Peroxisome Synthesis in the Absence of Preexisting Peroxisomes

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Abstract. Zellweger syndrome and related diseases are caused by defective import of peroxisomal matrix proteins. In all previously reported Zellweger syndrome cell lines the defect could be assigned to the matrix protein import pathway since peroxisome membranes were present, and import of integral peroxisomal membrane proteins was normal. However, we report here a Zellweger syndrome patient (PBD061) with an unusual cellular phenotype, an inability to import peroxisomal membrane proteins. We also identified human PEX16, a novel integral peroxisomal membrane protein, and found that PBD061 had inactivating mutations in the *PEX16* gene. Previous studies have suggested that peroxisomes arise from preexisting peroxisomes but we find that expression of *PEX16* restores the formation of new peroxisomes in PBD061 cells. Peroxisome synthesis and peroxisomal membrane protein import could be detected within 2–3 h of *PEX16* injection and was followed by matrix protein import. These results demonstrate that peroxisomes do not necessarily arise from division of preexisting peroxisomes. We propose that peroxisomes may form by either of two pathways: one that involves PEX11-mediated division of preexisting peroxisomes, and another that involves PEX16-mediated formation of peroxisomes in the absence of preexisting peroxisomes.

Key words: membrane biogenesis • Zellweger syndrome • peroxisomal membrane protein import • PEX16 • peroxisome biogenesis disorders

MEMBRANE-BOUND organelles appear to be synthesized by fission and fusion of homotypic organelles or budding from heterotypic organelles. These common modes of organelle biogenesis also represent attractive models for peroxisome biogenesis. The hypothesis that peroxisomes are derived from the ER was proposed nearly 30 yr ago (Novikoff and Shin, 1964). However, this hypothesis was largely abandoned when it was found that peroxisomal enzymes were imported directly from the cytoplasm to the peroxisome, rather than via the ER (Lazarow et al., 1982). Peroxisomal membrane proteins (PMPs)[1] are also imported directly from the cytoplasm (Fujiki et al., 1984), and together these results led to the currently favored hypothesis that peroxisomes form by division of preexisting peroxisomes (Lazarow and Fujiki, 1985). However, there is little direct evidence for how peroxisomes are synthesized.

To distinguish between these different models for peroxisome biogenesis we and others have searched for mutants that are unable to import PMPs into peroxisomes, and thus, fail to synthesize peroxisome membranes. In addition to studies in yeast (Höhfeld et al., 1991; Wiemer et al., 1996; Gotte et al., 1998) (Gould, S.J., and S. South, manuscript in preparation), we have examined human cell lines derived from patients who suffer from disorders of peroxisome biogenesis. The peroxisome biogenesis disorders (PBDs) include Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and rhizomelic chondrodysplasia punctata (Lazarow and Moser, 1995). This group of genetically heterogeneous, lethal diseases is characterized by severe mental retardation, loss of multiple peroxisomal metabolic functions, and defective import of one or more classes of peroxisomal matrix proteins. All previously characterized PBD cell lines import PMPs normally and contain numerous peroxisomes, leading to the hypothesis that the PBDs are caused by defects in peroxisomal matrix protein import and not the biogenesis of peroxisome membranes (Santos et al., 1988a). The cellular and molecular analysis of PBD cells has demonstrated that PMP import is fundamentally different from the matrix protein import pathways, but until now has done little to advance our understanding of peroxisome membrane biogenesis, or PMP import.

Here we describe a cell line from a Zellweger syndrome patient, PBD061, that differs from previously reported PBD cell lines. Specifically, it is unable to import PMPs, and lacks detectable peroxisome membranes. The charac-

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1. *Abbreviations used in this paper:* DPBS, Dulbecco's phosphate-buffered saline; PBD, peroxisome biogenesis disorders; PMP, peroxisomal membrane protein.

terization of PBD061 cells and the gene product defective in this cell line, PEX16, revealed the following: PEX16 is required for peroxisome synthesis, and peroxisomes can form in the absence of preexisting peroxisomes. Possible mechanisms for peroxisome synthesis in rescued PBD061 cells and in normal cells are discussed.

Materials and Methods

Tissue Culture, Cell Lines, and Antibodies

Skin fibroblasts from PBD patients were obtained from Ann and Hugo Moser (The Kennedy Krieger Institute, Baltimore, MD), and from the Coriell Cell Repository (Camden, NJ). All cell lines were cultured in high glucose DME that was supplemented with penicillin, streptomycin, and 10% fetal calf serum. Cell lines in this report are referred to by a unique PBD number with the exception of the normal human fibroblast cell line, 5756T (an SV-40–transformed derivative of GM5756; Coriell Cell Repository), and the hepatoblastoma cell line HepG2 (a gift from Michael Schrader, Baltimore, MD). The CG1 cell line, PBD009, has inactivating mutations on both *PEX1* alleles, and lacks detectable levels of *PEX1* mRNA and protein (Reuber et al., 1997). The CG2 cell line, PBD005, is homozygous for an inactivating mutation in *PEX5*, and lacks detectable levels of *PEX5* mRNA and protein (Dodt et al., 1995). The CG3 cell line, PBD097, is a compound heterozygote for two inactivating frameshift mutations in *PEX12* (Chang et al., 1997). The CG4 cell line, PBD105, is also a compound heterozygote for two inactivating frameshift mutations, except in the *PEX6* gene (Yahraus et al., 1996). The CG7 cell line, PBD100, is homozygous for a splice donor site mutation in *PEX10* and expresses a *PEX10* mRNA with a large internal deletion that lacks *PEX10* activity (Warren et al., 1998). The gene that is mutated in cells from complementation group 8 of the PBDs is unknown but the CG8 cell line used in this study, PBD109, was equally or more severely deficient in peroxisomal matrix protein import than any other CG8 cell line. The CG9 cell line, PBD061, is equivalent to GM6231, a Zellweger syndrome cell line from the Coriell Cell Repository. The CG10 cell line, PBD094, is homozygous for an inactivating nonsense mutation in the *PEX2* gene (Shimozawa et al., 1992). The CG13 cell line, PBD222, is the sole representative of this complementation group and was derived from a neonatal adrenoleukodystrophy patient. Again, the gene defective in this patient remains to be determined. Aside from PBD222, all cell lines used in this study were derived from severely affected Zellweger syndrome patients.

Antibodies to PMP70 were obtained from Gerardo Jimenez-Sanchez and David Valle (The Johns Hopkins University School of Medicine, Baltimore, MD). Antibodies to P70R were obtained from Wilhelm Just (University of Heidelberg, Heidelberg, Germany). mAbs to the myc epitope tag were obtained from the tissue culture supernatant of the hybridoma 1-9E10 (Evan et al., 1985). To generate antibodies to PEX16, we first expressed amino acids 145–336 of *PEX16* in fusion with the maltose binding protein. The resulting maltose binding protein–*PEX16* fusion was purified by affinity chromatography on an amylose resin according to the manufacturer's instructions (New England Biolabs Inc.). The purified fusion protein was then injected into rabbits and polyclonal sera were collected. The sera displaying the highest titer against in vitro synthesized PEX16 was used for all experiments. Fluorescently labeled secondary antibodies were obtained from commercial sources. Horseradish peroxidase–labeled anti–rabbit secondary antibodies were also obtained from commercial sources.

Immunofluorescence

Cells were cultured on glass coverslips and processed for indirect immunofluorescence as follows. Cells were washed twice in Dulbecco's phosphate buffered saline (DPBS; 0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8.0 g/liter NaCl, 21.6 g/liter Ha_2HPO_4, pH 7.1), and fixed for 15 min in 3.7% formaldehyde in DPBS, followed by two washes with DPBS. Unless otherwise noted, the cells were then permeabilized by incubation in 1% Triton X-100 in DPBS for 5 min to permeabilize all cellular membranes. Cells were washed twice in DPBS, and incubated with the primary antibody or antibodies for 15 min. All antibody incubations were performed in DPBS that was supplemented with 5% nonspecific goat serum. Next, the cells were washed 10 times with DPBS, and incubated with the appropriate secondary antibodies in 5% nonspecific goat serum and DPBS for 15 min. Cells were washed 10 times, and mounted on glass slides in a solution of 100 mM Tris-HCl, pH 8.5, 90% glycerol, and 0.1% *para*-phenylenediamine. For differential permeabilization experiments in which the plasma membrane is permeabilized but the peroxisome membrane remains intact, the Triton X-100 incubation was omitted and cells were permeabilized instead by incubation in 25 μg/ml digitonin in DPBS for 5 min. Cells were visualized on an Olympus fluorescence microscope and all images were collected on film (T-Maxx 400; Eastman-Kodak).

PEX16 Cloning, Mutation Detection, and Plasmids

Candidate cDNAs for the mammalian *PEX16* gene were identified by searching the database of expressed sequence tags using the TBLASTN algorithm. A mouse cDNA capable of encoding a protein similar to *Yarrowia lipolytica* PEX16 was identified. The sequence of the murine *PEX16* cDNA was used to identify a human *PEX16* cDNA by BLAST searches of the human database of expressed sequence tags. Clones encoding the human *PEX16* cDNA were obtained from Genome Systems Inc. and sequenced in their entirety. The *PEX16* ORF was also amplified from a full-length cDNA clone using *PEX16*-specific oligonucleotides 5′-CCCGGTACCATGGAGAAGCTGCGGCTCCTGGGC-3′ and 5′-CCAAGCGGCCGCAGTTTTATTAGGGAAGAGGGGC-3′. The resulting PCR products were digested with Asp718 and NotI, and cloned between the Asp718 and NotI sites of pcDNA3, creating pcDNA3-*PEX16.* The *PEX16* insert in this plasmid was sequenced in its entirety to ensure that no mutations were introduced during the cloning process. The cDNA and deduced protein sequences for human *PEX16* are available from GenBank (accession number AF118240).

Mutation detection was performed initially by RT-PCR. RNA was extracted from PBD061 cells and from a control human fibroblast and converted to *PEX16* cDNA as follows. Approximately 1 μg of total RNA from an unaffected individual and from PBD061 was used as template in a cDNA synthesis reaction using a *PEX16*-specific oligonucleotide (5′-CCAAGCGGCCGCAGTTTTATTAGGGAAGAGGGGC-3′) and Superscript reverse transcriptase (Life Sciences Inc.). 3 μl of the cDNA synthesis reaction products was used as template in PCR using the *PEX16*-specific oligonucleotides 5′-CCTAAGACCGGAAGCAGGAAGGAG-3′, and 5′-ACACCCTCCTTCCGGGAGGTCTGT-3′ that lie in the 5′ and 3′ untranslated regions of the *PEX16* cDNA. A product of the correct size was obtained from each reaction. However, the yield of *PEX16* cDNA from PBD061 RNA was significantly lower than that obtained from control RNAs, a result that was consistently observed in multiple experiments. The *PEX16* cDNAs from the control group, and PBD061 cells were sequenced directly and in their entirety. The control fragment matched the *PEX16* cDNA sequence exactly. In contrast, the *PEX16* cDNA from PBD061 had a 1-bp substitution in which the first nucleotide of the arginine 176 codon, CGA, was converted to a T, terminating the *PEX16* ORF about halfway through the coding region (data not shown).

The sequence chromatograph of the *PEX16* cDNA from PBD061 showed no evidence of the C at this position. However, a wide array of mutations may result in altered mRNA abundance and it is, therefore, common for a mutation to appear homozygous at the cDNA level even though there may be different mutations on the two alleles in the patient. To test whether PBD061 cells were homozygous for the R176ter mutation we first had to determine the *PEX16* genomic organization in the region of the mutation. Oligonucleotide primers flanking the site of mutation, and based on the cDNA sequence, were used in various combinations in PCR reactions with total human genomic DNA from an unaffected individual as a template. One set of oligonucleotides (5′-CAGCCCTGGCAACCATGAGCAGTC-3′ and 5′-CAAAAACTCTGCGATGGTCTCC-3′) amplified a DNA fragment that was ~100 bp larger than expected from the cDNA sequence, indicating that it spanned an intron and therefore would be derived from genomic DNA. Direct sequence analysis confirmed this hypothesis as the cDNA sequences of the amplicon were interrupted by a 134-bp segment that had all of the features of a human intron. These same oligonucleotides were used to amplify a *PEX16* gene fragment spanning the site of mutation from the genomic DNA of an unaffected individual and PBD061. The structure of each product was determined by direct sequence analysis.

The *PEX16* expression vector, pcDNA3-*PEX16*, was described above. The plasmid pcDNA3-*PEX16*/R176ter was created by T/A cloning the PEX16 RT-PCR product that was generated from PBD061 RNA into the pCR2.1 vector (Invitrogen Corp.). The resulting clone was sequenced in its entirety to ensure that no other mutations were introduced during the amplification process. The *PEX16*/R176ter cDNA was released from this plasmid, pCR2.1-*PEX16*/R176ter, by digestion with EcoRI and cloned

into the unique EcoRI site of pcDNA3, creating pcDNA3-*PEX16*/R176ter. The COOH-terminal myc-tagged derivative of the *PEX16* cDNA was generated by PCR using the wild-type *PEX16* cDNA as a template, the oligonucleotide 5′-CCCGGTACCATGGAGAAGCTGCGG-CTCCTGGGC-3′ at the 5′ end and the oligonucleotide 5′-CCAAAG-ATCTGCCCCAACTGTAGAAGTAGATTTTC-3′ at the 3′ end, which replaces the stop codon with a BglII site. The resulting fragment was cleaved with Asp718 and BglII and inserted between the Asp718 and BamHI sites of pcDNA3-myc (Yahraus et al., 1996), creating the plasmid pcDNA3-*PEX16myc.* This plasmid carries a modified *PEX16* ORF that encodes the 10–amino acid long c-myc epitope tag at the COOH terminus of PEX16. The NH_2-terminal myc-tagged derivative of the *PEX16* cDNA was generated by PCR using the wild-type *PEX16* cDNA as a template, the oligonucleotide 5′-CCCGTCGACGGAGAAGCTGCGGCTCCTGGG-CCTC-3′ at the 5′ end and the oligonucleotide 5′-CCAAGCGGCCGC-AGTTTTATTAGGGAAGAGGGGC-3′ at the 3′ end. This cDNA fragment was cleaved with SalI and NotI, and cloned between the XhoI and NotI sites of the NH_2-terminal myc tag vector, pcDNA3-N-myc. This plasmid carries a high efficiency initiation codon, and the c-myc epitope tag upstream of the XhoI and NotI sites. The resulting plasmid, pcDNA3-*mycPEX16*, encodes the 10–amino acid long c-myc epitope tag at the 5′ end of the PEX16 ORF. The plasmid that expresses the maltose binding protein–PEX16 fusion (pMBP-PEX16) was created by amplifying a subfragment of the *PEX16* cDNA by PCR using the *PEX16* cDNA as a template, and the oligonucleotides 5′-CCCGTCGACACTGGACAGAGAGAC-CCAGGCAC-3′, and 5′-CCAAGCGGCCGCAGTTTTATTAGGGAA-GAGGGGC-3′. This fragment encoding amino acids 145–336 of PEX16, was cleaved with SalI and NotI, and inserted between the SalI and NotI sites of pMBP, a modified version of pMALc2 (New England Biolabs Inc.) The plasmids designed to express PMP70myc, PEX12myc, PMP32myc, PEX3myc, PEX11αmyc, and PEX11βmyc all consist of the full-length human cDNA cloned into the mammalian expression vector pcDNA3-myc in such a way as to encode the 10–amino acid myc tag after the COOH-terminal amino acid of each protein, followed immediately by a stop codon.

Transfection and Microinjection

All cell lines were actively growing before transfection or injection. Transfections were performed by growing cells to near confluency on two T75 flasks, harvesting the cells by trypsinization, and electroporating them as described in Chang et al. (1997). Microinjections were performed with DNA at a concentration of 1 μg/ml in reverse phosphate buffered saline (4 mM $NaHPO_4$, 1 mM KH_2PO_4, 140 mM KCl, pH 7.3). DNAs were injected into the nucleus of cells at a pressure of 100 mmHg for 0.4 s.

Protein Preps, Fractionations, Protease Protection, and Western Blots

Total cellular proteins were extracted from cultured fibroblasts as described (Dodt and Gould, 1996). For subcellular fractionation experiments, postnuclear supernatants were prepared from HepG2 cells by homogenization in a Balch homogenizer followed by successive centrifugation steps to remove nuclei and unbroken cells. The resulting clarified supernatant was separated by density gradient centrifugation (Nycodenz; Nycomed Pharma) as described in Dodt et al. (1995). Equal amounts of each fraction were assayed for catalase, succinate dehydrogenase (SDH), and NADPH-cytochrome reductase (NCR) to follow the peroxisomes, mitochondria, and microsomes, respectively. Equal amounts of each fraction were also separated by SDS-PAGE and assayed for PEX16 levels by Western blot as described in Crane et al. (1994).

For membrane extractions, a light mitochondrial pellet was obtained by centrifugation of a HepG2 postnuclear supernatant at 25,000 *g* for 30 min. The pellet fraction was then extracted with 10 mM Tris, pH 8.3 on ice for 20 min with agitation. This sample was separated into a membrane pellet, and a soluble supernatant by centrifugation at 200,000 *g* for 30 min. The supernatant was saved and the pellet was subjected to extraction with 1 M NaCl on ice for 20 min with agitation. This sample was separated into a membrane pellet, and a soluble supernatant by centrifugation at 200,000 *g* for 30 min. The supernatant was saved, and the pellet was subjected to extraction with 100 mM Na_2CO_3 pH 11.5 on ice for 20 min with agitation. The carbonate extracted supernatant and pellet were separated by centrifugation at 200,000 *g* for 30 min. The levels of PEX16 in each sample were determined by semiquantitative Western blotting after separating equal proportions of the three supernatants and the carbonate extracted pellet by SDS-PAGE.

Protease protection experiments were performed as follows. A postnuclear supernatant was prepared from HepG2 cells and a light mitochondrial pellet was obtained as above. This was resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Equal proportions were aliquoted to eight different tubes. Triton X-100 was added to four of the tubes to a final concentration of 1%. Either 0, 15, 30, or 60 μg of the protease trypsin (Sigma Chemical Co.) was added to an aliquot that contained Triton X-100 and to an aliquot that lacked protease. These samples were incubated on ice for 20 min, and protease digestion was terminated by adding a twofold excess of bovine trypsin inhibitor. Equal proportions of each sample were separated by SDS-PAGE and PEX16 was detected by Western blot.

Results

PBD061 Cells Are Defective in PMP Import

Little is known about the mechanisms of PMP import or the synthesis of peroxisome membranes. To identify cell lines that are defective in these processes we screened skin fibroblasts from over 100 PBD patients for the presence of PMP-containing peroxisomal structures. Each cell line was processed for indirect immunofluorescence using antibodies specific for PMP70, an integral membrane protein of the peroxisome. Previous studies have reported that PBD cells contain peroxisomes and import PMPs (Santos et al., 1988a, 1988b) and we detected numerous PMP70-containing peroxisomes in virtually all cell lines examined. These included representatives of complementation groups 1, 2, 3, 4, 7, 8, 10, and 13 (Fig. 1). However, we did identify one cell line, PBD061, which failed to import PMP70 into peroxisomes (Fig. 1, I). This cell line was derived from a severely affected Zellweger syndrome patient and is the sole representative of PBD complementation group 9 (Moser et al., 1995). To test whether the absence of PMP70 staining in PBD061 cells reflected a generalized defect in PMP import or a peculiarity of PMP70 distribution, representatives of all complementation groups were also processed for indirect immunofluorescence using antibodies specific for other PMPs, including ALDP (Mosser et al., 1994), and P70R (Shani et al., 1997). These antibodies also revealed the presence of numerous PMP-containing peroxisomes in representatives of all complementation groups except for the CG9 cell line, PBD061 (data not shown).

As an independent test of this phenotype we followed the distribution of overexpressed PMPs in this cell line. Plasmids designed to express the integral peroxisomal membrane proteins PMP70myc, PEX12myc and PMP32myc (a human peroxisomal solute carrier) were transfected into PBD061 cells and a control PBD fibroblast line, PBD094, which is mutated in the *PEX2* gene (Shimozawa et al., 1992). We were unable to detect peroxisomal or vesicular staining for these PMPs in PBD061 cells, even though all three proteins were efficiently targeted to peroxisome membranes in PBD094 cells (Fig. 2). Similar rates of transfection in both cell lines were confirmed by cotransfection with a cytosolic marker expression vector (data not shown). Similar results were observed in cells transfected with expression vectors designed to express other human PMPs, including PEX3myc (Kammerer et al., 1998), PEX10myc (Warren et al., 1998), PEX11αmyc (Schrader et al., 1998), PEX11βmyc (Schrader et al., 1998), and PEX13myc (Gould

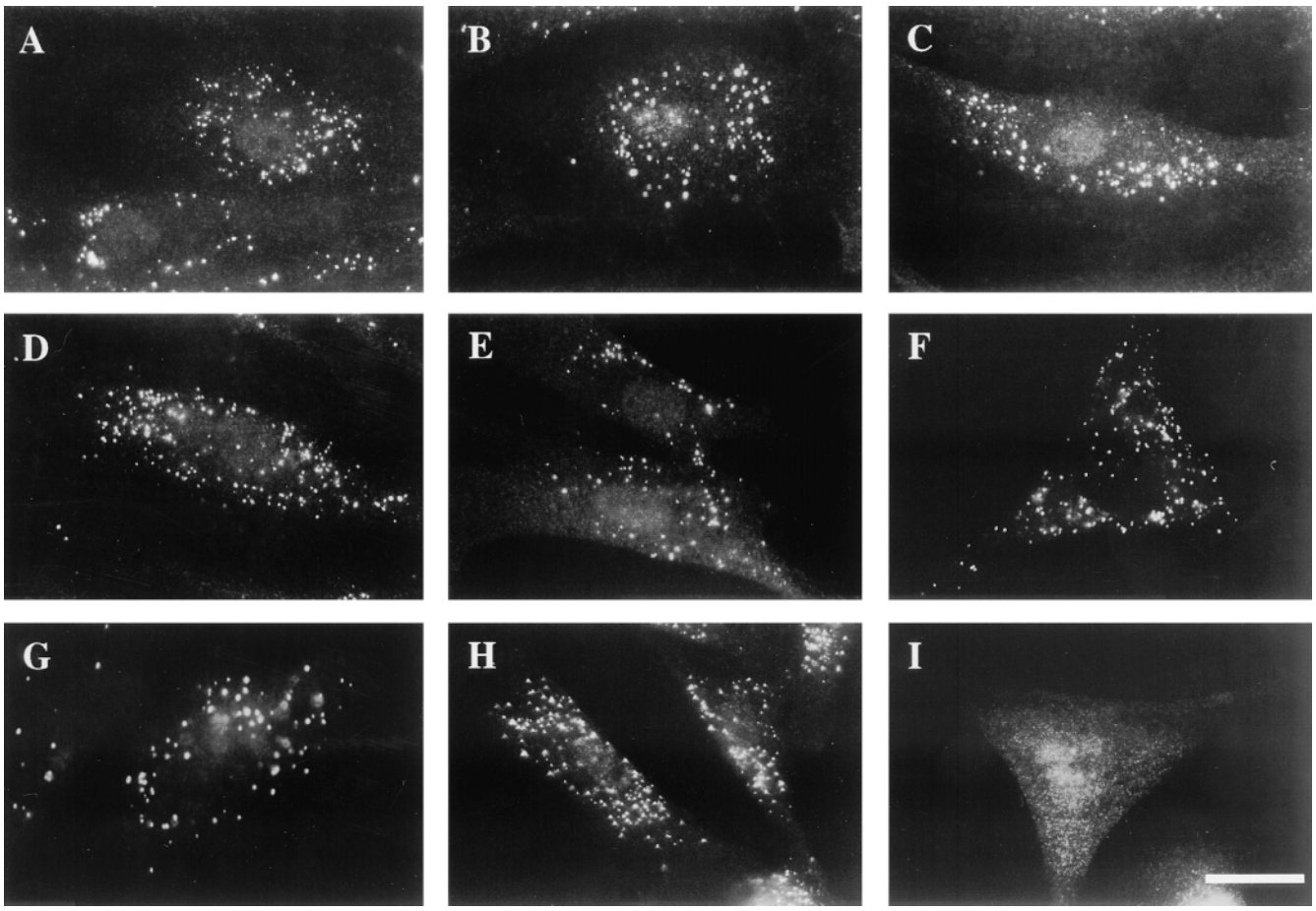

Figure 1. The CG9 cell line lacks detectable peroxisomes. Human fibroblasts from representatives of nine different complementation groups of the PBDs were processed for indirect immunofluorescence using rabbit antibodies specific for PMP70, and Texas red–labeled goat anti–rabbit IgG secondary antibodies. Numerous peroxisomal structures were detected in (A) the *PEX1*-deficient CG1 cell line, PBD009; (B) the *PEX5*-deficient CG2 cell line, PBD005; (C) the *PEX12*-deficient CG3 cell line, PBD097; (D) the *PEX6*-deficient CG4 cell line, PBD105; (E) the *PEX10*-deficient CG7 cell line, PBD100; (F) the CG8 cell line, PBD109; (G) the *PEX2*-deficient CG10 cell line, PBD094; and (H) the CG13 cell line, PBD222. In contrast, (I) the CG9 cell line, PBD061, lacks detectable peroxisomes. Bar, 10 μm.

et al., 1996) (data not shown). Although we were unable to detect peroxisomal or vesicular structures with any of the 11 PMPs tested, it is possible that some as yet unidentified PMPs may reside in some type of peroxisome-related structure in PBD061 cells.

To better understand the fates of PMPs in PBD061 cells, total cellular protein was extracted from the CG9 cell line, PBD061, and a CG10 cell line, PBD094, as well as from PBD061, and PBD094 cells that had been transfected with the PEX12myc expression vector. The abundance of the PMPs (PMP70, P70R, PEX14, and PEX12myc) were then determined by immunoblot with antibodies specific for each protein (Fig. 3). As evident from this experiment, levels of full-length PMP70 and P70R in PBD061 cells were below the limit of detection in PBD061 cells but were readily detected in PBD094 cells, indicating that these proteins might be degraded in PBD061 cells. This hypothesis is supported by the presence of anti–P70R-reactive products of lower molecular mass in PBD061 cells, but not in PBD094 cells. However, this was not the fate of all PMPs, since levels of PEX14 were similar in PBD061 cells and PBD094 cells, as were levels of PEX12myc. Thus, the fate of PMPs in a cell line defective in PMP import varies from protein to protein, just as the fate of peroxisomal matrix proteins varies in cells with specific defects in matrix protein import (Lazarow and Fujiki, 1985; Santos et al., 1988a, 1988b).

Identification of PEX16, the Gene Mutated in CG9 Cells

The prevailing model of peroxisome biogenesis holds that peroxisomes are formed by division of preexisting peroxisomes. Thus, it was an open question as to whether peroxisome synthesis could be restored in a cell line such as PBD061; and the identification of the gene defective in PBD061 cells would likely have a significant impact on our understanding of peroxisome biogenesis. We employed the homology probing strategy that has been used previously to identify human homologues of other yeast *PEX* genes (Dodt et al., 1996). The database of expressed sequence tags was searched for mammalian homologues of the *Y. lipolytica PEX16* gene and a single human homologue was identified (Fig. 4 A). This human *PEX16* cDNA

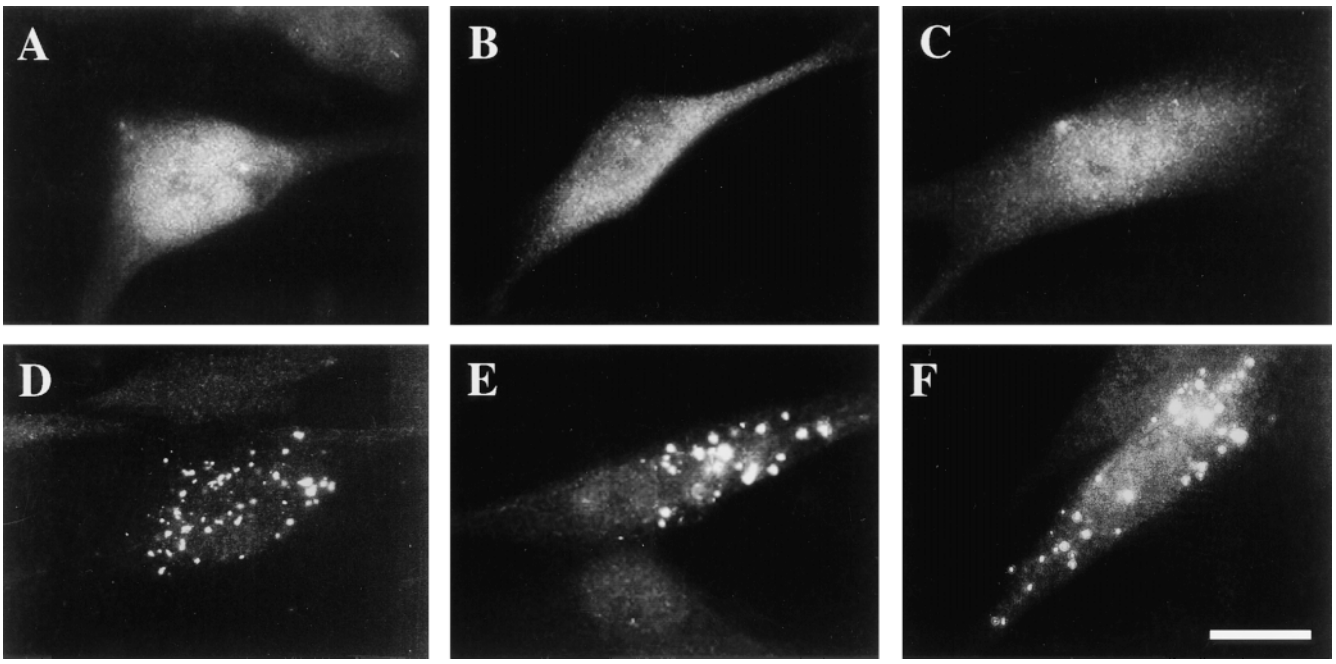

Figure 2. The PMP import defect of CG9 cells applies to numerous PMPs. The CG9 cell line, PBD061 (A–C); and the CG10 cell line, PBD094 (D–F), were transfected with vectors designed to express PMP70 myc (A and D), PEX12myc (B and E), and PMP32myc (C and F). 2 d after transfection the cells were processed for indirect immunofluorescence using antibodies specific for the myc tag. The peroxisomal nature of the punctate structures in PBD094 cells was confirmed by double label experiments with peroxisomal membrane markers (data not shown). Bar, 10 μm.

was inserted into a mammalian expression vector and the resulting plasmid, pcDNA3-*PEX16*, was transfected into PBD061 cells. 3 d after transfection the cells were processed for indirect immunofluorescence using antibodies specific for the peroxisome membrane marker, PMP70, and a peroxisomal matrix protein, catalase. Expression of *PEX16* rescued both PMP and matrix protein import in PBD061 cells, demonstrating that peroxisomes can form in the absence of preexisting peroxisomes (Fig. 4). No rescue was observed in cells transfected with the vector alone.

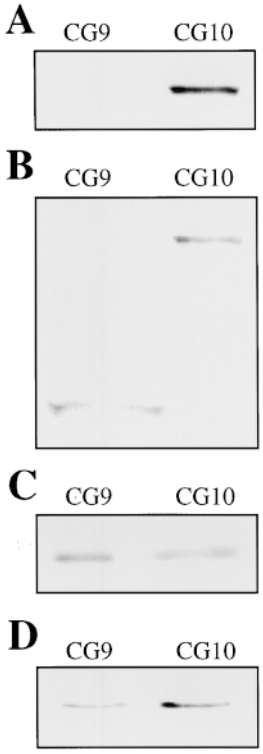

Figure 3. Different fates for different PMPs in PBD061 cells. Total cellular protein was extracted from the CG9 cell line, PBD061, and the CG10 cell line, PBD094. Equal amounts of each lysate (by protein) were separated by SDS-PAGE, and blotted with antibodies specific for the PMPs PMP70 (A), P70R (B); and PEX14 (C). In addition, total cellular protein was extracted from PBD061 and PBD094 cells that had been transfected with the PEX12myc expression vector. Equal amounts of each lysate (by protein) were separated by SDS-PAGE, and blotted with antibodies specific for the myc epotope tag (D).

The functional complementation experiments described above indicated that this patient would have mutations in the *PEX16* gene. The *PEX16* cDNA was amplified from control and PBD061 RNAs by RT-PCR, and direct sequence analysis revealed the presence of a nonsense mutation in the *PEX16*/PBD061 cDNA population (data not shown). This mutation, a C-to-T transition at a CpG dinucleotide, converts the arginine 176 codon to a nonsense codon (R176ter), and appeared to represent the only form of *PEX16* mRNA present in PBD061 cells. To determine whether this mutation was present at the genomic DNA level we amplified an appropriate fragment of the *PEX16* gene from control and PBD061 genomic DNAs. Direct sequence analysis showed only the mutant form of the gene in PBD061 (Fig. 5, A and B), indicating that this patient is homozygous for the R176ter mutation.

The R176ter mutation is predicted to delete the COOH-terminal half of *PEX16*, suggesting that it may have a severe effect on gene function. To test this directly we engineered the R176ter mutation into the *PEX16* cDNA expression vector and assayed the effects of this mutation by functional complementation. PBD061 fibroblasts were transfected with the expression vectors pcDNA3-*HsPEX16*/R176ter and pcDNA3-*HsPEX16.* After 3 d, cells from each population were processed for indirect immunofluorescence using anti-PMP70 and anticatalase antibodies to assess the recon-

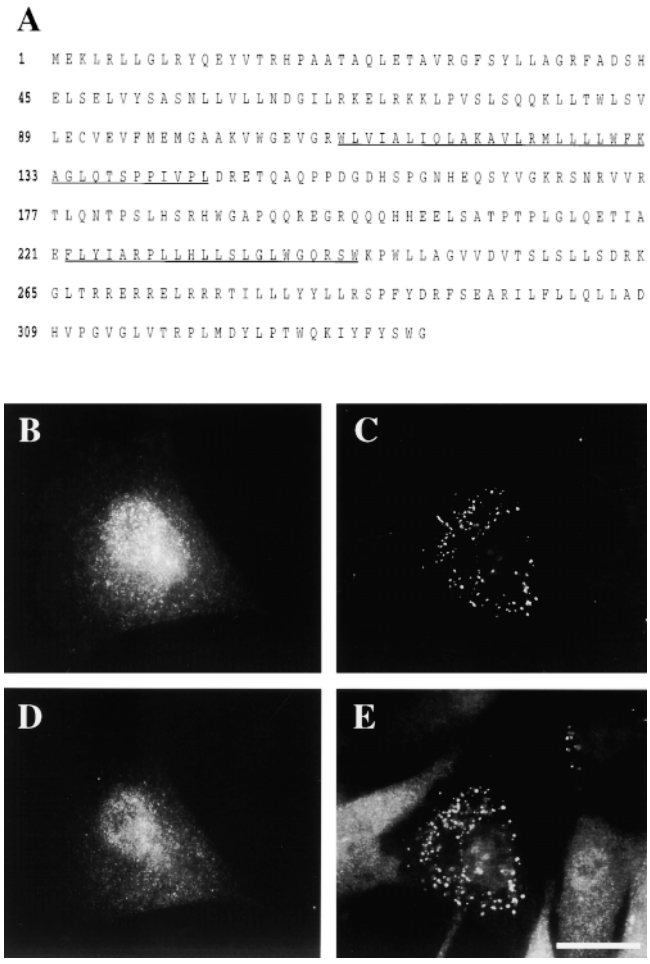

Figure 4. *PEX16* expression restores peroxisome synthesis in the CG9 cell line, PBD061. (A) The deduced amino acid sequence of human PEX16. Amino acid positions are noted on the left and the two predicted transmembrane domains are underlined. PBD061 cells were transfected with the plasmids pcDNA3 (B and D), and pcDNA3-*PEX16* (C and E). 3 d later the cells were processed for indirect immunofluorescence using rabbit antibodies specific for PMP70 (B and C), and sheep antibodies specific for catalase (D and E), followed by fluorescein-labeled goat anti–rabbit and Texas red–labeled goat anti–sheep secondary antibodies. Note the colocalization of PMP70 and catalase in the rescued cell (C and E). Bar, 10 μm.

stitution of peroxisome biogenesis. The *PEX16*/R176ter cDNA displayed no rescue activity, indicating that the R176ter mutation had a severe effect on *PEX16* function (Fig. 5, C–F).

PEX16 Encodes an Integral PMP with Its Termini Extending into the Cytoplasm

While the above results demonstrated that PEX16 is required for peroxisome biogenesis they did not reveal how PEX16 might perform this role. Since the physical location of proteins can provide useful insight into their function we first determined the subcellular distribution of PEX16. Antibodies to PEX16 were generated and recognized a polypeptide of 38 kD, the predicted molecular mass of PEX16, in human cell extracts (data not shown). These antibodies were used to localize PEX16 in subcellular fractionation experiments. A postnuclear supernatant was prepared from HepG2 cells and fractionated by Nycodenz density gradient centrifugation. Fractions were assayed for marker enzymes of peroxisomes, mitochondria, and microsomes, as well as PEX16 (Fig. 6 A). PEX16 behaved as a typical PMP, localizing only to the high-density, catalase-containing peroxisomal fractions (rupture of peroxisomes during homogenization and centrifugation leads to the presence of peroxisomal matrix proteins at the top of the gradient, demonstrated here by the presence of catalase activity in the low density fractions).

Sequence analysis predicted that the 336–amino acid-long PEX16 protein contained two transmembrane domains: one among amino acids 110–144, and another spanning amino acids 222–243 (Fig. 3 A). To test the hypothesis that PEX16 was a PMP we prepared another postnuclear supernatant from HepG2 cells and collected peroxisomes, mito-

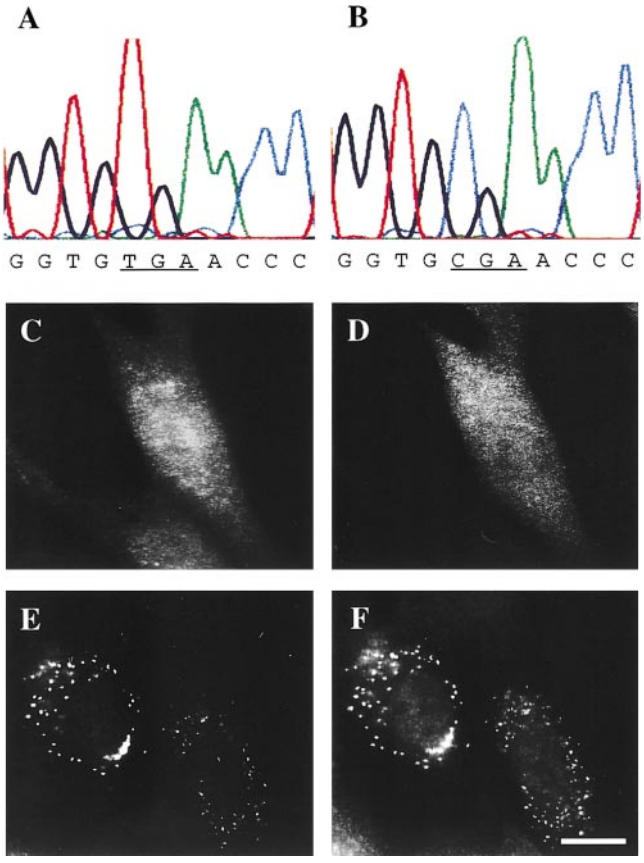

Figure 5. PBD061 cells have an inactivating mutation in PEX16. Direct sequence analysis of a *PEX16* fragment from (A) PBD061 and (B) an unaffected individual showing the CGA to TGA nonsense mutation in PBD061 genomic DNA. The homogeneous nature of the PBD061 sequence indicates that this patient is homozygous for the R176ter mutation. The effect of this mutation was assayed by transfecting PBD061 cells with pcDNA3-*PEX16*/R176ter (C and D) a plasmid designed to express the mutated form of the cDNA; and pcDNA3-*PEX16* (E and F). 2 d after transfection the cells were processed for indirect immunofluorescence using rabbit antibodies specific for PMP70 (C and E) and sheep antibodies specific for catalase (D and F), followed by fluorescein-labeled goat anti–rabbit and Texas red–labeled goat anti–sheep secondary antibodies. Bar, 10 μm.

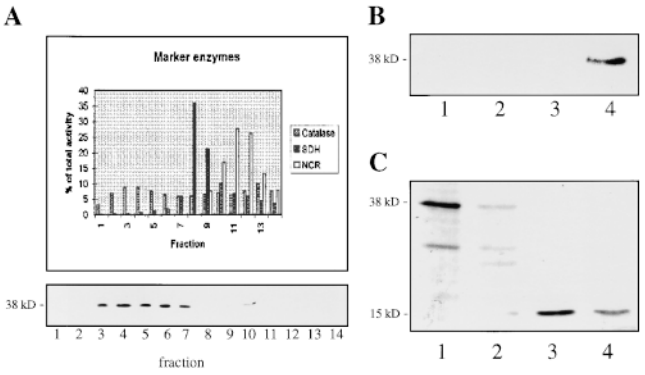

Figure 6. PEX16 encodes an integral peroxisomal membrane protein. (A) A postnuclear supernatant of HepG2 cells was fractionated by density gradient centrifugation. Equal proportions of each fraction were assayed for the marker enzymes catalase, a peroxisomal enzyme, SDH, a mitochondrial marker, and NCR, an ER-associated enzyme. Equal proportions of each fraction were also assayed for levels of PEX16 by immunoblot (bottom). These antibodies also detect an unknown protein of slightly higher mobility in fraction 10. (B) PEX16 is present in high pH carbonate-washed membranes. A light mitochondrial fraction from HepG2 cells was separated into a hypotonic extraction supernatant (lane 1); a high salt extraction supernatant (lane 2); a high pH carbonate supernatant (lane 3); and a pellet of carbonate-washed membranes (lane 4). Equal proportions of each fraction were separated by SDS-PAGE and assayed for levels of PEX16 by Western blot. (C) Protease protection experiments indicate that PEX16 spans the membrane twice with its NH_2 and COOH termini exposed to the cytoplasm. A light mitochondrial fraction was prepared from HepG2 cells and incubated with no trypsin (lane 1), 15 μg trypsin (lane 2), 30 μg trypsin (lane 3), and 60 μg trypsin (lane 4). PEX16 is reduced to a protease-resistant form of 15 kD, the size of the two transmembrane domains and the intermembrane loop. Digestion in the presence of detergent (1% Triton X-100) did not yield the 15-kD species (data not shown).

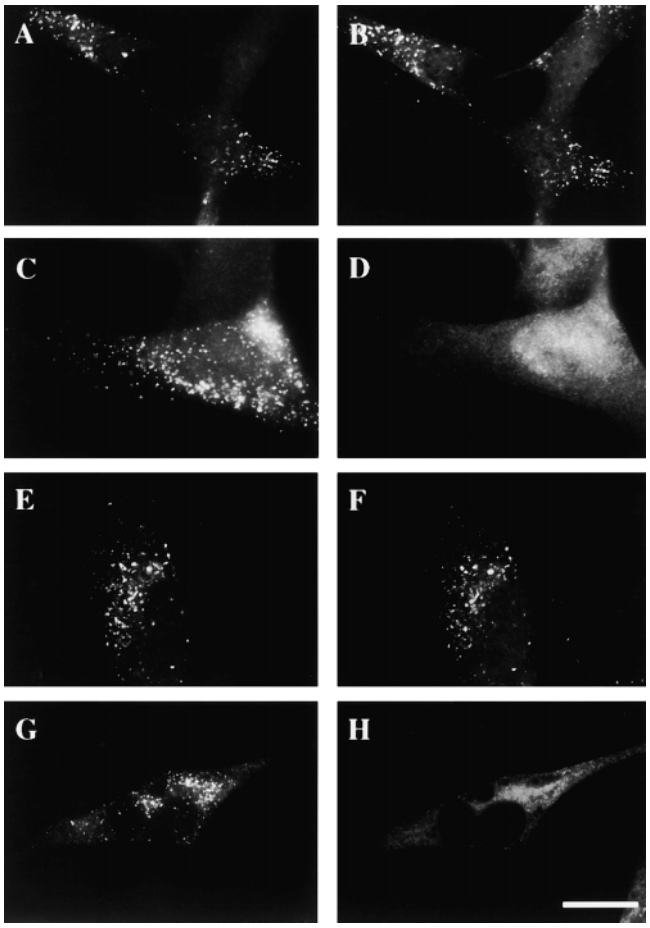

Figure 7. The NH_2 and COOH termini of PEX16 are exposed to the cytoplasm. Normal human fibroblasts were transfected with pcDNA3-*mycPEX16* (A–D) or pcDNA3-*PEX16myc* (E–H). 2 d after transfection cells were fixed and processed for indirect immunofluorescence under standard permeabilization conditions (A, B, E, and F) or under differential permeabilization conditions (C, D, G, and H). Cells were labeled with mouse mAbs specific for the myc epitope tag (A, C, E, and G) and with sheep antibodies specific for catalase (B, D, F, and H), followed by Texas red–labeled goat anti–mouse and fluorescein-labeled goat anti–sheep secondary antibodies. Bar, 10 μm.

chondria, and other large organelles by centrifugation at 25,000 *g.* This light mitochondrial fraction was subjected to successive extractions with hypotonic buffer (10 mM Tris, pH 8.5), high salt (1 M NaCl), and high pH (100 mM Na_2CO_3, pH 11.5). These treatments are designed to release soluble peroxisomal enzymes, electrostatically associated membrane proteins, and tightly associated membrane proteins, respectively (Fujiki et al., 1982). Integral PMPs are predicted to remain in the carbonate-extracted membrane pellet. Western blot analysis revealed that PEX16 behaved as an integral PMP, remaining in the membrane pellet even after carbonate extraction (Fig. 6 B).

To assess the topology of PEX16 in the peroxisome membrane we first performed protease protection experiments on a light mitochondrial fraction of HepG2 cells. Exogenous protease reduced PEX16 from 38 to 15 kD in the absence of detergent (Fig. 6 C), demonstrating that a significant portion of PEX16 extends into the cytoplasm. The anti-PEX16 antibodies used in this experiment were raised against its COOH-terminal 189 amino acids, which comprises the segment between the two transmembrane domains, the second transmembrane domain, and the 10-kD COOH-terminal tail. The only topology that is consistent with these results is for PEX16 to reside in the peroxisome membrane with its NH_2 and COOH termini extending into the cytoplasm and its intermembrane loop protected in the peroxisome lumen.

To test PEX16 orientation by an independent method we generated tagged versions of the *PEX16* cDNA expression vectors: one designed to express the c-myc tag at the NH_2 terminus of PEX16 (pcDNA3-*mycPEX16*), and one designed to express the c-myc tag at its COOH terminus (pcDNA3-*PEX16myc*). Normal human fibroblasts were transfected with these plasmids. After 2 d of incubation, the cells were processed for immunofluorescence using antibodies specific for the myc epitope tag and for catalase, a peroxisomal matrix protein (Fig. 7). The mycPEX16 and PEX16myc proteins both colocalized with catalase, confirming that PEX16 is a peroxisomal protein. Furthermore, the mycPEX16 and PEX16myc proteins were detected un-

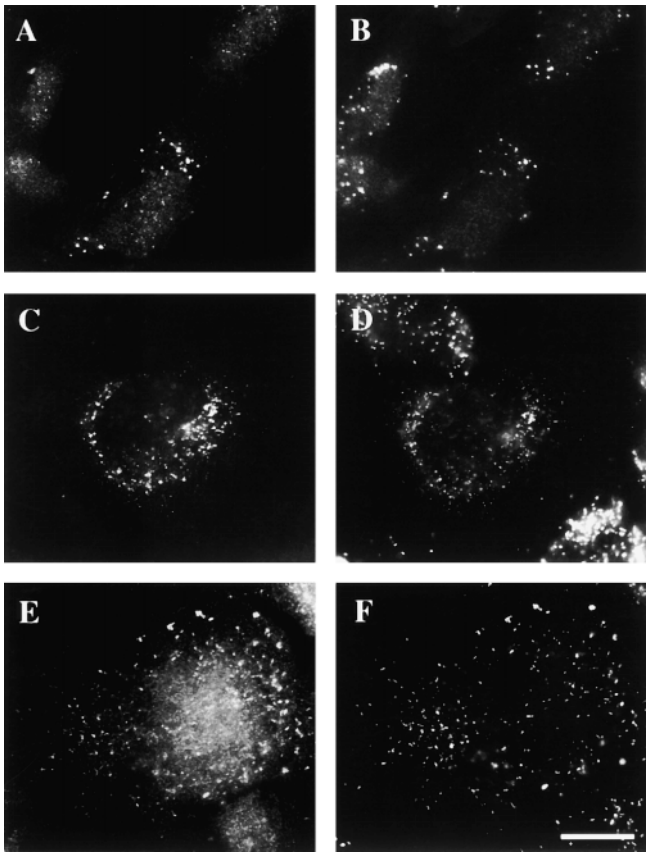

Figure 8. PEX16 is rapidly targeted to peroxisomes in a *PEX1*-independent and BFA-independent manner. The *PEX1*-deficient cell line, PBD009, was transfected with pcDNA3-*PEX16myc* (A and B), and processed for indirect immunofluorescence 2 d later using mouse mAbs specific for the myc epitope tag (A), and rabbit antibodies specific for PMP70 (B), followed by fluorescein-labeled goat anti–mouse and Texas red–labeled goat anti–rabbit secondary antibodies. The normal human fibroblast cell line, 5756T, was injected with pcDNA3-*PEX16myc* and processed for indirect immunofluorescence 2 h later using mouse mAbs specific for the myc epitope tag (C) and rabbit antibodies specific for P70R (D), followed by fluorescein-labeled goat anti–mouse and Texas red–labeled goat anti–rabbit secondary antibodies. (E and F) 5756T cells that had been pretreated for 30 min with 10 μg/ml BFA were injected with pcDNA3-*PEX16myc*, incubated for 2 h in 10 μg/ml BFA, and processed for immunofluorescence using mouse mAbs specific for the myc epitope tag (E) and rabbit antibodies specific for P70R (F), followed by fluorescein-labeled goat anti–mouse and Texas red–labeled goat anti–rabbit secondary antibodies. Bar, 10 μm.

der differential permeabilization conditions that preclude the detection of intraperoxisomal antigens, confirming that PEX16 is a peroxisomal membrane protein with its NH_2 and COOH termini extending into the cytoplasm. Surprisingly, overexpression of mycPEX16 or PEX16myc never induced peroxisome proliferation, suggesting that there may be a functional distinction between factors that are required for peroxisome membrane synthesis and those that are involved in peroxisome proliferation, such as PEX11α and PEX11β (Schrader et al., 1998). In control experiments, the mycPEX16 and PEX16myc expression vectors were able to rescue peroxisome synthesis in PBD061 cells, confirming that these localization studies were performed with functional forms of PEX16 (data not shown).

Biogenesis of PEX16

PEX16 was first identified in the yeast *Y. lipolytica.* Although previous studies of PMP biogenesis have reported that they are synthesized in the cytoplasm and imported into peroxisomes posttranslationally (Lazarow and Fujiki, 1985), the analysis of *Y. lipolytica* PEX16 suggested that this protein is inserted into the ER, and it traffics to peroxisomes in a *PEX1*- and *PEX6*-dependent manner (Eitzen et al., 1997; Titorenko et al., 1997; Titorenko and Rachubinski, 1998b). This model for PEX16 biogenesis is built on the following observations: YlPEX16 accumulates in the ER in the absence of either the *PEX1* or *PEX6* genes; a pulse of YlPEX16 expression can be detected first in the ER and subsequently in the peroxisome; and YlPEX16 is modified by N-linked glycosylation. Given that the loss of human *PEX16* results in the absence of peroxisomes from the cell, a similar biogenic pathway for human PEX16 would implicate the ER as a possible source of newly synthesized peroxisomes in rescued PBD061 cells.

We first examined the distribution of PEX16myc in cells that lack *PEX1.* PBD009 cells have inactivating mutations on both *PEX1* alleles, lack *PEX1* mRNA and protein (Reuber et al., 1997), and would be expected to accumulate PEX16myc in the ER. In contrast to the expected results, we found that PEX16myc colocalized with the peroxisomal membrane marker, P70R, in *PEX1*-deficient PBD009 cells (Fig. 8, A and B). Next, we replicated the pulsed expression experiments. However, because of the need to detect PEX16myc at the very earliest time points during its expression, we had to deliver the PEX16myc expression vector by nuclear microinjection rather than electroporation. The normal human fibroblast line 5756T was injected with pcDNA3-*PEX16myc* and then processed for indirect immunofluorescence at 0.5, 1, 2, and 3 h after injection. The first time point that we detected PEX16myc expression was 2 h after injection, at which point all of the PEX16myc appeared to colocalize with the peroxisomal membrane marker, P70R (Fig. 8, C and D). Examination of multiple cells at the 2- and 3-h time points showed a similar peroxisomal distribution of PEX16myc. The fact that human PEX16 lacks a consensus sequence for N-linked oligosaccharide addition, Asn-Xaa-Ser/Thr, made it impossible to follow N-linked glycosylation as a test of whether PEX16 entered the ER before its delivery to peroxisomes.

The recent evidence for ER involvement in peroxisome biogenesis is not limited to the biogenesis of PEX16 in *Y. lipolytica.* Other tantalizing evidence includes the observation that brefeldin A (BFA) blocks peroxisome biogenesis in *Hansenula polymorpha* (Solomos et al., 1997), and the finding that ARF and COPI may play a role in peroxisome proliferation in mammalian cells (Passreiter et al., 1998). Since PEX16 plays an essential role in peroxisome synthesis we tested whether BFA might block the targeting of PEX16 to peroxisomes in normal cells. Normal human fibroblasts were incubated in 10 μg/ml BFA for 30 min and then injected with the pcDNA3-*PEX16myc* expression vec-

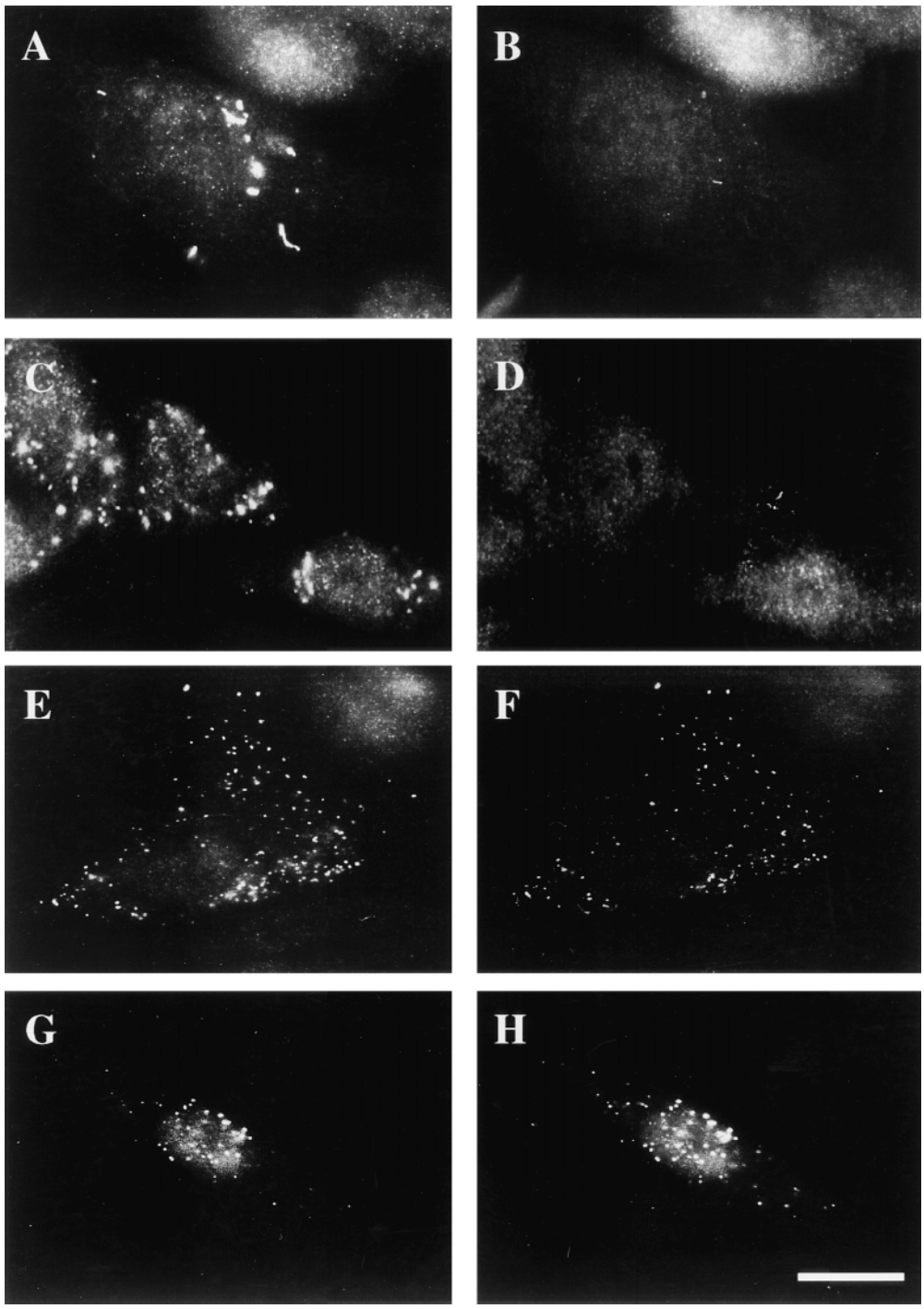

Figure 9. Rescue of peroxisome synthesis in PBD061 cells occurs in a multistep process. PBD061 cells were injected with pcDNA3-*PEX16* and processed for indirect immunofluorescence 3 h later using rabbit antibodies specific for P70R (A); and sheep antibodies specific for catalase (B); followed by fluorescein-labeled goat anti–rabbit and Texas red–labeled goat anti–sheep secondary antibodies. Note that many of the peroxisomes appeared to be undergoing elongation, the first step in PEX11β-mediated peroxisome proliferation (Schrader et al., 1998). Cells processed for 22 h (C and D) and 72 h (E and F) after introduction of pcDNA3-*PEX16* were also processed for indirect immunofluorescence using rabbit antibodies specific for P70R (C and E) and sheep antibodies specific for catalase (D and F), followed by fluorescein-labeled goat anti–rabbit and Texas red–labeled goat anti–sheep secondary antibodies. (G and H) PBD061 cells were preincubated for 30 min with 10 μg/ml BFA, injected with pcDNA3-*PEX16*, and incubated in 10 μg/ml BFA for 30 h. These cells were processed for immunofluorescence using rabbit antibodies specific for P70R (G); and sheep antibodies specific for catalase (H); followed by fluorescein-labeled goat anti–rabbit and Texas red–labeled goat anti–sheep secondary antibodies. Bar, 10 μm.

tor. The cells were maintained in the same concentration of BFA and processed for indirect immunofluorescence at various times after injection. Peroxisomal PEX16myc was detected in peroxisomes 2 h after injection, demonstrating that BFA did not inhibit the targeting of PEX16myc to peroxisomes (Fig. 8, E and F). We also tested whether incubation at 15°C, a treatment that blocks exit of proteins from the ER/Golgi intermediate compartment (Saraste and Kuismanen, 1984), had any effect on PEX16myc targeting to peroxisomes. We failed to detect any significant inhibition of PEX16myc targeting to peroxisomes in cells incubated at 15°C as compared to cells incubated at 37°C (data not shown).

These experiments failed to provide evidence for an ER role in PEX16myc biogenesis. Most proteins which enter the ER do so cotranslationally, and, thus, are never present in the cytosolic fraction before association with a sedimental organelle. We attempted to follow the biogenesis of PEX16 in pulse–chase experiments but were unable to incorporate enough radioactivity into newly synthesized PEX16 to detect the protein after even a long (1 h) pulse labeling period (data not shown). Therefore, we were un-

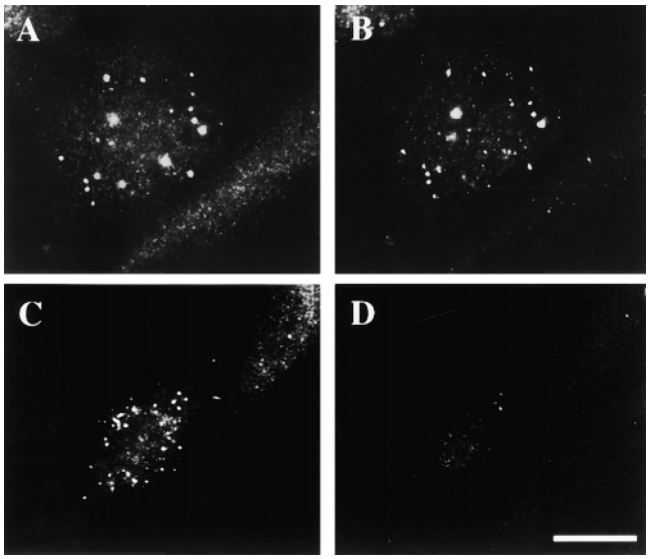

Figure 10. Import of PEX16myc can precede P70R import in peroxisomes of rescued PBD061 cells. PBD061 cells were injected with pcDNA3-*PEX16myc* and processed for indirect immunofluorescence 3 h later using mouse mAbs specific for the myc epitope tag (A and C) and rabbit antibodies specific for P70R (B and D), followed by fluorescein-labeled goat anti–mouse and Texas red–labeled goat anti–sheep secondary antibodies. Bar, 10 μm.

able to use pulse–chase experiments to determine whether PEX16 enters the peroxisome directly from the cytoplasm or indirectly through the ER.

Peroxisome Synthesis in Rescued CG9 Cells

The restoration of peroxisome synthesis in PBD061 cells demonstrated that peroxisomes do not necessarily arise by division of preexisting peroxisomes. To characterize the process by which this occurs we followed the appearance of peroxisomes at various times after injection of PBD061 cells with the *PEX16* expression vector. At each time point, cells were processed for indirect immunofluorescence using antibodies specific for the peroxisomal membrane marker, PMP70, and the peroxisomal matrix marker, catalase. We were unable to detect any peroxisomal structures 30 min, 1, or 2 h after injection. However, by 3 h after injection we started to observe P70R-containing structures that could be detected over and above background staining (Fig. 9, A and B). These structures generally did not contain the matrix protein catalase, and were much reduced in number as compared to normal cells or fully rescued PBD061 cells. In addition, peroxisomes detected at early time points often appeared to form elongated structures similar to those that form at early times in the PEX11β-mediated proliferation of human peroxisomes.

At later time points, ~18–24 h after injection or transfection, peroxisomes of more normal distribution and abundance could be detected, although these commonly lacked significant levels of catalase (Fig. 9, C and D). At later time points (36–72 h after injection or transfection), the proportion of rescued cells that contained both P70R and catalase increased (Fig. 9, E and F), although it never attained unity. In no instance did we observe punctate catalase in the absence of P70R-containing peroxisomes (data not shown). Taken together, these results suggest that peroxisome synthesis occurs in a multistep fashion beginning with the formation of a limited number of PMP-containing vesicles, followed by the elongation and proliferation of peroxisomal structures, and ending with the robust import of peroxisomal membrane and matrix proteins. However, it should be noted that low but detectable numbers of catalase-import-

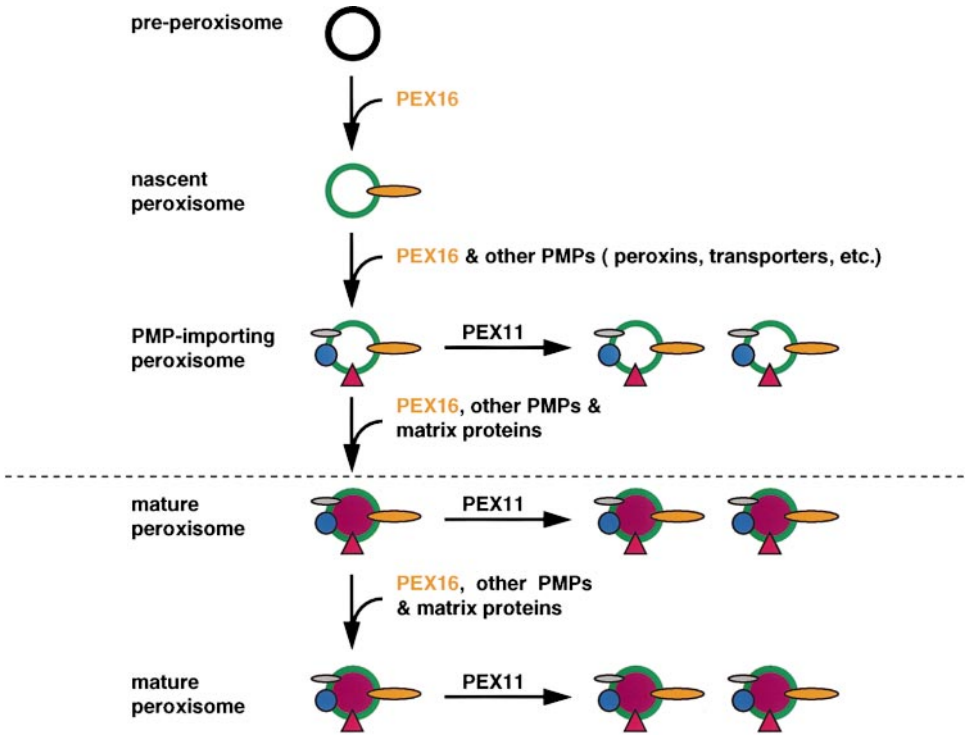

Figure 11. A model of peroxisome biogenesis in the absence (top) and presence (bottom) of preexisting peroxisomes. During rescue of PBD061 cells, PEX16 creates nascent peroxisomes, possibly from a preperoxisomal vesicle. These nascent PEX16-containing peroxisomes then import additional PMPs. The import of PEX11 proteins allows these structures to proliferate by fission or by budding, and the import of other peroxins leads to formation of a functional matrix protein import apparatus. Under normal conditions, peroxisomes would form primarily from preexisting peroxisomes in a PEX11-mediated process. Although the targeting of PEX16 to preperoxisomal structures may be enhanced in the absence of peroxisomal membranes, such a process may also occur under normal conditions.

ing cells could be detected at early time points after injection of the *PEX16* expression vector. Similar rates of rescue were observed in the presence of BFA, demonstrating that peroxisome synthesis is insensitive to this inhibitor of vesicular transport (Fig. 9, G and H).

We repeated these kinetic studies using the PEX16myc expression vector. PBD061 cells were injected with pcDNA3-*PEX16myc* and processed for indirect immunofluorescence using antibodies specific for the myc epitope tag and for P70R. As with the wild-type expression vector, rescue could not be detected until 3 h after injection. At this time point, some cells contained punctate structures that contained both PEX16myc and P70R, but a few cells were observed that contained only PEX16myc (Fig. 10).

Discussion

We have identified human *PEX16*, and established that an inactivating mutation in this gene abolishes the synthesis of peroxisomes in a Zellweger syndrome patient, PBD061. These results expand the range of phenotypes and genotypes that are associated with Zellweger syndrome, and thus, expand our understanding of this lethal disease. However, this study also enhances our understanding of the molecular events involved in formation of peroxisome membranes. Most importantly, the rescue of peroxisome synthesis in PBD061 cells by *PEX16* expression demonstrates that preexisting peroxisomes, defined here as PMP-containing vesicles, are not required for the formation of new peroxisomes. While the elucidation of PEX16 function clearly requires additional studies our results do provide some insight into the biogenesis of peroxisome membranes, at least in humans.

Perhaps the simplest model for peroxisome synthesis in rescued PBD061 cells would be that PEX16 expression allows the synthesis of peroxisomes from phospholipids and PMPs rather than preexisting vesicles. However, this appears unlikely since there is no precedent for the synthesis of biological membranes other than from a preexisting lipid bilayer. An alternative hypothesis is that PEX16, either alone or in conjunction with other proteins, converts a preperoxisomal vesicle to recognizable peroxisomes by mediating the import of other PMPs. This in turn would lead to assembly of the matrix protein import apparatus, and the subsequent import of peroxisomal matrix proteins, completing the formation of mature peroxisomes.

Although this may explain the formation of peroxisomes during the rescue of PBD061 cells it is questionable whether such a simple model can explain peroxisome synthesis under all conditions. For instance, if peroxisomes were typically formed by such a process we would expect that the biogenesis of peroxisomes in PBD061 cells would occur on roughly the same time course as the import of PEX16 into peroxisomes of normal cells. However, this is clearly not the case as PEX16 is imported into virtually all peroxisomes of normal cells in <2 h whereas the reconstitution of normal peroxisomes in PBD061 cells requires 18–24 h. Furthermore, this model fails to incorporate roles for the proliferation factors PEX11α and PEX11β (Schrader et al., 1998) in the formation of peroxisomes.

These and other lines of evidence suggested instead that peroxisome formation may occur by two distinct, yet overlapping pathways (Fig. 11). In this model, peroxisomes would typically form by division of preexisting peroxisomes in a process mediated by PEX11α and PEX11β, not PEX16. This would explain the hyperabundance of peroxisomes in cells overexpressing PEX11α or PEX11β, and the fact that overexpression of PEX16 does not induce any detectable proliferation of peroxisomes. In this model, PEX16 would function normally in importing PMPs into preexisting peroxisomes, and would itself be imported into preexisting peroxisomes. In the absence of PEX16, PMP import would cease, peroxisomes could not divide, and the organelle would eventually be lost from the cell population. In addition to this common route of peroxisome formation we propose that PEX16 may, at low efficiency, be targeted to an unknown preperoxisomal structure. The incorporation of PEX16 into such vesicles may convert them to nascent peroxisomes that are able to import other PMPs, and eventually to mature peroxisomes. This model would not only explain the ability of PEX16 to rescue PBD061 cells but would also explain: the presence of a few, large peroxisomes in mutants lacking the *PEX11* gene (Erdmann and Blobel, 1995; Marshall et al., 1995); the peroxisome proliferation promoting activity of PEX11 proteins; and the lack of peroxisome proliferation promoting activity for PEX16.

This model is consistent with virtually all available data on the formation of peroxisomes. However, it proposes the existence of a structure that has yet to be identified, the preperoxisome, and proposes an activity for PEX16 for which there is no direct evidence, PMP import. Nevertheless, this model serves as a useful starting point for considering how peroxisomes are formed. The preperoxisomal structure need not be a dedicated structure awaiting PEX16 for conversion into a nascent peroxisome, only a subcellular vesicle that happens to incorporate a PEX16 molecule. As for the function of PEX16, our only evidence that this protein is involved in PMP import is the inability of PBD061 cells to import PMPs into peroxisomes, a phenotype that is also consistent with a defect in the synthesis of the preperoxisomal structure of our model. Nevertheless, PEX16 has the appropriate structural requirements for a role in PMP import. PEX16 is itself an integral PMP and extends its NH_2 and COOH termini into the cytoplasm where they may facilitate the recognition of PMPs and/or their insertion into the peroxisome membrane. In addition, PEX16 appears to be transported efficiently to peroxisomes, and is detected only in peroxisomes, not some other compartment, at steady state. It should be noted that similar models have been proposed that implicate the ER as the source of the preperoxisomal structure (Kunau and Erdmann, 1998; Titorenko and Rachubinski, 1998a). However, our data do not provide evidence for a role of the ER in peroxisome biogenesis.

In addition to improving our understanding of peroxisome synthesis and the molecular basis of Zellweger syndrome, this report also makes clear that homologues of the same peroxisome biogenesis factor may have disparate roles in different organisms. Although we identified human PEX16 by its sequence similarity to *Y. lipolytica* PEX16, there were significant differences between these proteins. Prior reports have established that YlPEX16 is peripherally associated with the inner face of the peroxisome membrane,

and transported to peroxisomes via the ER in a *PEX1*- and *PEX6*-dependent manner. Despite it playing a role in the import of a subset of peroxisomal matrix proteins, YlPEX16 is not required for synthesis of peroxisome membranes nor PMP import (Eitzen et al., 1997; Titorenko et al., 1997; Titorenko and Rachubinski, 1998b). In contrast, human PEX16, an integral PMP with its NH_2 and COOH termini exposed to the cytoplasm, is required for PMP import and peroxisome synthesis, and appears to traffic from the cytoplasm to the peroxisome in a *PEX1*-independent manner. These differences emphasize the need for caution in extrapolating the functions of a particular peroxisome biogenesis factor to its homologue in divergent species. Discretion in this matter is emphasized further by the fact that the *Saccharomyces cerevisiae* genome lacks a PEX16 homologue, even though the YlPEX16 and human PEX16 proteins share 24% amino acid identity.

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