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The peroxisome biogenesis disorder group 4 gene, *PXAAA1*, encodes a cytoplasmic ATPase required for stability of the PTS1 receptor

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In humans, defects in peroxisome assembly result in the peroxisome biogenesis disorders (PBDs), a group of genetically heterogeneous, lethal recessive diseases. We have identified the human gene *PXAAA1* based upon its similarity to *PpPAS5*, a gene required for peroxisome assembly in the yeast *Pichia pastoris*. Expression of *PXAAA1* restored peroxisomal protein import in fibroblasts from 16 unrelated members of complementation group 4 (CG4) of the PBD. Consistent with this observation, CG4 patients carry mutations in *PXAAA1*. The product of this gene, Pxaaa1p, belongs to the AAA family of ATPases and appears to be a predominantly cytoplasmic protein. Substitution of an arginine for the conserved lysine residue in the ATPase domain of Pxaaa1p abolished its biological activity, suggesting that Pxaaa1p is an ATPase. Furthermore, Pxaaa1p is required for stability of the predominantly cytoplasmic PTS1 receptor, Pxr1p. We conclude that Pxaaa1p plays a direct role in peroxisomal protein import and is required for PTS1 receptor activity.

Keywords: AAA protein/expressed sequence tag/protein import/Zellweger syndrome

Introduction

Peroxisome biogenesis disorders (PBDs), including Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease and classical rhizomelic chondrodysplasia punctata, are autosomal recessive disorders with an aggregate incidence of approximately 1/50 000 live births. Their phenotypes include moderate to severe neurological and hepatic dysfunction, often leading to death within the first year (Lazarow and Moser, 1995). At the cellular level, PBD patients exhibit a defect in the import of one or more classes of peroxisomal matrix proteins into the organelle (Motley *et al.*, 1994; Slawecki *et al.*, 1995). Somatic cell fusion complementation analysis indicates that the PBDs can be caused by defects in any of at least 10 different genes (Shimozawa *et al.*, 1993; Moser *et al.*, 1995). Because these disorders are associated with a peroxisomal protein sorting defect, a major focus of PBD research has become the elucidation of the molecular mechanisms of protein import into peroxisomes.

Peroxisomal targeting signals (PTSs) are discrete amino acid sequences capable of directing proteins to the peroxisome. The majority of peroxisomal matrix proteins contain PTS1, a C-terminal tripeptide of the sequence SKL-COOH (single letter code) or a conservative variant (Gould *et al.*, 1989; de Hoop and Ab, 1992). In contrast, the N-terminal PTS2 consists of ~10 amino acids and has only been observed in three peroxisomal proteins (Osumi *et al.*, 1991; Swinkels *et al.*, 1991; Gietl *et al.*, 1994; Faber *et al.*, 1995). The only known human PTS2-containing protein is thiolase. Additional types of PTS have been reported (Small *et al.*, 1988; Kragler *et al.*, 1993), but their precise characteristics are not yet known. However, a PTS is not an absolute requirement for entry into the peroxisome lumen. Studies by Glover *et al.* (1994) and McNew and Goodman (1994) have revealed that proteins lacking a PTS can be imported by oligomerization with a PTS-containing protein. The observation that PTS1-coated gold particles can be imported into the peroxisome (Walton *et al.*, 1995) also raises the possibility that the translocation machinery can accommodate fully folded proteins.

Studies of the peroxisomal protein import capabilities of PBD cell lines (Motley *et al.*, 1994; Slawecki *et al.*, 1995) and yeast *pas/per/pay/peb* mutants (Erdmann *et al.*, 1989; Cregg *et al.*, 1990; Gould *et al.*, 1992; Nuttley *et al.*, 1993) have enriched our understanding of the protein import process and led to the description of five general classes of peroxisome assembly mutants. These may be defective in the PTS1 receptor (McCollum *et al.*, 1993; Van der Leij *et al.*, 1993; Dodt *et al.*, 1995), the PTS2 receptor (Marzioch *et al.*, 1994; Motley *et al.*, 1994; Slawecki *et al.*, 1995; Zhang and Lazarow, 1995), the translocation apparatus (Kalish *et al.*, 1995, 1996; Slawecki *et al.*, 1995; Gould *et al.*, 1996), factors required for high-efficiency protein import (Heyman *et al.*, 1994; Slawecki *et al.*, 1995) or factors involved in peroxisome membrane biogenesis (C.C.Chang, G.Dodt and S.J.Gould, in preparation). In humans and in the yeast *Pichia pastoris*, the first four classes of mutants contain peroxisome ghosts (Santos *et al.*, 1988). These ghosts consist of peroxisomal membranes containing integral and peripheral membrane proteins, but are unable to import the full complement of matrix proteins. In addition to the two PTS receptors, these studies have led to the identification of two AAA ATPases (Erdmann *et al.*, 1991; Spong and Subramani, 1993; Voorn-Brouwer *et al.*, 1993; Heyman *et al.*, 1994), a ubiquitin-conjugating enzyme (Wiebel and Kunau, 1992; Crane *et al.*, 1994), a farnesylated protein (Kunau *et al.*, 1993; James *et al.*, 1994), three zinc-binding integral membrane proteins (Tsukamoto *et al.*, 1991; Kunau *et al.*, 1993; Kalish *et al.*, 1995, 1996; Tan *et al.*, 1995) and several other proteins involved in peroxisome assembly (Kunau *et al.*, 1993).

The most extensively studied of these factors has been

the PTS1 receptor, encoded by *PXR1* in humans (Dodt *et al.*, 1995; Fransen *et al.*, 1995; Wiemer *et al.*, 1995), *PpPAS8* in *P.pastoris* (McCollum *et al.*, 1993) and *ScPAS10* in *Saccharomyces cerevisiae* (Van der Leij *et al.*, 1993). These orthologs contain eight tetratricopeptide repeats (TPRs) which comprise the C-terminal half of the protein and form the PTS1-binding domain (Brocard *et al.*, 1994; Dodt *et al.*, 1995). In humans, two functionally distinct isoforms of the PTS1 receptor are generated by alternative splicing of a single exon upstream of the TPR domains (Braverman *et al.*, 1996). Both isoforms are capable of rescuing the PTS1 protein-import defect of cells carrying mutations in *PXR1*. However, only the long form is essential for import of PTS2 proteins. We have found that the PTS1 receptor in both yeast and human cells is a predominantly cytoplasmic protein, and only associates with peroxisomes to a small extent at steady state (Dodt *et al.*, 1995; Gould *et al.*, 1996; G.Dodt and S.J.Gould, in preparation). Furthermore, the PTS1 receptor in human cells cycles between the cytoplasm and peroxisome, suggesting that it may act as a chaperone, recognizing newly synthesized PTS1-containing proteins in the cytoplasm and subsequently guiding them to the peroxisome (G.Dodt and S.J.Gould, in preparation).

PXR1 was originally identified by searching for human expressed sequence tags (ESTs) similar to the yeast *PAS8* gene (Dodt *et al.*, 1995). The continued application of this approach has led to the cloning and characterization of *PXAAA1*, a novel human gene. Mutations in *PXAAA1* are responsible for complementation group 4 (CG4) of the PBD. Import of both PTS1 and PTS2 proteins in CG4 fibroblasts is severely impaired, whereas synthesis of peroxisomal membranes is not affected. The product of *PXAAA1*, Pxaaa1p, is a member of the AAA family of ATPases and appears to be a predominantly cytoplasmic protein. Furthermore, loss of Pxaaa1p results in a pronounced destabilization of the PTS1 receptor. These results suggest that Pxaaa1p interacts with the PTS1 receptor and plays a direct role in peroxisomal protein import.

Results

Identification of PXAAA1 as the probable human homolog of the P.pastoris PAS5 gene

To identify the genes responsible for the various complementation groups of the PBD, we used the BLAST algorithm (Altschul *et al.*, 1990) to screen the database of expressed sequence tags (Adams *et al.*, 1993) (dbEST) for human genes similar to yeast *PAS* (Peroxisome ASsembly) genes. We identified a 97 nucleotide expressed sequence tag (GenBank accession number R00950) with the capacity to encode a protein similar to the product of the *P.pastoris PAS5* gene (PpPas5p) (Spong and Subramani, 1993). PpPas5p is a putative ATPase of the AAA protein family (Confalonieri and Duguet, 1995) and is essential for normal import of proteins into peroxisomes. The deduced product of EST R00950 shared 16 identical and seven similar residues with amino acids 1023–1053 of PpPas5p and was more similar to PpPas5p than any other known protein. EST R00950 was also similar to the *PpPAS5* orthologs from the yeasts *S.cerevisiae* (*ScPAS8*) and *Yarrowia lipolytica* (*YlPAY4*) (Voorn-Brouwer *et al.*, 1993; Nuttley *et al.*, 1994).

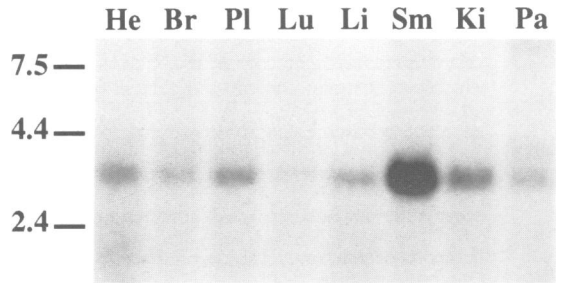

Fig. 1. *PXAAA1* is a ubiquitously expressed gene. A multiple tissue Northern blot (Clontech) was probed with [α-^{32}P]dCTP-labeled cDNA corresponding to EST R00950 using standard procedures. An ~3.5 kb mRNA for *PXAAA1* was detected in (He) heart, (Br) brain, (Pl) placenta, (Lu) lung, (Li) liver, (Sm) skeletal muscle, (Ki) kidney and (Pa) pancreas. High levels of 18S and 28S rRNAs were also detected in the muscle lane (data not shown), suggesting that unequal loading is at least partly responsible for the much stronger signal for *PXAAA1* mRNA in this lane.

Because peroxisomes are constituents of all human cells (except mature erythrocytes), PBD genes should be expressed in all tissues. A Northern blot of RNA from eight human tissues was probed with the 0.6 kb cDNA corresponding to EST R00950 (Figure 1). A single mRNA of ~3.4–3.7 kb was detected in all lanes. Next, cDNA clones for this gene were isolated from a human fetal brain cDNA library. From these, a cDNA containing the entire open reading frame (ORF) was assembled (Figure 2). We designated this gene *PXAAA1* based on (i) sequence similarity between its deduced product and the products of the PeroXisome assembly genes *P.pastoris PAS5*, *S. cerevisiae PAS8* and *Y.lipolytica PAY4* (probability of random match $<1e^{-111}$) and (ii) similarity with the AAA family of putative ATPases. The degree of similarity was highest over a 250 amino acid segment from the C-terminal third of the proteins which contain the conserved ATPase domain (Figure 3). The N-terminus of Pxaaa1p is 120 amino acids shorter than PpPas5p and shares little similarity. However, the orthologs from *S.cerevisiae* (Pas8p) and *Y.lipolytica* (Pay4p) also differ significantly from Pxaaa1p and PpPas5p over the N-terminal 300–400 residues of each protein.

PXAAA1 is defective in complementation group 4 of the PBD

We utilized a transient transfection assay to test whether *PXAAA1* could rescue the peroxisomal protein import defect in fibroblasts from PBD patients. A fragment of the *PXAAA1* cDNA encompassing the complete ORF was inserted downstream of the cytomegalovirus promoter in the expression vector pcDNA3. The resulting plasmid, pTY3, was transfected into fibroblasts from PBD patients. Two days after transfection, rescue was assessed by indirect immunofluorescence using an anti-SKL antibody specific for multiple PTS1-containing proteins (Gould *et al.*, 1990), or an antibody against thiolase, a PTS2 protein. We found that *PXAAA1* expression restored peroxisomal protein import in all 16 CG4 cell lines (Figure 4A–D), but not in fibroblasts from CG1, 2, 3, 7, 8, 9 or 10 (Table I). Furthermore, *PXAAA1* expression also restored the import of thiolase, a PTS2 protein (Figure 4E and F). No punctate staining for anti-SKL-reactive proteins was detected in digitonin permeabilized *PXAAA1*-

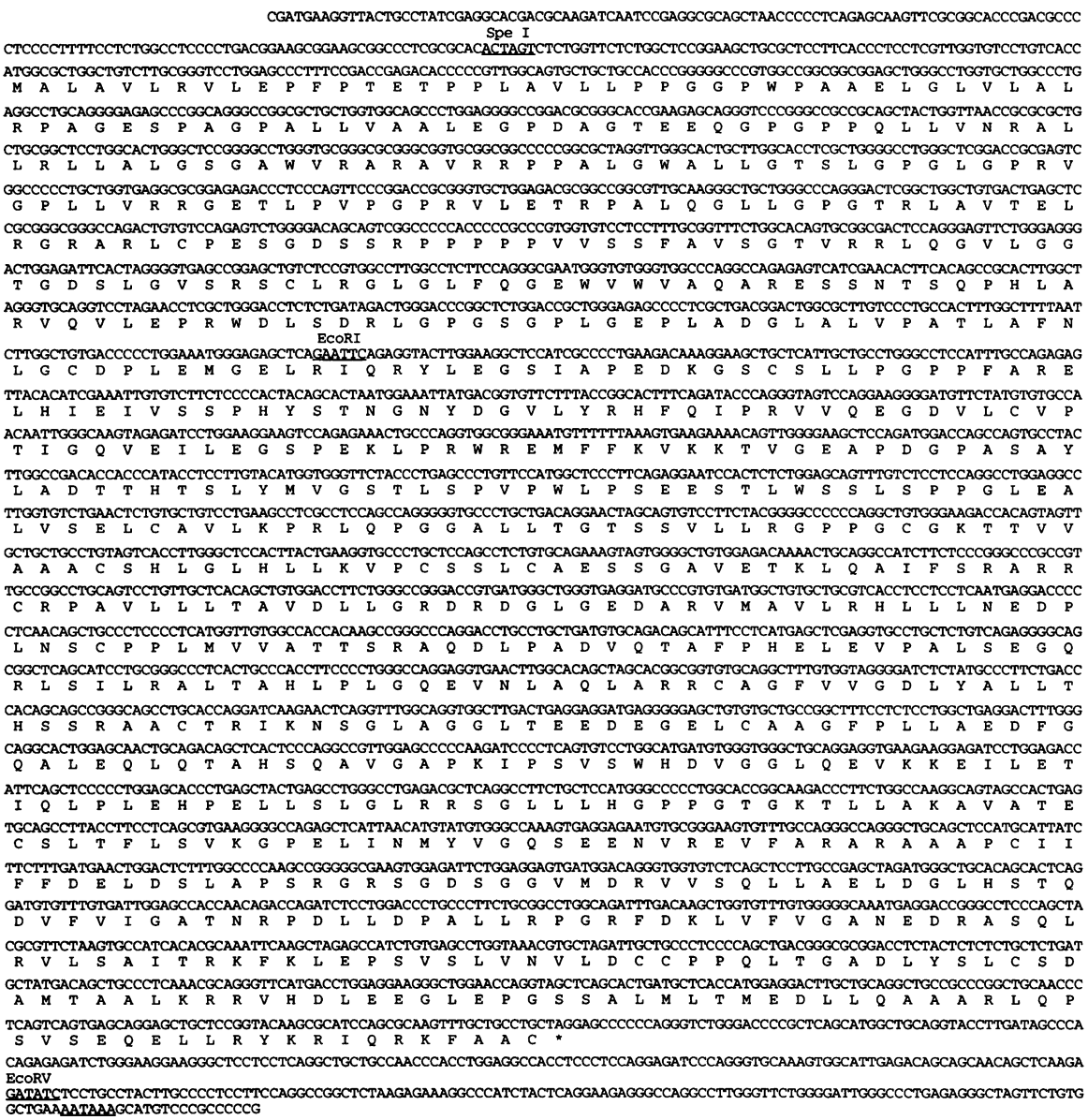

Fig. 2. Nucleotide and deduced amino acid sequences of *PXAAA1* and its product, Pxaaa1p. The first in-frame ATG of the open reading frame is surrounded by a perfect match to the Kozak consensus for mammalian initiator codons, RYYAYYATGG (Kozak, 1992). A consensus polyadenylation signal is located 16 nucleotides upstream of the site of polyadenylation and is underlined. The first nucleotide of the predicted initiator codon is designated as position 1 of the sequence. The *Spe*I, *Eco*RI and *Eco*RV sites used in subcloning are labeled. This sequence is available under GenBank accession number U56602.

expressing cells, indicating that matrix proteins were indeed sequestered within the peroxisome (Figure 4G and H). Interestingly, cells from CG4 patients normally import a small amount of both PTS1 and PTS2 proteins (Slawecki *et al.*, 1995), suggesting that the peroxisome ghosts in these cells are translocation competent.

The restoration of peroxisomal protein import in all 16 CG4 cell lines led us to search for *PXAAA1* mutations in CG4 patients. A gene-specific primer was used to synthesize *PXAAA1* cDNA from normal and CG4 fibroblasts. Fragments of the *PXAAA1* ORF were then amplified by PCR and subjected to single-strand conformational polymorphism (SSCP) analysis (Michaud *et al.*, 1992). *PXAAA1* SSCP migration variants were detected in nine of the 13 CG4 patients examined, even though only 40% of the ORF had been examined. Direct sequencing of RT-PCR-generated cDNA fragments revealed that patient PBD106 was heterozygous for an 8 bp deletion (1962–1969) and a 20 bp deletion (2398–2417)/single T insertion. Direct sequencing of amplified genomic DNA revealed that one allele contained a G→A transition at the –1 position of a splice acceptor site. This resulted in the use of a cryptic splice acceptor site 8 bp downstream of the normal site, generating the 8 bp deletion observed in the cDNA. The father was heterozygous for this splice site mutation. The mother was found to be heterozygous for the 20 bp deletion/1 bp insertion found on the patient's other *PXAAA1* allele. This inheritance pattern was consistent with the recessive nature of the disorder. We concluded that PBD106 is a compound heterozygote for two different mutations in *PXAAA1*.

Both of the mutations in PBD106 shifted the reading

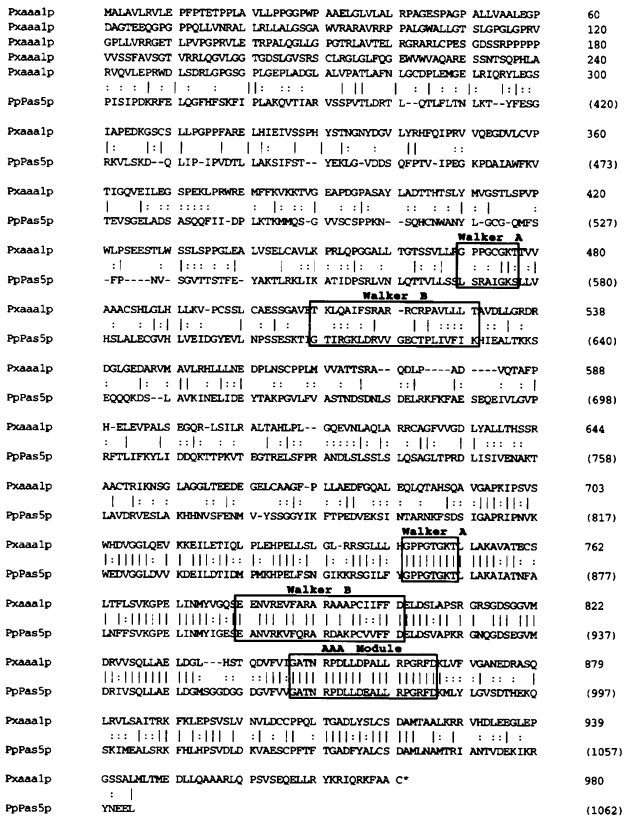

Fig. 3. Deduced amino acid sequence alignment between Pxaaa1p and PpPas5p. The two sequences (single letter code) were aligned using MacVector alignment with pam250 matrix. Vertical lines represent identities while colons represent similarities. The Walker A and B ATP-binding motifs and the AAA box are outlined.

frame of the *PXAAA1* mRNA. The product of the first allele would lack the C-terminal 326 amino acids of Pxaaa1p and instead contain the three amino acids WLD (single letter code) in their place. The product of the second allele would lack the C-terminal 181 amino acids of Pxaaa1p and instead contain the amino acids SLWPQAGGEVEILEE (single letter code). Previous studies have demonstrated that mutations which affect the reading frame of an mRNA commonly result in mRNA instability and decreased steady-state levels of mRNA (10–30% of normal) (Maquat, 1995). We observed that the steady-state level of *PXAAA1* mRNA in PBD106 was below the level of detection by Northern blot (data not shown). Furthermore, it is unlikely that the protein products would be functional since they lack large segments of the most highly conserved region of Pxaaa1p.

PXAAA1 is located on chromosome 6

To determine the chromosomal location of *PXAAA1*, genomic DNA from a panel of mouse–human hybrid cell lines (Drwinga *et al.*, 1993) was probed with the full-length *PXAAA1* cDNA. Hybridizing fragments specific for human genomic DNA were detected only in the cell lines containing human chromosome 6 (data not shown). The *PXAAA1* cDNA was also used to determine the map position of the mouse ortholog of *PXAAA1*. The inheritance of *PXAAA1* was followed by RFLP analysis of the BSS interspecific backcross DNA panel (Rowe *et al.*, 1994) (The Jackson Laboratories, Bar Harbor, ME, USA). This analysis located the murine *PXAAA1* ortholog on chromosome 17 between the markers D17/Mit16 and 1APLS1-3 (data not shown). This region of mouse chromosome 17 has homology of synteny with human chromosome 6p11–6p22. There are no known disease loci on mouse chromosome 17 near *PXAAA1*.

PXAAA1 appears to encode a predominantly cytoplasmic ATPase

In order to determine the cellular localization of Pxaaa1p, a modified form of Pxaaa1p containing the c-myc epitope tag at its C-terminus was transiently expressed in CG4 cells. Indirect immunofluorescence analysis indicated that Pxaaa1p–myc protein was distributed throughout the cytoplasm (Figure 5A). This cytoplasmic distribution was observed not only in cells expressing high levels of Pxaaa1p–myc, but also in cells expressing very low or intermediate levels of the protein. In contrast, heterologously expressed PMP70myc, an integral peroxisomal membrane protein, was detected only in peroxisomes (Figure 5B). The myc-tagged version of Pxaaa1p restored peroxisomal protein import in CG4 cells (Figure 5C and D) at the same frequency as wild-type Pxaaa1p (38% rescue for *PXAAA1–myc*, 31% for wild-type *PXAAA1*).

Amino acid sequence analysis revealed that Pxaaa1p is a member of the AAA family. These ATPases Associated with diverse cellular Activities (Confalonieri and Duguet, 1995) share a region of ~200 amino acids, including both Walker A and B nucleotide binding motifs (Walker *et al.*, 1982) and an AAA box. In order to test the importance of ATP-binding in Pxaaa1p function, we substituted an arginine for the invariant lysine residue of the Walker A motif (GXXGXGKT). This residue is essential for biological activity in other ATPases. The resultant *PXAAA1-K750R* cDNA was expressed in CG4 cells and assayed for its ability to rescue the PTS1 and PTS2 protein import defects of CG4 cell lines (Figure 6A to D). Unlike wild-type *PXAAA1*, the *PXAAA1-K750R* allele did not restore import of either PTS1 or PTS2 proteins in CG4 cells.

The peroxisome assembly factor encoded by human PXAAA1 and P.pastoris PAS5 is required for stability of the PTS1 receptor

The apparent cytoplasmic distribution of Pxaaa1p was reminiscent of the predominantly cytoplasmic distribution of Pxr1p, the PTS1 receptor. Furthermore, it suggested that Pxaaa1p might play a role in transporting newly synthesized peroxisomal proteins from the cytoplasm to the peroxisome (also the postulated role for Pxr1p). Although no physical association between Pxaaa1p and Pxr1p has yet been detected, we observed that the levels of Pxr1p were reduced to <15% of normal in CG4 patients (Figure 7A) but were not reduced in the CG10 cell line PBD094 (data not shown). Pulse–chase experiments revealed a significantly shorter half-life for Pxr1p in CG4 cell lines (Figure 7B; $t_{1/2}$ = 1 h in CG4 cells, 6 h in normal fibroblasts), demonstrating that the reduced steady-state levels of Pxr1p are caused, at least in part, by an increased rate of degradation.

If the instability of Pxr1p in CG4 cells is indicative of an interaction between Pxr1p and Pxaaa1p, a similar interaction would be expected for the yeast orthologs of

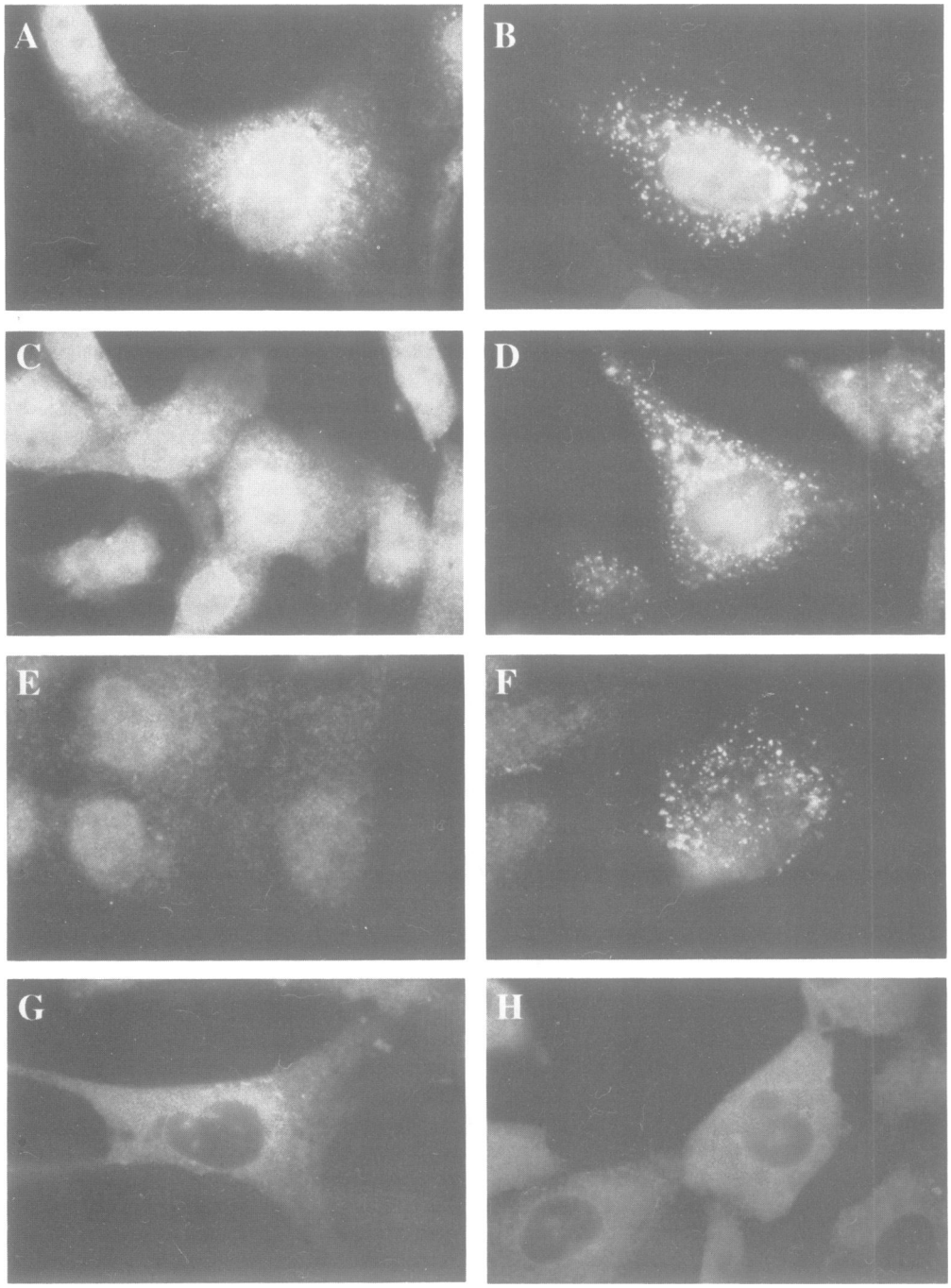

Fig. 4. Heterologous expression of *PXAAA1* rescues the PTS1 protein import defect of CG4 cell lines. The CG4 cell line PBD108 was transfected with (**A**) pcDNA3 alone or (**B**) pTY3, the *PXAAA1* expression vector. Two days post-transfection the cells were processed for indirect immunofluorescence by permeabilizing the fixed cells with 1% Triton X-100 and using anti-SKL antibodies to detect PTS1-containing proteins (Gould *et al.*, 1990). PBD105 cells were also transfected with (**C**) pcDNA3 and (**D**) pTY3 and processed for indirect immunofluorescence with anti-SKL antibodies. PBD108 cells transfected with (**E**) pcDNA3 and (**F**) pTY3 were stained with anti-thiolase antibodies, demonstrating that *PXAAA1* also rescued the PTS2 protein import defect of CG4 cells. No punctate staining was found in *PXAAA1*-transfected (**G**) PBD108 and (**H**) PBD105 cells processed for immunofluorescence by permeabilization with digitonin instead of Triton X-100. Detection of the fluorescein-labeled goat anti-rabbit secondary antibodies was performed with an Olympus fluorescence microscope.

these proteins. Wild-type, *pas5* and *pas6* cells of *P.pastoris* (Gould *et al.*, 1992) were incubated in methanol-containing medium for 16 h. Equal amounts of total cell protein were separated by SDS–PAGE and the levels of Pas8p were determined by immunoblot. Steady-state levels of Pas8p were reduced to <30% of wild-type in the *pas5* mutant but were not reduced in the *pas6* mutant (Figure 7C). In order to further address the issue of functional identity between human *PXAAA1* and *P.pastoris PAS5*, we expressed human *PXAAA1* in the *P.pastoris pas5* mutant. Although *PXAAA1* was unable to rescue any of the *pas5* phenotypes (data not shown), the significance of this non-complementation remains to be determined. Cross-species complementation of *pas* mutants with *PAS* or PBD genes has been attempted in several instances with no success (Heyman *et al.*, 1994; Nuttley *et al.*, 1994; Dodt *et al.*, 1995; van der Klei *et al.*,

Table I. Pxaaa1 rescue of PBD import defects

Complementation group	Patient	Rescue?	
		Yes/no	%
CG1	PBD121	no	0
CG2	PBD005	no	0
	PBD018	no	0
CG3	PBD095	no	0
	PBD006	no	0
CG4	PBD010	yes	21
	PBD011	yes	n.d.
	PBD039	yes	27
	PBD041	yes	12
	PBD048	yes	23
	PBD049	yes	28
	PBD101	yes	18
	PBD102	yes	48
	PBD103	yes	32
	PBD104	yes	31
	PBD105	yes	36
	PBD106	yes	26
	PBD107	yes	26
	PBD108	yes	31
	PBD115	yes	30
	PBD119	yes	28
CG7	PBD054	no	0
CG8	PBD111	no	0
CG9	PBD061	no	0
CG10	PBD094	no	0

1995), even between the closely related yeasts *P.pastoris* and *Pichia angusta* (*Hansenula polymorpha*).

Discussion

We have identified *PXAAA1* based on its similarity to the *P.pastoris PAS5* gene. Transient expression of *PXAAA1* rescued the peroxisomal protein import defects of cells from all 16 CG4 patients and led to the discovery that mutations in *PXAAA1* are responsible for group 4 of the PBD. These results demonstrate that *PXAAA1*, like the *P.pastoris PAS5* gene, is required for normal peroxisome biogenesis. Yet what is the role of Pxaaa1p in this process? As with any peroxisome assembly factor, it is possible that Pxaaa1p could play a role in peroxisomal membrane synthesis or protein import. In order to distinguish between these possibilities, examination of the phenotype of CG4 cells and the properties of Pxaaa1p is required.

Previous studies have demonstrated that peroxisomes exist in many PBD cells, even those unable to import any peroxisomal matrix proteins (Santos *et al.*, 1988). As part of an independent study, we have found that CG4 cells contain >100 peroxisomes per cell (C.C.Chang, G.Dodt and S.J.Gould, in preparation). Thus, Pxaaa1p is clearly not required for synthesis of peroxisome membranes.

The recent observation that the PTS1 receptor is a predominantly cytoplasmic protein (Dodt *et al.*, 1995; Gould *et al.*, 1996) that can cycle between the cytoplasm and peroxisome (G.Dodt and S.J.Gould, in preparation) indicates that peroxisomal protein import involves two separate yet overlapping processes: (i) receptor-mediated transport of newly synthesized peroxisomal proteins from the cytoplasm to the peroxisome, and (ii) subsequent translocation of these proteins across the peroxisomal membrane. Two lines of evidence argue that Pxaaa1p is involved in the first process. First, peroxisomes in CG4 cell lines are capable of importing small amounts of both PTS1- and PTS2-containing proteins, indicating that Pxaaa1p is not required for protein translocation across the membrane. Second, Pxaaa1p appears to be a cytoplasmic protein and, therefore, is more likely to be involved in a cytoplasmic aspect of import. Consistent with this hypothesis, the severe instability of Pxr1p in CG4 cells suggests that there is an important interaction between Pxaaa1p and the predominantly cytoplasmic PTS1 receptor.

Assuming that Pxaaa1p is involved in protein transport to the peroxisome, there are two possible explanations for the residual protein import observed in CG4 cells: (i) the mechanism of protein transport from the cytoplasm to peroxisomes is the same in both normal and CG4 fibroblasts and loss of Pxaaa1p simply reduces transport efficiency; or (ii) the loss of Pxaaa1p abolishes the normal protein transport process and reveals the existence of a distinct mechanism for protein transport from the cytoplasm to the peroxisome. This second possibility could be accounted for by diffusion of PTS-containing proteins (or PTS receptor–ligand complexes) from the cytoplasm to the peroxisome. Although *in vitro* import studies have suggested that cytoplasmic factors are absolutely required for peroxisomal protein import (Soto *et al.*, 1993; Wendland and Subramani, 1993), these assays may simply lack the sensitivity to detect the residual import observed in CG4 cells.

The involvement of cytoplasmic factors in protein targeting to the peroxisome (Dodt *et al.*, 1995), nucleus (Gorlich *et al.*, 1995), mitochondria (Hachiya *et al.*, 1994) and endoplasmic reticulum (ER) (Walter and Lingappa, 1986) suggests that diffusion of newly synthesized organellar proteins from the cytoplasm to organelle surfaces is incompatible with efficient protein import into organelles. If this is true, there is no reason to believe that diffusional processes alone could account for the transport of receptor–ligand complexes to the organelle surface. Thus, the existence of these cytoplasmic receptors implies that there is a mechanism for directed transport of cytosolic receptor–ligand complexes to the organelle. Continued analysis of *PXAAA1* may help elucidate this mechanism as it applies to peroxisomal protein import.

Amino acid sequence analysis indicates that Pxaaa1p is a member of the AAA family of ATPases. We found that both mutant alleles from patient PBD106 encode products which lack large segments of the AAA ATPase domain. Furthermore, substitution of arginine for lysine in the second Walker A motif of Pxaaa1p eliminated biological activity. Similar substitutions in other ATPases do not appear to affect protein folding and the effects of these mutations have been attributed to decreased nucleotide binding (Thomas *et al.*, 1992). The role of the first putative ATP-binding domain of Pxaaa1p remains to be addressed. It may be that, like the *S.cerevisiae PAS1* gene which also encodes a AAA protein with two ATP-binding motifs, only the second motif is required for biological activity (Krause *et al.*, 1994).

Interestingly, an ATP requirement for protein import into peroxisomes was demonstrated previously (Imanaka *et al.*, 1987; Wendland and Subramani, 1993b) but was attributed to the transmembrane translocation step, rather

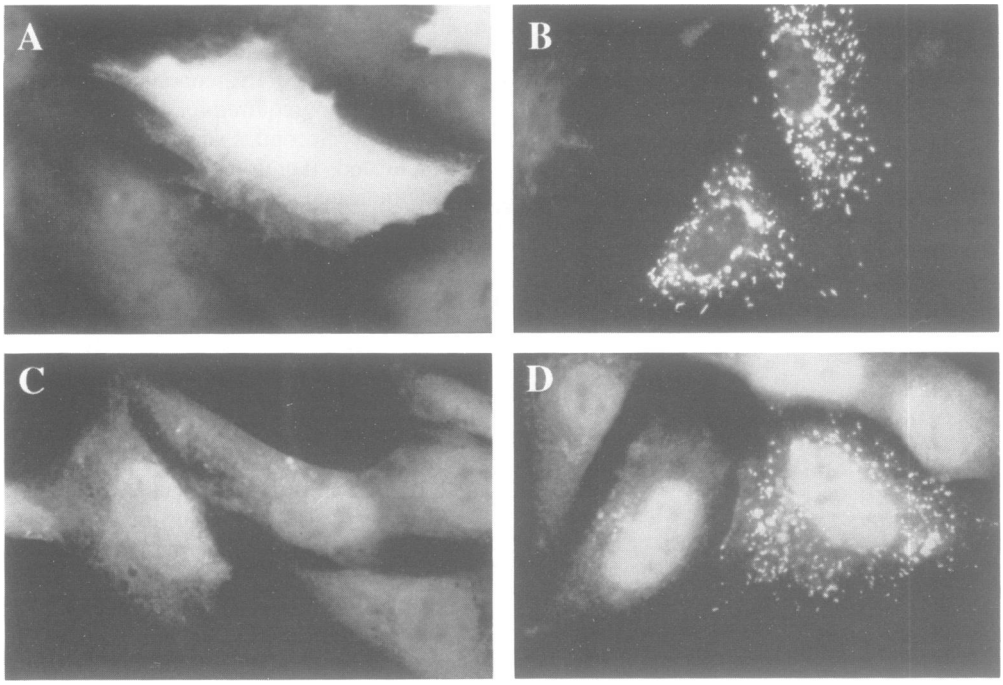

Fig. 5. Pxaaa1p is a cytoplasmic protein. The CG4 cell line PBD108 was transfected with (**A**) the *PXAAA1–myc* expression vector pTY9 and (**B**) the *PMP70-myc* expression vector pcDNA3-*PMP70*-myc (S.J.Gould, unpublished data). Two days after transfection the cells were processed for immunofluorescence with the anti-myc monoclonal antibody 1-9E10 and fluorescein-labeled goat anti-mouse secondary antibodies. The CG4 cell line, PBD103, was transfected with (**C**) pcDNA3 and (**D**) pTY9 and processed for immunofluorescence with anti-SKL antibodies and fluorescein-labeled goat anti-rabbit secondary antibodies.

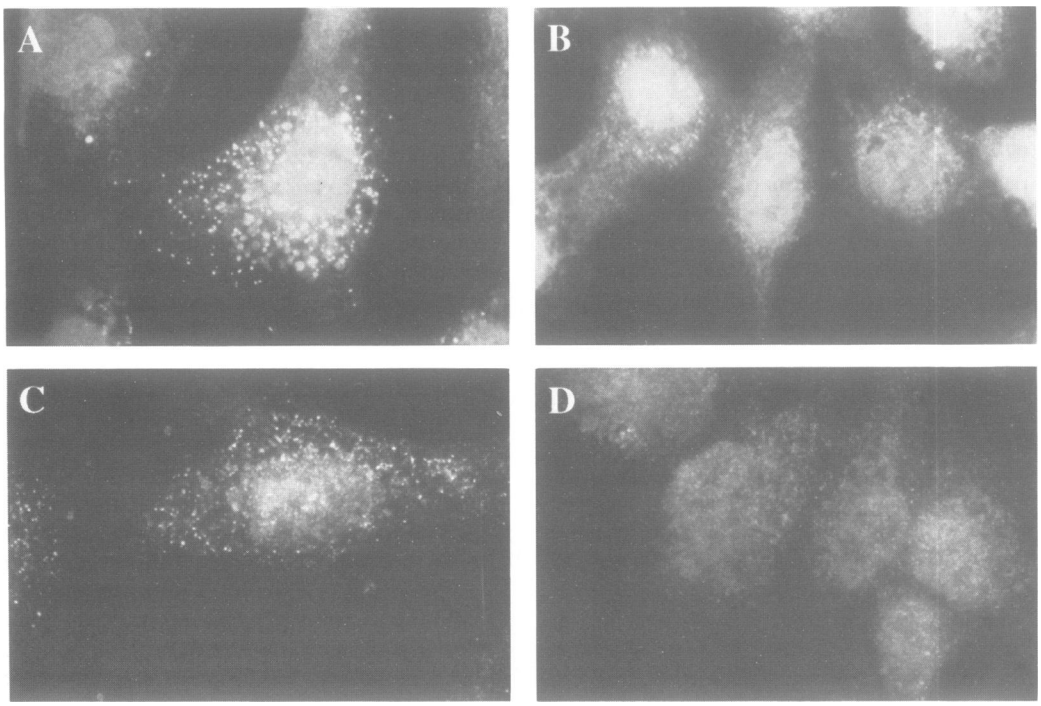

Fig. 6. The K750R mutation abolishes the ability of *PXAAA1* to rescue the PTS1 and PTS2 protein import defects of CG4 cells. The CG4 cell line PBD108 was transfected with (**A** and **C**) the *PXAAA1*-expression vector pTY3 and (**B** and **D**) pTY11, an equivalent vector expressing the K750R mutant. Two days post-transfection, the cells were processed for indirect immunofluorescence using (A and B) anti-SKL and (C and D) anti-thiolase antibodies.

than transport of proteins from the cytoplasm to the peroxisome. Our data suggest that ATP binding and hydrolysis are likely to be essential for Pxaaa1p activity and that this activity may be involved in protein transport from the cytoplasm to peroxisomes. Thus, ATP may be required at several points in the peroxisomal protein import pathway. This idea is also supported by the observation that hsp70 ATPases can play a role in peroxisomal protein

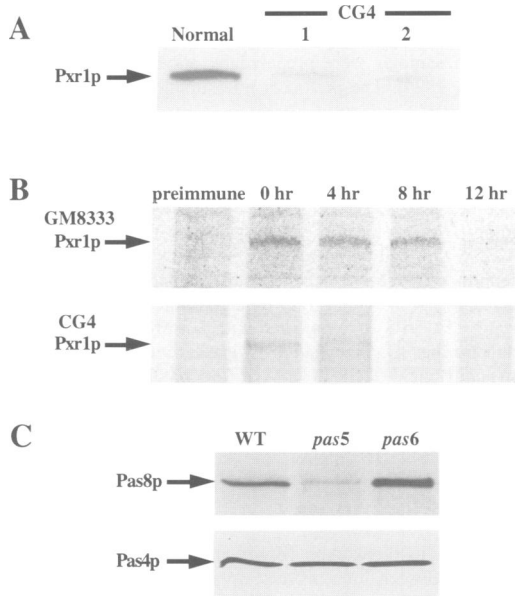

Fig. 7. Loss of *PXAAA1/P.pastoris PAS5* leads to instability of the PTS1 receptor. (**A**) Steady-state levels of Pxr1p in normal and CG4 fibroblasts. Equivalent amounts of total fibroblast cell protein from normal individuals and two CG4 patients (PBD039 and PBD041) were separated by SDS–PAGE, transferred to nylon membranes and blotted with anti-Pxr1p antibodies. Relative to GM8333 fibroblasts (N) which are from unaffected individuals, the CG4 cell lines PBD039 (1) and PBD041 (2) contained only 12% and 14% of Pxr1p. (**B**) Half-life of Pxr1p in normal and CG4 fibroblasts. The normal fibroblast line, GM8333, and the CG4 line, PBD039, were pulse labeled with [^{35}S]methionine for 1 h and then incubated for 0, 4, 8 and 12 h in medium containing excess unlabeled methionine. At each time point, the cells were lysed and subjected to immunoprecipitation with anti-Pxr1p serum or pre-immune serum. (**C**) Steady-state levels of Pas8p in WT, *pas5* and *pas6* cells compared with levels of Pas4p. Equivalent amounts of total cell protein from wild-type, *pas5* and *pas6* strains of *P.pastoris* (each induced in methanol-containing medium) were separated by SDS–PAGE and transferred to nylon membranes. These samples were blotted with affinity-purified antibodies directed against Pas8p, the *P.pastoris* PTS1 receptor, and Pas4p, a ubiquitin-conjugating enzyme that is also required for normal peroxisomal protein import. While the *pas5* and *pas6* mutants have no effect on steady-state levels of Pas4p, levels of Pas8p are severely reduced in the *pas5* mutant (<30% compared with levels detected in wild-type cells).

import (Walton *et al.*, 1994) and that a second AAA ATPase is required for peroxisome assembly (Erdmann *et al.*, 1991; Heyman *et al.*, 1994).

An understanding of the roles that AAA ATPases play in other cellular pathways may provide further insight into the role of Pxaaa1p. AAA proteins are involved in a wide variety of cellular functions: *PAS1* and *PAS5* are required for normal peroxisomal protein import; *SEC18*/NSF are components of a cytosolic 20S particle required for vesicle transport from the ER to Golgi; *TBP1* activates transcription of multiple target genes, possibly via proteolysis of inhibitory factors; *CDC48* is a component of a ring structure similar to 19S chaperone–proteasome complexes and is required for progression through the cell cycle and homotypic ER membrane fusion; and finally, AAA proteins of the 19S proteasome subunit may regulate proteasome specificity (Confalonieri and Duguet, 1995; Latterich *et al.*, 1995). Common features of these proteins include their association with large multi-subunit complexes and their chaperone-like roles in particle/vesicle targeting. These and other features of AAA proteins have led to the proposal that the AAA module functions as an ATP-dependent protein clamp (Confalonieri and Duguet, 1995).

Bellion and Goodman (1987) demonstrated that newly synthesized peroxisomal matrix proteins associate transiently with a high molecular mass complex prior to import. Furthermore, depletion of ATP trapped these proteins in the complex, suggesting that an ATPase is involved in dissociation of the complex. This ATP-dependent transport of a high molecular mass complex from the cytoplasm to the peroxisome correlates with the chaperone role proposed for AAA proteins. We have found that Pxr1p binds PTS1-containing proteins (Dodt *et al.*, 1995) and can form multimers via its TPR domains (unpublished observations). It will be interesting to determine whether a receptor–ligand complex lies at the core of the putative peroxisomal targeting particle identified by Bellion and Goodman. Based on the hypothesis that AAA ATPases may function as ATP-dependent protein clamps, it is also conceivable that Pxaaa1p could be involved in formation, targeting or binding of such a complex to the peroxisome membrane, and/or its dissociation. The next step in understanding the role of Pxaaa1p in peroxisomal protein import will require determination of whether Pxaaa1p interacts directly with Pxr1p and/or a receptor–ligand complex, as well as whether any such interactions are regulated by ATP binding and/or hydrolysis.

Materials and methods

Strains, cell lines and antibodies

All plasmids were propagated in the *Escherichia coli* strain DH10B (Grant *et al.*, 1990). The wild-type, *pas5* and *pas6* strains of the yeast *P.pastoris* were originally described in Gould *et al.* (1992). Skin fibroblasts were obtained from PBD patients by informed consent. Culturing and cell fusion complementation analysis of each line was performed as described (Moser *et al.*, 1995). To obtain transformed derivatives of these cell lines, each was transfected with the plasmid pRSV-SV40T (Dodt *et al.*, 1995). Three to four weeks after transfection, foci of transformed cells were collected and pooled. All transformed PBD cell lines are referred to by patient number (Table I). Rabbit polyclonal antibodies directed against the C-terminal tripeptide SKL_{COOH} recognize multiple peroxisomal proteins (Gould *et al.*, 1990). Rabbit polyclonal antibodies directed against rat peroxisomal thiolase were a generous gift from Dr Richard Rachubinski. The monoclonal antibody 1-9E10 which recognizes the c-myc epitope tag was obtained from BABCO (Berkeley, CA, USA). Affinity-purified rabbit polyclonal anti-Pas8p antibodies were raised against a bacterially expressed form of Pas8p (Dodt *et al.*, 1995; Gould *et al.*, 1996). Labeled secondary antibodies were obtained from standard commercial sources.

cDNA cloning, sequencing and plasmid construction

The original cDNA corresponding to EST R00950 was obtained from Lawrence Livermore National Laboratories (Livermore, USA). The 0.6 kb cDNA was used to probe a λZAP human fetal brain cDNA library (Stratagene, San Diego, CA, USA) at high stringency (Sambrook *et al.*, 1989). Fourteen independent clones that hybridized with the EST R00950 probe were excised and sequenced from both ends of the cDNA. Of these, clone 8.2 extended from nucleotides –212 to 2094 and clone 13.3 extended from nucleotides 692–3268. The 942 bp *Spe*I–*Eco*RI fragment of *PXAAA1* was excised from clone 8.2 and inserted between the *Spe*I and *Eco*RI sites of pGEM9 (Promega Co., Madison, WI, USA), creating pTY1. The 2248 bp *Eco*RI–*Eco*RV fragment from clone 13.3 was inserted between the *Eco*RI and *Eco*RV sites of pcDNA3 (Invitrogen, San Diego, CA, USA), creating pTY2. Next, the 950 bp *Hin*dIII–*Eco*RI fragment from pTY1 (a *Hin*dIII site lies just upstream from the *Spe*I site in pTY1) was inserted between the *Hin*dIII and *Eco*RI sites of pTY2. The resulting plasmid, pTY3, contained nucleotides –67 to 3127

of the *PXAAA1* cDNA (containing the entire ORF) inserted downstream of the strong cytomegalovirus promoter in pcDNA3.

Site-directed mutagenesis of the lysine 750 codon to an arginine codon was performed by PCR. A non-mutagenic sense primer (5′-CAGGACCTGCCTGCTGATG-3′) and mutagenic anti-sense primer (5′-GCTGCACTCAGTGGCTACTGCCTTGGCGAGAAGGGTAMGGCC-GGTGCCAGG-3′) were used to amplify a fragment spanning the unique *Nhe*I and *Dra*III sites of the *PXAAA1* cDNA. This fragment was cleaved with *Nhe*I and *Dra*III and inserted between the *Nhe*I and *Dra*III sites of the *PXAAA1* cDNA in pTY3, creating pTY11. Sequence analysis confirmed that the K750R mutation in pTY11 was the only mutation introduced during this procedure with the exception of a silent G→C transition used to eliminate an *Msc*I site and allow restriction digest identification of recombinant clones.

Appending the myc tag to the 3′ end of the *PXAAA1* ORF was also accomplished by PCR. A fragment stretching from upstream of the unique *Mlu*I site to the end of the *PXAAA1* ORF was amplified using a sense non-mutagenic primer (5′-GTGGTAGGGGATCTCTATGC-3′) and a mutagenic anti-sense primer (5′-CGAGGATCCGCAGGCAGCAAAC-TTGCGCTGGATG-3′). This fragment contained a *Bam*HI site in place of the *PXAAA1* stop codon. This fragment was cleaved with *Mlu*I and *Bam*HI. This 301 bp *Mlu*I–*Bam*HI fragment was combined with the 2720 bp *Hin*dIII–*Mlu*I *PXAAA1* fragment from pTY3 and the two were inserted between the *Hin*dIII and *Bam*HI sites of pcDNA3-myc, creating pTY9. pcDNA3-myc contains 36 nucleotides (5′-GGATCCGAGGAGC-AGAAGCTGATCTCCGAGGAGGACCTGTAGTCTAGA-3′) between the *Bam*HI and *Xba*I sites of pcDNA3, which encode the c-myc epitope followed by a stop codon.

Northern, Southern, RFLP and mutational analysis

A multiple tissue Northern blot (Clontech Palo Alto, CA, USA) was probed (Sambrook *et al.*, 1989) with the 0.6 kb EST R00950 cDNA. Southern blot analysis (Sambrook *et al.*, 1989) was used to map the *PXAAA1* gene in humans and mice. To identify the chromosomal location of *PXAAA1*, genomic DNAs from the NIGMS human–rodent mapping panel #2 (Drwinga *et al.*, 1993) were digested with a restriction enzyme that cleaves the *PXAAA1* locus of each organism into fragments with distinct sizes. For determining the chromosomal location of the mouse *PXAAA1* gene using the BSS backcross panel (Rowe *et al.*, 1994), a restriction enzyme that would detect an RFLP between C57BL/6J and *Mus spretus* genomic DNA was identified. Genomic DNAs from all 94 N2 animals in the BSS backcross were digested with this enzyme and the presence of the C57BL/6J and *M.spretus PXAAA1* alleles in each animal was determined by Southern blot and compared with the inheritance of known markers. Mutational analysis of PBD106 was performed as described previously (Dodt *et al.*, 1995). Briefly, 15 overlapping primer sets were used to generate cDNA fragments of the *PXAAA1* ORF by RT-PCR from patient mRNA. Mutations were detected based on altered migration patterns by SSCP analysis (Michaud *et al.*, 1992).

Transfections and immunofluorescence microscopy

Human fibroblasts were grown in Dulbecco's modified Eagle's medium high glucose (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin (DMEM/10% FCS/PS). Transfections were performed using lipofectamine (Gibco/BRL, Bethesda, MD, USA). Three micrograms of plasmid DNA were mixed with 250 µl OptiMEM (Gibco/BRL) and combined with 250 µl OptiMEM containing 30 µg lipofectamine. After mixing gently, the solution was incubated 15–45 min at room temperature. Simultaneously, cells (60–80% confluent on a 25 mm^2 flask) were washed twice with 5 ml Hanks' balanced salt solution and once with 5 ml OptiMEM. One milliliter of OptiMEM was then added to each flask. The 500 µl DNA/lipofectamine mixture were added to each flask and the cells incubated for 4–6 h at 37°C in a humidified incubator. The solution was removed, the cells were washed gently two times with DMEM/10% FCS/PS and then 5 ml DMEM/10% FCS/PS were added to each flask. The next morning the medium was changed again. Approximately 2 h later, the cells were trypsinized and seeded onto glass coverslips in 60 mm dishes. Cells were processed for indirect immunofluorescence microscopy as described (Slawecki *et al.*, 1995) using appropriate primary and secondary antibodies.

Immunoblots and immunoprecipitations

Protein samples were prepared from human skin fibroblasts by growing cells on a 60 mm dish to 80–90% confluency. After washing three times with 5 ml Hanks' balanced salt solution, cells were lysed by the addition of 300 µl H_2O and rapidly scraped from the plate. An aliquot was removed for protein determination and the remainder was immediately mixed with reducing SDS–PAGE sample buffer and frozen at −80°C. Yeast protein samples were prepared by alkaline lysis of intact cells (Crane *et al.*, 1994). The protocols for immunoblot analysis have been described earlier (Crane *et al.*, 1994).

Pulse–chase experiments were performed as follows. Skin fibroblasts from unaffected individuals and skin fibroblasts from various PBD patients were grown on 60 mm dishes to ~70–90% confluency in DMEM/10% FCS/PS. The cells were washed twice with DMEM lacking methionine (DMEM–met) and then incubated for 1 h in DMEM–met/10% FCS/PS that had been supplemented with 1 mCi [^{35}S]methionine. The medium was removed, cells were washed once in DMEM/10% FCS/PS supplemented with 1 mM methionine and then incubated in DMEM/10% FCS/PS supplemented with 1 mM methionine for varying amounts of time. The incubations were terminated by removing the medium and lysing the cells in RIPA buffer containing protease inhibitors (Harlow and Lane, 1988). Pxr1p was immunoprecipitated from each lysate using anti-Pxr1p antibodies (Dodt *et al.*, 1995), separated by SDS–PAGE, and detected by fluorography.

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While this manuscript was in review, Tsukamoto *et al.* published the cloning and sequencing of rat *PAF2* by functional complementation of a peroxisome-deficient Chinese hamster ovary cell line (*Nature Genet.*, 1996, **11**, 395–401). The products of the rat *PAF2* and human *PXAAA1* genes are 87% identical and expression of rat *PAF2* complemented the peroxisomal protein import defects of PBD group 4 (Japanese PBD group C) patients. For these reasons, we believe that *PAF2* is the rat ortholog of *PXAAA1*.