Identification of three distinct peroxisomal protein import defects in patients with peroxisome biogenesis disorders

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SUMMARY

Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum’s disease, and classical rhizomelic chondrodysplasia punctata are lethal genetic disorders caused by defects in peroxisome biogenesis. We report here a characterization of the peroxisomal matrix protein import capabilities of fibroblasts from 62 of these peroxisome biogenesis disorder patients representing all ten known complementation groups. Using an immunofluorescence microscopy assay, we identified three distinct peroxisomal protein import defects among these patients. Type-1 cells have a specific inability to import proteins containing the PTS1 peroxisomal targeting signal, type-2 cells have a specific defect in import of proteins containing the PTS2 signal, and type-3 cells exhibit a loss of, or reduction in, the import of both PTS1 and PTS2 proteins. Considering that the common cellular phenotype of Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum’s disease has been proposed to be a complete defect in peroxisomal matrix protein import, the observation that 85% (40/47) of the type-3 cell lines imported a low but detectable amount of both PTS1 and PTS2 proteins was surprising. Furthermore, different cell lines with the type-3 defect exhibited a broad spectrum of different phenotypes; some showed a complete absence of matrix protein import while others contained 50-100 matrix protein-containing peroxisomes per cell. We also noted certain relationships between the import phenotypes and clinical diagnoses: both type-1 cell lines were from neonatal adrenoleukodytrophy patients, all 13 type-2 cell lines were from classical rhizomelic chondrodysplasia punctata patients, and the type-3 import defect was found in the vast majority of Zellweger syndrome (22/22), neonatal adrenoleukodystrophy (17/19), and infantile Refsum’s disease (7/7) patients.

Our finding that all type-1 cell lines were from the second complementation group (CG2), all 13 type-2 cell lines were from CG11, and that cells from the eight remaining complementation groups only exhibit the type-3 defect indicates that mutations in particular genes give rise to the different types of peroxisomal protein import defects. This hypothesis is further supported by correlations between certain complementation groups and particular type-3 sub-phenotypes: all patient cell lines belonging to CG3 and CG10 showed a complete absence of peroxisomal matrix protein import while those from CG6, CG7, and CG8 showed some peroxisomal matrix proteins. However, the fact that cell lines from within particular complementation groups (CG1, CG4) could have different matrix protein import characteristics suggests that allelic heterogeneity also plays an important role in generating different import phenotypes in certain patients. These same types of peroxisomal protein import defects and genotype/phenotype correlations have been described for yeast pas (peroxisome assembly) mutants, suggesting that peroxisome biogenesis disorders may be caused by mutations in human homologs of yeast PAS genes.

Key words: peroxisome, peroxisome biogenesis disorder, protein import
proposes that the organelle arises by fission of pre-existing peroxisomes (Lazarow and Fuji ki, 1985). Although there is little direct evidence for this hypothesis, several aspects of peroxisome biogenesis have been established which provide circumstantial evidence in support of this view. Peroxisomal proteins are synthesized on cytosolic polyribosomes and are imported into the organelle post-translationally. This is true for both matrix and membrane proteins. In vitro import studies have demonstrated that ATP is required for this process and that import occurs in a time- and temperature-dependent manner (Subramani, 1993). In addition, a variety of studies have shown that proteins destined for this organelle are distinguished by the fact that they contain peroxisomal targeting signals (PTSs). These signals are both necessary and sufficient for directing newly synthesized peroxisomal proteins into the peroxisome. Two types of signals for peroxisomal matrix proteins have been studied extensively, the PTS1 and the PTS2.

More than 80% of all known peroxisomal matrix proteins are imported via the carboxy-terminal tripeptide PTS1 (de Hoop and Ab, 1992). Mutational analysis of this signal in mammalian cells revealed that serine, alanine, and cysteine are acceptable at the −3 position, that lysine, arginine, and histidine can function at the penultimate residue, and that the carboxy-terminal position requires either leucine or methionine (Gould et al., 1989; Swinkels et al., 1992; Miyazawa et al., 1989). Although this consensus sequence appears to include most if not all forms of the PTS1 that function in mammalian cells, additional variations in the sequence of this signal are acceptable in other eukaryotes such as yeast and protozoans (Aitchison et al., 1991; Didion and Roggenkamp, 1992; Hansen et al., 1992; Sommer et al., 1992, 1993; Blattner et al., 1992). The PTS-2 signal is distinct from the PTS-1 signal and is found at the aminoterminus of peroxisomal thiolases from mammals to yeast (Swinkels et al., 1991; Glover et al., 1994) and the watermelon glyoxysomal malate dehydrogenase (Gietl et al., 1994). This signal conforms to the sequence R/KHX3Q/HL and is located anywhere from 2 to 12 amino acids from the aminoterminus of the protein (Swinkels et al., 1991). Although this signal may be cleaved after import, cleavage is not required for import and does not appear to be coupled to the import reaction (Swinkels et al., 1991). In addition to the PTS1 and PTS2 signals, there is evidence for the existence of additional types of PTSs (Small et al., 1988; McCammon et al., 1994), but these remain incompletely characterized.

Although the peroxisome does not appear to be essential for cellular viability, it is found in almost all eukaryotic cells examined. In humans, defects in peroxisome assembly are thought to be the primary cause of Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum’s disease (IRD). These closely related diseases are inherited in an autosomal recessive manner and are characterized by pronounced neurological, hepatic and renal abnormalities, severe mental retardation, and premature death. Multiple peroxisomal enzymatic pathways are disrupted in these patients and the resulting accumulation of very-long-chain fatty acids, reduction of plasmalogens (ether lipids), and inability to oxidize phytic acid are all markers for these diseases (Lazarow and Moser, 1994). Because of these common characteristics these three diseases have been designated as the generalized peroxisomal disorders. They occur in approximately one out of every 50,000 live births (Lazarow and Moser, 1994).

It was originally suggested that ZS patient cells lacked peroxisomes entirely (Goldfischer et al., 1973) but Santos et al. (1988) demonstrated that peroxisome ‘ghosts’ containing integral peroxisomal membrane proteins exist within cells from some of these patients. The assumption that all ZS, NALD, and IRD patient cells are completely unable to import peroxisomal matrix proteins has also been challenged with the observations that certain patient cells import some peroxisomal matrix proteins. This has been reported for a PTS2-containing protein (thiolase) (Balfi et al., 1990) as well as a PTS1-containing protein (catalase) (Santos et al., 1992). However, matrix protein import has been reported for only a very limited set of cell lines from these patients and in no case was it clear whether the cells were able to import only PTS1-containing proteins, only PTS2-containing proteins, or both. These results have led to some confusion regarding the general nature of the peroxisomal protein import defect in these disorders. As for the genes that are responsible for these diseases, somatic cell fusion experiments have uncovered at least nine different complementation groups amongst the patient cell lines, suggesting that defects in any of nine genes can cause these diseases (Brul et al., 1988; Roscher et al., 1989; Shimo zawa et al., 1993; Moser et al., 1995). The fact that ZS, NALD, and IRD patients can all be found within particular complementation groups indicates that these related diseases can all be caused by defects in the same gene. One of the nine (or more) genes responsible for these diseases has been identified, PAF-1 (Tsukamoto et al., 1991; Shimo zawa et al., 1992, 1993). This gene encodes an integral peroxisomal membrane protein (PMP) of 35 kDa, but its role in peroxisome assembly has not been determined.

While ZS, NALD, and IRD appear to be related diseases that may differ only by the severity of the causative mutation, rhizomelic chondrodysplasia punctata (RCDP) is clearly a distinct disease (Lazarow and Moser, 1994). Nevertheless, this autosomal recessive disorder also results in severe mental retardation and premature death. At the biochemical level, RCDP patients have abnormally low peroxisomal plasmalogen biosynthetic activity in common with ZS, NALD, and IRD patients, but there is no apparent defect in very-long-chain fatty acid oxidation (Heymans et al., 1986; Heikoop et al., 1990, 1992). Two forms of RCDP exist, classical and non-classical, which have indistinguishable clinical presentations but differ in their biochemical manifestations. All patients with the classical form of RCDP are defective in phytic acid oxidation and proteolytic processing of peroxisomal thiolase to its mature form, as well as in plasmalogen biosynthesis (Heikoop et al., 1990, 1992). The fact that classical RCDP appears to be inherited in an autosomal recessive manner and manifests deficiencies in multiple peroxisomal metabolic functions suggests that, like ZS, NALD and IRD, RCDP also may be caused by a defect in peroxisome biogenesis. Thus, we refer to ZS, NALD, IRD and classical RCDP as peroxisome biogenesis disorders (PBDs).

Cell fusion complementation analysis suggests that defects in only one gene lead to all cases of classical RCDP (Moser et al., 1995). Non-classical RCDP patients do not manifest any biochemical abnormalities other than low plasmalogen biosynthesis and have been placed into two complementation groups distinct from the one populated by classical RCDP patients.
(Moser et al., 1995). The defect in these non-classical RCDP patients appears to lie in either of the two peroxisomal enzymes responsible for plasmalogen biosynthesis.

In addition to studies in human cells, several investigators have used yeast as a model system with which to attempt to understand the molecular basis of these peroxisome biogenesis disorders. At least 15 different genes are required for peroxisome assembly in yeast (Erdmann et al., 1989; Cregg et al., 1990; Gould et al., 1992; Liu et al., 1992; van der Leij et al., 1992; Elgersma et al., 1993), a number which seems in proportion to the ten known complementation groups in humans. While all pas mutants are defective in peroxisome assembly, biochemical and electron microscopic analysis of these mutants has revealed that some pas mutants are capable of importing small amounts of peroxisomal matrix proteins while others are completely defective in matrix protein import (Spong and Subramani, 1994; Heyman et al., 1994; Kalish and Gould, unpublished observations). In general, these studies suggest that defects in some PAS genes completely abolish protein import while others may only greatly reduce the efficiency with which it occurs. We used an immunofluorescence microscopy assay to determine the peroxisomal protein import capabilities in normal human skin fibroblasts, as well as in skin fibroblasts from 62 PBD patients. These cell lines represent all ten complementation groups of PBD cell lines that have been identified to date (Shimozawa et al., 1993; Moser et al., 1995).

**MATERIALS AND METHODS**

**Cell lines and antibodies**

All human cell lines were obtained and cultured as described (Moser et al., 1980). The antibodies directed against the carboxy-terminal tripeptide serine-lysine-leucine-COOH recognize multiple proteins with the PTS1 signal and have been described previously (Gould et al., 1990; Keller et al., 1991). The antisera to peroxisomal thiolase from rat liver was a generous gift from Dr. R. Ruchubisni (University of Alberta). Fluorescein-conjugated goat anti-rabbit IgG antibody was purchased from Gibco/BRL.

**Immunofluorescence microscopy**

Cells were seeded onto sterile glass coverslips 1-2 days prior to fixation for immunofluorescence. For experiments using the anti-SKL antibodies, all manipulations were performed in phosphate-buffered saline (PBS). Cells were fixed by incubation in 3% formaldehyde/PBS for 20 minutes at room temperature. Permeabilization of all intracellular membranes was performed by incubation of the fixed cells in 1% Triton X-100/PBS for 5 minutes, followed by 2 washes with PBS. Permeabilization of just the plasma membrane was performed by incubation of the fixed cells in 25 µg/ml digitonin/PBS for 5 minutes followed by 2 washes with PBS. After permeabilization, the cells were incubated with anti-SKL serum (diluted 1:300 in PBS) for 30 minutes, washed 8 times with PBS, incubated with a fluorescein-conjugated goat anti-rabbit IgG antibody (diluted 1:100 in PBS) for 30 minutes, washed 8 times with PBS, and then mounted on glass slides in 90% glycerol, 100 mM Tris-HCl, pH 8.5, 0.1% p-phenylene diamine.

For indirect immunofluorescence using anti-thiolase antibodies, the procedure was identical to that described above except that Triton-buffered saline (TBS) was used in place of PBS and that anti-thiolase serum was used at a dilution of 1:200. After mounting the coverslips on glass slides, cells were observed using a Leitz Aristoplan microscope. Micrographs were taken using Kodak Ektachrome 400 film.

**Biochemical assays**

Relative peroxisomal very-long-chain fatty acid (VLCFA) oxidation of human skin fibroblast cell lines was assessed by comparing the ratio of VLCFA oxidation (in µg/mg protein), an exclusively peroxisomal process, to long chain fatty acid (LCFA) oxidation (in µg/mg protein), a predominantly mitochondrial process (Moser et al., 1995). Values for relative VLCFA oxidation in cell lines from normal individuals averaged 0.08±0.03 (the units cancel). Peroxisomal plasmalogen biosynthesis was determined by the method of Roscher et al. (1985) and is also represented by a ratio. In this case it is the ratio of incorporation of [1-14C]hexadecanol into plasmalogens, which requires both the peroxisomal and endoplasmic reticulum enzymes, to that of the incorporation of [9,10-3H]hexadecaglycerol into plasmalogens, which requires only the enzymes of the endoplasmic reticulum which perform the latter steps of the pathway (Moser et al., 1985). Values for relative plasmalogen biosynthesis in cell lines from normal individuals averaged 0.67±0.19.

**RESULTS**

Human skin fibroblast cell lines were examined by immunofluorescence microscopy to determine their ability to import PTS1- and PTS2-containing peroxisomal matrix proteins. An antibody directed against the carboxy-terminal tripeptide serine-lysine-leucine-COOH (SKL, a form of the PTS1 signal) has been shown previously to recognize multiple PTS1-containing proteins in mammalian cells (Gould et al., 1990). In this study the anti-SKL antibody was used in immunofluorescence microscopy experiments to determine the ability of human skin fibroblast cells to import endogenous PTS1-containing proteins into peroxisomes. To determine the import of PTS2 proteins in these cells, the immunofluorescence analysis was repeated using an antibody to peroxisomal thiolase, the only known PTS2 protein in human cells. In addition, immunofluorescence microscopy analysis of each cell was determined after permeabilization of both the plasma membrane and the peroxisomal membrane (Triton X-100 permeabilization) as well as after permeabilization of just the plasma membrane (digitonin permeabilization). Proteins inside the peroxisome are detected after permeabilization with Triton X-100 whereas proteins detected after digitonin permeabilization are either cytosolic or associated with the outer surface of the peroxisome (Swinkels et al., 1991).

Normal human skin fibroblast cells (line N1) permeabilized with Triton X-100 and processed for indirect immunofluorescence microscopy using either the anti-SKL or anti-thiolase antibodies showed the typical peroxisomal distribution of these two classes of proteins (Fig. 1A,B). The fact that no staining was observed with these antibodies when cells were permeabilized with digitonin demonstrated that the proteins recognized by these antibodies were inside the peroxisome (Fig. 1C,D). Indistinguishable staining patterns were observed with the seven other control human fibroblast cell lines (Table 1).

**A loss or reduction of both the PTS1 and PTS2 peroxisomal protein import pathway typifies the generalized peroxisomal disorders**

Skin fibroblast cell lines from 49 generalized peroxisomal disorder patients representing nine different complementation groups were analyzed by immunofluorescence microscopy using both the anti-SKL and anti-thiolase antibodies. In
contrast to what was observed in the eight control cell lines examined, 47 of these cell lines exhibited either a complete or a partial defect in the import of both PTS1 and PTS2 proteins. This result was not surprising, considering that the classic phenotype of ZS is defective protein import into peroxisomes (Goldfischer et al., 1973). However, it was quite surprising to observe that only seven of the 47 cell lines (15%) were completely defective in the import of matrix proteins into peroxisomes (Fig. 2; Table 1). The staining for two such PBD cell lines are shown here. Although these lines showed no punctate staining for either PTS1 or PTS2 proteins, all cells examined did contain vesicle-associated peroxisomal membrane proteins (data not shown).

Surprisingly, the most common phenotype of fibroblasts from ZS, NALD, and IRD patients (40/47 cell lines) showed punctate staining with both the anti-SKL and anti-thiolase antibodies (Fig. 3; Table 1). Because neither the PTS1 nor PTS2 marker proteins was detected when these cells were permeabilized with digitonin (for example, see Fig. 3E,F), we conclude that the PTS1 and PTS2 marker proteins were inside a membrane-bound compartment. The observation that so many of these patients were capable of importing at least some peroxisomal matrix proteins does not mean that peroxisomal protein import was similar to that detected in normal fibroblasts. Cell lines from these patients were easily distinguishable from normal cells: they usually had significantly fewer peroxisomes, the staining for the peroxisomal protein was often of lower intensity, and in many instances the cells had unusually large peroxisomes. It will be of interest to determine whether the efficiency of matrix protein import in these cell lines is related to the number and/or size of the peroxisomes detected. While the extent of peroxisomal matrix protein import varied greatly among these 40 partially defective PBD cell lines, the ability of cells from a particular patient to import PTS1 proteins closely mirrored their ability to import thiolase, the PTS2 marker protein.

The general characteristics of a loss or reduction in import of both PTS1 and PTS2 peroxisomal proteins define the type-3 peroxisomal protein import defect and were observed in 47/49 ZS, NALD, and IRD patients. Furthermore, individual cell lines imported, or failed to import, both PTS1 and PTS2 proteins to the same degree. Although some variability existed in the staining from one cell to another within any given cell line, these differences were minor for all but one cell line. Isolated from the patient PBD061, the majority of cells in this population did not import any PTS1 or PTS2 proteins (Fig. 4A,C) but approximately one out of every 50 cells did demon-
Although 47 of the 49 ZS, NALD, and IRD cell lines were defective in lysosomal protein (data not shown).

These unusual cell lines (PBD018 and PBD093) from com-

manent peroxisome-defective pedigrees revealed the following

Table 1. The peroxisomal protein import phenotypes and peroxisomal enzymatic capacities of human skin fibroblast cells from 73 normal and affected individuals

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disease</th>
<th>CG</th>
<th>PT1 import</th>
<th>PT2 import</th>
<th>VCLFA oxidation</th>
<th>Plasmalogen synthesis</th>
</tr>
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<tbody>
<tr>
<td>N1</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>N2</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>0.07</td>
<td>0.67</td>
</tr>
<tr>
<td>N3</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>N4</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>0.08</td>
<td>0.55</td>
</tr>
<tr>
<td>N5</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>0.10</td>
<td>0.63</td>
</tr>
<tr>
<td>N6</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+++</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>PBD002</td>
<td>ZS</td>
<td>1</td>
<td>+/-</td>
<td>+/-</td>
<td>0.96</td>
<td>9.48</td>
</tr>
<tr>
<td>PBD009</td>
<td>ZS</td>
<td>1</td>
<td>none</td>
<td>none</td>
<td>0.98</td>
<td>4.65</td>
</tr>
<tr>
<td>PBD022</td>
<td>ZS</td>
<td>1</td>
<td>+/-</td>
<td>+/-</td>
<td>1.26</td>
<td>5.29</td>
</tr>
<tr>
<td>PBD036</td>
<td>ZS</td>
<td>1</td>
<td>+/-</td>
<td>+</td>
<td>0.82</td>
<td>5.88</td>
</tr>
<tr>
<td>PBD044</td>
<td>NALD</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>1.62</td>
<td>4.47</td>
</tr>
<tr>
<td>PBD086</td>
<td>IRD</td>
<td>1</td>
<td>+/-</td>
<td>+/-</td>
<td>0.35</td>
<td>1.35</td>
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<tr>
<td>PBD088</td>
<td>IRD</td>
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<td>+/-</td>
<td>+/-</td>
<td>0.83</td>
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<tr>
<td>PBD089</td>
<td>IRD</td>
<td>1</td>
<td>+/-</td>
<td>+/-</td>
<td>0.59</td>
<td>1.31</td>
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<td>IRD</td>
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<td>+/-</td>
<td>+/-</td>
<td>0.64</td>
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<tr>
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<td>ZS</td>
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<td>+/-</td>
<td>+/-</td>
<td>2.18</td>
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<tr>
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<td>1</td>
<td>+</td>
<td>+</td>
<td>0.52</td>
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</tr>
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<td>1</td>
<td>+</td>
<td>+</td>
<td>0.41</td>
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<td>none</td>
<td>+</td>
<td>1.37</td>
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<tr>
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<td>2</td>
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<td>+</td>
<td>1.36</td>
<td>445.00</td>
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<td>3</td>
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<td>none</td>
<td>1.70</td>
<td>25.90</td>
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<td>none</td>
<td>none</td>
<td>1.07</td>
<td>10.71</td>
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<td>ZS</td>
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<td>+</td>
<td>+</td>
<td>1.58</td>
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<tr>
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<td>none</td>
<td>2.80</td>
<td>3.90</td>
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<tr>
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<td>ZS</td>
<td>4</td>
<td>+/-</td>
<td>+/-</td>
<td>1.14</td>
<td>5.30</td>
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<tr>
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<td>ZS</td>
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<td>+</td>
<td>+/-</td>
<td>1.59</td>
<td>3.30</td>
</tr>
<tr>
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<td>NALD</td>
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<td>+</td>
<td>+/-</td>
<td>0.21</td>
<td>0.69</td>
</tr>
<tr>
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<td>+</td>
<td>0.91</td>
<td>0.93</td>
</tr>
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<td>NALD</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>1.13</td>
<td>2.50</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>1.14</td>
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</tr>
<tr>
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<td>NALD</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>0.55</td>
<td>3.40</td>
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<tr>
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<td>NALD</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>0.18</td>
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<td>+</td>
<td>+</td>
<td>0.59</td>
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<td>+</td>
<td>0.16</td>
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<td>8</td>
<td>+</td>
<td>+/-</td>
<td>1.64</td>
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<td>8</td>
<td>+</td>
<td>+/-</td>
<td>1.00</td>
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<td>PBD057</td>
<td>NALD</td>
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<td>+</td>
<td>+/-</td>
<td>0.62</td>
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<td>+</td>
<td>+/-</td>
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<td>PBD059</td>
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<td>4.96</td>
</tr>
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<td>PBD107</td>
<td>ZS</td>
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<td>none</td>
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<td>0.67</td>
<td>16.77</td>
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<tr>
<td>PBD027</td>
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<td>N.D.</td>
<td>+</td>
<td>+/-</td>
<td>1.29</td>
<td>1.59</td>
</tr>
<tr>
<td>PBD063</td>
<td>NALD</td>
<td>N.D.</td>
<td>+</td>
<td>+/-</td>
<td>1.33</td>
<td>1.45</td>
</tr>
</tbody>
</table>

Import of PTS1 and PTS2 marker proteins was assessed by immunofluorescence microscopy. The criteria for assessing the degree of import were as follows: +/- indicates that some punctate structures were detected (5 or more in a single cell) and that the intensity of staining was very weak; cell lines with import categorized as + had a greater number of peroxisomes per cell (5-20) and intensity of staining was weak; ++ indicates that >20 peroxisomes were observed in the cells and that the intensity of staining was strong; +++ indicates that there were hundreds of peroxisomes per cell and that the staining was very intense. It should be noted that these ratings do not reflect a % import. CG stands for complementation groups, patient identification numbers are designated PBDxxx, and patients with single-enzyme defects in plasmalogen synthesis are identified by NCRCDP numbers. The relative VLCFA oxidation capacity is expressed as a ratio of C26:22 fatty acid oxidation activity of the cell line. Using this assay, higher ratios of C26:22 fatty acid oxidation indicate lower peroxisomal VLCFA oxidation activity (Moser and Moser, 1991). Plasmalogen biosynthesis capacity is also expressed as a ratio, in this case a ratio of the second half of the pathway (ER-based) to the total pathway (peroxisomal steps plus ER steps). Again, the higher the ratio, the lower the activity of the peroxisomal enzymes (Roscher et al., 1985). The VLCFA oxidation and plasmalogen biosynthesis data in this table are in press (Moser et al., 1995). N.D., not determined.

Two neonatal adrenoleukodystrophy patients exhibit a PTS1-specific peroxisomal protein import defect

Although 47 of the 49 ZS, NALD, and IRD cell lines were as defective in PTS2 import as they were in PTS1 import, cells from two NALD patients differed from this pattern. These unusual cell lines (PBD018 and PBD093) from complementation group 2 (CG2; Table 1) imported thiolese but did not import SKL-containing proteins into a detectable structure (Fig. 5). The fact that vesicular thiolese could not be detected when PBD018 cells were permeabilized with digitonin instead of Triton X-100 suggested that the thiolese was within a membrane-bound compartment. Because the anti-SKL antibody recognizes at least 10 different proteins in mammalian cells (Gould et al., 1990), the complete absence of punctate staining with this antibody suggested that this cell line had a severe defect in the PTS1-dependent peroxisomal protein import pathway. This phenotype is defined as the type-1 peroxisomal protein import defect. PBD018 and...
PBD093 also shared an unusual biochemical phenotype: while β-oxidation was severely reduced, plasmalogen biosynthetic capacity was only mildly impaired (Table 1). Most other ZS, NALD, and IRD patients with a severe defect in very-long-chain fatty acid β-oxidation express a more pronounced defect in plasmalogen biosynthesis than PBD018 and PBD093 (Moser et al., 1995). Surprisingly, the third cell line belonging to CG2 (PBD005) exhibited a type-3 defect, not the PTS1-specific import defect we observed for PBD018 and PBD093.

**PTS2-dependent protein import is defective in all classical rhizomelic chondrodysplasia punctata patient cell lines**

Cell lines from 18 RCDP patients were also tested for their ability to compartmentalize PTS1 and PTS2 proteins. Of these 18 cell lines, 13 were from classical RCDP patients and five were from non-classical RCDP patients. Although the classical and non-classical forms of RCDP are indistinguishable at the clinical level, the classical form of the disease is characterized by multiple peroxisomal enzymatic deficiencies whereas the non-classical form manifests only a plasmalogen biosynthetic defect. Each of these 13 cell lines from classical RCDP patients demonstrated an inability to import peroxisomal thiolase into peroxisomes, but normal peroxisomal import of PTS1 proteins (Fig. 6). No punctate staining was observed with the anti-SKL antibody in digitonin-permeabilized cells, demonstrating that the PTS1-containing proteins were inside the peroxisomes in these cells (Fig. 6). These staining characteristics define the type-2 peroxisomal protein import defect. Cell fusion complementation analysis has demonstrated that all 13 classical RCDP cell lines fall within the same complementation group, CG11 (Moser et al., 1995). In addition, this phenotype was never observed in any ZS, NALD or IRD patient cell lines. The five cell lines from non-classical RCDP patients (defective only in plasmalogen biosynthesis) were indistinguishable from normal human fibroblasts in their ability to import PTS1 and PTS2 proteins into peroxisomes (data not shown), strongly suggesting that there is no peroxisomal protein import defect in cells from these patients.

**DISCUSSION**

Earlier studies, summarized by Lazarow and Moser (1994), have led to the hypothesis that ZS, NALD, IRD and classical RCDP are caused by defects in peroxisome assembly. Nevertheless, because the peroxisomal protein import capacities have...
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Cellular phenotypes of PBD patients

not been determined in a sufficient number of PBD patient cells, there has been significant confusion regarding which peroxisomal protein import pathways are defective in these patients, as well as the extent of these import defects (Balfe et al., 1990; McCollum et al., 1993). The study presented here was designed to clarify this issue.

The type-1 peroxisomal protein import defect

The type-1 peroxisomal protein import defect was exhibited by two cell lines from NALD patients belonging to complementation group 2 (CG2). An analogous defect in import of PTS1 proteins has also been identified in a yeast peroxisome assembly mutant, pas8 from Pichia pastoris. Because of this
phenotype, and the fact that the protein encoded by the PAS8 gene binds PTS1-containing peptides, McCollum et al. (1993) have proposed that PAS8 encodes the receptor for PTS1-containing proteins. We have recently found that a mutation in PXR1, the human homolog of the P. pastoris PAS8 gene, is the cause of the PTS1-specific peroxisomal protein import defect in PBD018 (Dodt et al., 1995) and PBD093.

In addition to the inability of the type-1 cells to import PTS1 proteins, they contained peroxisomes of abnormally large size and low abundance. Peroxisomes of similarly abnormal morphology can be detected in cells from patients defective in the peroxisomal β-oxidation enzymes, acyl-CoA oxidase (Poll-The et al., 1988) or bifunctional enzyme (unpublished observations), but not in cells lacking plasmalogen biosynthetic enzymes from non-classical RCDP patients. These results raise the interesting possibility that the size, number, and distribution of peroxisomes in human skin fibroblasts are dependent upon the presence or absence of a functional peroxisomal β-oxidation pathway. Although both type-1 cell lines were severely defective in β-oxidation of very-long-chain fatty acids, each had a relatively mild plasmalogen biosynthesis defect. Although several other PBD patient fibroblasts had biochemical properties similar to this cell line (reduced β-oxidation but only a mild plasmalogen biosynthesis deficiency), none demonstrated a complete defect in PTS1-specific protein import as determined by our immunofluorescence assay.

Curiously, a third cell line from complementation group 2 (PBD005) did not exhibit the type-1 phenotype, but rather was unable to import both PTS1 and PTS2 proteins, thus exhibiting a severe type-3 import defect. Since PXR1 is also defective in PBD005 (Dodt et al., 1995), the mutation in this patient must somehow affect the PTS2-dependent peroxisomal matrix protein import pathway as well. The fact that all type-1 mutants fall within a single complementation group in both yeast and humans provides genetic evidence that the PTS1 receptor is the only factor involved specifically in PTS1 protein import.

The type-2 peroxisomal protein import defect

Thirteen PBD fibroblasts were identified that exhibited a specific inability to import thiolase, a PTS2-containing protein, but normal ability to import PTS1 proteins. This type-2 peroxisomal protein import defect was observed for all cell lines from classical RCDP patients but never in cells from ZS, NALD or IRD patients. Unlike the analysis of PTS1 protein import where the test antibody detected multiple PTS1-containing proteins, it was not possible to examine the distribution of another PTS2-containing protein because thiolase is the only

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**Fig. 4.** PBD061 expresses a heterogeneous protein import phenotype. PBD061 fibroblasts were fixed, permeabilized with Triton X-100, and stained with anti-SKL (A and B) and anti-thiolase (C and D) antibodies. Note the rare cells demonstrating punctate distribution of peroxisomal marker proteins in B and D. Bar, 10 µm.
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Neither of which involves peroxisomal thiolase, argues against the possibility that classical RCDP is caused by a defect in peroxisomal thiolase. On the basis of our results, we propose that classical RCDP is caused by a defect in import of PTS2-containing proteins. This hypothesis predicts that: (a) the import of all PTS2-containing proteins is reduced in cells from classical RCDP patients; and (b) both plasmalogen biosynthesis and phytanic acid alpha-oxidation must involve at least one PTS2-containing peroxisomal enzyme, since these pathways are defective in classical RCDP.

In spite of the fact that thiolase is not imported into peroxisomes of classical RCDP patients, the β-oxidation of VLCFAs appears to be normal. What can explain this paradox? One model is that a small, undetectable amount of thiolase is imported into the peroxisome in classical RCDP cells and that this quantity of peroxisomal thiolase is sufficient to complete the β-oxidation spiral. Because kinetic data for the peroxisomal VLCFA β-oxidation enzymes (Hashimoto, 1982) suggest that acyl-CoA oxidase is the rate-limiting enzyme of this pathway, a decrease in thiolase levels would not necessarily result in a decrease in VLCFA β-oxidation. As for mechanisms that would allow a small amount of thiolase import in these cells, one possibility is that the PTS2 protein import pathway may be only partially defective in classical RCDP patients. Alternatively, it may be that PTS2 protein import pathway is completely defective in these patients and that the PTS1-specific protein import pathway has a small capacity for importing proteins with the PTS2 signal. This last model is not without precedent. In the import of mitochondrial proteins, two distinct receptors for mitochondrial targeting signals have been identified, MAS20/MOM19 and MAS70/MOM72 (Ramage et al., 1993; Moczko et al., 1994). Some proteins that are normally imported by MAS20 can, in the absence of MAS20, be partly imported into mitochondria via MAS70.

A second general model to explain the normal VLCFA β-oxidation in classical RCDP is that thiolase is not present in the peroxisome but is able to complete the ‘peroxisomal’ VLCFA β-oxidation spiral in the cytosol. Substrates for thiolase would be transported from the peroxisome into the cytosol, cleaved by the mislocalized thiolase, and the shortened fatty acid would be further oxidized after transport back into the peroxisomes or mitochondria, depending upon its length.

A third possible model is that human peroxisomes contain another thiolase enzyme which is imported via a PTS1 signal. Sterol carrier protein-X (SCPx) contains a canonical PTS1 signal and after it is imported into peroxisomes it is cleaved to release sterol-carrier protein 2 (SCP-2) and a 44 kDa of previously unknown function. A recombinant form of rat SCPx has recently been shown to have thiolase activity against C18 fatty acids, although with 20% of the activity relative to that measured against C8 fatty acids (Seedorf et al., 1994). It may be that this protein, or another as yet unidentified peroxisomal thiolase, is able to complete the VLCFA β-oxidation spiral in classical RCDP cells.

Like the type-1-specific protein import defect, the type-2 protein import defect has also been described for a yeast peroxisome assembly mutant, Saccharomyces cerevisiae pas7 (Marzioch et al., 1994). The fact that only a single complementation group of cells with the type-2 phenotype exists in yeast and humans suggests that there is one peroxisome assembly factor that is required for PTS2 protein import. Given

Fig. 5. PBD018 shows an inability to import PTS1 proteins but no loss of PTS2 protein import. PBD018 fibroblasts were fixed, permeabilized with Triton X-100, and stained with antibodies specific for SKL-containing proteins (A) and thiolase (B). These cells were also fixed, permeabilized with digitonin and stained with the anti-thiolase antibodies (C). Bar, 10 µm.
Fig. 6. Fibroblasts from classical RCDP patients show normal import of PTS1 proteins but no import of the PTS2 marker protein thiolase. Fibroblasts from three different classical RCDP patients, PBD070 (A, B and C), PBD076 (D, E and F), and PBD077 (G, H and I), were each fixed, permeabilized with Triton X-100, and stained with the anti-SKL (A, D and G) and anti-thiolase (B, E and H) antibodies. In addition, these cell lines were also fixed, permeabilized with digitonin, and stained with the anti-SKL antibodies (C, F and I). Bar, 10 µm.
that there must be a receptor for the PTS2 signal, it seems that defects in the putative PTS2 receptor are likely to be responsible for the type-2 peroxisomal protein import defect.

The type-3 peroxisomal protein import defect is usually associated with residual matrix protein import

The absence or reduction of both PTS1 and PTS2 protein import was observed in the vast majority (47/49) of ZS, NALD, and IRD patient cells but not in any cells from RCDP patients. At the initiation of this study we expected that all ZS, NALD, and IRD patients would have the type-3 phenotype, since these diseases were postulated to be caused by the absence of peroxisomal matrix protein import. Furthermore, we expected that the common phenotype would be a complete absence of matrix protein import. While the detection of the two patients with the type-1 import defect was surprising, it was not nearly as surprising as our observation that 85% (40/47) of the type-3 cell lines imported detectable quantities of peroxisomal matrix proteins. We refer to this phenotype as the partial type-3 peroxisomal protein import defect.

While most studies have supported the notion that cells from ZS, NALD, and IRD patients lack the ability to import peroxisomal matrix proteins, a few exceptions have been noted. Unfortunately, these studies are open to numerous interpretations because they only looked at import of one class of peroxisomal matrix protein (only catalase (Santos et al., 1992) or because the studies were performed on a very small number of cell lines (Balfe et al., 1990; Santos et al., 1992). One mistaken conclusion has been that the general phenotype of Zellweger syndrome patients is the ability to import PTS2 proteins but not PTS1 proteins (McCollum et al., 1993), what we have referred to as the type-1 phenotype. On the contrary, not a single Zellweger syndrome patient exhibited this phenotype out of the 22 that were examined; both type-1 cell lines were from neonatal adrenoleukodystrophy patients.

Correlations between particular complementation groups and certain type-3 sub-phenotypes

The analysis of the many type-3 cell lines revealed that certain phenotypes are quite rare, particularly the complete absence of peroxisomal matrix protein import. Nevertheless, the various gradations of the type-3 phenotype do not appear to be distributed randomly, but rather cluster within particular complementation groups (Table 2). For example, the two cell lines comprising CG3 and the two cell lines from CG10 exhibited a complete absence of PTS1 and PTS2 import (the severe type-3 defect), a phenotype shared by only 3 of the 43 other type-3 cell lines (Balfe et al., 1990; Santos et al., 1992). One mistaken conclusion has been that the general phenotype of Zellweger syndrome patients is the ability to import PTS2 proteins but not PTS1 proteins (McCollum et al., 1993), what we have referred to as the type-1 phenotype. On the contrary, not a single Zellweger syndrome patient exhibited this phenotype out of the 22 that were examined; both type-1 cell lines were from neonatal adrenoleukodystrophy patients.

Table 2. Summary of the import defects observed among different PBD complementation groups

<table>
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<th>Complementation group</th>
<th>Type-1 defect</th>
<th>Type-2 defect</th>
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<th>Partial type-3 defect</th>
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*This cell line (PBD061) expressed the heterogeneous type-3 defect.

Table 2. Summary of the import defects observed among different PBD complementation groups

Partial complementation groups can exhibit distinct peroxisomal protein import characteristics, it is also true that a single complementation group can be composed of cell lines with distinct peroxisomal protein import phenotypes (Table 1). From CG1, PBD009 cells show no import of either PTS1 or PTS2 marker proteins whereas PBD047 imports a small amount of both PTS1 and PTS2 proteins. Similar examples are found for members of CG4. In these cases the severely affected cell lines might carry loss of function mutations in the peroxisome assembly gene while the cell lines showing some slight import capacity might have one or two partly functional copies of the gene in question. Variations in phenotype among members of particular PBD complementation groups can also be observed clinically, as evidenced by the fact that ZS, NALD, and IRD patients can all belong to the same complementation group (Roscher et al., 1989).

It has been noted previously that there is a rough, positive correlation between the severity of the biochemical defects of a patient, the severity of the disease in that individual, and the clinical diagnosis as either ZS or NALD (Moser et al., 1995). Our data also suggest correlations between certain peroxisomal protein import defects and clinical presentations. While the most obvious was between the type-2 phenotype and classical RCDP, the fact that both type-1 cell lines were from NALD patients suggests that this import defect may result in a predictable set of clinical phenotypes. The fact that all seven type-3 cell lines devoid of PTS1 and PTS2 protein import were from Zellweger syndrome patients suggests that the more severe peroxisomal protein import defects may result in more severe clinical phenotypes.

**Peroxisome biogenesis disorder patients have the same peroxisomal protein import defects as yeast pas mutants**

A key finding of this report is that cell lines from PBD patients exhibit the same type-1, type-2, and type-3 peroxisomal protein import defects described for the yeast pas mutants (McCollum et al., 1993; Elgersma et al., 1993; Kunau et al., 1993; van der Leij et al., 1992). This observation has also been made by Motley et al. (1994) in a recent analysis of peroxisomal protein import in cell lines from 5 PBD patients. We have confirmed this basic observation and extended it by reporting the frequency of the peroxisomal matrix protein import phenotypes of 62 PBD cell lines representing 10 complementation groups. The comprehensive nature of this study has also
allowed us to draw correlations between certain genotypes and particular peroxisomal protein import defects in the cell lines from PBD patients. The most basic of these is the finding that cell lines with the type-1 defect all belong to a single complementation group (CG2), cell lines with the type-2 defect all fall within one other complementation group (CG11), and all remaining complementation groups are composed of cell lines with the type-3 phenotype. This again is similar to what has been observed for yeast pas mutants, where the type-3 defect occurs in 13/15 known complementation groups of yeast pas mutants (S. cerevisiae pas1-6, 8, 9, 11, 12, 20-22 and P. pastoris pas1-7, 9-11), all type-1 mutants belong to a single complementation group (P. pastoris pas8 and S. cerevisiae pas10) and only one complementation group of type-2 mutants (S. cerevisiae pas7) (McCollum et al., 1993; Elgersma et al., 1993; Marzioch et al., 1994). The similarities between the human PBD cell lines and the yeast pas mutants even extend to the association between particular complementation groups and certain sub-phenotypes of the type-3 defect: in the yeast P. pastoris, the pas1 (Heyman et al., 1994), pas4 (Kalish and Gould, unpublished observations), and pas5 (Spong and Subramani, 1993) mutants are able to import small quantities of matrix proteins into peroxisomes whereas the pas7 and pas10 mutants appear to be completely defective in protein import (Kalish and Gould, unpublished observations). These similarities suggest that the process of peroxisome assembly might occur by the same mechanism in both yeast and human cells. Furthermore, these results suggest that defects in the human equivalents of the yeast PAS genes might be the cause of the peroxisome biogenesis disorders. The recent cloning of PXR1, the human homolog of yeast PAS8 (Dodt et al., 1995) provides even more direct evidence for this model.

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