Inhibitors of COPI and COPII Do Not Block PEX3-mediated Peroxisome Synthesis

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Abstract. In humans, defects in peroxisome biogenesis are the cause of lethal diseases typified by Zellweger syndrome. Here, we show that inactivating mutations in human PEX3 cause Zellweger syndrome, abrogate peroxisome membrane synthesis, and result in reduced abundance of peroxisomal membrane proteins (PMPs) and/or mislocalization of PMPs to the mitochondria. Previous studies have suggested that PEX3 may traffic through the ER en route to the peroxisome, that the COPI inhibitor, brefeldin A, leads to accumulation of PEX3 in the ER, and that PEX3 overexpression alters the morphology of the ER. However, we were unable to detect PEX3 in the ER at early times after expression. Furthermore, we find that inhibition of COPI function by brefeldin A has no effect on trafficking of PEX3 to peroxisomes and does not inhibit PEX3-mediated peroxisome biogenesis. We also find that inhibition of COPII-dependent membrane traffic by a dominant negative SAR1 mutant fails to block PEX3 transport to peroxisomes and PEX3-mediated peroxisome synthesis. Based on these results, we propose that PEX3 targeting to peroxisomes and PEX3-mediated peroxisome membrane synthesis may occur independently of COPI- and COPII-dependent membrane traffic.

Key words: Zellweger syndrome • membrane biogenesis • protein import • vesicle traffic • peroxisome biogenesis disorders

Introduction
Eukaryotic cells contain numerous subcellular organelles. The division of cellular metabolism among these specialized compartments allows for the concentration of related activities, segregation of competing functions, and the formation of unique microenvironments. However, the assembly and maintenance of these organelles is complex and involves several interrelated processes. These include the recognition of proteins destined for particular organelles, transport of these proteins to and into the organelle lumen, and the biogenesis of the organelle membrane (Schatz and Dobberstein, 1996). Peroxisomes are present in virtually all eukaryotes and their assembly is thought to involve these same general processes. However, our knowledge of peroxisome biogenesis, particularly those aspects that relate to formation of peroxisome membranes, is far from complete.

Peroxisomes lack nucleic acids and must therefore import all of their protein content (Lazarow and Fujiki, 1985). Peroxisomes have a single membrane, are ~0.1-1 μm in diameter, and have a dense proteinaceous matrix. The metabolic roles of peroxisomes can vary considerably, but human peroxisomes contribute primarily to the α-oxidation and β-oxidation of fatty acids, as well as the synthesis of ether-linked phospholipids and isoprene compounds (Wanders and Tager, 1998). The peroxisomal enzymes involved in these processes are encoded by nuclear genes, are synthesized in the cytoplasm, and imported in a post-translational fashion (Lazarow and Fujiki, 1985). Two different targeting signals have been identified for matrix enzymes, the COOH-terminally located PTS1, which directs most proteins to peroxisomes, and the NH2-terminal PTS2, which directs only a small number of proteins into the peroxisome (Subramani, 1993).

Genetically determined, lethal diseases are caused by defects in any of several different peroxisomal enzymes, underscoring the importance of peroxisomal contributions to metabolism (Wanders and Tager, 1998). However, Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and rhizomelic chondrodysplasia punctata represent a different set of lethal peroxisomal disorders. Rather than lacking a single peroxisomal enzyme, these patients lack virtually all peroxisomal metabolic functions, a phenotype that results from defects in the import of one or more classes of peroxisomal matrix proteins (Lazarow and Moser, 1995). Theoretically, the peroxisomal matrix protein import defects observed in
these peroxisome biogenesis disorder (PBD)\textsuperscript{1} patients could be caused by mutations in either of two different types of genes, those that encode peroxisomal matrix protein import factors (Chang et al., 1999a), and those that encode factors necessary for peroxisome membrane biogenesis (Honsho et al., 1998; Matsuzono et al., 1999; South and Gould, 1999; Sacksteder et al., 2000). Cellular studies have revealed that most PBD patients display an isolated defect in peroxisomal matrix protein import (Santos et al., 1988a,b; Chang et al., 1999a). Cells from these patients contain nearly a hundred peroxisomes and import peroxisomal membrane proteins (PMPs) normally, even though they display mild to severe defects in peroxisomal matrix protein import. This phenotype is genetically heterogeneous and can be caused by loss of any of at least eight different peroxins (proteins required for peroxisome biogenesis). These include the receptors for newly synthesized peroxisomal matrix proteins, PEX5 and PEX7, as well as other peroxins that are required for peroxisomal matrix protein import (PEX1, PEX2, PEX6, PEX10, PEX12, and PEX13; Chang et al., 1999a).

A different phenotype recently has been described for a few rare Zellweger syndrome patients. These patients lack detectable peroxisomes altogether and are unable to import integral peroxisomal membranes into recognizable peroxisome-like structures (Honsho et al., 1998; Matsuzono et al., 1999; South and Gould, 1999; Sacksteder et al., 2000). This phenotype could result from either a defect in PMP import, which is distinct from peroxisomal matrix protein import, or a defect in peroxisome membrane synthesis. In humans, this phenotype can be caused by mutations in PEX16 (Honsho et al., 1998; South and Gould, 1999) or PEX19 (Matsuzono et al., 1999; Sacksteder et al., 2000), and reexpression of either gene results in the reformation of peroxisomes in the mutant cells. A through these results demonstrate that peroxisome synthesis can occur in the absence of preexisting peroxisomes, the mechanism for this mode of peroxisome membrane synthesis remains obscure. One hypothesis is that peroxisomes may arise from some other endomembrane of the cell (South and Gould, 1999), perhaps the ER (Titorenko and Rachubinski, 1998a), and may involve COPI-dependent processes (Solomons et al., 1997; Passreiter et al., 1998; Muller et al., 1999). Here, we show that two Zellweger syndrome patients who lack detectable peroxisomes are each homozygous for inactivating mutations in PEX3, which itself encodes a PMP (Honfeld et al., 1991; Kammerer et al., 1998; Soukupova et al., 1999). Studies of PEX3 import fail to identify peroxisomal, mitochondrial, and microsomal fractions, respectively. Equal amounts of each fraction were also separated by SDS-PAGE and assayed for catalase, succinate dehydrogenase (SDH), and NADPH-cytochrome reductase (NCR) to identify peroxisomal, mitochondrial, and microsomal fractions, respectively. Equal amounts of each fraction were also separated by SDS-PAGE and assayed for PEX3 levels by Western blot as described (Crane et al., 1995). Equal amounts of each fraction were assayed for catalase, succinate dehydrogenase (SDH), and NADPH-cytochrome reductase (NCR) to identify peroxisomal, mitochondrial, and microsomal fractions, respectively. Equal amounts of each fraction were also separated by SDS-PAGE and assayed for PEX3 levels by Western blot as described (Crane et al., 1994).

Materials and Methods

Tissue Culture, Cell Lines, Antibodies, and Reagents

Skin fibroblasts were obtained from Ann and Hugo Moser (The Kennedy Institute, Baltimore, MD), the Coriell Cell Repository (Camden, NJ), and Manuel Santos (The Catholic University of Chile, Santiago, Chile). HepG2 cells were obtained from Michael Schrader (The Johns Hopkins University, Baltimore, MD). All cell lines were cultured in high glucose DME supplemented with penicillin, streptomycin, and 10% FCS. The mutations identified in the PBD patient cell lines used for the PE X3 immunoblot have been described elsewhere (South and Gould, 1999).

A nontoxicity to catalase were obtained commercially (The Binding Site, Inc.) and antibodies to PMPs were generated to a COOH-terminal 18 amino acid peptide conjugated to maleimide-activated keyhole limpet hemocyanin (Pierce Chemical Co.) and injected into either sheep or guinea pigs. A nontoxicity to PEX3 was generated to a COOH-terminal 13 amino acid peptide conjugated to maleimide-activated keyhole limpet hemocyanin and injected into rabbits. A nontoxicity to PEX3 and PEX14 was generated by expressing a portion of each protein missing its putative N\textsubscript{t}-terminal transmembrane domain (amino acids 147-373 and 127-377, respectively) in fusion with maltose-binding protein. The resulting fusion proteins were purified by affinity chromatography on amylose resin (New England Biolabs, Inc.) and injected into rabbits. A nontoxicity to PEX11b was generated by fusing glutathione S-transferase to amino acids 227 and expressing the fusion protein in bacteria and isolating the inclusion body. The inclusion body was washed with 1% NP-40, then with 2M urea, and finally with PBS. The resulting protein lyasate was analyzed by SDS-PAGE and was found to be ~80% PEX11b fusion protein, and was then injected into rabbits. Polyclonal sera was collected and was either used directly or was purified by affinity chromatography using either purified fusion protein or peptide conjugated to cyanogen bromide sepharose beads (Sigma Chemical Co.). mAbs to the myc epitope were obtained from the tissue culture supernantant of the hybridoma 1-9E10 (Evans et al., 1985). Polyclonal antibodies to the myc and HA epitopes and mAb bs to the vsg epitope were obtained from commercial sources. Fluorescently labeled secondary antibodies and HRP-labeled anti-rabbit secondary antibodies were also obtained from commercial sources (Jackson Immunoresearch Laboratories). MitoTracker and FITC-C\textsubscript{1}-ceramide were incorporated into living cells according to the manufacturer's instructions (Molecular Probes).

Transfection, Microinjection, and Immunofluorescence

All cell lines were actively growing before transfection or microinjection. Transfections were performed by growing cells to near confluency, harvesting the cells by trypsinization, and electroporating them as described (Chang et al., 1997). Microinjections were performed with DNA at a concentration of 10 ng/ml in reverse PBS (4 mM Na\textsubscript{2}PO\textsubscript{4}, 1 mM KH\textsubscript{2}PO\textsubscript{4}, 140 mM KCl, pH 7.3). DNA was injected into the nucleus of cells at a pressure of 100 mm Hg for 0.4 s. Cells were grown on glass coverslips and processed for indirect immunofluorescence as described (South and Gould, 1999) with the exception that digitonin permeabilization was necessary for visualization of PEX11b (Schrader et al., 1998).

Protein Preps, Western Blots, and Fractionations

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, followed by centrifugation to remove nuclei and unbroken cells. The clarified supernatant was separated by density gradient centrifugation using Nycodenz (Nycomed Pharma) as described (Dodt et al., 1995). Equal amounts of each fraction were assayed for catalase, succinate dehydrogenase (SDH), and NADPH-cytochrome reductase (NCR) to identify peroxisomal, mitochondrial, and microsomal fractions, respectively. Equal amounts of each fraction were also separated by SDS-PAGE and assayed for PEX3 levels by Western blot as described (Crane et al., 1994).

Clonings and Mutation Detection

pcDNA3-PEX3 was created by PCR amplification of a human liver cDNA library using the PEX3-specific oligonucleotides 5'-CCAGTGCTGAGGTCTGTATGGAATTTTC-3' and 5'-CCAAATCGTCCAATTTTCTCAGCAGTGGAGGG-3'. The resulting PCR product was digested with A\textsubscript{sp}71B and SalI and cloned between the A\textsubscript{sp}71B and XhoI sites of pcDNA 3 (Invitrogen). pcDNA3-PEX3 was created by PCR amplification of a human liver cDNA library using the PEX3-specific oligonucleotides 5'-CCAGTGCTGAGGTCTGTATGGAATTTTC-3' and 5'-CCAAATCGTCCAATTTTCTCAGCAGTGGAGGG-3'. The resulting PCR product was digested with A\textsubscript{sp}71B and SalI and cloned between the A\textsubscript{sp}71B and XhoI sites of pcDNA 3 (Invitrogen). pcDNA3-PEX3 was created by PCR amplification of a human liver cDNA library using the PEX3-specific oligonucleotides 5'-CCAGTGCTGAGGTCTGTATGGAATTTTC-3' and 5'-CCAAATCGTCCAATTTTCTCAGCAGTGGAGGG-3'. The resulting PCR product was digested with A\textsubscript{sp}71B and SalI and cloned between the A\textsubscript{sp}71B and XhoI sites of pcDNA 3 (Invitrogen). pcDNA3-PEX3 was created by PCR amplification of a human liver cDNA library using the PEX3-specific oligonucleotides 5'-CCAGTGCTGAGGTCTGTATGGAATTTTC-3' and 5'-CCAAATCGTCCAATTTTCTCAGCAGTGGAGGG-3'. The resulting PCR product was digested with A\textsubscript{sp}71B and SalI and cloned between the A\textsubscript{sp}71B and XhoI sites of pcDNA 3 (Invitrogen).
TGCTGAGGG-3' at the 3' end, which replaces the stop codon with a BglII site. The resulting PCR product was digested with A sp718 and BglII and cloned between the A sp718 and BamHI sites of a modified pcDNA3 vector encoding the ten-amino acid c-myc epitope. The resulting plasmid contained the full-length cDNA sequence of PEX3 fused at its COOH terminus to the myc epitope, followed immediately by a stop codon. The plasmids designed to express PEX12myc, PEX13myc, A LD Pmyc, and PMP34myc all consist of the full-length human cDNA cloned into the same modified pcDNA-myc-containing vector. The sequence of mouse SAR1a was used to identify human SAR1 through BLAST searches of the human database of expressed sequence tags. Clones encoding the human SAR1 were obtained from G enome Systems Inc., and were sequenced in their entirety. Human SAR1 was first amplified with the oligonucleotides 5'-CCCGTACATGTCTTCTTCTGCTGTTACTC-3' and 5'-CAATATTGAAAACTGAGGCAAACGAC-3' and then cloned into a modified pcDNA3 vector encoding the six-amino acid vsvg epitope such that the resulting plasmid, pcDNA3-SAR1, contained the full-length sequence of human SAR1 with its COOH terminus fused to the vsvg epitope, immediately, followed by a stop codon. pcDNA 3-SAR1/T39N/vsvg was created by PCR site-directed mutagenesis using the oligonucleotides 5'-CCCGTACATGTCTTCTTCTGCTGTTACTC-3' and 5'-GGAAAATGTGGCGCAAATACGTCTTCTGCTGTTACTC-3', which creates a PCR fragment identical to the wild-type (WT) human SAR1 sequence, with the exception of an A CC to A AT codon change that creates the threonine at position 39 in the amino acid sequence to asparagine. This PCR fragment was cut with A sp718 and A fII and used to replace its complementary sequence in pcDNA3-SAR1, also cut with A sp718 and A fII. pcDNA3-SAR1/T39N/vsvg was sequenced to determine that only the T39N mutation was introduced during the amplification process. pcDNA3-GALT3xHA was created by PCR amplification using the oligonucleotides 5'-CCGGTACATGTCTTCTTCTGCTGTTACTC-3' and 5'-CCCCAGATGTCCCTCCGGTCCGGAGCTCCCC-3', and pECFP-Golgi vector (CLONTECH Laboratories, Inc.) as template. The resulting PCR product was digested with A sp718 and BglII and cloned into a modified pcDNA3 vector encoding three copies of the nine-amino acid human influenza virus hemagglutinin, HA epitope, such that the resulting plasmid contained the targeting sequence from human β1,4-galactosyltransferase (GALT) fused at the COOH terminus to three copies of the HA epitope immediately followed by a stop codon. All PCR-generated clones were sequenced in their entirety to ensure that no mutations were introduced during the amplification process.

Mutation detection was performed initially by reverse transcriptase (RT)-PCR. RNA was extracted from normal human fibroblasts, PBD 400 fibroblasts, and PBD 401 fibroblasts, and were converted to PEX cDNA as follows. A proximally 1 μg of total RNA from control and patient samples was used as template in a cDNA synthesis reaction using the PEX3-specific oligonucleotide 5'-CCAACGGGCGGAGTTTTGGTAAACAAACTGTGTCATCTC-3', which is located in the 3' untranslated region, using Superscript reverse transcriptase (Life Sciences Inc.). 3 μl of the cDNA synthesis reaction products were used as templates in PCRs using the PEX3-specific oligonucleotide 5'-CCAACGGGCGGAGTTTTGGTAAACAAACTGTGTCATCTC-3'. The resulting PCR products were digested with A sp718 and BglII and cloned into a modified pcDNA 3 vector encoding three copies of the nine-amino acid human influenza virus hemagglutinin, HA epitope, such that the resulting plasmid contained the targeting sequence from human β1,4-galactosyltransferase (GALT) fused at the COOH terminus to three copies of the HA epitope immediately followed by a stop codon. All PCR-generated clones were sequenced in their entirety to ensure that no mutations were introduced during the amplification process.

Results

**PEX400 and PBD401 Cells Are Defective in Peroxisome Membrane Synthesis**

Complementation group 12 of the PBDs was identified previously by cell complementation studies (Poulos et al., 1995). We examined the peroxisomes in two cell lines from CG12, PBD 400, and PBD 401. Immunofluorescent staining demonstrated that these cells failed to compartmentalize the peroxisomal matrix protein marker catalase (Fig. 1, A–C), the common phenotype of cells from Zellweger Syndrome patients (Santos et al., 1988a,b; Chang et al., 1999a). However, these cells also lacked detectable PM-containing peroxisome membranes, shown here by the absence of staining for the membrane marker protein, PM70 (Fig. 1, D–F). We tested whether peroxisomes might be detected using antibodies to other integral PMPs by staining WT, PBD 400, and PBD 401 cells with antibodies specific for PEX13 (Gould et al., 1996; Bjorkman et al., 1998) and PEX11b (Schrader et al., 1998). Although PEX13 and PEX11b are readily detected in peroxisomes of WT cells, they are not visible in PBD 400 or PBD 401 cells (Fig. 1, G–L). This phenotype is unusual for PBD cell lines, as null mutations in most PEX genes affect only matrix protein import, but this has been observed previously in Zellweger Syndrome patient cells mutated for PEX16 (Honsho et al., 1998; South and Gould, 1999) or PEX19 (Matsuzono et al., 1999; Sacksteder et al., 2000).

The lack of punctate staining for PM70, PEX13, and PEX11b in PBD 400 and PBD 401 cells was not accompanied by any detectable staining for these PMPs in the cytoplasm or any other cellular compartment, indicating that the absence of these PMPs may be reduced in PBD 400 and PBD 401 cells. We previously established that levels of PM70 and another PMP, P70R, fall below the limit of detection in PEX16-deficient cells (South and Gould, 1999). Furthermore, the low levels of PM70 reported in PEX19-deficient mammalian cells appear to be caused by rapid proteolysis of this integral PMP (Kinoshita et al., 1998; Matsuzono et al., 1999). Here, we examined the levels of another integral PMP, PEX13, in PBD 400 cells, as well as in human fibroblasts with inactivating mutations in various PEX genes (Fig. 2). PEX13 levels were similar in WT cells and in cells from PBD patients with inactivating mutations in the PEX1 (PBD009; Reuber et al., 1997; Collins and Gould, 1999), PEX12 (PBD097; Chang et al., 1997), PEX6 (PBD 106; Yahrus et al., 1996), PEX10 (PBD 100; Warren et al., 1998), and PEX2 (PBD 094; Shimozawa et al., 1992) genes, consistent with the presence of numerous PMP-containing peroxisomes in these cells (Chang et al., 1999a).

In contrast, PEX13 could not be detected in cells with activating mutations in PEX16 (PBD 061; Honsho et al., 1998; South and Gould, 1999) or in the CG12 cell line, PBD 400.
While many PMPs appear to be degraded in human cells that lack detectable peroxisomes, some PMPs are stable. For example, PEX14 levels are similar in PEX16-deficient human fibroblasts and fibroblasts from normal individuals (South and Gould, 1999). PEX14 levels were also normal in PBD400 and PBD401 cells (data not shown). However, the PEX14 in these cells is not located in peroxisome-like structures, but rather is found in the mitochondria (Fig. 3). A mitochondrial distribution was also observed for over-expressed versions of the integral PMPs, PEX13 and PMP34 (data not shown). A similar mislocalization of PMPs to the mitochondria has been reported in PEX19-deficient cells (Sacksteder et al., 2000) and observed in PEX16-deficient cells (data not shown).

**Peroxisome Synthesis in the Absence of Preexisting Peroxisomes**

To identify the gene defective in PBD400 and PBD401...
cells, we expressed all known human PEX genes in these cells and then assayed the transfected cell populations by immunofluorescence microscopy using antibodies specific for peroxisomal membrane and matrix proteins. A mammalian plasmid designed to express the PEX3 cDNA, pcDNA3-PEX3, was found to restore peroxisome biogenesis in both PBD400 and PBD401 cells (Fig. 5). This is shown here by immunofluorescence experiments in which PBD400 and PBD401 cells were transfected with either pcDNA3 or pcDNA3-PEX3, grown for three days, and then processed for immunofluorescence using antibodies specific for PMP70, as well as for both PEX14 and the matrix protein marker catalase. Not only do PMP70-containing vesicles reappear, but PEX14 is now detected in peroxisomes that also contain catalase. The putative human PEX3 gene previously was identified based on the sequence similarity of its product to that of fungal PEX3 proteins (Kammerer et al., 1998; Soukupova et al., 1999), all of which play an essential role in peroxisome biogenesis (Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996).

**PBD400 and PBD401 Have Inactivating Mutations in PEX3**

Extragenic suppression and extragenic noncomplementation have been observed within the PBDs (Geisbrecht et al., 1998; Chang et al., 1999b). Therefore, it was necessary...
to examine the PEX3 gene structure from PBD400 and PBD401 to determine if they actually had mutations in the PEX3 gene. We first examined the sequence of PEX3 cDNAs from these patients. Total RNA was extracted from PBD400, PBD401, and control fibroblasts, PEX3 cDNA was synthesized by RT-PCR, and the sequence of the PCR products was determined directly. These studies revealed the presence of mutations in the PEX3 cDNA from both patients, but not from the control individual (data not shown). The PEX3 cDNA from PBD400 had a single nucleotide insertion following nt 542 of the ORF, c.542insT, which shifts the reading frame and terminates translation after ten out-of-frame codons. The PEX3 cDNA from PBD401 had a nonsense mutation at codon 53, R53ter. There was no evidence for the WT sequence in PEX3 cDNAs from either PBD400 or PBD401. This indicates that these patients may be homozygous for the c.542insT and R53ter mutations, respectively. However, many mutations can dramatically reduce the steady-state abundance of mRNAs (Maquat, 1995), and we therefore sequenced the mutated regions of the PEX3 gene from these individuals. Total genomic DNA was extracted from control, PBD400, and PBD401 fibroblasts, and the regions surrounding each mutation were amplified by PCR using primers based on the genomic sequence of this gene (GenBank/EMBL/DDJB accession number AL031320). Each PCR product was then sequenced directly. No mutations were detected in the control genomic DNA, and only the mutant alleles were detected in each patient (Fig. 6, A and B). These results suggest that PBD400 and PBD401 are homozygous for their respective mutations, a result that is consistent with their consanguineous origins.

The predicted products of the PEX3 alleles in PBD400 and PBD401 would lack approximately the COOH-terminal 3/7 and 6/7 of the protein, respectively. Such severe frameshift and nonsense mutations are expected to completely eliminate gene activity. To actually test the effects of these mutations on PEX3 activity, each mutation was engineered into the PEX3 expression vector, pcDNA3-PEX3. The resulting plasmids, pcDNA3-PEX3/c.542insT and pcDNA3-PEX3/R53ter, were transfected into PBD400 and PBD401 cells and assayed three days later for their ability to restore peroxisome biogenesis with pcDNA3 and pcDNA3-PEX3 as controls. None of the cells transfected with either of the mutated PEX3 expression vectors displayed any PEX3 activity (Fig. 6, C and D), as well as the vector only control (data not shown). In contrast, the PEX3 expression vector efficiently rescued peroxisome biogenesis in a matched population of patient cells (data not shown). These results demonstrate that the PEX3 mutations in PBD400 and PBD401 abrogate PEX3 gene function.

We next assessed the kinetics of PEX3-mediated rescue of PBD400 cells. PEX3-deficient PBD400 cells were transfected with pcDNA3-PEX3 and processed for indirect immunofluorescence at various times. No evidence of phenotypic rescue could be observed at 4, 8, or 12 h after introducing these plasmids. The earliest time point at which we could detect PM70-containing vesicles in any cells of the transfected population was at 24-28 h after transfection, and this was only in a limited number of the transfected cells (data not shown). The proportion of transfected cells containing recognizable peroxisomes was greater by 48 h after transfection, but was not maximal until 72 h after transfection. As reported previously for PEX16- and PEX19-mediated synthesis of peroxisomes in mammalian cells (Matsuzono et al., 1999; South and Gould,

Figure 4. PBD400 cells mislocalize PEX13myc and ALDPmyc to mitochondria. Vectors designed to express PEX13myc (A–C) or ALDPmyc (D–F) were transfected into normal human fibroblasts (A and D) or PBD400 cells (B, C, E, and F), and visualized with antibodies to the myc epitope. PEX13myc and ALDPmyc showed a peroxisomal distribution in normal cells (A and D). In contrast, PBD400 cells mislocalized PEX13myc (B) and ALDPmyc (E) to mitochondria, shown here by their colocalization with the mitochondrial marker MitoTracker (C and F). Bar, 10 μm.
1999), the reconstitution of peroxisome biogenesis in peroxisome-deficient cells proceeds in a stepwise fashion, with PMP-containing vesicles detected first, followed by the import of peroxisomal matrix proteins. We also observed evidence for this during PEX3-mediated rescue of peroxisome biogenesis in PBD400 cells (data not shown).

**PEX3 Encodes a Peroxisomal Protein**

Previous studies have examined the subcellular distribution of yeast PEX3 and found that it is an integral PMP (Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996). A similar subcellular distribution has been suggested for the human protein, based on experiments with tagged versions of PEX3 (Kammerer et al., 1998; Soukupova et al., 1999). We used subcellular fractionation experiments to test whether endogenously expressed mammalian PEX3 displayed a similar subcellular distribution. A postnuclear supernatant was generated from HepG2 cells and separated by Nycodenz gradient centrifugation. Equal amounts of each fraction were then assayed for peroxisomal, mitochondrial, and microsomal markers, as well as for PEX3 by immunoblot (Fig. 7). PEX3 was detected exclusively in the peroxisomal fractions, confirming the previous localization of this protein to peroxisomes.

**PEX3 Transport to Peroxisomes Is Rapid and Independent of COPI and COPII**

The critical role of PEX3 in human peroxisome membrane
biogenesis mirrors its function in yeast, where it is also essential for peroxisome membrane biogenesis (Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 1996). Studies in the yeast Hansenula polymorpha revealed that certain PEX3 fusion proteins accumulate in the ER (Baerends et al., 1996), and studies in mammalian cells have reported that PEX3 overexpression leads to dysmorphogenesis of the ER (Kammerer et al., 1998). These data have led to speculation that PEX3 may transit through the ER before import into peroxisomes (Kunau and Erdmann, 1998). Therefore, we examined the distribution of human PEX3 at various times after its synthesis. A plasmid designed to express a COOH-terminally myc-tagged form of PEX3, pcDNA3-PEX3myc, was generated and tested for PEX3 activity. The pcDNA3-PEX3myc plasmid rescued peroxisome biogenesis in PBD400 cells as efficiently as the WT expression vector, demonstrating that the addition of the myc tag had no effect on PEX3 function (data not shown). We then microinjected the pcDNA3-PEX3myc expression vector into normal human fibroblasts and followed its expression and subcellular distribution. At 15 or 30 min after DNA microinjection, we were unable to see expression of PEX3myc in either the cytosol, the ER, or peroxisomes. However, by 1 h after injection, PEX3myc staining was readily detected in the peroxisomes of microinjected cells (Fig. 8, A and B). Furthermore, PEX3myc was detected only in peroxisomes at this and all subsequent time points, regardless of whether a cell was expressing low, moderate, or high levels of PEX3myc. We also stained cells expressing PEX3myc at the 1 h time point with antibodies to the ER resident protein, BiP. However, in no cell did we detect any colocalization of PEX3myc and BiP (data not shown).

A additional evidence that PEX3 may transit through the ER en route to the peroxisome comes from a study in the yeast H. polymorpha in which brefeldin A (BFA), an inhibitor of COP1 coat formation, caused the accumulation of PEX3 in the ER and the inhibition of peroxisome biogenesis in general (Solomons et al., 1997). Therefore, we tested whether BFA affected PEX3 import into peroxisomes by incubating normal human fibroblasts in medium containing either a mock control solution (10 μM/ml methanol) or 10 μg/ml BFA for 30 min and then microinjecting the PEX3myc expression vector. Mock solution or BFA was maintained throughout the entire experiment. Controls confirmed that BFA had the appropriate effect on FITC-C5-ceramide distribution in mock and BFA-treated cells (Fig. 8, C and D). Furthermore, PEX3myc was detected in PMP70-containing peroxisomes 1 h after microinjection in similar numbers of cells in both mock- and BFA-treated cell populations (Fig. 8, E–H).

The fact that PEX3 import was refractory to BFA indicated that any role for the secretory pathway in the transport of PEX3 to peroxisomes would manifest at a step before COPI-mediated vesicular transport. COPII mediates the exit of membrane vesicles from the ER and is regulated by SAR1, a small ER-associated GTPase (Barlowe et al., 1993, 1994; Kuge et al., 1994; Aridor et al., 1995). Dominant negative mutants of SAR1 have been useful in the analysis of COPII-mediated processes, particularly in mammalian cells (Kuge et al., 1994). To determine whether COPII-mediated processes are important for the
trafficking of PEX3 to peroxisomes, we first cloned and sequenced the human SAR1 gene. Human SAR1 is virtually identical to hamster SAR1, and is highly similar to yeast SAR1 (Fig. 9). Previous studies have established that substitution of asparagine for threonine at position 39 of human SAR1 should prevent guanine nucleotide exchange, trapping SAR1 in the GDP-bound state, and preventing COPII-mediated membrane budding from the ER (Kuge et al., 1999). Previous studies in yeast have suggested that PEX3 plays an essential role in the biogenesis of peroxisome membranes (Höhfeld et al., 1991; Baerends et al., 1996; Wiemer et al., 2000). However, the function of particular peroxins can vary widely between yeast and humans, particularly those that participate in membrane biogenesis (Eitzen et al., 1997; South and Gould, 1999; Hettema et al., 2000). However, the function of particular peroxins can vary widely between yeast and humans, particularly those that participate in membrane biogenesis (Eitzen et al., 1997; South and Gould, 1999; Tabak et al., 1999). By demonstrating that two Zellweger...
syndrome patients who lack detectable peroxisomes have inactivating mutations in PEX3, we establish that human PEX3 also plays an essential role in peroxisome membrane biogenesis. Furthermore, we find that reexpression of PEX3 in PEX3-deficient cells leads to the reformation of peroxisomes, demonstrating that peroxisomes can be synthesized in the absence of preexisting peroxisomes. These results mirror results obtained from studies of PEX16- and PEX19-deficient human cells (Honsho et al., 1998; Matsuzono et al., 1999; South and Gould, 1999), as well as from the analysis of yeast pex3 and pex19 mutants (Hettema et al., 2000).

The most widely accepted model for peroxisome biogenesis involves growth and division, growth by uptake of proteins from the cytoplasm, followed by division of preexisting peroxisomes. The biogenic route for peroxisomal matrix proteins clearly follows this model (Lazarow and Fujiki, 1985), and studies of two mammalian PMPs, PMP22 (Fujiki et al., 1984) and PMP70 (Imanaka et al., 1996), suggest that integral PMPs are also imported directly from the cytoplasm. If this model is correct, we expect that integral PMPs in PEX3-deficient cells would be degraded or mistargeted to other cellular membranes, given that integral membrane proteins are generally not soluble. Alternatively, it has been hypothesized that some, and perhaps many, integral PMPs shuttle through the ER en route to the peroxisome (Kunau, 1998; Titorenko and Rachubinski, 1998a; Mullen et al., 1999). If this latter
model is correct, we should detect an accumulation of integral PMPs in the ER. Our analysis of PEX3-deficient cells revealed that the levels of several integral PMPs were reduced in abundance, whereas others were mislocalized to the mitochondria. These results do not rule out the possibility that some small number of integral PMPs reach the peroxisome via the ER. However, they do support the hypothesis that many PMPs are imported directly from the cytoplasm.

The synthesis of peroxisomes in the absence of preexisting peroxisomes raises a number of interesting issues, the most important being the source of membrane for the nascent peroxisomes. Prior studies have suggested that PEX3 may transit through the ER en route to the peroxisome (Baerends et al., 1996), and may mediate the formation of peroxisome membranes from the ER (Baerends et al., 1996; Solomons et al., 1997; Kammerer et al., 1998; Kunau, 1998; Titorenko and Rachubinski, 1998a). Some of the observations that have fueled this hypothesis include the targeting of PEX3 fusion proteins to the ER (Baerends et al., 1996), the accumulation of PEX3 in the ER of cells treated with the COPI inhibitor, BFA (Solomons et al., 1997), the inhibition of peroxisome biogenesis and PMP import by BFA (Solomons et al., 1997; Mullen et al., 1999), aberrant ER morphology in cells overexpressing PEX3 (Kammerer et al., 1998), and the detection of other PMPs in the ER (Elgersma et al., 1997; Titorenko and Rachubinski, 1998b).

Figure 9. Alignment of human, CHO, and yeast homologues of SAR1. Identical residues are shaded, and the asterisk identifies threonine 39. The sequence of HsSAR1 is available from GenBank/EMBL/DDBJ under accession number AF261717.

Figure 10. PEX3 targeting to peroxisomes is independent of COPII-mediated ER export. Normal human fibroblasts were cotransfected with either pcDNA3-GALT3xHA and pcDNA3 (A) or pcDNA3-GALT3xHA and pcDNA3-SAR1/T39N/vsvg (B and C) and processed 1 d later for indirect immunofluorescence with antibodies to the HA epitope (A and B) and the vsvg epitope (C). Note the accumulation of GALT in the ER in cells expressing the SAR1 mutant. Normal human fibroblasts were also transfected with pcDNA3-SAR1/T39N/vsvg, incubated for 1 d, and then microinjected with pcDNA3-PLEX3myc (D and E). 1 h later, the cells were processed for double indirect immunofluorescence with antibodies to the myc epitope (D) and the vsvg epitope (E). Note that PEX3 is still targeted to peroxisomes, even in cells expressing high level of the SAR1 mutant. Bar, 10 μm.
If PEX3 does transit through the ER en route to the peroxisomes, then we should detect PEX3 first in the ER and only later in the peroxisome. We employed a DNA microinjection system to assess the kinetics of PEX3 import in normal human fibroblasts. A fully functional myc-tagged version of PEX3, PEX3myc, was detected in peroxisomes just one hour after DNA microinjection. It was never observed in any other subcellular structures before that time, or at any subsequent time, in any of hundreds of injected cells that were examined. The technical limitations of these experiments cannot rule out the possibility that PEX3 might move extremely rapidly through the ER, precluding our ability to detect any ER-associated PEX3. However, it has been reported that BFA causes an accumulation of PEX3 in the ER, and inhibits peroxisome biogenesis in general (Solomons et al., 1997). These earlier reports predict that BFA-treated cells should accumulate newly synthesized PEX3 in the ER and be resistant to PEX3-mediated peroxisome biogenesis. In contrast, concentrations of BFA that fully inhibited COPI failed to inhibit the targeting of PEX3 to peroxisomes in normal fibroblasts, did not lead to an accumulation of PEX3 in the ER, and had no effect on PEX3-mediated peroxisome membrane synthesis in PEX3-deficient cells.

The hypothesis that peroxisome membranes arise directly from the ER does not necessarily mean that the process is dependent upon COPI. Vesicle budding from the ER occurs in a COPII-dependent process (Kuehn and Schekman, 1997), and it was formally possible that PEX3 targeting and/or PEX3-mediated peroxisome membrane synthesis involved this early-acting coat protein complex. In fact, one model for an ER role in peroxisome biogenesis invokes COPII as an essential component (Titorenko and Rachubinski, 1998a). COPII-dependent vesicle formation requires SARS1, a small ER-associated GTPase (Barlowe et al., 1993; Kuge et al., 1994). Dominant negative SARS1 mutants that are defective in guanine nucleotide exchange and lock the protein in the GDP-bound state prevent vesicle budding from the ER (Kuge et al., 1994). We cloned the human SARS1 gene and found that overexpression of this SARS1 mutant had no detectable effect on PEX3 targeting to peroxisomes in normal human fibroblasts, and failed to prevent PEX3-mediated peroxisome membrane synthesis.

Not all vesicle budding processes in the early secretory organelles depend upon COPII or COPI. Homotypic membrane budding and fusion may occur independently of coat protein complexes (Latterich et al., 1995), and it is formally possible that peroxisomes may arise by budding of homotypic vesicles from the ER. This hypothesis is sup-
ported in part by the detection of COPI components on peroxisomal membranes (Passreiter et al., 1998). However, homotypic membranes are competent to fuse, as evident from the rapid fusion of Golgi membranes with the ER when COPI coats are removed by treatment with BFA (Klausner et al., 1992). Therefore, if peroxisomes form by homotypic budding from the ER, we would expect significant mixing of peroxisomal and ER proteins, particularly after BFA treatment, yet this does not seem to be the case. Numerous studies have established that peroxisomal proteins are distinct from those found in the ER and are not subject to ER-specific modifications, such as N-linked glycosylation (Lazarow and Fujiki, 1985). Furthermore, BFA had no effect on the structural integrity of peroxisomes in human cells, even after prolonged exposure to the drug, and had no effect on PEX3 targeting or PEX3-mediated peroxisome membrane synthesis.

The results presented here fail to support the hypothesis that peroxisomes arise from the ER, and in fact argue against homotypic-, COPII-, and COPI-dependent mechanisms of ER-to-peroxisome transport of membranes. The two-pathway model of peroxisome biogenesis proposed previously (South and Gould, 1999) can, however, provide a framework for directing future studies of PEX3 function. This model suggests that peroxisomes typically arise by the growth and division of preexisting peroxisomes, with direct uptake of both matrix and membrane proteins from the cytoplasm (Lazarow and Fujiki, 1985). This model also suggests that peroxisomes may form by an independent pathway from some preperoxisomal vesicle, presumably another endomembrane of the cell. This process is thought to involve the formation or conversion of a membranous structure into a PMP-importing vesicle. This allows the import of PEX11 proteins, which mediate peroxisome division (Passreiter et al., 1998; Schrader et al., 1998), as well as the formation of a matrix protein import apparatus, matrix protein import, and metabolic activity. This second pathway requires the integral PMPs, PEX3 and PEX16 (South and Gould, 1999), as well as a predominantly cytoplasmic, partly peroxisomal PMP-binding protein, PEX19 (Matsuzono et al., 1999; Sacksteder et al., 2000). PEX19 appears to participate primarily in PMP import, either as a PMP-targeting signal receptor or as a chaperone for newly synthesized PMPs (Sacksteder et al., 2000). Within the context of this model, the simplest hypothesis is that PEX3 and PEX16 normally participate in PMP import, but can, at some low rate, also mediate the formation of nascent peroxisomes. Alternatively, one or both of these peroxins

Figure 12. PEX3-mediated peroxisome synthesis is independent of COPII. PBD400 cells were cotransfected with either pcDNA3-GALT3xHA and pcDNA3 (A) or pcDNA3-GALT3xHA and pcDNA3-3-SAR1/T39N/vsvg (B and C), and were then processed 2 d later for indirect immunofluorescence with antibodies to the HA epitope (A and B) and the vsvg epitope (C). PBD400 cells were also cotransfected with pcDNA3-3-SAR1/T39N/vsvg and pcDNA3-PEX3 (D and E) and processed 2 d later for double indirect immunofluorescence with antibodies to PMP70 (D) and the vsvg epitope (E). Note that in PBD400 cells expressing the SAR1 mutant, GALT accumulates in the ER, yet PEX3 is still able to rescue peroxisome synthesis. Bar, 10 μm.
could participate in peroxisome membrane synthesis under all conditions.

In addition to the implications for peroxisome biogenesis, our results resolve the molecular basis of disease in complementation group 12 of the PBDs. PBD 400 and PBD 401 both display the severe phenotypes of Zellweger syndrome, as expected for patients who are unable to compartmentalize even a small amount of peroxisomal matrix enzymes. This study brings the number of known PBD genes to 11, and brings the number of genes that can cause the Zellweger spectrum diseases: Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease, to ten. The genetic basis of disease has yet to be resolved in only one of the known groups of PBD patients, CG8, though the rapid rate of PBD gene identification makes it likely that it too will soon be known.

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