Characterization and Analysis of Conserved Motifs in a Peroxisomal ATP-binding Cassette Transporter*

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The adrenoleukodystrophy protein (ALDP) and the 70-kDa peroxisomal membrane protein are half ATP-binding cassette (ABC) transporters in the human peroxisome membrane. Both are implicated in genetic disorders of peroxisome biogenesis and function. Proteins homologous to ALDP and the 70-kDa peroxisomal membrane protein have been discovered in other eukaryotic organisms and form a growing group of peroxisomal half ABC transporters. Amino acid sequence alignment of these and other ABC transporters reveals several protein motifs that are highly conserved both in sequence and location. Here we characterize two of these, designated the EAA-like and the loop1 motifs. We study them by introducing missense mutations in Pxa1p, a *Saccharomyces cerevisiae* ortholog of ALDP, and show that both motifs are important for Pxa1p function. Interestingly, missense mutations in corresponding amino acids in ALDP cause adrenoleukodystrophy in humans. We conclude that these motifs are important for ABC transporter function and that the yeast protein Pxa1p is a useful system for understanding the molecular basis of adrenoleukodystrophy.

ATP-binding cassette (ABC)[1] 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 transmembrane domain (TMD) with multiple transmembrane (TM) segments and a nucleotide binding domain (NBD) with Walker A and B consensus motifs (3). Mammalian ABC transporters are found either as complete transporters (*e.g.* the multiple drug resistance (MDR) transporter and the cystic fibrosis transmembrane regulator proteins) or as half transporters (*e.g.* the TAP1 and TAP2 proteins, which dimerize to form the active TAP transporter) (4).

Two half ABC transporters have been identified in the human peroxisome membrane: the adrenoleukodystrophy protein (ALDP) and the 70-kDa peroxisomal membrane protein (PMP70) (5, 6). Mutations in the adrenoleukodystrophy gene cause X-linked adrenoleukodystrophy, an inborn error of peroxisomal β-oxidation of very long chain fatty acids (7, 8). We identified mutations in the PMP70 genes of two patients with Zellweger syndrome (9), an inborn error of peroxisome biogenesis, although the role PMP70 plays in this disease remains uncertain (10). *PXA1* and *YKL741* are *Saccharomyces cerevisiae* genes that encode homologs of ALDP and PMP70. Pxa1p, an ortholog of ALDP, is involved in peroxisomal β-oxidation of fatty acids (11). *YKL741*, an open reading frame found by the yeast genome sequencing project (12), also encodes a half ABC transporter with high similarity to ALDP and PMP70. We have reported genetic evidence suggesting that the *YKL741* protein heterodimerizes with Pxa1p (11) and, in recent work, have shown that it has the expected peroxisome association.[2] For these reasons, we designated the *YKL741* gene as *PXA2*. However, the exact function(s) and physical interaction(s) of both the human and yeast peroxisomal ABC transporters are still unclear.

EAA motifs are conserved sequences of approximately 30 residues between TM4 and TM5 of prokaryotic ABC transporters (13–15). Alignment of prokaryotic ABC transporters reveals several residues that are highly conserved in the core of this motif (13, 14), and missense mutations altering one of these, a central glycine, result in loss of function in bacterial transporters (14). Sequence analysis of bacterial EAA motifs suggested that they predict substrate specificity (13). We recently reported that certain eukaryotic ABC transporters possess a 15-amino acid motif resembling the central core of the prokaryotic EAA motif that we designated an EAA-like motif (11). Mutations in a conserved glutamic acid residue in the EAA-like motif of the gene encoding ALDP have been reported in four unrelated adrenoleukodystrophy patients (8, 16–18). A deletion of 19 amino acids in this region of cystic fibrosis transmembrane regulator influences the stability of Cl^- channel conductance (19).

In *S. cerevisiae*, fatty acid β-oxidation takes place only in the peroxisomes (20). Yeast *pxa1* mutants have impaired growth on oleic acid medium and reduced ability to oxidize oleate (11). Wild type growth on oleic acid and β-oxidation of oleate can be restored by expressing the wild type *PXA1* gene in the *pxa1::URA3* mutant yeast (11). We have used this expression system to assess the functional consequences of missense mutations in the EAA-like motif and in a newly characterized loop1 motif on eukaryotic ABC transporter function.

EXPERIMENTAL PROCEDURES

Strains—We used a *pxa1::URA3* mutant of *S. cerevisiae* CH1305 (11) as our control strain. All wild type and mutated *PXA1* genes were expressed in this strain. Plasmids were propagated in *Escherichia coli* DH5α. Phage were propagated in *E. coli* MV1190 except to obtain uracil containing DNA, in which case *E. coli* CJ236 was used.

Yeast Culture Conditions—All yeast media were as described (11,

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[1] The abbreviations used are: ABC, ATP-binding cassette; ALDP, adrenoleukodystrophy protein; PMP70, 70-kDa peroxisomal membrane protein; MDR, multiple drug resistance; NBD, nucleotide binding domain, TM, transmembrane; TMD, transmembrane domain; C, conserved.

[2] N. Shani and D. Valle, manuscript in preparation.

21). For growth on oleic acid medium, we cultured yeast to logarithmic phase in YPD and then pelleted and washed the cells in sterile water. 10-fold dilutions were then plated on fresh oleic acid medium containing 0.1% oleic acid and 0.5% Tween-40.

Mutagenesis and Plasmids—For expression of wild type and mutated PXA1 genes in yeast, we used the low copy number plasmid, pSG448-*PXA1* (11). For site-directed mutagenesis, we subcloned a *Bam*HI–*Mun*I DNA fragment from the pSG448-*PXA1* construct (containing 1410 base pairs upstream of the *Mun*I site in the *PXA1* gene) into the *Bam*HI and *Eco*RI sites of the M13mp18 vector. The *Eco*RI/*Mun*I junction was then corrected by site-directed mutagenesis to generate a full *Mun*I restriction site. All site-directed mutagenesis was carried out in this M13-*PXA1* construct using the oligonucleotides indicated in Table I as described by Kunkel *et al.* (22). The desired mutations were transferred to the pSG448-*PXA1* construct by replacing the wild type *Bam*HI–*Mun*I DNA fragment with the corresponding mutant fragment. Both DNA strands of the manipulated fragments were sequenced to confirm each mutation.

TABLE I

Synthetic oligonucleotides	Mutation
5′-CAAGAATTCGCAGGGATTCTACTCAAAATT-3′	L70G
5′-TTCCTGGTGATGTTAACTTGGCTCTC-3′	R108L
5′-CCTGGTGATGAAAACTTGGCTCTC-3′	R108K
5′-CATGATAAATAATAGCGATGAGATCGCATTTTACC-3′	E294D
5′-GATCGCATTTTACCTAGGAACAGCAGTGG-3′	Q300L
5′-GATCGCATTTTACCAACCAACAGCAGTGGAAAGG-3′	G301P
5′-CGCATTTTACCAAGCAACAGCAGTGGAAAG-3′	G301A

β-Oxidation of [^{14}C]Oleic Acid—After overnight growth in YPD, yeast were cultured for 24 h in induction medium and then transferred to induction medium with 2.5 μCi/ml [1-^{14}C]oleic acid (New England Nuclear). The amount of radiolabeled water-soluble products was analyzed after 20 h by the partition method of Folch *et al.* (23). The β-oxidation activity dependent on Pxa1p was calculated by subtracting the activity observed in yeast lacking an intact *PXA1* gene from the activity of the same mutant yeast expressing either a wild type or the indicated mutant allele of *PXA1*. In general, the β-oxidation measured in the *pxa1* mutant yeast averaged about 50% of the activity in yeast expressing the wild type *PXA1* allele.

Cellular Fractionation and Antibodies—High density organelle isolation by differential centrifugation was as described (11, 24). For immunoblot analysis (25) we used a 1:3000 dilution of a rabbit antiserum raised against a hybrid protein formed by fusing the maltose-binding protein (New England Biolabs) fused to the C-terminal 163 amino acids of Pxa1p (11).

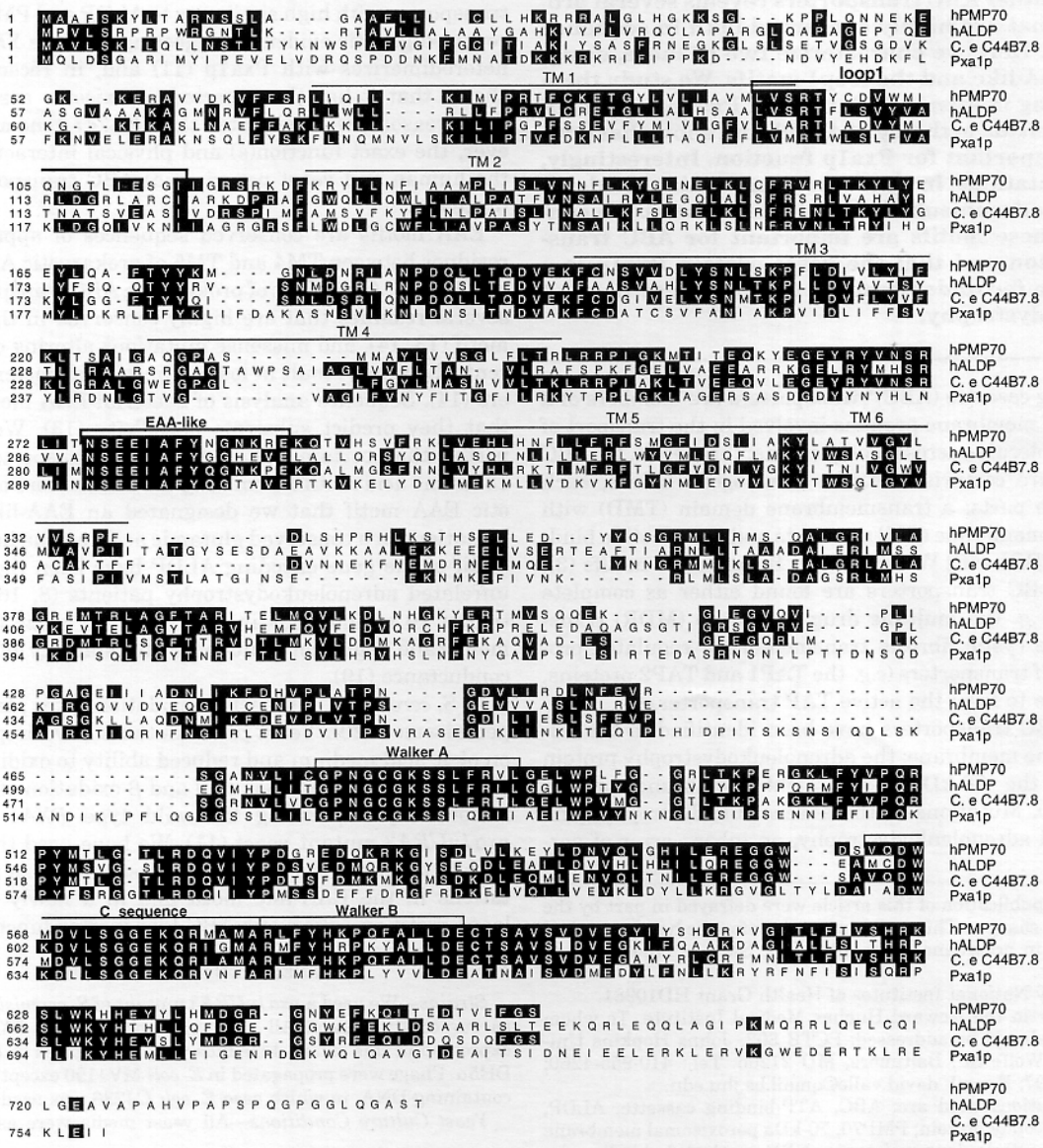

FIG. 1. **Alignment of peroxisomal ABC transporters.** The amino acid sequences of the human PMP70 and ALDP are aligned with a putative peroxisomal transporter encoded by a *C. elegans* open reading frame (*C.e C44B7.8*) and *S. cerevisiae* Pxa1p. Amino acids present in a plurality of these sequences are *boxed* in *black*. The six putative transmembrane domains (*TM*) segments are indicated by *overlines*. The loop1 and the EAA-like protein motifs as well as the Walker-A, Walker-B, and the C sequences of the nucleotide binding fold are indicated.

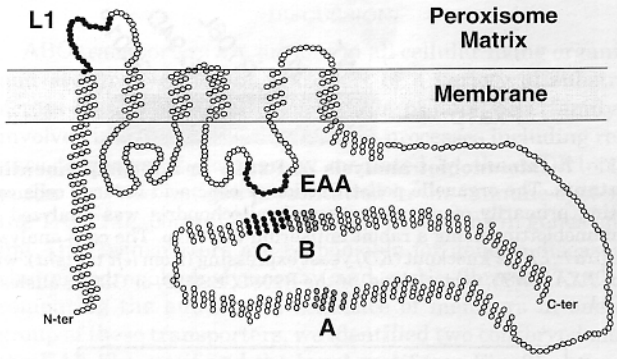

FIG. 2. **A hypothetical model of Pxa1p showing the transmembrane segments and the locations of the protein motifs.** *L1*, loop1 motif; *EAA*, EAA-like motif; *A*, Walker A motif; *B*, Walker B motif; *C*, nucleotide binding fold conserved sequence; *N-ter*; N-terminal.

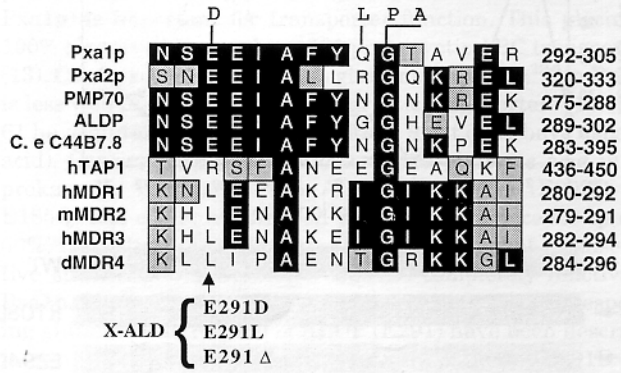

FIG. 3. **Alignment of the EAA-like motifs of eukaryotic ABC transporters and the location of mutations.** Listed from *top* to *bottom* are the *S. cerevisiae* Pxa1p, *S. cerevisiae* Pxa2p, human PMP70, human ALDP, *C. elegans* open reading frame C44B7.8 (*C.e C44B7.8*), human TAP1 (*hTAP1*), human MDR1 (*hMDR1*), murine MDR2 (*mMDR2*), human MDR3 (*hMDR3*), and *Drosophila* MDR4 (*dMDR4*) proteins. Amino acids present in a plurality of these sequences are *boxed* in *black*, and conservative amino acid substitutions (27) from this plurality are *boxed* in *gray*. Mutations in ALDP that cause adrenoleukodystrophy are indicated *below*. Missense mutations introduced in the *PXA1* gene are indicated *above*.

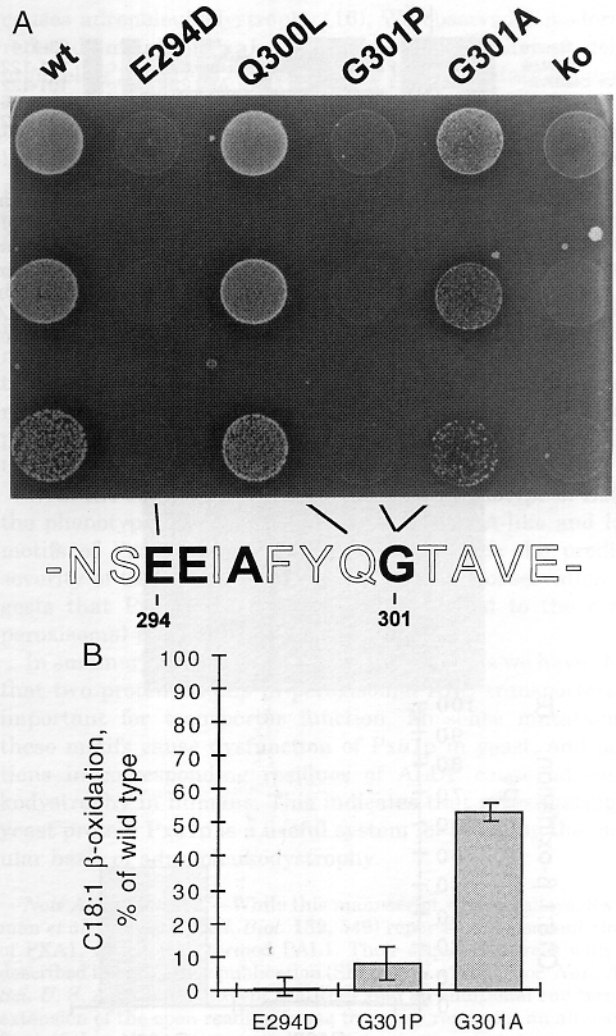

FIG. 4. **Phenotype of missense mutations in the EAA-like motif of Pxa1p.** *A*, growth of *pxa1::LEU2* knockout (*ko*) yeast transformed with (from *left* to *right*): wild type, E294D, Q300L, G301P, or G301A alleles of PXA1 or vector alone. 10-fold dilutions (*top* to *bottom*) of the transformants were inoculated on medium with 0.1% oleic acid as a sole carbon source. The amino acid sequence of the EAA-motif from Pxa1p is indicated on the *bottom* with conserved residues in *bold letters*. *B*, β-oxidation of [^{14}C]oleic acid by *pxa1::LEU2* knockout yeast expressing from *left* to *right*: E294D, G301P, or G301A alleles of PXA1.

RESULTS

Conserved Protein Motifs in Peroxisomal ABC Transporters—A data base search for homologs of the human ALDP and PMP70, using the BLAST algorithm (BLASTP 1.4.7MP) (26) detected several other peroxisomal ABC transporters. Three are rodent orthologs of ALDP and PMP70 (mouse and rat), two are the yeast proteins Pxa1p and Pxa2p, and two are putative peroxisomal proteins encoded by *Caenorhabditis elegans* genes recently entered into the data base (C44B7.8 and C44B7.9). Interestingly, an open reading frame from the cyanobacterium *Synechocystis* sp. (D64002) also codes for a half ABC transporter with high homology to the peroxisomal proteins (28.7% of the amino acids identify to the human ALDP). To identify conserved motifs we aligned representatives of the human, rodent, *C. elegans*, and *S. cerevisiae* proteins (Fig. 1). As with other ABC transporters, these proteins exhibit the highest similarity in the region surrounding the NBD (1, 2). The alignment also detected other blocks of conserved amino acid sequences, and we selected two for further investigation. One of them, the EAA-like motif, has the next highest similarity after the NBD and is located between TM4 and TM5. The second, a region we named the loop1 motif, is a conserved amino acid sequence located just C-terminal to TM1. The location of these motifs in a model of Pxa1p is shown in Fig. 2.

Characterization of the EAA-like Protein Motif—Fig. 3 shows alignment of EAA-like motifs from several eukaryotic ABC transporters (from human, mouse, *C. elegans*, *Drosophila*, and yeast) including five from the peroxisomal family of ABC transporters and four from the MDR family. As in prokaryotes, the most conserved amino acids are the central alanine and glycine separated by three less conserved residues. Because substitutions for the central glycine cause transporter dysfunction in bacteria (14), we chose to mutate the corresponding glycine (Gly^{301}) in the EAA-like motif of Pxa1p, making the evolutionarily severe and conservative substitutions G301P and G301A, respectively (27) (Fig. 3). Additionally, we changed the conserved glutamic acid (Glu^{294}) to aspartic acid (E294D). The corresponding residue in ALDP (Glu^{291}) is a site of recurrent mutations causing adrenoleukodystrophy, including one example of the same conservative missense mutation (E291D) (8, 16–18) (Fig. 3). We also changed a nonconserved glutamine residue adjacent to the conserved central glycine 301 to leucine (G300L) (Fig. 3).

Analysis of the EAA-like Protein Motif—To study the effect of these mutations on Pxa1p function, we expressed the mutant PXA1 alleles in *pxa1::URA3* cells and analyzed their ability to

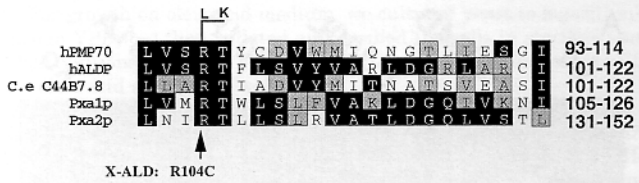

FIG. 5. **Alignment of loop1 motifs and position of mutations.** Listed from *top* to *bottom* are PMP70 (*hPMP70*) and ALDP (*hALDP*), the *C. elegans* open reading frame C44B7.8 (*C.e C44B7.8*), Pxa1p, and Pxa2p. Amino acids present in a plurality of these sequences are *boxed* in *black*, and conservative amino acid substitutions (27) from this plurality are *boxed* in *gray*. Missense mutations produced in the *PXA1* gene are indicated *above*. A mutation in ALDP that causes adrenoleukodystrophy is indicated *below*.

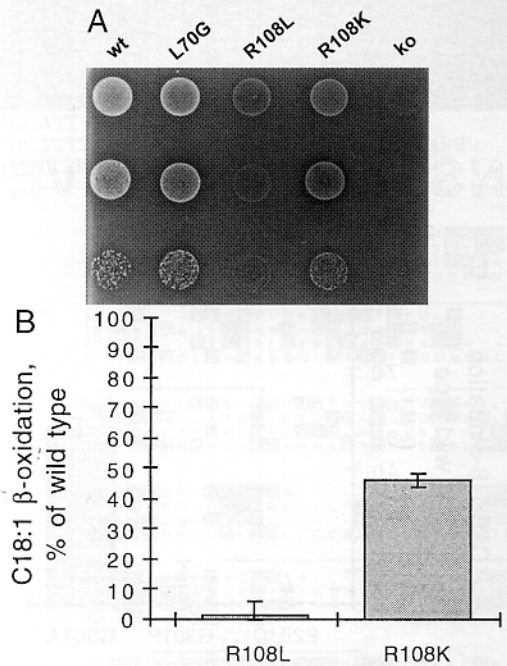

FIG. 6. **Phenotype of missense mutations in the loop1 motif.** *A*, phenotypes of *pxa1::LEU2* knockout (*ko*) yeast expressing (from *left* to *right*): wild type (*wt*), L70G, R108L, and R108K alleles of PXA1 or vector alone. 10-fold dilutions (*top* to *bottom*) of the different cells were inoculated on medium containing 0.1% oleic acid as a sole carbon source. *B*, β-oxidation of [^{14}C]oleic acid by *pxa1::LEU2* knockout yeast expressing R108L or R108K alleles of PXA1.

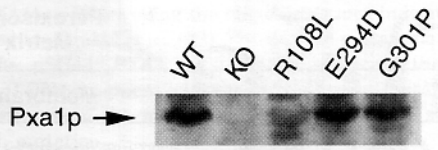

FIG. 7. **Immunoblot analysis of Pxa1p in noncomplementing mutants.** The organelle pellet fraction of oleic acid-induced cells consisting primarily of peroxisomes and mitochondria was analyzed by immunoblotting using a rabbit antiserum to Pxa1p. The cells analyzed are *pxa1::LEU2* knockout (*KO*) yeast expressing (from *left* to *right*): wild type *PXA1* (*WT*), vector alone, or the R108L, E294D, or G301P alleles of PXA1.

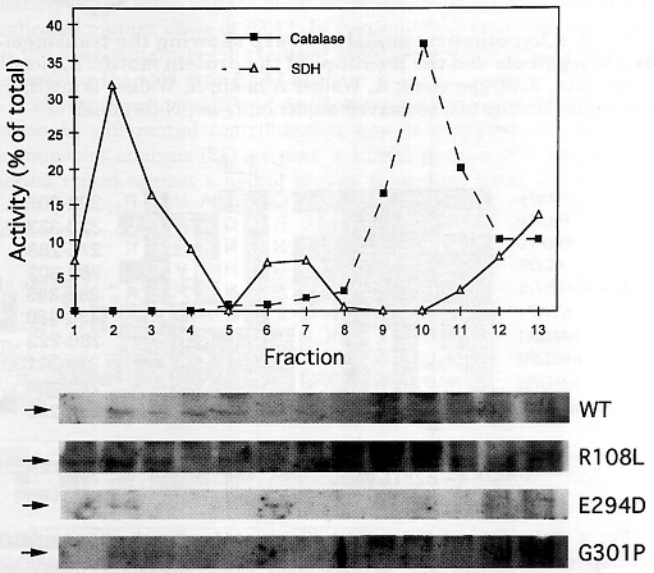

FIG. 8. **Localization of mutant Pxa1ps to peroxisomes.** *pxa1::LEU2* mutant yeast expressing the wild type (*WT*) or the indicated *PXA1* alleles (R108L, E294D, and G301P) were induced by oleic acid. The high density organelle pellets consisting primarily of peroxisomes and mitochondria were fractionated on Nycodense gradients. Gradient fractions were assayed for a peroxisome marker (*Catalase*) and a mitochondrial marker (succinate dehydrogenase (*SDH*)) activity. Only the profile of the fractionation of yeast expressing the wild type *PXA1* allele is shown. All others had a similar profile with the peaks of catalase and SDH activity in the same fractions as the wild type (data not shown). Shown *below* are immunoblots in which equal volumes of fractions from each of the indicated gradients were probed with a rabbit antiserum to Pxa1p. The position of Pxa1p is indicated by an *arrow*.

restore growth on oleic acid medium and to β-oxidize radiolabeled oleate. The results from these two assays were in good agreement. The E294D and G301P mutations resulted in virtually complete loss of Pxa1p function in both growth and β-oxidation assays; the G301A allele conferred an intermediate phenotype in both (Fig. 4, *A* and *B*). By contrast, the phenotype of the Q300L allele was indistinguishable from wild type (Fig. 4*A*).

Characterization of the loop1 Protein Motif—Fig. 5 shows an alignment of putative loop1 motifs in five peroxisomal ABC transporters. Three residues (Leu^{105}, Arg^{108}, and Thr^{109} in Pxa1p) are conserved in all five proteins (Fig. 5). The corresponding arginine in ALDP (R104) is the site of a mutation causing adrenoleukodystrophy (16) (Fig. 5). To study if the loop1 motif is important for Pxa1p function, we mutated the arginine 108 codon to encode either leucine or lysine as evolutionarily severe or conservative substitutions, respectively (27). We also changed a nonconserved amino acid near the loop1 motif (L70) to glycine because in previous work we suggested that this residue might play a role in fatty acid binding (11)

Analysis of the loop1 Protein Motif—Alterations of the conserved loop1 arginine have an effect on Pxa1p function (Fig. 6); the R108L mutation has a null phenotype, as measured by growth on oleic acid and the β-oxidation assay; R108K has an intermediate phenotype in both assays (approximately 50% of wild type), whereas the growth phenotype of L70G was indistinguishable from wild type (Fig. 6) (β-oxidation not performed).

Pxa1p Is Expressed in the Noncomplementing Mutants—To determine if the severe consequences of the R108L, E294D, and G301P mutations were due to instability or mistargeting of the mutant Pxa1p, we performed immunoblots of high density organelle pellets from cells expressing these alleles. In all three cases the mutant Pxa1p is detectable; the amount of G301P and E294D Pxa1p were equivalent to wild type, whereas the amount of R108L Pxa1p was reduced but consistently detectable (Fig. 7). High density organelle pellets of yeast consist mainly of peroxisomes and mitochondria (24). To determine if the mutant proteins were correctly targeted, we centrifuged the high density organelle pellet on Nycodense gradients. The distribution of the mutant proteins was identical to that of the wild type Pxa1p and, with the activity of catalase, a peroxisomal marker (Fig. 8). The results indicate that the mutant forms of Pxa1p are correctly targeted to peroxisomes.

DISCUSSION

ABC transporters are present in all cellular living organisms and are involved in the transport of a variety of substrates across membranes. Members of this protein superfamily are involved in many medically relevant processes including resistance to cancer chemotherapy mediated by the MDR1 transporter (28) and antigen presentation in the immune system by the TAP transporter (29). Moreover, mutations in genes encoding ABC transporters are responsible for genetic diseases including adrenoleukodystrophy and cystic fibrosis (7, 30). By comparing the amino acid sequence of members in one subgroup of these transporters, we identified two conserved motifs, the EAA-like motif and the loop1 motif (see Fig. 2).

We utilized site-directed mutagenesis in the gene encoding Pxa1p, a yeast peroxisomal ABC transporter, to further analyze these motifs. We found that as in prokaryotic ABC transporters, the central glycine in the EAA-like motif (Gly^{301} of Pxa1p) is important for transporter function. This glycine is 100% conserved in a survey of 61 prokaryotic ABC transporters (13). Conversely, the consensus glutamic acid (Glu^{294} in Pxa1p) is less well conserved in the prokaryotic transporters (16 out of 61 have glutamic acid at this position; 9 out of 61 have aspartic acid). Conservative substitutions of this glutamic acid in the prokaryotic PstC and PstA ABC transporters (E202Q and E185Q) had either no effect on transport or reduced it by only 50%, respectively (31). Despite this, we found that a conservative substitution at this site (E294D) completely inactivates Pxa1p. Similarly, missense mutations altering the corresponding glutamic acid residue of ALDP (E291) have been described in two unrelated adrenoleukodystrophy patients (E291D and E291L) (8, 16–18).

Several recent studies suggest that the substrate binding site of ABC transporters is located in the TMD (1, 32–34). In contrast, Sheps *et al.* (33) and Beaudet *et al.* (30) found that missense mutations in the NBD conserved (C) sequence also influence substrate specificity. The C sequence is a 19-mer located immediately N-terminal to the Walker B motif of the NBD and is a hallmark of ABC transporters (1, 30). Because there is interaction between the TMD and the NBD (36), it is possible that the C sequence mediates this interaction and exerts its effect on substrate specificity in this manner. Gottesman *et al.* (37) have recently suggested that the C sequence of MDR1 interacts with the TMD. The exact function of the EAA motif is not known. However, based on the evolutionary conservation in prokaryotes, Kerppola and Ames suggested that the EAA motif was the segment of the TMD that interacts with the NBD (15). Moreover, Saurin *et al.* (13) presented evidence that the substrate specificity of prokaryotic ABC transporters can be predicted by sequence variation in their EAA-motifs. Our results suggest that the EAA-like motif of peroxisomal ABC transporters is important for function but not for stability or targeting. Similar results have been described for prokaryotic EAA motifs (14). Watkins *et al.* (8), however, found that the E291D allele of ALDP was antigen negative by immunohistochemistry. As shown in Fig. 3, some sequence conservation in this region is specific for certain groups of eukaryotic ABC transporters. Taking all these results together, we suggest that the C region of the NBD interacts with the TMD through the EAA and EAA-like motifs.

We also recognized a conserved sequence in the region between TM1 and TM2 that we designated the loop1 motif. Three residues (Leu^{105}, Arg^{108}, and Thr^{109} in Pxa1p) are completely conserved in our alignment of peroxisomal ABC transporters. Changing Arg^{108} to either leucine or lysine reduces Pxa1p function. This result agrees with the observation that a missense mutation in the corresponding residue in ALDP (Arg^{104}) causes adrenoleukodystrophy (16). We observed a moderately reduced amount of Pxa1p (approximately 20%) in high density organelles from cells expressing the R108L mutation, suggesting that this mutation does influence Pxa1p targeting or stability. A role for the loop1 motif in targeting is also suggested by the report that an N-terminal fragment of the ABC transporter Ste6p functions as a signal sequence (38). Interestingly, in addition to TM1 of Ste6p, this fragment also contains the first loop between TM1 and TM2. Leucine and arginine residues are positioned similarly relative to TM1 in both Ste6p (Leu^{47} and Arg^{50}) and Pxa1p (Leu^{105} and Arg^{108}). If the loop1 motif is important for correct cellular placement, its function may involve orientation of the protein in the membrane rather than targeting because Ste6p is targeted to the endoplasmic reticulum (39) rather than the peroxisome. The importance of positive residues in membrane protein loops for correct insertion in the membrane is well recognized (40).

With the striking exception of the null phenotype of E294D, the phenotypes of mutations in both the EAA-like and loop1 motifs of Pxa1p were closely correlated with the predicted severity of the amino acid substitution. This observation suggests that Pxa1p function is tightly coupled to the overall peroxisomal β-oxidation process in yeast.

In summary, using site-directed mutagenesis we have shown that two protein motifs in peroxisomal ABC transporters are important for transporter function. Missense mutations in these motifs cause dysfunction of Pxa1p in yeast, and mutations in corresponding residues of ALDP cause adrenoleukodystrophy in humans. This indicates that investigating the yeast protein Pxa1p is a useful system for studying the molecular basis of adrenoleukodystrophy.

Note Added in Proof—While this manuscript was in galleys, Swartzman *et al.* ((1996) *J. Cell. Biol.* **132,** 549) reported independent cloning of PXA1, which they termed PAL1. Their sequence agrees with that described in our earlier publication (Shani *et al.* (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6012) except that they find an additional end terminal extension of the open reading frame that may represent an alternative form of the protein.

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