

Metabolic control of peroxisome abundance

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Accepted 25 February; published on WWW 22 April 1999

SUMMARY

Zellweger syndrome and related disorders represent a group of lethal, genetically heterogeneous diseases. These peroxisome biogenesis disorders (PBDs) are characterized by defective peroxisomal matrix protein import and comprise at least 10 complementation groups. The genes defective in seven of these groups and more than 90% of PBD patients are now known. Here we examine the distribution of peroxisomal membrane proteins in fibroblasts from PBD patients representing the seven complementation groups for which the mutant gene is known. Peroxisomes were detected in all PBD cells, indicating that the ability to form a minimal peroxisomal structure is not blocked in these mutants. We also observed that peroxisome abundance was reduced fivefold in PBD cells that are defective in the *PEX1*, *PEX5*, *PEX12*, *PEX6*, *PEX10*, and *PEX2* genes. These cell lines all display a defect in the import of proteins with the type-1 peroxisomal

targeting signal (PTS1). In contrast, peroxisome abundance was unaffected in cells that are mutated in *PEX7* and are defective only in the import of proteins with the type-2 peroxisomal targeting signal. Interestingly, a fivefold reduction in peroxisome abundance was also observed for cells lacking either of two PTS1-targeted peroxisomal β -oxidation enzymes, acyl-CoA oxidase and 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase. These results indicate that reduced peroxisome abundance in PBD cells may be caused by their inability to import these PTS1-containing enzymes. Furthermore, the fact that peroxisome abundance is influenced by peroxisomal β -oxidation activities suggests that there may be metabolic control of peroxisome abundance.

Key words: Peroxisome biogenesis disorder, Peroxisomal β -oxidation, Peroxisome abundance

INTRODUCTION

The peroxisome biogenesis disorders (PBD) represent a group of genetically heterogeneous syndromes that are caused by a defect in peroxisomal matrix protein import (Lazarow and Moser, 1995). These often lethal disorders include Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD), and classical rhizomelic chondrodysplasia punctata (cRCDP). Of these, ZS, NALD and IRD appear to represent a continuum of related diseases, referred to here as the Zellweger spectrum. Within this continuum, ZS patients display the most severe phenotypes. These include an inability to import proteins that are targeted to the peroxisome matrix by either the PTS1 or PTS2, the loss of most peroxisomal metabolic pathways, and an array of hepatic, renal and neurologic dysfunctions, including mental retardation. ZS patients rarely survive the first year. NALD patients display similar but slightly less severe phenotypes and are often capable of slight protein import into the peroxisome lumen. IRD patients are the most mildly affected individuals in the ZS spectrum and may survive into their second, third, and in rare instances, fourth decade (Moser et al., 1995a). The

PBD also include rhizomelic chondrodysplasia punctata (RCDP). This distinct disease is characterized by a specific defect in import of PTS2 proteins but normal import of PTS1 proteins, loss of metabolic processes that involve PTS2-targeted enzymes, and clinical phenotypes that include a characteristic shortening and calcification of the proximal limbs, neurological dysfunction, and mental retardation.

Goldfischer et al. (1973) were the first to recognize that ZS patients are defective in peroxisome biogenesis. Peroxisomes were originally thought to be absent from cells of ZS patients. However, subsequent studies demonstrated the existence of peroxisomal structures in cells from several ZS patients (Santos et al., 1988a,b). These minimal peroxisomes failed to import peroxisomal matrix proteins but contained the normal complement of peroxisomal membrane proteins (PMPs). These results provided the first evidence that: (1) peroxisomal matrix protein import is fundamentally distinct from PMP import; and (2) ZS cells have a specific defect in peroxisomal matrix protein import. However, these earlier studies failed to explain why peroxisomes of ZS cells are reduced in abundance and are abnormally large. Furthermore, these studies were limited to just a few cell lines and were undertaken prior to the

identification of the genes that are defective in the PBD. The genes responsible for seven of the eleven known complementation groups of the PBD and more than 90% of PBD patients are now known. *PEX5*, which encodes the PTS1 receptor, is mutated in complementation group 2 (CG2) (Dodt et al., 1995; Wiemer et al., 1995). *PEX7*, which encodes the PTS2 receptor, is mutated in CG11 (Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997), a group comprised solely of RCDP patients. *PEX1* and *PEX6* encode a pair of interacting ATPases that are required for stabilization of *PEX5* (Dodt and Gould, 1996; Yahraus et al., 1996) and are mutated in CG1 and CG4, respectively (Fukuda et al., 1996; Portsteffen et al., 1997; Reuber et al., 1997; Yahraus et al., 1996). *PEX2*, *PEX10*, and *PEX12* all encode zinc-binding integral peroxisomal membrane proteins and are mutated in CGs 10, 7, and 3, respectively (Chang et al., 1997; Okumoto et al., 1998; Shimozawa et al., 1992; Warren et al., 1998). Here we extend the earlier studies on the nature of peroxisomes in PBD cells by following the distribution of PMPs in PBD fibroblasts representing the seven complementation groups of the PBD for which the genetic defect is known. Many peroxisomal structures were detected in cells lacking the *PEX1*, *PEX2*, *PEX5*, *PEX6*, *PEX7*, *PEX10* or *PEX12* genes. We also find that peroxisome abundance is reduced in cells with defects in either of two peroxisomal fatty acid β -oxidation enzymes and that overexpression of an enzyme expected to inhibit peroxisomal fatty acid oxidation, thioesterase, also reduces peroxisome abundance. Our results indicate that there may be metabolic control of peroxisome abundance and that the aberrant peroxisome abundance of ZS cells can be explained solely by their matrix protein import defects.

MATERIALS AND METHODS

Cell lines and cell culture

Cells were cultured in Dulbecco's modified Eagle's medium with high glucose and were supplemented with 10% fetal calf serum and penicillin/streptomycin. All cell lines used in this study were human skin fibroblasts. The control cell lines GM5659, GM5756, and GM8333 were obtained from the Coriell Cell Repository (Camden, NJ), as were the PBD cell lines GM228 and GM4340. The CG1 cell line PBD009 was isolated from a ZS patient and has a splice site mutation in *PEX1* (Reuber et al., 1997). PBD009 cells fail to express *PEX1* mRNA (Reuber et al., 1997) or protein (B. Geisbrecht and S. J. Gould, unpublished observations). The CG2 cell line PBD005 was derived from a ZS patient and is homozygous for a nonsense mutation in *PEX5* (R390ter) (Dodt et al., 1995). PBD005 cells lack detectable levels of *PEX5* mRNA or protein (Dodt et al., 1995). PBD097 cells were isolated from a ZS patient who is a compound heterozygote for frameshift mutations in the *PEX12* gene, both of which inactivate *PEX12* (Chang et al., 1997). *PEX6*-deficient PBD106 cells were isolated from a ZS patient who lacks detectable *PEX6* mRNA and has frameshift mutations on both alleles of the *PEX6* gene (Yahraus et al., 1996). PBD100 cells were derived from a ZS patient, are homozygous for a splice site mutation in *PEX10*, and express only internally deleted, nonfunctional *PEX10* mRNAs (Warren et al., 1998). PBD094 cells were derived from a ZS patient and are homozygous for a nonsense mutation in *PEX2* (R119ter) which abrogates *PEX2* activity (Shimozawa et al., 1992). PBD070 cells are derived from a severely affected RCDP cell line with inactivating mutations in *PEX7* (Braverman et al., 1997), as are PBD073 and PBD076 cells. PBD052 cells were derived from a mildly affected NALD patient and display

significant PTS1 and PTS2 protein import. This cell line is a compound heterozygote for two mutations in *PEX10*, R125ter and H290Q. Both mutations reduce *PEX10* activity but neither eliminates function of the gene (Warren et al., 1998).

The fibroblast cell lines ACX001-006 were derived from patients with peroxisomal acyl-CoA oxidase deficiency. All acyl-CoA oxidase deficient patients have known mutations in the acyl-CoA oxidase gene (Fournier et al., 1994) or belong to the same complementation group as cells with mutations in this gene, as determined by cell fusion analysis. The fibroblast cell lines MFE2001-004 were derived from patients with peroxisomal 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase deficiency and have mutations in the D-specific 2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene, MFE2 (Suzuki et al., 1997; van Grunsven et al., 1998). The X-linked adrenoleukodystrophy cell lines were from X-ALD patients with inactivating mutations in the X-ALD gene (Kok et al., 1995; Mosser et al., 1993). The alkyl-dihydroxyacetonephosphate synthase-deficient patient has an inactivating mutation in the structural gene for this enzyme (de Vet et al., 1998). The dihydroxyacetonephosphate acyltransferase-deficient patients have inactivating mutations in the dihydroxyacetonephosphate acyltransferase gene (Ofman et al., 1998). The Refsum disease patients had inactivating mutations in the Refsum disease gene, *PAHX*, which encodes peroxisomal phytanoyl-CoA α -hydroxylase (Jansen et al., 1997; Mihalik et al., 1997). C26:C22 ratios were determined as described (Moser et al., 1995a).

Immunofluorescence and transfections

Cells were grown on 18 mm glass circle coverslips for 24-48 hours to 60-80% confluency and then processed for indirect immunofluorescence (Slawewski et al., 1995). Cells were washed twice with 5 ml Dulbecco's modified phosphate buffered saline, pH 7.4 (DPBS; Gibco/BRL, Bethesda, MD) and fixed by incubation in 3.7% formaldehyde in DPBS for 15-30 minutes at ambient temperature. Cells were then washed twice in DPBS and incubated for 5 minutes in 1% Triton X-100 in DPBS. Cells were again washed twice with DPBS and then incubated with primary antibodies, either rabbit anti-PMP70 sera (diluted 1:900 in DPBS) or affinity purified rabbit anti-*PEX14* antibodies (diluted 1:200 in DPBS) for 15-30 minutes at ambient temperature. Cells were then washed 10 times with DPBS and incubated with Texas Red-conjugated goat anti-rabbit secondary antibodies for 15-30 minutes. Cells were washed 10 times with DPBS and mounted on glass slides in a solution of 90% glycerol, 100 mM Tris-HCl, pH 8.5, 0.1% *para*-phenylenediamine. Polyclonal antibodies specific for PMP70 were obtained from Dr Suresh Subramani (San Diego, USA). Polyclonal antibodies specific for *PEX14* were generated against a bacterially synthesized form of human *PEX14*. Labeled secondary antibodies were obtained from commercial sources. The number of peroxisomes per cell was quantitated using a fluorescence microscope by counting the number of PMP70-containing or *PEX14*-containing vesicles per cell. Peroxisome abundance was determined in 20 randomly selected cells from each line and the averages and standard deviations were calculated for all cell lines. No data was excluded in the statistical analysis. Peroxisome abundance measurements were made by at least 2 individuals for each cell line.

Plasmids were prepared using standard procedures (Sambrook et al., 1989). Transfection of PBD100 cells was by electroporation (Chang et al., 1997) using pcDNA3 or pcDNA3-*PEX11* β myc (Schradler et al., 1998). One day after transfection the cells were seeded onto glass coverslips. On the second day after transfection the cells were processed for double indirect immunofluorescence using anti-PMP70 antibodies and the anti-myc monoclonal antibody, 1-9E10 (Evan et al., 1985). Secondary antibodies were obtained from commercial sources. Antibodies specific for acyl-CoA oxidase and MFE-2 were obtained from Dr Paul Watkins (The Kennedy Krieger Institute). The Nmyc-PTE1 expression vector has been described (Jones et al., 1999).

RESULTS

Peroxisomes in PBD groups 1-4,7,10, and 11

The existence of peroxisomal structures in cells from PBD patients has been established previously (Santos et al., 1988a,b). However, the PBD are genetically heterogeneous and the fate of PMPs in cells from different PBD complementation groups with defined genotypes has yet to be addressed. Here we examined the distribution of integral PMPs in cell lines representing the seven PBD complementation groups for which the defective gene is known. We examined only those cell lines which: (1) exhibited little or no peroxisomal matrix protein import; (2) were derived from severely affected patients; and

(3) had mutations that have been shown previously to abrogate gene function.

Control and PBD fibroblast cells were cultured under standard conditions, fixed, permeabilized, and processed for indirect immunofluorescence using antibodies specific for an integral PMP, PMP70. PMP70 has been shown previously to be a common component of peroxisomes in mammalian cells (Kamijo et al., 1990). Peroxisomes of normal cells were readily detected using the PMP70 antibodies (Fig. 1A). Furthermore, numerous peroxisomal structures were detected in PBD cell lines from 7 different complementation groups, including a *PEX1*-deficient CG1 patient (Fig. 1B), a *PEX5*-deficient CG2 patient (Fig. 1C), a *PEX12*-deficient CG3 patient (Fig. 1D), a

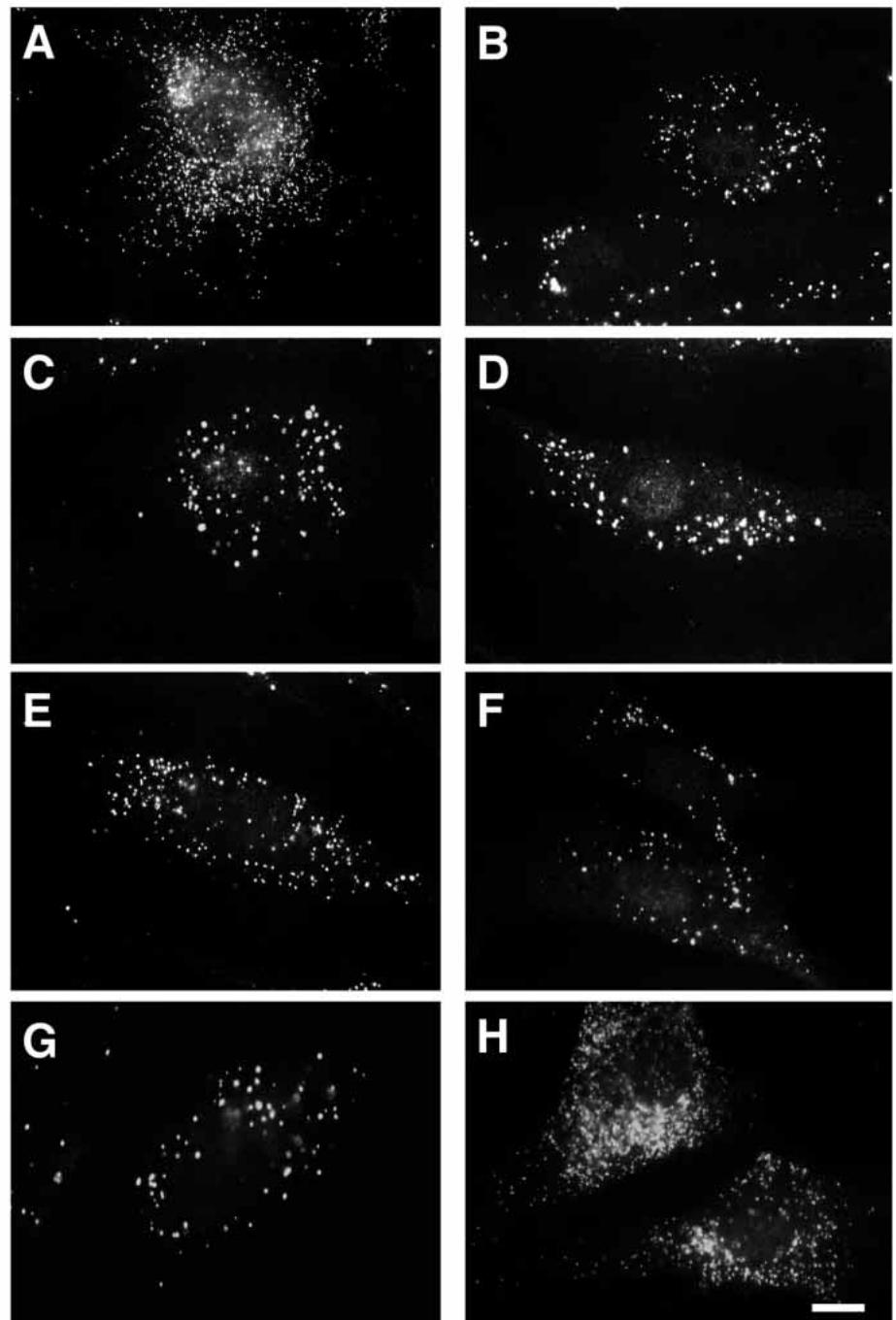


Fig. 1. Peroxisomes in PBD fibroblasts. Human skin fibroblasts were grown as described and processed for indirect immunofluorescence using a rabbit polyclonal antibody directed against PMP70, followed by Texas Red-labeled secondary antibodies. The distribution of PMP70 is shown in (A) the control cell line, GM5756; (B) the *PEX1*-deficient CG1 cell line, PBD009; (C) the *PEX5*-deficient CG2 cell line, PBD005; (D) the *PEX12*-deficient CG3 cell line, PBD097; (E), the *PEX6*-deficient CG4 cell line, PBD106; (F) the *PEX10*-deficient CG7 cell line, PBD100, (G) the *PEX2*-deficient CG10 cell line, PBD094, and (H) the *PEX7*-deficient CG11 cell line, PBD073. Bar, 25 μ m.

PEX6-deficient CG4 patient (Fig. 1E), a *PEX10*-deficient CG7 patient (Fig. 1F), a *PEX2*-deficient CG10 patient (Fig. 1G) and a *PEX7*-deficient CG11 patient (Fig. 1H). The genetic defects in these cell lines are described in Materials and Methods.

Reduced peroxisome abundance in PBD fibroblasts

A casual examination of these images indicated that the peroxisomes of most PBD fibroblasts were less numerous than those of control fibroblasts. They also appeared to be larger than peroxisomes of normal cells. The difference in peroxisome size has been quantitated previously by Santos et al. (1988b, 1992), who found that peroxisomes of ZS cells have approximately two times the radius of peroxisomes from normal cells. However, the difference in peroxisome abundance between normal and PBD cells had only been mentioned as an ancillary observation and has not previously been quantitated. Furthermore, there is no data in the literature on the generality of aberrant peroxisome abundance amongst the different complementation groups of the PBD. To address these issues we measured the abundance of peroxisomal structures in control fibroblasts and PBD fibroblasts from complementation groups 1, 2, 3, 4, 7, 10, and 11. Each line was coded, processed for indirect immunofluorescence using antibodies specific for PMP70, and then examined in a blind fashion by 2-3 different observers. Peroxisome abundance was determined by counting the number of distinct PMP70-labeled structures in a minimum of 20 randomly chosen cells. The control human fibroblasts all contained a similar number of peroxisomes: 554 ± 116 peroxisomes/cell in the GM5756 line, 514 ± 104 peroxisomes/cell in the GM5659 line, and 501 ± 106 peroxisomes/cell in the GM8333 line. In contrast, the abundance of PMP70-containing peroxisomal structures in the PBD cell lines were 98 ± 49 for PBD009 cells, 89 ± 63 for PBD005 cells, 95 ± 79 for PBD097 cells, 98 ± 48 for PBD106 cells, 88 ± 66 for PBD100 cells, 40 ± 11 for PBD094 cells, 551 ± 152 for PBD070 cells, 582 ± 193 for PBD073 cells, and 520 ± 206 for PBD076 cells. Thus, cells representing PBD complementation groups 1-4, 7, and 10 all displayed a significant decrease in the abundance of peroxisomal structures, to less than 20% the level observed in normal fibroblasts (Fig. 2).

The only PBD cell line which did not exhibit a decrease in peroxisome abundance was the CG11 cell line, PBD070, which had a normal amount of peroxisomal structures (551 ± 152). We examined peroxisome abundance in two other CG11 cell lines, PBD073 and PBD076, both of which were derived from severely affected RCDP patients. These lines also contained a normal number of peroxisomes (Fig. 2). It is worth noting that the CG11 lines import PTS1 proteins normally and have normal peroxisome abundance whereas PBD cell lines which do not import PTS1 proteins have reduced peroxisome abundance.

Although PMP70 is known to be a common constituent of peroxisome membranes and is used widely as a peroxisomal marker, it was formally possible that the reduced peroxisome abundance was limited to PMP70-containing structures. Therefore, we re-examined the PBD fibroblasts using antibodies specific for a different, unrelated integral PMP. PMP70 is a member of the ABC transporter superfamily, is thought to mediate transport of at least some class of fatty acids, and is required for peroxisomal oxidation of

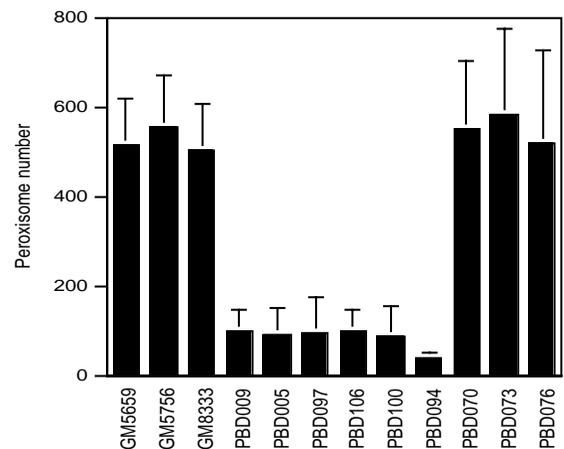


Fig. 2. Peroxisome abundance in control and PBD cells. Cells were processed for indirect immunofluorescence using antibodies specific for the peroxisomal marker PMP70. Samples were examined by fluorescence microscopy and the numbers of peroxisomes present in 20 randomly selected cells were counted. Results are presented as the average peroxisome abundance \pm one standard deviation.

dicarboxylic fatty acids (G. Jimenez-Sanchez and D. Valle, personal communication). In contrast, human *PEX14* is an integral PMP which lacks similarity to any class of known proteins and is involved in protein import into peroxisomes, probably as a component of the PTS1-receptor docking apparatus on the peroxisome membrane (Fransen et al., 1998). The distribution of *PEX14* in PBD cells was determined by indirect immunofluorescence (Fig. 3). Cells were examined by fluorescence microscopy and the number of peroxisomes present in 20 randomly selected cells was determined. Results are presented as the average peroxisome abundance \pm one standard deviation. The abundance of *PEX14*-containing peroxisomal structures per cell was 536 ± 114 for GM5756 cells, 94 ± 47 for PBD009 cells, 80 ± 57 for PBD005 cells, 93 ± 68 for PBD097 cells, 108 ± 51 for PBD106 cells, 87 ± 68 for PBD100 cells, and 40 ± 12 for PBD094 cells.

A metabolic basis for reduced peroxisome abundance

One possible explanation for the reduced abundance of peroxisomes in CG1, CG2, CG3, CG4, CG7, and CG10 cell lines is that the *PEX1*, *PEX5*, *PEX12*, *PEX6*, *PEX10*, and *PEX2* genes all play roles in the regulation of peroxisome abundance. However, these cell lines also exhibit pronounced defects in multiple peroxisomal metabolic processes (Lazarow and Moser, 1995). Therefore, it was also possible that reduced abundance of peroxisomal structures in PBD cells might be a secondary consequence of their peroxisomal metabolic deficiencies. In an attempt to distinguish between these possibilities, we examined peroxisome abundance in fibroblasts from patients with defects in single peroxisomal enzymes.

Although mitochondria are the site of most fatty acid oxidation in human cells, the peroxisomal β -oxidation pathway is required for the oxidation of very-long, branched and dicarboxylic fatty acids (Lazarow and Moser, 1995). Defects in peroxisomal β -oxidation are lethal and fall within 4 complementation groups (Wanders et al., 1996). The genes

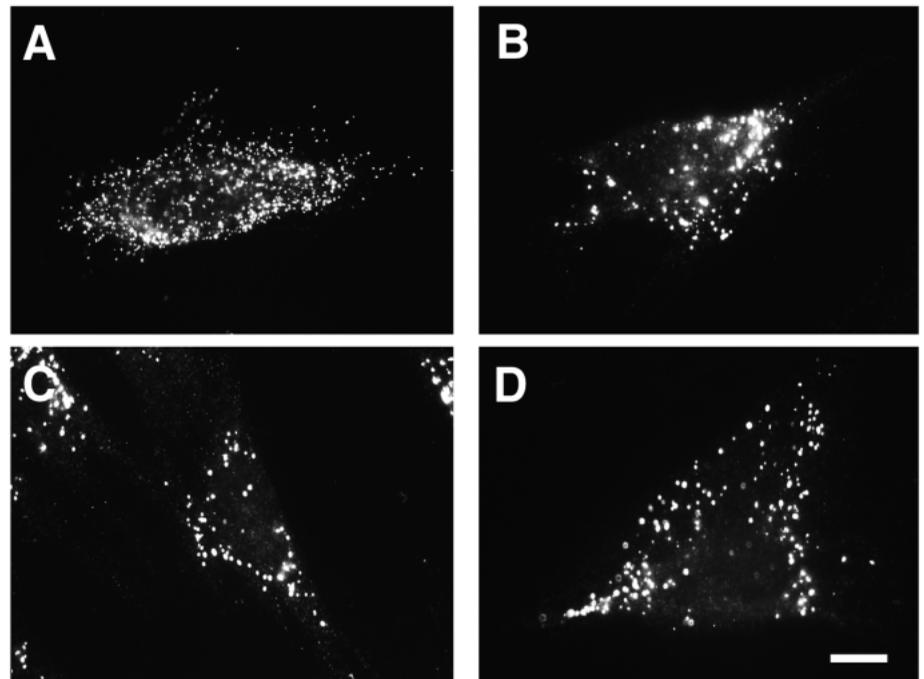
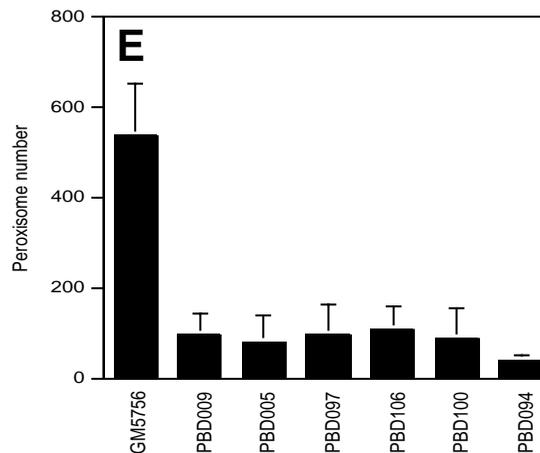


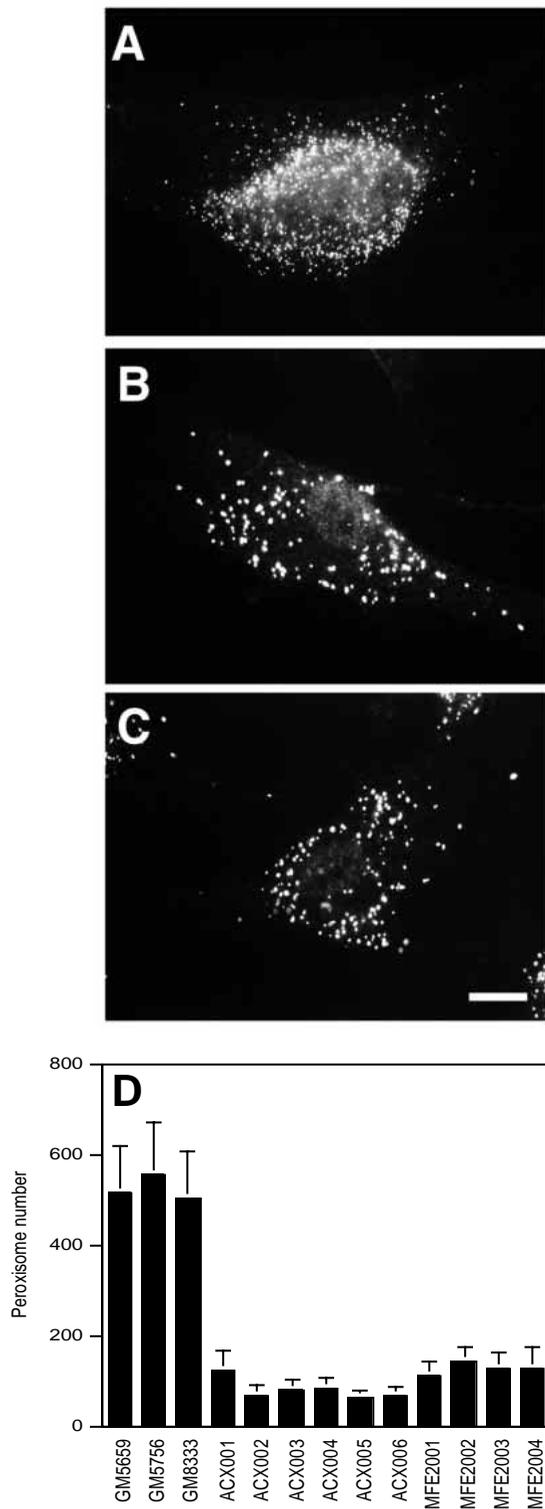
Fig. 3. PEX14-containing peroxisomes of control and ZS cells. Human skin fibroblasts were grown as described and processed for indirect immunofluorescence using a rabbit polyclonal antibody directed against human PEX14 and Texas Red-labeled secondary antibodies. The distribution of PEX14 is shown in (A) the control cell line, GM5756, (B) the *PEX1*-deficient CG1 cell line, PBD009, (C) the *PEX12*-deficient CG3 cell line, PBD097, and (D) the *PEX10*-deficient CG7 cell line, PBD100. Bar, 25 μ m. E, Peroxisome abundance in control and PBD cells as determined using PEX14 as the peroxisome marker.



mutated in the two most common groups of peroxisomal β -oxidation deficient patients are known and encode peroxisomal acyl-CoA oxidase and peroxisomal 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase (*MFE2*). Surprisingly, cells from patients with mutations in these genes displayed a pronounced reduction in peroxisome abundance (Fig. 4). Average peroxisome abundance in six acyl-CoA oxidase-defective cell lines was 67 ± 30 peroxisomes per cells and the average peroxisome abundance in four cell lines that are defective in the 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase gene was 120 ± 30 peroxisomes per cell. In addition, the peroxisomes of these β -oxidation-deficient cells appeared larger than in control cells, another phenotype shared by PBD cells that fail to import PTS1 proteins.

We next tested whether this phenotype was specific for cells with defects in peroxisomal β -oxidation or resulted from loss of any peroxisomal enzyme. The distribution of PMPs was examined in cells lacking other peroxisomal proteins (Fig. 5). The most common peroxisomal disorder is X-linked adrenoleukodystrophy (X-ALD), a degenerative neurological

disorder which is associated with reduced peroxisomal oxidation of very-long chain fatty acids (Moser et al., 1995b). However, the defect in peroxisomal fatty acid oxidation in X-ALD is restricted to very long chain fatty acids: peroxisomal oxidation of long chain fatty acids, branched chain fatty acids, and dicarboxylic fatty acids is unaffected in these patients (Lazarow and Moser, 1995; Moser et al., 1995b). The ALDP gene is mutated in X-ALD patients and encodes an integral PMP (Mosser et al., 1993, 1994). Although its precise role has yet to be determined, ALDP is a member of the ABC transporter family of proteins and the phenotypes of X-ALD patients suggest that it may be involved in the import of very-long chain fatty acids into peroxisomes. Peroxisome abundance was normal in fibroblasts from ALD patients (Fig. 5). The first three steps in the synthesis of plasmalogens are peroxisomal and mutations in the structural genes for enzymes that catalyze two of these reactions, alkyl-dihydroxyacetonephosphate synthase (de Vet et al., 1998) and dihydroxyacetonephosphate acyltransferase (Ofman et al., 1998), result in lethal diseases. Cells from patients who are



mutated in these genes contained a normal number of peroxisomes per cell. The α -oxidation of phytanic acid, a major dietary fatty acid, is also a peroxisomal process and mutations in the gene encoding peroxisomal phytanoyl-CoA α -hydroxylase are the cause of Refsum disease (Jansen et al., 1997; Mihalik et al., 1997). We also observed normal peroxisome abundance in fibroblasts from Refsum disease patients (data not shown). Thus, reduced peroxisome

Fig. 4. Aberrant peroxisome abundance and morphology in cells lacking single enzymes of peroxisomal β -oxidation. Human skin fibroblasts were processed for indirect immunofluorescence using a rabbit polyclonal antibody directed against PMP70, followed by Texas Red-labeled secondary antibodies. Representative images are shown for (A) a control cell line (GM5756), (B) an acyl-CoA oxidase-deficient patient (ACX003), and (C) a 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase-deficient patient (MFE2002). Bar, 25 μ m. D, Peroxisome abundance in control, acyl-CoA oxidase-deficient patients (ACX001-006), and 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase-deficient patients (MFE2001-004). Cells were examined by fluorescence microscopy and the number of peroxisomes present in 20 randomly selected cells was determined. Results are presented as the average peroxisome abundance \pm one standard deviation.

abundance is a phenotype specific to cells with generalized defects in peroxisomal β -oxidation.

These data indicate that reduced peroxisome abundance is related to loss of peroxisomal acyl-CoA oxidase and/or 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase activities, and perhaps peroxisomal β -oxidation in general. If true, peroxisome abundance in PBD cells should be inversely correlated with the severity of the PTS1 protein import defect observed in PBD cells. As noted earlier, NALD cell lines generally display at least some matrix protein import and have less severe biochemical deficits, due primarily to less severe mutations in the corresponding *PEX* gene. Warren et al. (1998) have described cells from two patients with mutations in *PEX10*, PBD100 and PBD052. PBD100, the CG7 cell line examined above, was derived from a ZS patient and displays no detectable matrix protein import. PBD052 cells were derived from an NALD patient and display significant import of PTS1 proteins. Mutation studies revealed that PBD100 is homozygous for a splice site mutation which causes a large internal deletion in the *PEX10* mRNA and eliminates *PEX10* activity. In contrast, PBD052 is a compound heterozygote for missense and nonsense mutations, neither of which eliminate *PEX10* activity. We find here that PBD052 cells contain 391 ± 140 peroxisomes/cell, a significant difference from the abundance of peroxisomes in PBD100 cells (88 ± 66 peroxisomes/cell). Similar relationships between peroxisomal protein import capacities and peroxisome abundance were also observed in cells from complementation groups 1, 3, and 4 (data not shown).

Reduced abundance of peroxisomal structures and peroxisomal β -oxidation

The reduced abundance of peroxisomal structures in cell lines with direct or indirect defects in peroxisomal fatty acid β -oxidation raised the issue of whether the fatty acid oxidation activities of these cells correlated directly with peroxisome abundance. Although we do not have data on peroxisomal fatty acid β -oxidation activities for these cell lines, measurements of very long chain fatty acids have been determined for most of the cell lines that we examined in this report (Moser et al., 1995a). These are presented here as the ratio of C26 to C22 fatty acids in each cell line and are shown together with the peroxisome abundance of each line (Table 1). Although the ratio of C26 to C22 fatty acids reflects the peroxisomal oxidation of only very long chain fatty acids, just one of several substrates of peroxisomal fatty acid β -oxidation (Lazarow and

Moser, 1995), they are at least informative. The primary exceptions are the X-ALD cells, which are defective only in the oxidation of this one peroxisomal substrate but appear to be normal for peroxisomal dicarboxylic and branched chain fatty acid oxidation (Moser et al., 1995b). Control cells have C26:C22 values of 0.08 ± 0.03 and the cell lines with defects in very long chain fatty acid oxidation have a much higher ratio, often between 0.5 and 2. Interestingly, the mildly affected *PEX10*-deficient cell line, PBD052, has a nearly normal C26:C22 ratio whereas the severely affected cell line, PBD100, has a very high C26:C22 ratio. In addition, the fact that X-ALD patients fail to display reduced peroxisome abundance suggests that the flux of very long chain fatty acids through the peroxisomal fatty acid β -oxidation pathway is not involved in the regulation of peroxisome abundance. The absence of data for the three RCDP cell lines, PBD070, PBD073, and PBD076, reflects the well-established fact that RCDP patients do not display any defects in peroxisomal fatty acid oxidation (Braverman et al., 1997; Lazarow and Moser, 1995).

Reduced peroxisome abundance is not due to loss of β -oxidation proteins

Although peroxisome abundance was clearly reduced in fibroblasts derived from patients with mutations in the acyl-CoA oxidase gene or the MFE2 gene, it was not clear whether these were the result of defective enzyme activities or the loss

of these proteins from the cell. To distinguish between these alternatives we tested whether any of the acyl-CoA oxidase-defective patients contained the protein in their peroxisomes or whether any of the MFE2-defective fibroblasts expressed peroxisomal MFE2. Indirect immunofluorescence experiments revealed that two of the acyl-CoA oxidase-deficient cell lines (ACX001 and ACX002) contained significant levels of peroxisomal acyl-CoA oxidase (Fig. 6A,B). Similar experiments revealed that the *MFE2*-deficient patient, MFE2003, lacked detectable staining for MFE2 but that another patient, MFE2004, contained significant levels of peroxisomal MFE2 protein (Fig. 6C, D). Western blot experiments (Fig. 7A) confirmed that the levels of acyl-CoA oxidase protein in ACX001 and ACX002 were similar to the levels in normal human fibroblasts. They also confirmed that MFE2003 lacked MFE2 protein but that MFE2 protein was abundant in MFE2004 cells (Fig. 7B). It should be noted that the abundance of peroxisomal structures in MFE2003 and MFE2004 are indistinguishable even though one of these lines contains normal levels of MFE2 and the other lacks MFE2.

The hypothesis that peroxisomal β -oxidation may control peroxisome abundance predicts that any generalized disruption of this process will lead to reduced peroxisome abundance. We recently identified a human peroxisomal thioesterase, PTE1 (Jones et al., 1999), an enzyme which catalyzes the conversion of acyl-CoAs to free fatty acids and CoASH. Fatty acid oxidation requires the prior esterification of fatty acids with CoA and overexpression of PTE1 would be expected to inhibit peroxisomal fatty acid β -oxidation by degrading the substrates of this pathway. A myc-tagged form of PTE was transfected into human skin fibroblasts and the cells were subsequently processed for indirect immunofluorescence using antibodies specific for the myc tag. Cells which overexpressed Nmyc-PTE1 contained only a few, large peroxisomes (Fig. 8A). To ensure that this was not an artifact associated with the overexpression of any peroxisomal matrix protein, we expressed an unrelated human peroxisomal enzyme, alpha-hydroxy acid oxidase, in the same cell line. Cells overexpressing this protein displayed normal peroxisome abundance (Fig. 8B).

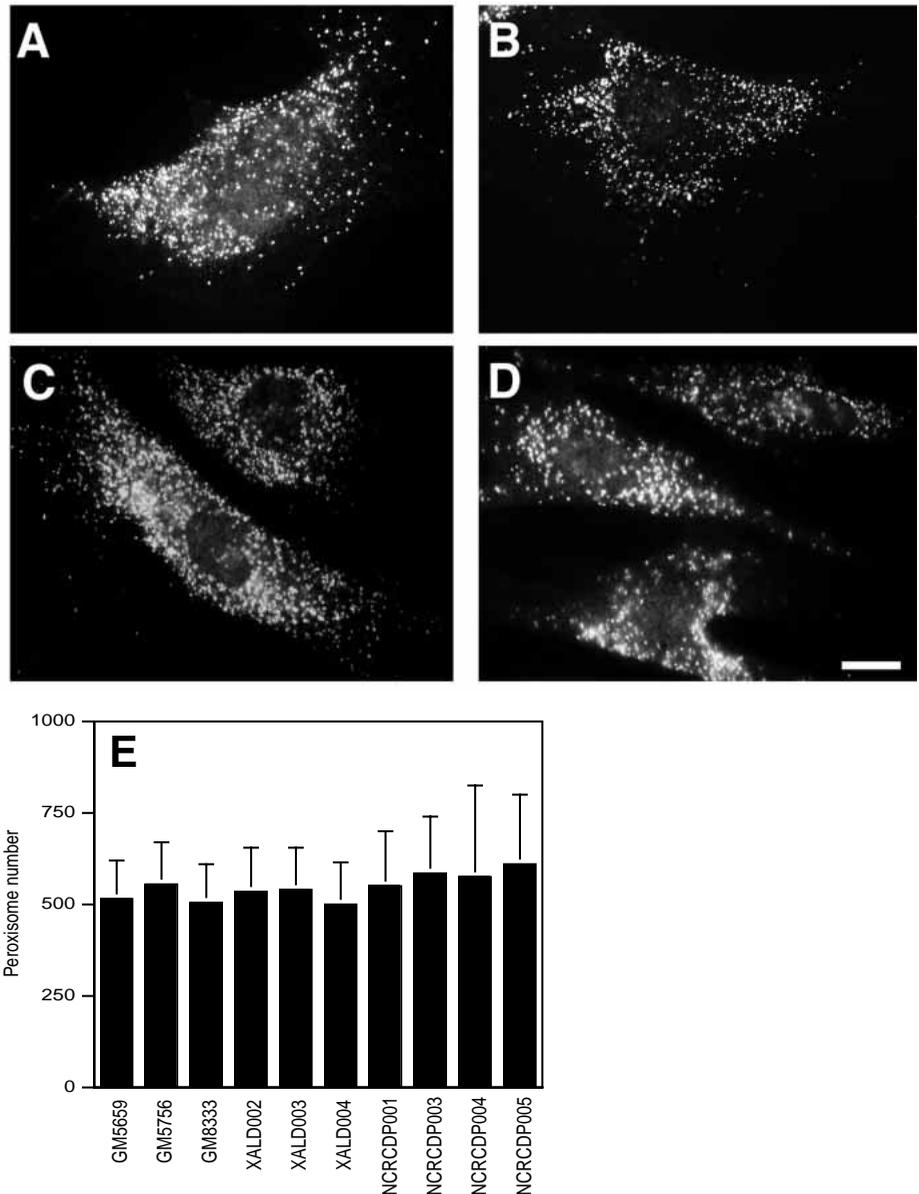
PEX11-mediated peroxisome proliferation in PBD cells

Peroxisome abundance in yeast is controlled, at least in part, by the expression of *PEX11* (Erdmann and Blobel, 1995; Marshall et al., 1995). This also appears to be true for human cells, which express two distinct *PEX11* genes, *PEX11 α* and *PEX11 β* (Schrader et al., 1998). One of many possible explanations for the reduced abundance of peroxisomes in PBD and β -oxidation-defective fibroblast cells is that they may be unable to proliferate peroxisomes in response to *PEX11*. We have demonstrated previously that overexpression of human *PEX11 β* or *PEX11 β myc* can induce peroxisome proliferation in normal human fibroblasts (Schrader et al., 1998). To determine whether peroxisome proliferation could be induced in ZS cells, the severely affected *PEX10*-deficient line PBD100 was transfected with a control plasmid and a plasmid designed to express *PEX11 β myc*. Two days after transfection, the abundance of peroxisomes was determined by indirect immunofluorescence using antibodies to PMP70 and *PEX11 β myc*. PBD100 cells that were transfected with vector

Table 1. Peroxisome abundance in fibroblast cell lines from unaffected individuals and patients with different peroxisomal disorders

Cell lines	PMP70-containing peroxisomes (n=20)	PEX14-containing peroxisomes (n=20)	C26:C22 levels
GM5659	514±104	n.d.	n.d. (0.08±0.03)
GM5756	554±116	536±114	n.d.
GM8333	501±106	n.d.	n.d.
PBD009	98±49	94±47	0.98
PBD005	89±63	80±57	1.36
PBD097	95±79	93±68	0.9
PBD106	98±48	108±51	0.93
PBD100	88±66	87±68	1.55
PBD052	391±140	n.d.	0.18
PBD094	40±11	40±12	0.8
PBD070	551±152	n.d.	n.d.
PBD073	582±193	n.d.	n.d.
PBD076	520±206	n.d.	n.d.
ACX001	124±41	n.d.	1.58
ACX002	66±25	n.d.	0.5
ACX003	78±24	n.d.	0.95
ACX004	84±23	n.d.	0.50
ACX005	61±18	n.d.	1.23
ACX006	67±19	n.d.	0.86
MFE2001	112±29	n.d.	1.63
MFE2002	141±35	n.d.	1.70
MFE2003	128±36	n.d.	1.26
MFE2004	128±48	n.d.	0.60
XALD002	534±121	n.d.	0.50
XALD003	539±113	n.d.	0.57
XALD004	500±111	n.d.	0.53
NCRCDP001	548±152	n.d.	0.059
NCRCDP003	582±155	n.d.	n.d.
NCRCDP004	575±248	n.d.	n.d.
NCRCDP005	609±187	n.d.	0.079

n.d., not determined.



alone showed the same number of peroxisomes as untransfected PBD100 cells (Fig. 9A). In contrast, overexpression of *PEX11* β myc in PBD100 cells resulted in a dramatic increase in peroxisome abundance (Fig. 9B and C). Thus, the reduction of peroxisome abundance in ZS cells is not due to an inability of these cells to respond to *PEX11* expression. Control experiments confirmed that the increase in peroxisome abundance was not due to restoration of peroxisomal matrix protein import: PBD100 cells expressing *PEX11* β myc were still defective in matrix protein import (data not shown).

DISCUSSION

In this report we examined the distribution of PMPs in cells that are defective in each of seven known PBD genes: *PEX1*, *PEX5*, *PEX12*, *PEX6*, *PEX10*, *PEX2*, and *PEX7*. These patients all have inactivating mutations in the corresponding *PEX*

genes. Peroxisomal structures were detected in all seven PBD cell lines. However, the abundance of peroxisomes was reduced fivefold in the six cell lines with mutations in the *PEX1*, *PEX5*, *PEX12*, *PEX6*, *PEX10*, or *PEX2* genes. Interestingly, the fivefold reduction in peroxisome abundance in ZS cells coincides with a 2- to 4-fold increase in peroxisome diameter. Assuming a roughly spherical shape for the peroxisome this would translate to a 4- to 16-fold increase in the surface area of individual peroxisomes, which may explain why levels of PMPs are not reduced in PBD cells (Espeel et al., 1995; Lazarow et al., 1986; Santos et al., 1988b; Small et al., 1988; Suzuki et al., 1987) (S. South and S. J. Gould, unpublished observations).

The properties of peroxisomes in ZS cells reflects the cellular processes that are affected by loss of these *PEX* genes and it is important to provide molecular explanations for these properties. A defect in the peroxisomal matrix protein import apparatus may be the simplest explanation for the phenotypes of ZS cells, as noted first by Santos et al. (1988a). However,

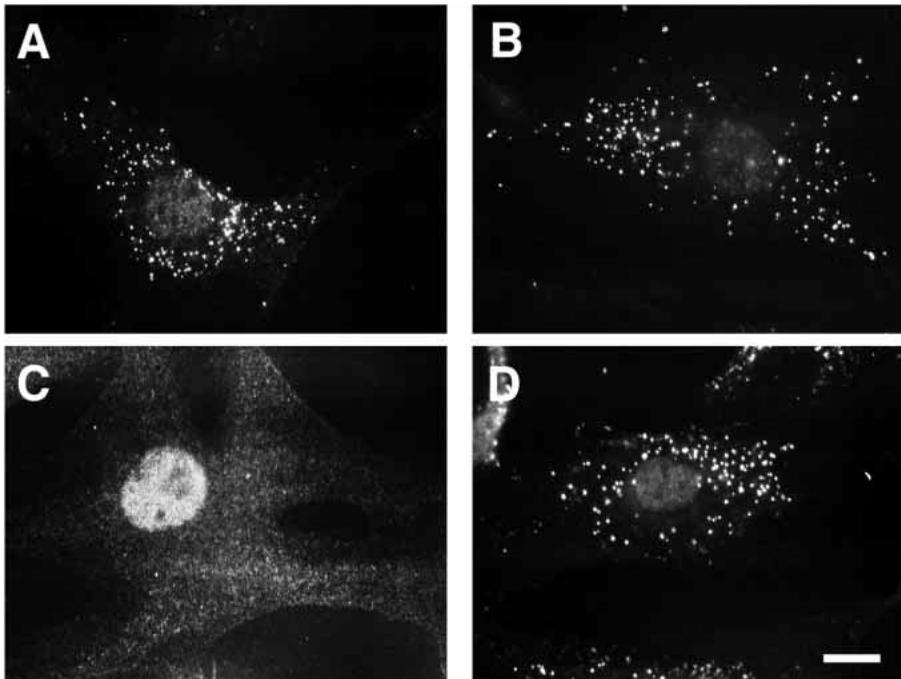


Fig. 6. Acyl-CoA oxidase and MFE2 distribution in acyl-CoA oxidase-defective and MFE2-defective cell lines. Acyl-CoA oxidase-defective cell lines ACX001 (A) and ACX002 (B) were stained with antibodies specific for acyl-CoA oxidase. MFE2-defective cell lines MFE003 (C) and MFE004 (D) were stained with antibodies specific for MFE2. Bar, 25 μ m.

this hypothesis cannot explain why these cells have reduced peroxisome abundance. In fact, the aberrant morphology and abundance of peroxisomal structures in ZS cells has raised the possibility that the matrix protein import defects might be a secondary consequence of membrane biogenesis defects (Titorenko and Rachubinski, 1998a). The data in this report provide strong support for the hypothesis that these ZS cells are defective only in matrix protein import.

We find that the reduced peroxisome abundance in ZS cells is shared by cells lacking either of two peroxisomal β -oxidation enzymes, acyl-CoA oxidase or peroxisomal 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase. In addition, we show that the reduced peroxisome abundance in these peroxisomal β -oxidation-deficient cell lines correlates only with loss of enzymatic activities: several acyl-CoA oxidase-defective and MFE2-defective cell lines have normal levels of the affected proteins and import these defective proteins into peroxisomes. Independent evidence to support the hypothesis that some MFE2-defective cell lines contain normal levels of MFE2 protein comes from mutational studies of MFE2, which demonstrate that some MFE2-defective patients are defective in only the hydratase or dehydrogenase activities of this bifunctional protein (van Grunsven et al., 1998). Not coincidentally, acyl-CoA oxidase and MFE2 are targeted to peroxisomes via PTS1 signals and all PBD cells with reduced peroxisome abundance display a defect in PTS1 protein import. Furthermore, PBD cells which import greater amounts of PTS1 proteins contain greater numbers of peroxisomal structures and cells that are defective in only PTS2 protein import contain normal levels of peroxisomes. The only hypothesis that is consistent with all of these data is that the aberrant morphology of peroxisomes in PBD cells is a secondary consequence of their PTS1 protein import defects, particularly the inability to import acyl-CoA oxidase and MFE2.

Implications for peroxisome biogenesis

By removing the last significant objection to the hypothesis that ZS patients are defective in peroxisomal matrix protein

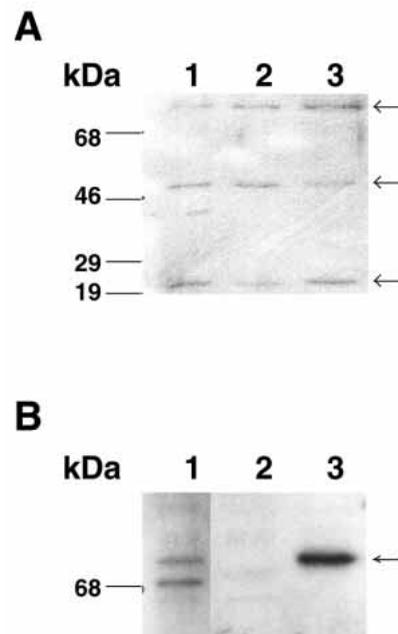


Fig. 7. Acyl-CoA oxidase and MFE2 levels in acyl-CoA oxidase-defective and MFE2-defective cell lines. (A) Western blot analysis of acyl-CoA oxidase in total cellular protein extracts of normal human fibroblasts (lane 1), ACX001 fibroblasts (lane 2), and ACX002 fibroblasts (lane 3). Arrows denote the positions of precursor acyl-CoA oxidase, as well as the 50 kDa and 20 kDa proteolytic fragments of acyl-CoA oxidase that are normally produced in the peroxisome lumen. (B) Western blot analysis of MFE2 levels in total cellular protein extracts of normal human fibroblasts (lane 1), MFE2003 fibroblasts (lane 2), and MFE2004 fibroblasts (lane 3).

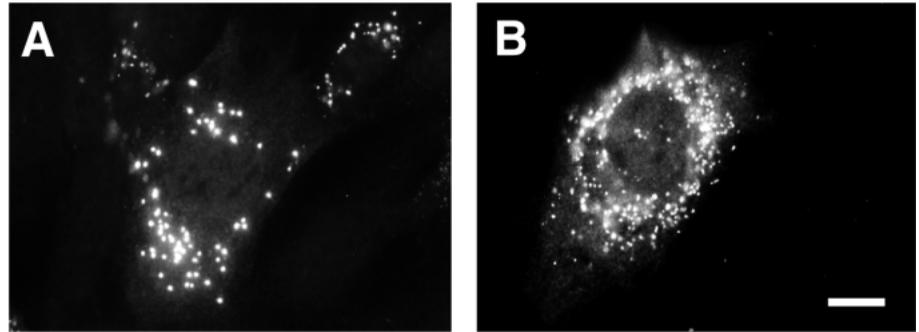


Fig. 8. Overexpression of PTE1 reduces peroxisome abundance. Human skin fibroblasts were transfected with plasmids designed to express (A) Nmyc-PTE1 or (B) Nmyc-alpha-hydroxy acid oxidase. Two days later they were processed for indirect immunofluorescence using antibodies specific for the myc epitope tag.

import, our results help to clarify the function (s) of the genes that are mutated in these patients. Many reports have chronicled the PTS1 receptor activity of PEX5 and the matrix protein import defects of cells with mutations in this gene (Dodt et al., 1995; Dodt and Gould, 1996; Terlecky et al., 1995; Wiemer et al., 1995). We now know why *PEX5*-deficient PBD005 cells also display reduced peroxisome abundance and increased peroxisome size along with their inability to import peroxisomal matrix proteins. Data from prior studies have also implicated both *PEX2* (Shimozawa et al., 1992; Waterham et al., 1996) and *PEX12* (Chang et al., 1997; Kalish et al., 1996) in matrix protein import and the phenotypes of the *PEX2*-deficient PBD094 and *PEX12*-deficient PBD097 cell lines reflects such a function. However, prior reports from fungal systems have concluded that *PEX1* and *PEX6*, the genes mutated in PBD009 and PBD106 cells, are required for the biogenesis of peroxisome membranes (Erdmann et al., 1991; Faber et al., 1998; Titorenko et al., 1997; Titorenko and Rachubinski, 1998b). The detailed studies by Santos et al. (1988a,b) on peroxisomes of ZS cells were performed primarily with two cell lines, GM228 and GM4340, and we have found that these cells are mutated in *PEX1* and *PEX6*, respectively (data not shown). The extensive biochemical and morphological data available from these earlier studies, together with those presented here, indicate that loss of *PEX1* or *PEX6* does not affect the ability of cells to synthesize peroxisomes or import PMPs but instead causes a specific defect in peroxisomal matrix protein import. Other results that implicate human *PEX1* and *PEX6* in matrix protein import are: (1) the fact that loss of either *PEX1* or *PEX6* results in destabilization of PEX5, the PTS1 receptor (Dodt and Gould, 1996; Portsteffen et al., 1997; Reuber et al., 1997; Yahraus et al., 1996); and (2) the observations that peroxisomal matrix protein import is an ATP-dependent process (Imanaka et al., 1987; Wendland and Subramani, 1993) and that *PEX1* and *PEX6* encode the only ATPases that are known to be required for peroxisomal matrix protein import (Distel et al., 1996). Although we cannot rule out the possibility that human *PEX1* and *PEX6* may play some ancillary role in membrane biogenesis, we find no evidence for such a function in the phenotypes of ZS cells.

Previous studies on *PEX10* have offered differing views of this peroxin as well. In *Pichia pastoris*, loss of *PEX10* results in a pronounced defect in import of peroxisomal matrix proteins but no significant defect in the synthesis or proliferation of peroxisome membranes, results that are consistent with a role for *PEX10* in peroxisomal matrix protein import (Kalish et al., 1995). In the closely related yeast *Pichia*

angusta (*Hansenula polymorpha*), loss of *PEX10* was associated with a complete absence of peroxisomal structures, a very different phenotype which suggested a role in the

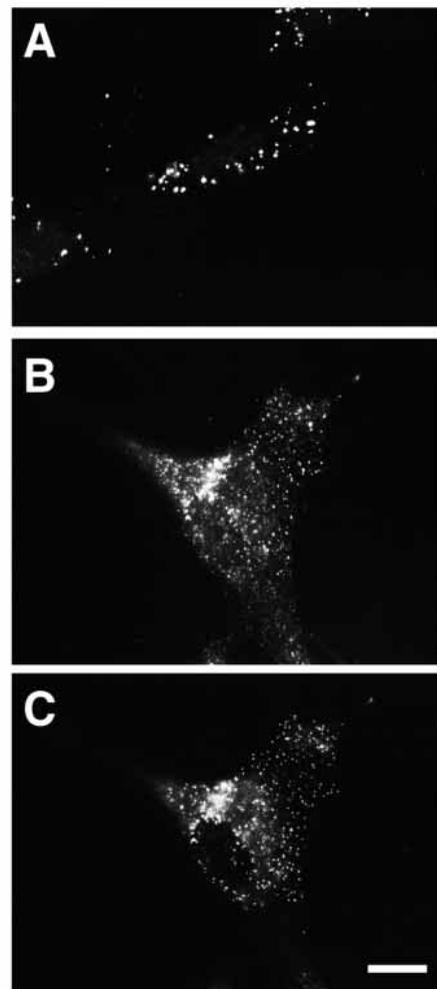


Fig. 9. ZS cells are competent for peroxisome proliferation. The *PEX10*-deficient PBD100 cell line was transfected with pcDNA3 and pcDNA3-*PEX11βmyc*. Two days later the cells were processed for indirect immunofluorescence using rabbit polyclonal antibodies specific for PMP70 and a mouse monoclonal antibody specific for the myc tag present on *PEX11βmyc*, followed by Texas Red labeled goat anti-rabbit IgG antibodies and fluorescein-labeled goat anti-mouse IgG antibodies. A, PMP70 staining in PBD100 cells transfected with pcDNA3. Cells transfected with pcDNA3-*PEX11βmyc* showed an increase in (B) the number of PMP70-containing peroxisomes in cells which (C) expressed *PEX11βmyc*.

biogenesis of peroxisome membranes (Tan et al., 1995). We report here that human cells lacking *PEX10* (PBD100 cells) contain nearly a hundred peroxisomes per cell, import PMPs, and can proliferate peroxisomes in response to *PEX11* expression, data that is consistent with the phenotypes of *P. pastoris pex10* cells. Thus, it appears that *PEX10* is involved in matrix protein import in both human cells and *P. pastoris* but may have an additional role in membrane biogenesis in *P. angusta*.

Is peroxisome abundance under metabolic control?

In addition to their implications for *PEX* gene function, the data presented here also suggest that there may be a novel mechanism for the control of peroxisome abundance. Specifically, our results show that defects in either of two peroxisomal metabolic enzymes, acyl-CoA oxidase or 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase (MFE2), cause a fivefold reduction in peroxisome abundance. This reduction is specific to defects in peroxisomal β -oxidation enzymes since defects in other peroxisomal enzymes had no effect on the numbers of peroxisomes per cell. These data are highly statistically significant, have been confirmed by multiple independent observers, and represent strong evidence that there is metabolic control over peroxisome abundance. Additional support for the hypothesis that peroxisome abundance may be under control of peroxisomal fatty acid β -oxidation comes from the fact that overexpression of PTE1, a peroxisomal thioesterase that would be expected to inhibit fatty acid oxidation by eliminating acyl-CoA substrates, led to reduced peroxisome abundance in otherwise normal human fibroblasts. However, many questions regarding this phenomenon remain to be answered. These include how the defect in these enzymes is transmitted to the proliferation process and whether it involves either of the human *PEX11* genes. Prior studies have established that *PEX11* genes can regulate the abundance of peroxisomes (Erdmann and Blobel, 1995; Marshall et al., 1995) and this is also true in human cells. Humans have two *PEX11* genes, *PEX11 α* and *PEX11 β* (Schrader et al., 1998). We tested whether cells with reduced peroxisome abundance might have lost the ability to respond to *PEX11* expression but this does not appear to be the case. We have also tested whether the expression of *PEX11* genes might be affected in ZS or peroxisomal β -oxidation-deficient cells. However, we find no evidence for differential expression of either the *PEX11 α* or *PEX11 β* genes in fibroblasts with reduced peroxisome abundance as opposed to those with normal peroxisome abundance (data not shown). Therefore, if the metabolic control of peroxisome abundance observed in ZS and peroxisomal β -oxidation-deficient cells is mediated by *PEX11 α* or *PEX11 β* , it does not appear to involve regulation at the level of mRNA abundance.

This work was supported by grants from the National Institutes of Health (DK45787 and HD10891).

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