstration of the heterodimeric structure of the PXA transporter, suggests that ALDP and PMP70 may be subunits of the same transporter. If this was the case, however, an ALD phenotype might be expected from mutations in the PMP70 gene. Virtually all ALD patients studied to date have mutations in ALDP (11–13). Moreover, the two patients identified with PMP70 mutations had Zellweger syndrome, a more severe phenotype with impaired assembly of the peroxisome (14). A possible explanation for this disparity is that like the *white* protein of *Drosophila*, PMP70 may be the more promiscuous subunit, capable of dimerization with more than one partner. In that case, mutations in the PMP70 gene would have more profound consequences and yield a clinical phenotype distinct from that caused by mutations in ALDP.

Note Added in Proof. While our paper was in press, a report from Hetttema *et al.* (44) was published describing results similar to ours localizing Pxa2p (which they designate Pat1p) to the peroxisomal membrane and confirming its role in fatty acid oxidation. They hypothesize that Pat1p together with Pat2p (their designation for

Pxa1p) heterodimerize to form a peroxisomal fatty acyl-CoA transporter.

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