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Pex3p-Dependent Peroxisomal Biogenesis Initiates in the Endoplasmic Reticulum of Human Fibroblasts

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ABSTRACT

The mechanisms of peroxisomal biogenesis remain incompletely understood, specially regarding the role of the endoplasmic reticulum (ER) in human cells, where genetic disorders of peroxisome biogenesis lead to Zellweger syndrome (ZS). The Pex3p peroxisomal membrane protein (PMP) required for early steps of peroxisome biogenesis has been detected in the ER in yeast but not in mammalian cells. Here, we show that Pex3p-GFP expressed in a new ZS cell line (MR), which lacks peroxisomes due to a mutation in the PEX3 gene, localizes first in the ER and subsequently in newly formed peroxisomes. Pex3p bearing an artificial *N*-glycosylation site shows an electrophoretic shift indicative of ER targeting while en route to preformed peroxisomes in normal fibroblast. A signal peptide that forces its entry into the ER does not eliminate its capability to drive peroxisome biogenesis in ZS cells. Thus, Pex3p is able to drive peroxisome biogenesis from the ER and its ER pathway is not privative of ZS cells. Cross-expression experiments of Pex3p in GM623 cells lacking Pex16p or Pex16p in MR cells lacking Pex3p, showed evidence that Pex3p requires Pex16p for ER location but is dispensable for the ER location of Pex16p. These results indicate that Pex3p follows the ER-to-peroxisomal route in mammalian cells and provides new clues to understand its function. J. Cell. Biochem. 107: 1083–1096, 2009.

KEY WORDS: PEROXISOME; BIOGENESIS; ENDOPLASMIC RETICULUM

Our understanding of peroxisome biogenesis has been evolving dramatically during recent years. The previously controversial notion of an intermediary step in the endoplasmic reticulum (ER) is now relatively well accepted [Schrader and Fahimi, 2008], but still certain aspects need to be defined in mammalian cells. Most of the basic elements involved in peroxisome biogenesis mechanisms have been identified and their function elucidate mainly as the result of studies on a group of human genetic disorders derived from defects in peroxisome biogenesis, including the prototypic Zellweger syndrome (ZS) [Brosius and Gartner, 2002]. Peroxisomes, like mitochondria and chloroplasts, have been considered for a long-time autonomous organelles, which multiply exclusively by growth and division of their pre-existing parental organelles [Lazarow and Fujiki, 1985]. This early paradigm is supported by evidence showing that both peroxisomal matrix and PMPs are synthesized on free ribosomes and imported posttranslationally into preexisting peroxisomes [Heiland and Erdmann, 2005]. Kinetic studies in vivo also support this model [Lazarow and Fujiki,

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1985]. However, later studies revealed a more complex scenario, demonstrating a de novo pathway for peroxisome generation involving insertion of at least certain PMPs in the ER [Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005]. Since then, the role of ER as platform for an initial peroxisomal biosynthetic event has been under intense scrutiny. Accumulated evidence from yeast, plants and mammalian cells strongly supports the ER hypothesis [Titorenko and Mullen, 2006; Tabak et al., 2008]. In mammalian cells, however, the kind of peroxisomal proteins that are incorporated first into the ER while en route to peroxisomes remains only partially defined.

Although former observations in liver biopsies suggested that patients with ZS lack peroxisomes [Goldfischer et al., 1973], later studies in Zellweger fibroblasts discovered membranes containing peroxisomal membrane proteins (PMPs), but lacking most of the matrix proteins, thus called "peroxisomal membrane ghosts" [Santos et al., 1988a,b, 2000]. This finding suggested that ZS entails defects in the peroxisomal import machinery for matrix proteins [Santos et al., 1988b]. Up to now, at least 14 different complementation groups (CG) have been described among ZS patients, most of them displaying peroxisomal ghosts [Brosius and Gartner, 2002]. These studies together with genetic studies in yeast have identified at least 32 proteins, called peroxins, and their corresponding PEX genes, as required for peroxisome biogenesis [Heiland and Erdmann, 2005; Platta and Erdmann, 2007]. Remarkably, only cells that carry a mutation in any of three PEX genes, namely PEX3 (CG12), PEX16 (GC9), and PEX19 (CG 14), lack peroxisomes, peroxisome ghosts and any peroxisomal membrane [Shimozawa et al., 2004]. Each of these three genes, which respectively encode the peroxines Pex3p, Pex16p, and Pex19p, has the ability to restore the biogenesis of peroxisomes when reintroduced to mutant cells lacking peroxisomes [South and Gould, 1999; South et al., 2000; Honsho et al., 2002; Heiland and Erdmann, 2005; Kragt et al., 2005; Tam et al., 2005]. This seminal observation suggests the capability for de novo synthesis of peroxisomes, and posit the challenge of elucidating the mechanism of action of Pex3p, Pex16p, and Pex19p.

We know now that Pex3p, Pex16p, and Pex19p act in early stages of peroxisome biogenesis [Heiland and Erdmann, 2005], more precisely in peroxisomal membrane formation and initial protein import. Pex19p is found distributed in both cytosol and peroxisomes, reflecting its mechanism of function by binding nascent PMPs in the cytosol and targeting them into the peroxisomal membrane [Heiland and Erdmann, 2005]. Thus, import of most PMPs, including Pex16 [Sacksteder et al., 2000; Jones et al., 2001; Jones et al., 2004], depends on Pex19p [Jones et al., 2004]. Pex16p is an integral membrane protein crucial for peroxisomal biogenesis at least in mammalian cells. The lack of Pex16p expression completely abrogates peroxisome biogenesis and determines development of a ZS disease [Honsho et al., 1998; South and Gould, 1999]. The role of Pex16p is not completely clear. Some evidence suggests that it might act as a membrane translocator component upstream of Pex3p [Honsho et al., 2002; Kim et al., 2006]. However, its role is dispensable at least in certain yeasts. Pex3p is the only PMP known to be imported independently of Pex19p and, therefore, currently defines a mechanistically distinct PMP import pathway [Jones et al., 2004]. Pex3p is a peroxisomal integral membrane protein that functions as a docking factor for Pex19p, recruiting complexes of Pex19p and newly synthesized PMPs [Heiland and Erdmann, 2005]. Its role is then essential for Pex19p-dependent import of PMPs [Fang et al., 2004]. Elucidating the biogenetic pathways and functional mechanisms of Pex3p constitute a crucial requirement to understand the peroxisome biogenesis problem.

The role of ER as a platform for the peroxisomal biosynthetic machinery is mainly sustained by the detection of crucial peroxisomal proteins in the ER and the demonstration that they can drive peroxisome biogenesis from this location [Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005; Mullen and Trelease, 2006]. In plants, Pex16p has been consistently reported in the ER, as well as in peroxisomes [Mullen and Trelease, 2006]. In Arabidopsis thaliana, Pex16p has been detected as more concentrated in discrete regions of the ER, suggesting that specific domains of the ER might be involved in peroxisomal biogenesis [Mullen and Trelease, 2006]. In certain yeast strains, *N*-glycosylation has been detected on Pex16p and Pex2p, indirectly indicating trafficking through the ER [Titorenko and Rachubinski, 1998a]. In mammalian cells, the most clear evidence involving the ER in peroxisome biogenesis has been obtained by live imaging studies in COS cells, which showed Pex16p targeting first the ER and then budding off in vesicles that evolve into peroxisomes [Kim et al., 2006]. Also, PEX13 and PMP70 have been reported in discrete regions of the ER in mouse dendritic cells [Geuze et al., 2003]. Regarding Pex3p, complementation experiments in yeast lacking Pex3p, and thus lacking peroxisomes, demonstrated that certain structures growing out from the ER, and containing Pex3p-GFP, constitute peroxisomal precursors [Hoepfner et al., 2005; Tam et al., 2005]. Also in yeast, experiments with a recombinant Pex3p bearing an attached signal peptide and, therefore, forced to enter the ER, restores peroxisome biogenesis and becomes integrated into peroxisomes [Kragt et al., 2005], demonstrating that Pex3p is capable of promoting peroxisomal biogenesis from the ER. Observations in yeast might not be entirely extensible to mammalian cells. For instance, Pex16p seems to be dispensable in certain yeasts, such as *Saccharomyces cerevisiae* [Eitzen et al., 1997], but is absolutely required for peroxisome biogenesis in mammalian cells. Despite the evidence in yeast, the experiments in plants have been incongruent with the possibility that Pex3p uses the ER-to-peroxisome pathway [Mullen and Trelease, 2006] and previous studies in mammalian cells failed to detect Pex3p in the ER [South et al., 2000; Voorn-Brouwer et al., 2001], even in cells lacking peroxisomes [Fang et al., 2004], unless Pex16p is overexpressed [Kim et al., 2006]. These incongruent observations prompted us to re-evaluate the sorting behavior of Pex3p in mammalian cells.

We took advantage of a new fibroblast cell line obtained from a Chilean patient with ZS in which we found a nonsense mutation in the PEX3 gene and a complete lack of peroxisome remnants. We first found that these Pex3p-lacking cells as well as the well known ZS fibroblast cell line GM6231 lacking Pex16p [South and Gould, 1999] both have detectable levels of endogenous PMP70 in the ER, not previously reported in PBD. Thus, this feature represents a new cellular phenotype in ZS. Our ZS fibroblast cell line not only restored peroxisomal biogenesis upon exogenous expression of Pex3p (GFP

tagged), but also showed that Pex3p-GFP reached first the ER and afterwards the newly formed peroxisomes. Furthermore, Pex3p bearing a signal peptide to force its entry into the ER also led to peroxisomal appearance, as previously shown in yeast [Kragt et al., 2005]. We also provide evidence indicating that Pex3p requires Pex16p for its ER location. These results provide definitive evidence that Pex3p has an ER-mediated pathway to peroxisomes and can promote peroxisomal biogenesis from the ER in mammalian cells.

MATERIALS AND METHODS

CLINICAL CASE

MR was the first child of a young consanguineous couple without family disease history. In the neonatal period he was hypotonic and dysmorphic (flat face, flattened supraciliary arches, wide sutures, broad forehead). He also had hepatomegaly, congenital cardiopathy (small auricular communication and persistent Ductus), hypospadias, central neurological damage, and diffuse hepatic damage, compatible with atresia of the biliary tract. Aminoacidemia, aminoaciduria, lactic acid and ammonium were normal; karyotype normal: 46,XY; electromyography: normal. The nuclear magnetic resonance and brain scanner showed non-specific alterations. The diagnosis of ZS was based on the following findings: (1) Increase of the very long chain fatty acid in fibroblasts: C22 g/mg protein: 1.259 (control: 0.9 ± 0.4); C26:0 g/mg protein: 0.706 (control: 0.07 ± 0.04); C26:1 g/mg protein: 0.622 (control: 0.09 ± 0.07); C26/C22: 0.585 (control: 0.08 ± 0.03). (2) Severe deficiency in the synthesis of plasmalogens in culture fibroblasts: $^{3}H/^{14}C$: 19.54 (control: 0.67 ± 0.19); $^{14}C/^{3}H$: 0.05 (1.65 ± 0.66). (3) Marked deficiency in the oxidation of phytanic acid: 0.730 pmol/h/mg protein (control: 31.6 ± 6.9 pmol/h/mg protein). (4) Matrix proteins mislocalized to the cell cytosol. The patient died of respiratory insufficiency at the age of 3 months.

CELL CULTURE

Skin fibroblasts were from control subjects, from our Chilean patient (MR) and from two other ZS patients previously characterized, GM4340 and GM6231, belonging to complementation groups 4 and 9, respectively, obtained from the Mutant Cell Repository (New Jersey). These cells were cultured in DMEM supplemented with antibiotics and 10% fetal calf serum in 5% CO_2 in air at 37°C.

TRANSFORMATION OF CONTROL AND ZS FIBROBLASTS

Transformation was carried out with a recombinant SV40 adenovirus as described by Van Doren and Gluzman [1984].

FRACTIONATION OF FIBROBLASTS

Cells grown in 10 cm culture dish were washed with phosphate-buffer saline (PBS), trypsinized, homogenized and fractionated to obtain a nuclear pellet and a post-nuclear (PNS), according to Santos et al. [1988a].

ENZYME ASSAYS

Established procedures were employed for the determination of catalase [Leighton et al., 1968], cytochrome *c* oxidase [Cooperstein and Lazarow, 1951]; *N*-acetylglucosaminidase [Sellinger et al., 1960]. Proteins were measured by the Bio-Rad protein assay method (Bio-Rad Laboratories).

PLASMIDS

pPEX3-GFP, pcmyc-PEX3 and pcmyc-GLY-PEX3: These plasmids were created by PCR using specific primers (Table I) and the

TABLE I. Primer Sequences Used in this Study

Plasmid/gene	Primers
pPEX3-GFP	Forward: 5′-TCAGCTAGCATGCTGAGGTCTGTA-3′ Reverse: 5′-GCAGGTACCTCTTTCTCCAGTTGCTG-3′
cDNA hPEX16	Forward: 5′-ATGGAGAAGCTGCGGCTCCTG-3′ Reverse: 5′-TCAGCCCCAACTGTAGAAGTA-3′
pPEX16-GFP	Forward: 5′-ATAAAGCTTCATGGAGAAGCTGCGG-3′ Reverse: 5′-GAGGAATTCGCCCCAACTGTAGAA-3′
Calreticulin	Forward: 5′-TATTGCTAGCATGCTGCTATCCGTGCCGCTGCTG-3′ Reverse: 3′-GGTCCGGTTCCTGCTCGACATCCTTAAGATTAT-5′
SP-pex3	Forward: 5′-TATTGCTAGCATGCTGCTATCCGTGCCGCTGCTG-3′ Reverse: 5′-ATTCCATACAGACCTCAGGGCGGGCTCGGCGACGGC-3′
sp-PEX3	Forward: 5′-GCCGTCGCCGAGCCCGCCCTGAGGTCTGTATGGAAT-3′ Reverse: 5′-GCAGGTACCTCTTTCTCCAGTTGCTG-3′
pSP-PEX3-GFP	Amplicon SP-pex3 Amplicon sp-PEX3 Forward: 5′-TATTGCTAGCATGCTGCTATCCGTGCCGCTGCTG-3′ Reverse: 5′-GCAGGTACCTCTTTCTCCAGTTGCTG-3′
SP-pex16	Forward: 5′-TATTGCTAGCATGCTGCTATCCGTGCCGCTGCTG-3′ Reverse: 5′-CAGGAGCCGCAGCTTCTCGGCGGGCTCGGCGACGGC-3′
sp-PEX16	Forward: 5′-GCCGTCGCCGAGCCCGCCGAGAAGCTGCGGCTCCTG-3′ Reverse: 5′-GAGGAATTCGCCCCAACTGTAGAA-3′
pSP-PEX16-GFP	Amplicon SP-pex16 Amplicon sp-PEX16 Forward: 5′-TATTGCTAGCATGCTGCTATCCGTGCCGCTGCTG-3′ Reverse: 5′-GAGGAATTCGCCCCAACTGTAGAA-3′
pcmyc-PEX3	Forward: 5′-TATACCATGGAGATGCTGAGGTCTGTATGGAATTTTC-3′ Reverse: 5′-TATAGCGGCCGCTCATTTCTCCAGTTGCTGAGGG-3′
pcmyc-GLY-PEX3	Forward: 5′-TATACCATGGAGATGAACAGGTCTGTATGGAATTTTC-3′ Reverse: 5′-TATAGCGGCCGCTCATTTCTCCAGTTGCTGAGGG-3′

pcDNA3-PEX3 plasmid. For pcmyc-GLY-PEX3, a specific change in the 2L to N was produced, in order to generate an *N*-glycosylation site NRS. The amplification product PEX3-GFP was digested with *Nhe* I and *Kpn* I enzymes, and ligated to the pEGFP-N1 plasmid (Clontech), and PEX3 and GLY-PEX3 were digested with *Nco* I and *Not* I enzymes, and ligated into the vector pCS2 + MT. This vector has the myc sequence immediately after the promoter, and allow fuse it to the 5′ of our genes.

pPEX16-GFP: The human PEX16 gene was obtained by RT-PCR using mRNA from human control fibroblasts and specific primers (Table I). For the generation of pPEX16-GFP, the cDNA of human PEX16 was used as template in a new PCR, using specific primers (Table I). The amplification product was digested with *Hin*d III and *Eco*R I enzymes, and ligated to the pEGFP-N2 plasmid (Clontech).

pSP-PEX3-GFP: This plasmid was created using the overlapping PCR technique to fuse the signal peptide (SP) of the human protein calreticulin, to the entire sequence of human Pex3 gene. The calreticulin gene was obtained from human fibroblast by RT-PCR, using specific primers (Table I). The SP of the calreticulin gene was obtained by PCR, and a tail corresponding of the first six codons (without the ATG) of the human PEX3 gene was added to it. The primers used to obtain this amplicon (SP-pex3) are indicated in Table I. On the other hand, the last six codons of the SP of calreticulin gene was added to the 5′- of the PEX3 gene by PCR. The primers used to obtain this new amplicon (sp-PEX3) are indicated in Table I. Next, in order to obtain the complete construct *Nhe*I-PS-PEX3-*Kpn*-I, an overlapping PCR was performed, using the primers and sequences shown in Table I. Finally, this construct was digested with *Nhe* I and *Kpn* I enzymes, and ligated to the pEGFP-N1 plasmid (Clontech).

pSP-PEX16-GFP: the same protocol used to generate pSP-PEX3-GFP, was employed to produce this construction. The sets of primers used to generate the different amplicons are listed in Table I. Finally, the gene *Nhe*I-PS-PEX16-*Eco*R-I was digested with *Nhe* I and *Kpn* I enzymes, and ligated to the pEGFP-N1 plasmid (Clontech).

INDIRECT IMMUNOFLUORESCENCE, ANTIBODIES, TRANSFECTION, AND MITOCHONDRION-SELECTIVE DYE

Cultured control and ZS fibroblasts were subjected to immunofluorescence according to Santos et al. [1988b]. The following primary antibodies were used: monospecific affinity-puriffied antibodies against PMP70 and PMP22 [Santos et al., 1988b]; rabbit polyclonal antibody against GFP, human autoantibody against the Golgi protein p230 [Mardones and Gonzalez, 2003]; mouse monoclonal antibody against the ER protein disulfide isomerase (PDI) (StressGen Biotechnologies Corp., Canada); rabbit antisera against catalase or human PMPs [Santos et al., 1988b]; polyclonal antibody against Thiolase. Secondary antibodies used were: FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis), Texas Red-conjugated goat anti-mouse IgG (KPL Laboratories), Rhodamine-conjugated donkey anti-human IgG (Sigma Chemical Co.).

Cells growing in coverslips were transfected with Lipofectamin 2000 (Invitrogen), according to manufacturer's instructions. After 48 h, the cells were fixed with 2% parafolmaldehyde and mounted. MitoTracker (Molecular Probes, Oregon), a mitochondrion-selective dye, were used following manufacturer's instructions.

MICROINJECTIONS

The plasmid pPEX3-GFP (25 μg/ml) alone or with Texas Red-conjugated antibodies (40 μg/ml), were microinjected into the fibroblasts nucleus using a workstation for microinjection into adherent cells (InjectMan NI 2 with Transjector 5246 (Eppendorf, USA), coupled to a Zeiss microscope Axiovert S100 (Carl Zeiss, Germany) as described [Cancino et al., 2007]. After different time periods, the cells were processed for immunofluorescence with FICT-conjugated antibodies against GFP to enhance the signal. Digital images of cells with Texas Red and FITC signal, were then analyzed with MetaMorph 6.1 imaging software (Universal Imagining, West Chester, PA) as described in Soza et al. [2004].

WESTERN BLOTTING

Membranes were isolated by the alkaline carbonate method [Fujiki et al., 1982], and the proteins were separated by SDS–PAGE (0.1% SDS and 11% polyacrilamide), transferred to nitocellulose membranes, incubated with polyclonal antibodies and then with secondary antibody conjugated to horseradish peroxidase (HRP) (Calbiochem-Novabiochem Intl., La Jolla, CA) and the chemiluminescene reagent plus kit (NEN™ Life Science Products Inc., USA). Membranes were exposed to Hyperfilm™ ECL™ chemiluminescence film (Amershan Life Science) and the film was developed in a Kodak X-OMAT 1000 processor.

Transfected fibroblasts with pcmyc-PEX3 and pcmyc-GLY-PEX3 were cultured for 48 h, and then trypsinized and lyzed with 80 μl of lysis buffer (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% Glycerol, 2 mM EDTA, and 10 mg/ml PMSF). Total proteins were separated in SDS–PAGE (0.1% SDS and 15% polyacrilamide), transferred to nitocellulose membranes, incubated with monoclonal antibodies against cmyc (sc-40, Santa Cruz, USA), and processed as mentioned above.

ELECTRON MICROSCOPY, CATALASE CYTOCHEMISTRY, AND IMMUNOCYTOCHEMISTRY

Confluent cultures of fibroblasts were fixed in situ with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1 h at room temperature. The fixed cells were removed with a rubber scraper, washed with 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon for conventional EM. Ultrathin sections were further stained with uranyl acetate and lead citrate and examined in a Phillips electron microscope. Cytochemistry for catalase was carried out using the alkaline 3,3-diamonibenzidine (Sigma) procedure as described [Santos et al., 1994].

For immunocytochemical localization of PMPs, fibroblasts were fixed for 1 h at 4°C in 2% paraformaldehyde, 0.01 M periodate, 0.075 M lysine and 0.037 M phosphate buffer pH7.4. Cell sections were incubated in a solution of anti-PMP antibody diluted in TCT (0.25% Triton X-100, 0.7% carrageine and 40 mM Tris phosphate buffer pH 7.6) for 18 h at 4°C. After washings, cells were incubated in anti-mouse IgG (Sigma), for 1 h, and then were incubated for 1 h in the complex peroxidase-antiperoxidase (Sigma). After washings, the

samples were histochemically stained with diaminobenzidine for 3 h at 37°C [Graham and Karnovsky, 1966]. Cells were then post fixed for 30 min in 1% OsO_4 in 0.1 M cacodylate buffer pH 7.2, dehydrated in graded ethanol solutions and embedded in epoxy resin.

For electron microscopy thin sections were analyzed in unstained sections and in sections contrasted with lead citrate, in a Joel electron microscope operate at 80 kV.

CONFOCAL LASER SCANNING MICROSCOPY

Cells were examined using an Axiovert 100M inverted microscope (Carl Zeiss, Jena, Germany) with an LSM 510 confocal laser scanning module (Carl Zeiss) equipped with both argon and helium/neon laser and the samples were examined under a 63×/1.25 n.a. oil objective lens. The focal plane of maximal peroxisomal (or peroxisomal ghost) abundance (i.e., the greatest number of peroxisomes per unit area) within the cell was selected to assess colocalization with the different organelle markers.

RESULTS

SUBCELLULAR DISTRIBUTION OF PEROXISOMAL MATRIX PROTEINS: CATALASE AND THIOLASE

To characterize the phenotype of the fibroblasts of the MR patient with ZS, we first performed cell fractionation and compared the distribution of peroxisomal enzymes with those of the characterized cell line GM6231. Both MR and GM6231 fibroblasts showed catalase (peroxisomal matrix marker enzyme containing PTS-1) mislocalized to the cytosol, whereas mitochondria and lysosomal markers fractionated in the organellar pellet, as expected (Table II). Thiolase (peroxisomal matrix enzyme containing PTS-2) was also largely recovered in the cytosolic fraction (data not shown). Immunofluorescence confirmed the distribution of catalase and thiolase in the cytoplasm of MR and GM6231 fibroblasts (Fig. 1B,E, and C,F, respectively). Thus, MR fibroblasts, similarly to GM6231 fibroblasts, have a peroxisomal biogenetic defect.

TABLE II. Fractionation of Matrix Peroxisomal Enzymes in Control, MR, and GM6231 Fibroblasts

	Proportion of catalase (peroxisomes), nabgase (lysosomes), and cytochrome *c* oxidase (mitochondria) in cytosolic fraction[a] from fibroblasts		
	Catalase	Nabgase	Cytochrome *c* oxidase
Control	5–10	7–11	6–8
Patient MR	>90	12	8–10
GM6231	>90	10	8–10

Postnuclear supernatants were subjected to cell fractionation to obtain fractions P (subcellular organelles) and S (cytosol), in which enzymatic activities were measured. Table shows the proportion of catalase, cytochrome *c* oxidase and nabgase in the S fraction of control, MR and GM6231 cells. Only catalase was mislocalized to cytosol in MR and GM6231 cells.
[a]% of total activity in cytoplasm.

PRESENCE AND SUBCELLULAR DISTRIBUTION OF PMPS

Previous studies have shown that PMPs are mainly degraded when fibroblasts do not have peroxisomal membrane remnants [South and Gould, 1999; South et al., 2000]. However, immunoblots clearly demonstrated the presence of PMP22 and PMP70 in membrane

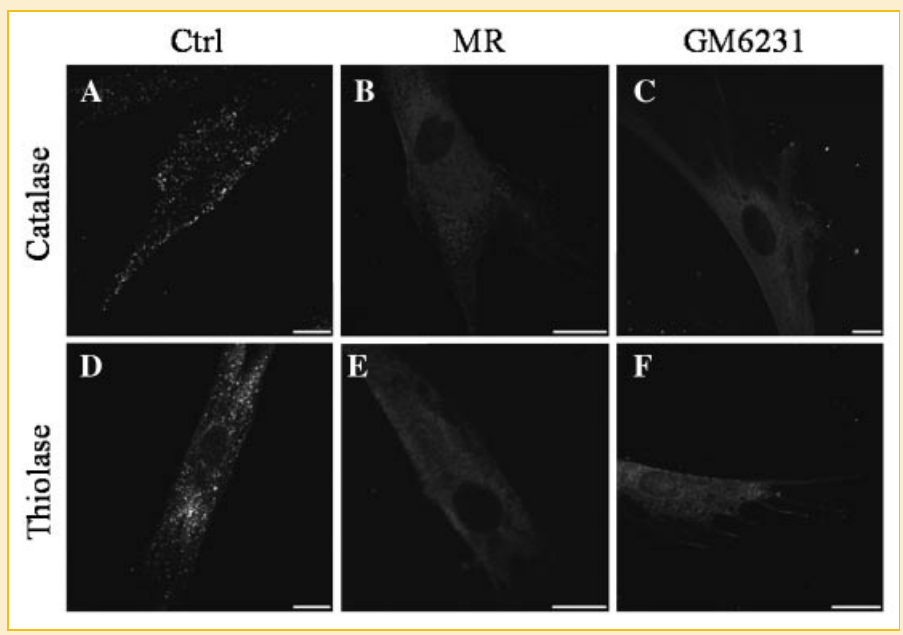

Fig. 1. Subcellular distribution of matrix peroxisomal enzymes in normal and ZS fibroblasts. Indirect immunofluorescence of Control (A,D), MR (B,E), and GM6231 (C,F) fibroblasts incubated with antiserum against Catalase (A–C) or Thiolase (D–F) followed by FITC-conjugated secondary antibodies conjugated. Normal peroxisomes are only observed in control cells, whereas MR and GM6231 fibroblasts show catalase and thiolase staining in the cytosol. Bar: 20 μm.

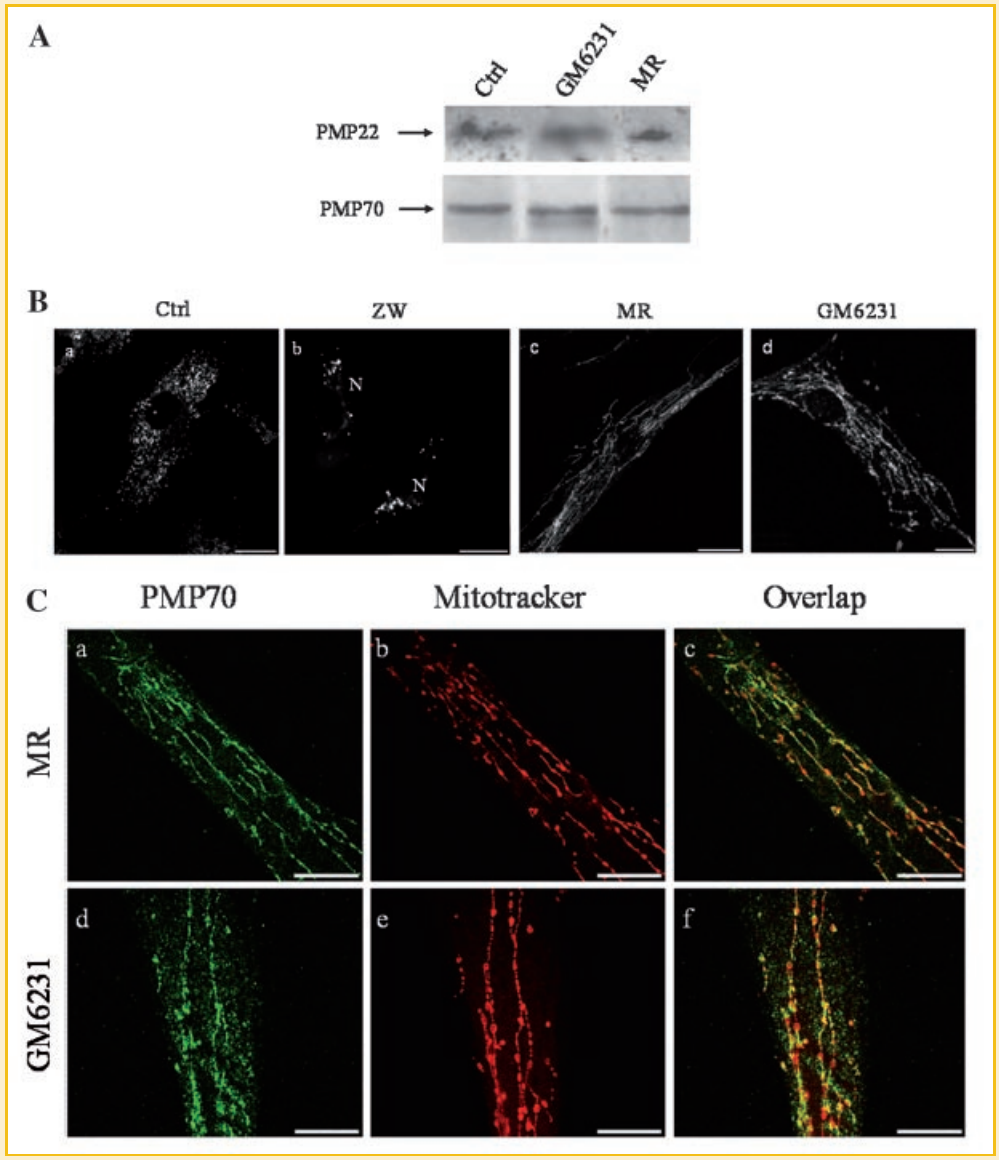

Fig. 2. Presence and subcellular localization of PMPs in normal and ZS fibroblasts. A: Western blot of membranes (100 μg of protein) from organellar pellets of control, MR and GM6231 fibroblasts subjected to SDS–PAGE. Immunoblots were carried out using antibodies against human PMP22 and PMP70, and secondary antibodies conjugated to peroxidase. PMP22 and PMP70 are detected in both mutant fibroblasts. B: Immunofluorescence of PMP70 in Control (a), GM4340 (b), MR (c) and GM6231 (d) fibroblasts. Cells were incubated with anti-PMP70 affinity purified antibodies. Normal peroxisomes are observed in control cells (a). Peroxisomal ghosts are shown in Zellweger syndrome cells (b). In contrast, MR (c) and GM6231 (d) fibroblasts have PMP70 assembled in membranous tubular structures. C: Mitocondrial distribution of PMP70 in ZS fibroblasts. MR (a–c) and GM6231 (d–f) fibroblasts were first incubated at 37°C with the mitochondrial dye Mitotracker (b,e) and then treated for indirect immunofluorescence of PMP70 (a,d). Fluorescence images obtained by confocal laser scanning microscopy show PMP70 containing tubules in MR and GM6231 fibroblasts colocalizing with mitochondria (c,f). N: nucleus. Bar: 20 μm.

fractions from MR and GM6231 fibroblasts (Fig. 2A). The finding of both PMPs in our ZS fibroblasts prompted us to analyze the distribution of these class I PMPs.

Indirect immunofluorescence of PMP70 showed the typical pattern of peroxisomes in control cells (Fig. 2Ba) and peroxisomal membrane ghosts in GM 4340 ZS fibroblasts, in which the original description of peroxisomal membrane ghosts was made [Santos et al., 1988b] (Fig. 2Bb). In contrast, MR (Fig. 2Bc) and GM6231 (Fig. 2Bd) cells displayed PMP70 in tubular membranous structures different from classic peroxisomal ghosts. Similar results were obtained with PMP53 using laser confocal microscopy (data not shown). Colocalization experiments of MR (Fig. 2Cc) and GM6231 (Fig. 2Cf) cells revealed PMP70 mainly in mitochondria, as shown by colocalization with mitotracker.

Immunolabeling at the electron microscopy level showed results congruent with the immunofluorescence. We first examined the regular morphology of control and MR fibroblasts by conventional transmission electron microscopy. Control fibroblasts display some peroxisome-like structures (Fig. 3A, arrowheads). Cytochemical detection of catalase makes peroxisomes easily recognizable

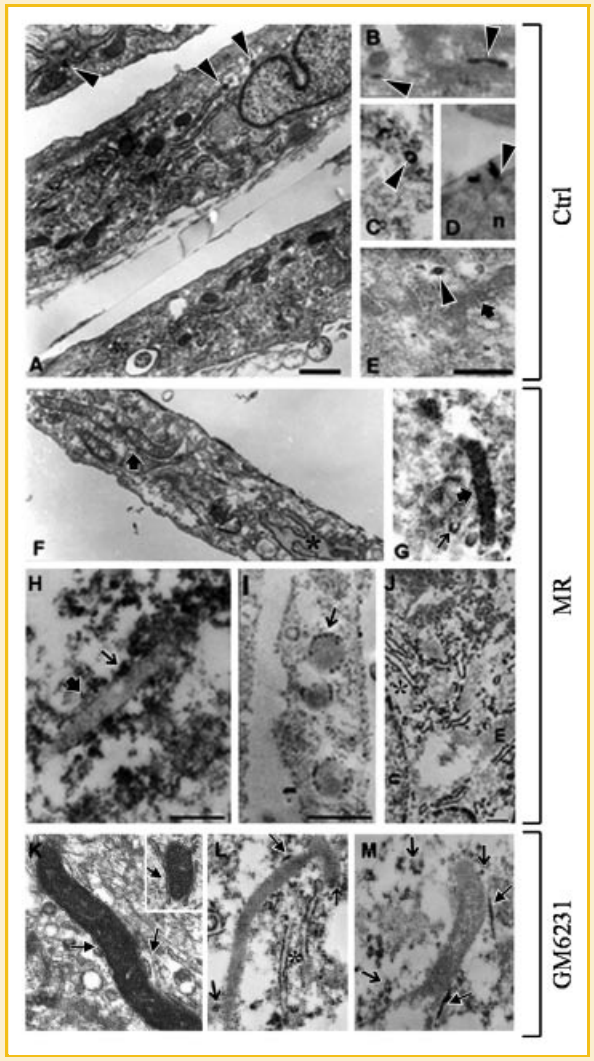

Fig. 3. Ultrastructural, cytochemical and immunoelectron microscopy studies. Control (A–E), MR (F–J), and GM6231(K–M) fibroblasts were fixed in situ and subjected to conventional transmission electron microscopy (A,F,K), cytochemistry for catalase detection (B) or immunoelectron microscopy with anti-PMPs serum and peroxidase-anti-peroxidase detection (C–E, control; G–J, MR; L,M GM6231). Only the ultrathin sections for conventional ultrastructure (A,F,K) were counterstained. Cytochemistry (arrowheads in B) and immunocytochemistry (arrowheads in C–E) show peroxisomes in control but not in MR and GM6231 fibroblasts. Immunocytochemistry shows PMPs located in mitochondrial membranes (arrows in G,H), small vesicles (thin arrows in G–I) and ER cisternae (asterisk in J) in MR fibroblasts, as well as in small vesicles (thin arrows in M), ER (asterisk in L) and thin membranous tubules in GM6231 fibroblasts (arrows in L,M). n: nucleus. Bar: 0.5 μm.

(Fig. 3B, arrowheads). In contrast, MR fibroblasts did not show any recognizable peroxisome-like structures, whereas they showed an enlarged ER (Fig. 3F, asterisk) and long-tubular mitochondria (Fig. 3F, arrow). We made similar observations in GM6231 cells. In these cells, thin membranous tubular projections from the ER cisternae were found closely associated with mitochondria (Fig. 3K, arrow).

The subcellular localization of a great variety of PMPs can be studied with a serum that specifically recognizes several human PMPs [Santos et al., 1988a]. This serum immunolabeled the peroxisomes of control cells (Fig. 3C–E, arrowheads). In MR cells, our antibody showed a PMP-labeling mainly in mitochondrial membranes (Fig. 3G, wide arrow), and in vesicles associated with this organelle (Fig. 3H, thin arrows), as well as some labeling in small cytoplasmic vesicles of unknown origin (Fig. 3G, thin arrow, and Fig. 3I). PMPs have been described in mitochondria when there is absence of peroxisomal membranes [Muntau et al., 2000; South et al., 2000]. More recently, mitochondria derived vesicles accounting for a novel vesicular traffic between this organelle and peroxisomes was reported [Schumann and Subramani, 2008]. Therefore, the small vesicles that we found positive for PMPs in MR cells could have mitochondrial origin.

We also found specific PMP staining in ER cisternae of MR fibroblasts, (Fig. 3J, asterisk), similarly to GM6231 cells. In these fibroblasts, we found thin membranous tubules and vesicles containing PMPs associated with mitochondria (Fig. 3L,M, thin arrows) and ER membranes (Fig. 3L, asterisk). These results not only indicate mislocalization of PMPs in MR and GM6231 cells, but for the first time reveal localization of a small pool of PMPs in the ER of human fibroblasts lacking any peroxisomal membranes.

GENETIC DEFECTS IN MR FIBROBLASTS

Mutations in the PEX3 gene in yeast and humans have been shown to produce a cellular phenotype that lacks peroxisome membrane ghosts and any detectable peroxisomal remnant [South et al., 2000]. We sequenced the PEX3 gene from MR cells and found an inactivating nonsense mutation that generates a stop codon at position 53 (Fig. 4A), thus mimicking previously reported PEX3 deficient human cells [South et al., 2000]. As expected from previous studies [Ghaedi et al., 2000a; Muntau et al., 2000; South et al., 2000], transfection of human PEX3 in MR cells led to the appearance of peroxisomes in 48 h (Fig. 4B). These newly generated peroxisomes imported matrix enzymes such as catalase and thiolase (Fig. 4C,D). Expression of recombinant Pex3p-GFP also restored peroxisome biogenesis in MR cells (Fig. 4E,F), as described in yeast and mammalian cells [Ghaedi et al., 2000b; Hoepfner et al., 2005]. This mimicked the described effect [Voorn-Brouwer et al., 2001] of expressing Pex16p-GFP in a complementation group 9 fibroblasts (Fig. 4G and H, respectively), which carry a mutation in the PEX16 gene [Voorn-Brouwer et al., 2001].

TIME COURSE AND ER INVOLVEMENT OF Pex3p AND Pex16p DRIVEN PEROXISOME BIOGENESIS

Previous studies in PEX3 deficient cells have shown reestablishment of peroxisomes after 3 h of PEX3 expression [South et al., 2000], but did not analyze shorter time periods. We performed similar nuclear microinjection experiments to express Pex3p-GFP in MR fibroblasts and assess its sorting behavior as soon as it became detectable. Because GFP signal was barely detectable, we used indirect immunofluorescence with an anti-GFP antibody. In this way, we could easily detect newly made peroxisomes 4 h post-microinjection (Fig. 5Ac,d). Strikingly, within the first hour, we could clearly detect Pex3p-GFP mainly in the ER (Fig. 5Aa,b). Quantitative colocalization analysis with the ER marker PDI indicated that roughly 70% of

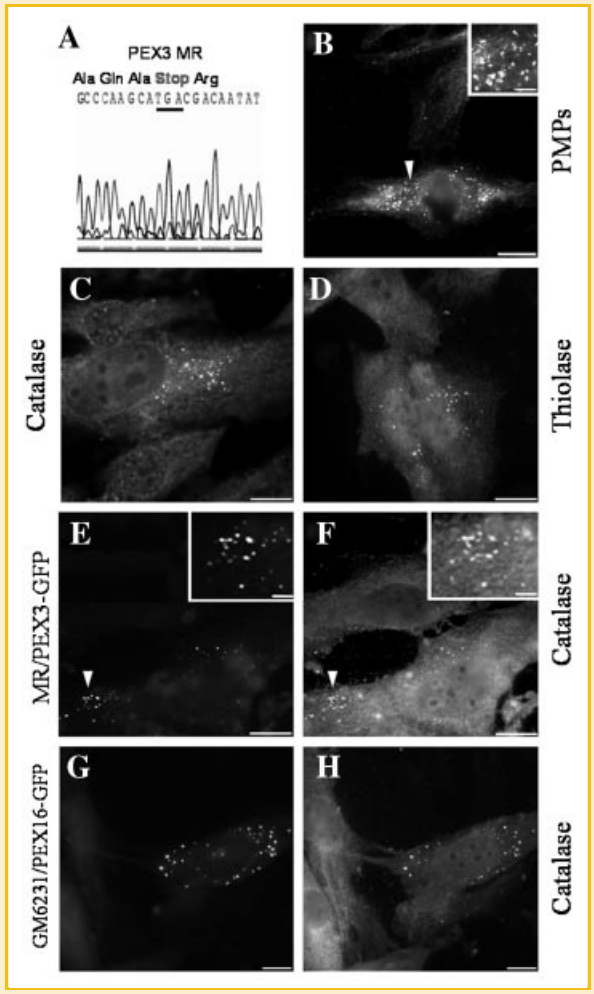

Fig. 4. PEX3 and PEX16 genes restore peroxisomal biogenesis in MR and GM6231 fibroblasts, respectively. A: PEX3 gene sequence of MR fibroblasts showing a stop mutation at codon number 53. MR fibroblasts were transfected with the wild human PEX3 gene (B–D). After 48 h post-transfection, cells were subjected to immunofluoresce using the following antibodies: (B) anti-PMPs, (C) anti-Catalase, and (D) anti-Thiolase. Restoration of peroxisome biogenesis also occurs 48 h after transfection with PEX3-GFP in MR fibroblasts (E,F) and PEX16-GFP in GM6231 fibroblasts (G,H), as revealed by the peroxisomal immunofluorescence pattern of catalase and thiolase. Arrow in (E) and (F) show zoomed region. Bar: 20 μm. Inset bar: 5 μm.

Pex3p-GFP protein distributed to the ER at this early time point (Fig. 5Ag). After 4 h, only peroxisomes showed Pex3p-GFP staining.

We then asked whether this Pex3p obligated targeting to the ER is due to the lack of peroxisomes in ZS or constitutes a normal pathway. We inserted a *N*-glycosylation site at the N-terminal of Pex3p, so that its entrance into the ER lumen can be easily detected by a shift in electrophoretic migration, as previously described in yeast [Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005]. This chimeric protein (myc-GLY-Pex3p) showed an increased molecular mass when expressed in normal wild type human fibroblasts (Fig. 5Ah). Therefore, Pex3p is targeted to the ER even in cells that contain and manufacture peroxisomes normally.

To evaluate further the role of the ER in Pex3p-dependent generation of new peroxisomes, we engineered the human PEX3 cDNA by adding the signal peptide (SP) of the ER resident protein calnexin (SP-Pex3p-GFP). Transfection experiments in wild type human fibroblasts showed this chimeric protein primarily localized in the ER, with only a minor peroxisomal distribution (data not shown). Transfected MR cells also disclosed SP-Pex3p-GFP mainly in the ER (Fig. 5Bc). Detection of vesicles containing PMPs (Fig. 5Bf) indicated that peroxisome biogenesis was restored upon SP-Pex3-GFP expression. These results indicate that Pex3 artificially addressed to the ER is able to promote peroxisomal biogenesis and to become incorporated into newly generated peroxisomes. Taken together, all these results provide for the first time evidence in human fibroblasts that Pex3p follows the ER-to-peroxisomal pathway and is able to promote de novo peroxisomal biogenesis from the ER.

This sorting behavior of Pex3p mimicked that of Pex16p previously described in mammalian cells [Kim et al., 2006]. For comparison, we transfected Pex16p-GFP in GM6231 cells and found that it localized in the ER for several hours (5–15 h) (Fig. 6Ac). After 15 h post-transfection, we also detected a punctate staining of Pex16p-GFP around the nucleus (Fig. 6Ae), very likely corresponding to newly manufactured peroxisomes, as judged by PMPs detection (Fig. 6Af). Later, 45 h posttransfection, the Pex16p-GFP signal clearly distributed in peroxisomes (data not shown). These results are congruent with previous studies in PEX16 mutant cells [South and Gould, 1999; Voorn-Brouwer et al., 2001]. Furthermore, in congruency with a previous report [Kim et al., 2006], Pex16p-GFP expressed in human wild-type fibroblasts also showed clear colocalization with the ER marker calnexin (data not shown). In addition, we engineered the human PEX16 gene by adding the signal peptide (SP) of calnexin (SP-Pex16p-GFP). In wild type fibroblasts, this chimeric protein primarily localized in the ER (data not shown), with only a minor peroxisomal distribution (Fig. 6Bf). In GM 6231 cells, the PS-Pex16p-GFP also showed an ER location (Fig. 6Bc), and restored peroxisome biogenesis (Fig. 6Bi).

Finally, our MR fibroblasts and the cell line GM6231 offered the opportunity to test the interrelationship and temporal–functional hierarchy between these two proteins. MR fibroblasts transfected with the PEX16-GFP plasmid and examined 15 h later, showed the Pex16p-GFP protein mainly distributed in the ER (Fig. 7C,D), indicating that its targeting to ER membranes does not require Pex3p. In contrast, GM6231 cells transfected to express Pex3p-GFP distributed this protein only to mitochondria (Fig. 7E,F), indicating that Pex16p is required for Pex3p incorporation into the ER.

DISCUSSION

Here we provide the first evidence that human Pex3p is targeted to the ER during de novo biogenesis of peroxisomes in PEX3 mutant ZS fibroblasts, as well as when it is en route to preformed peroxisomes in normal human fibroblasts, thus mimicking the Pex3p yeast homolog [Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005]. We also show that Pex3p targeting to the ER requires Pex16p expression. Unexpectedly, fibroblasts lacking peroxisomes due to

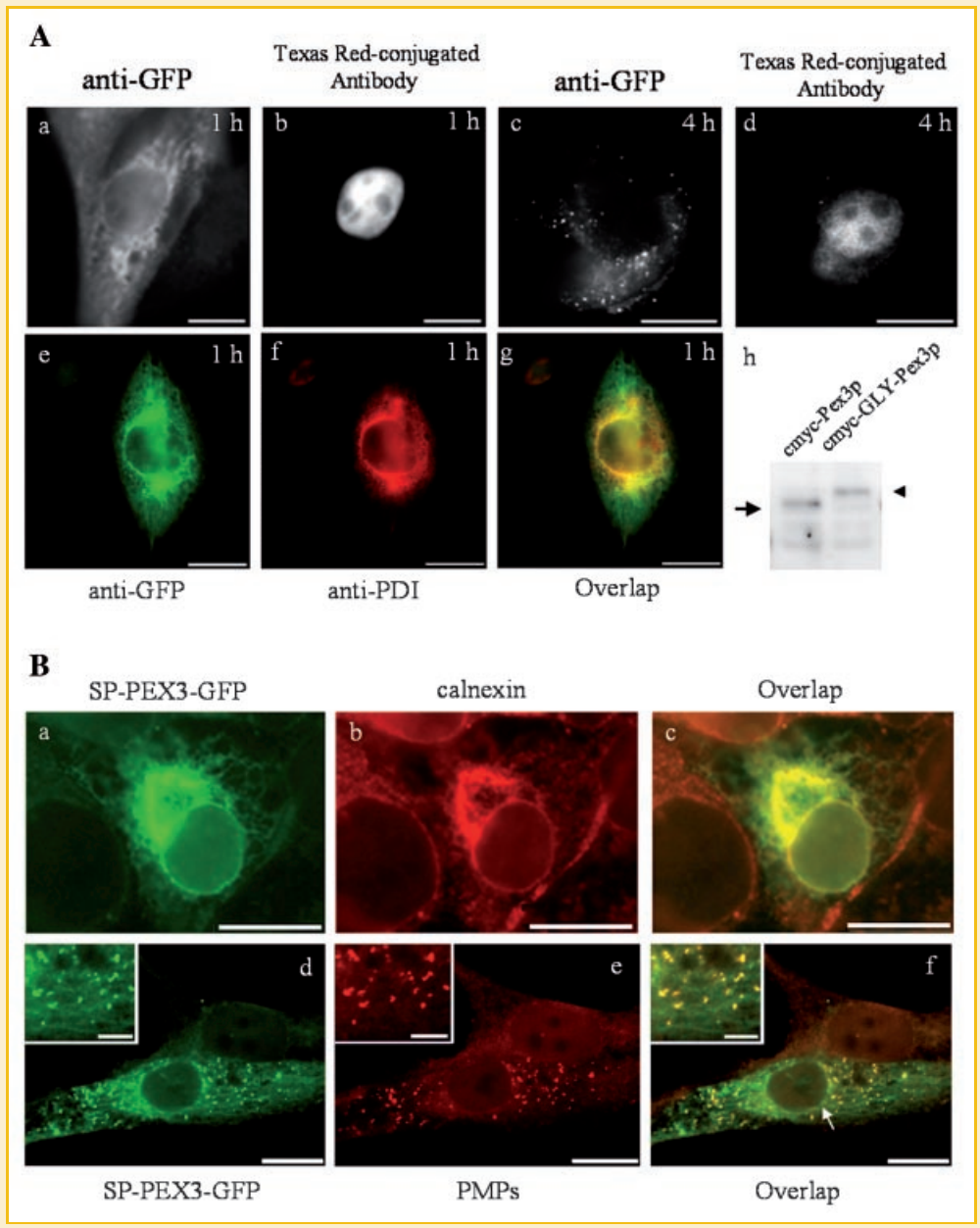

Fig. 5. Pex3p is first targeted to the ER in MR fibroblasts. A: MR fibroblasts were microinjected with the plasmid pPEX3-GFP and a Texas-Red labeled antibody (b,d), fixed at the indicated times and subjected to immunofluoresce with an anti-GFP antibody (a–d). Within 1 h, Pex3p-GFP is found in the perinuclear area and in cytosol (a,b), whereas 4 h post-injection peroxisome-containing Pex3p-GFP are easily found (c,d). An analysis with antibodies against PDI (an ER marker) shows 70% Pex3p-GFP colocalizing with this marker in the ER within the first hour of microinjection (g). h: Western blotting of recombinant Pex3p bearing an *N*-glycosylation sequence at the N-terminal. Control fibroblasts were transfected with c-*myc*-tagged PEX3 (lane 1) or GLY-PEX3 (lane 2). After 28 h, the cells were lysed and subjected to SDS–PAGE (15%). The immunoblot with antibodies against the c-*myc* epitope shows an electrophoretic shift of c-*myc*-GLY-Pex3p (arrowhead) as compared with c-*myc*-Pex3p (arrow). B: Pex3p-GFP bearing a signal peptide (SP) is targeted to the ER but sill restores peroxisome biogenesis in MR fibroblasts. MR fibroblasts transfected with the plasmid pSP-PEX3-GFP for 24 h were subjected to immunoflourescence for calnexin (b) or PMPs (e). The chimeric protein showed a colocalization with the ER (c), and restores the peroxisome biogenesis defect in these cells (f). Arrow in (f) show zoomed region. Bar: 20 μm. Inset bar: 5 μm.

mutations in either PEX3 or PEX16 showed detectable levels of PMPs in the ER, thus revealing a novel feature within ZS cell phenotypes. These results are congruent with the notion that the ER plays a central role in peroxisomal biogenesis in mammalian cells.

Lazarow and Fujiki [1985] postulated that peroxisomes form by growth and division of pre-existing peroxisomes. Supporting this hypothesis is the fact that peroxisomal matrix and membrane proteins are synthesized on free ribosomes and become imported posttranslationally into pre-existing organelles. However, the ZS complementation groups 9 (PEX16 gene defect), 12 (PEX3 gene defect) and 14 (PEX19 gene defect) do not contain any peroxisomal remnants in their cells, but reestablish functional peroxisomes upon

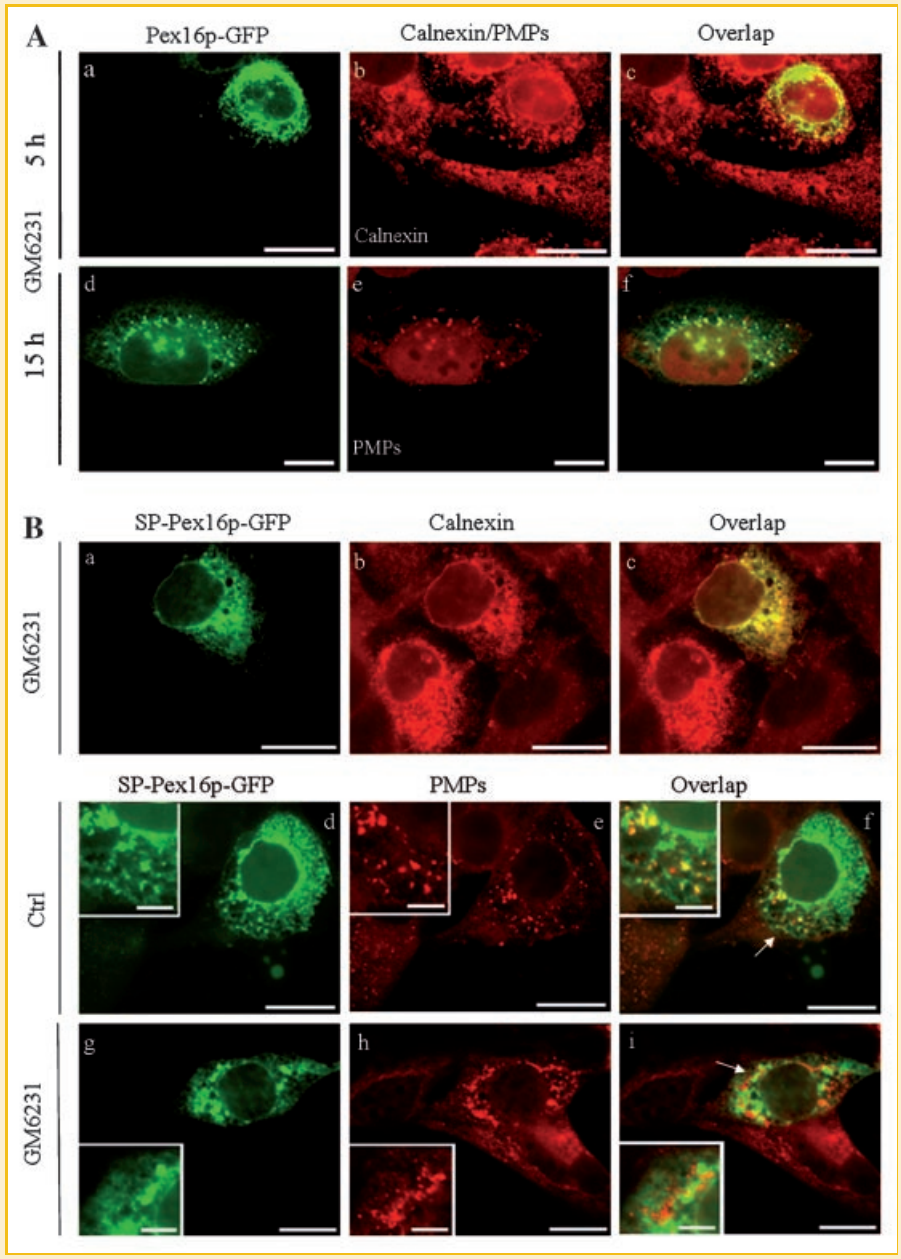

Fig. 6. PEX16 is targeted to the ER in GM6231. A: Kinetics of peroxisome restoration. Fibroblasts were transfected with the plasmid pPEX16-GFP. At 5 (a–c) or 15 h (d–f) of expression, cells were subjected to colocalization experiments using antibody against the ER marker calnexin (b) or PMPs (e). A similar ER pattern with Pex16p-GFP was detected after 5 h posttransfection (c). A punctate signal around the nucleus colocalizing with PMPs (f), was detected only at 15 h. B: SP-PEX16-GFP can restore peroxisome biogenesis in GM6231 cells. Control (Ctrl; d,f) and GM6231 fibroblasts (a–c and g–i) were transfected with the plasmid pSP-PEX16-GFP. After 24 h, the cells were subjected to immunoflourescence with the ER marker calnexin (b) or PMPs (e,h) antibodies. In GM6231 fibroblasts, pSP-PEX16-GFP shows main colocalization with calnexin (c) and restores the peroxisome biogenesis (i). This construction was also detected in peroxisomes of control fibroblasts (f). Arrow in (f) and (i) shows zoomed region. Bar: 20 μm. Inset bar: 5 μm.

expression of their respective wild type PEX genes [Schrader and Fahimi, 2008]. Therefore, de novo peroxisomal synthesis is possible [Geuze et al., 2003; Tabak et al., 2003; Hoepfner et al., 2005; Schekman, 2005; Kim et al., 2006] and challenges the "growth and division" model of peroxisome biogenesis from preexisting organelles.

A growing body of evidence now sustains a role of the ER in peroxisome biogenesis, perhaps representing an alternative route for at least some peroxisomal proteins [reviewed in Hoepfner et al., 2005]. For example, in *Yarrowa lipolytica*, Pex2p and Pex16p are first targeted from the cytosol to the ER, become *N*-glycosilated and transported to peroxisomes [Titorenko and Rachubinski, 1998b]. In

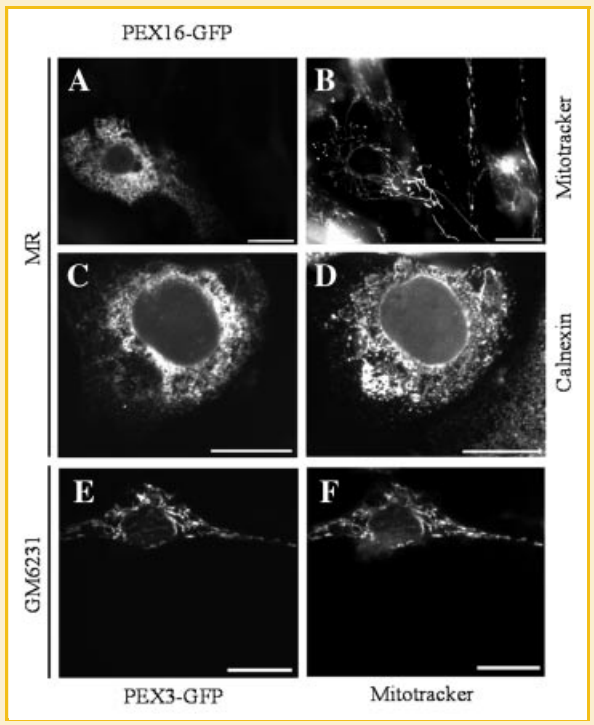

Fig. 7. Biogenesis of Pex3p-GFP and Pex16p-GFP in MR and GM6231 fibroblasts. MR fibroblasts (A–D) were transfected with the plasmid pPEX16-GFP (A,C) for 15 h and then incubated with Mitotracker (B) or subjected to immunofluorescence for calnexin (D). Pex16p-GFP colocalizes with calnexin but not with Mitotracker. In contrast, pPEX3-GFP expression in GM6231 fibroblasts (E,F) lacking Pex16p (E) colocalized with Mitotracker (F). Bar: 20 μm.

Hansenula polymorha, Pex3p, Pex8p, and Pex14p accumulate in the ER in the presence of BFA, being targeted to peroxisomes after BFA removal [Salomons et al., 1997]. In *S. cerevisiae*, Hoepfner et al. [2005] showed that Pex3p and Pex19p are synthesized in the ER and then move to peroxisomes. In mammalian cells, ER-connected reticular structures containing Pex13 and PMP70 in dendritic cells [Geuze et al., 2003] and the synthesis of PMP50 in ER-bound ribosomes in rat liver [Bodnar and Rachubinski, 1991], are congruent with a role of the ER in peroxisome biogenesis. Furthermore, imaging analysis revealed that Pex16p transits through the ER while en route to peroxisomes in COS7 cells and restores peroxisomal biogenesis in GM6231 cells even when forced to target the ER by an engineered ER sorting signal [Kim et al., 2006].

Under this scenery, it might be expected that Pex3p, Pex16p and pex19p have similar sorting behaviors respect to ER targeting in mammalian cells [reviewed in Hoepfner et al., 2005]. These proteins are required for PMPs insertion into the peroxisomal membrane, which in turn mediates import of matrix proteins [Schrader and Fahimi, 2008]. However, previous studies in mammalian cells had failed to detect Pex3p in the ER [Ghaedi et al., 2000b; Muntau et al., 2000; South et al., 2000], unless pex16p is overexpressed [Kim et al., 2006]. Targeting of Pex3p to the ER had only been reported in yeast [Hoepfner et al., 2005; Kragt et al., 2005; Tam et al., 2005], but observations in yeasts may not be completely extensive to mammalian cells. For instance, Pex16p is not essential for peroxisomal biogenesis in *S. cerevisiae* [Hettema et al., 2000], although is clearly required in mammalian cells [South and Gould, 1999; Kim et al., 2006]. Therefore, we reassessed here the sorting behavior of Pex3p in fibroblasts from a new ZS patient carrying a PEX3 gene mutation (MR fibroblasts), using as a reference GM6231 fibroblasts previously reported to carry a PEX16 gene mutation [Honsho et al., 1998].

Cells carrying either PEX3 or PEX16 mutations completely lack peroxisomes, peroxisomal ghosts and any vestige of peroxisomal membrane [reviewed in Schrader and Fahimi, 2008]. In congruency with previous reports [Muntau et al., 2000; Sacksteder et al., 2000], both MR and GM6231 mutant cells display the majority of PMPs missorted to mitochondrial membranes, suggesting that PMPs contain a cryptic and weak mitochondrial signal. However, we also detected specific PMP staining in ER cisternae of MR and GM6231 fibroblasts. These results reveal for the first time localization of a small pool of PMPs in the ER of human fibroblasts lacking any peroxisomal membranes.

In MR cells (PEX3 deficient), microinjection experiments showed that Pex3p-GFP distributes in the ER within the first hour of expression and 4 h later appeared only in newly generated peroxisomes. A SP-Pex3p-GFP chimeric protein artificially forced to target the ER displays a similar peroxisome biogenesis restoring capability as the wild type protein.

We also obtained evidence that Pex3p targeting to the ER constitutes a normal pathway. The chimeric protein myc-GLY-Pex3p bearing an engineering *N*-glycosylation site increased its molecular mass when expressed in control human fibroblasts, indicating that even cells that contain and manufacture peroxisomes address Pex3p normally to the ER. Therefore, there is a sorting pathway for Pex3p that includes an ER station and from this location Pex3p is able to complement the peroxisomal biosynthetic machinery as to generate new peroxisomes.

The functional relationship between Pex3p and Pex16p has not been entirely clarified. Pex16p interacts with Pex19p in the cytosol and its peroxisomal targeting requires Pex19p similarly to other PMPs [Fang et al., 2004; Jones et al., 2004; Shibata et al., 2004]. In contrast, peroxisomal import of Pex3p is unique in that it does not require Pex19p [Jones et al., 2004], although a Pex16p and Pex19p-dependent peroxisomal import of this peroxin has recently been shown by Matsuzaki and Fujiki [2008]. Because Pex3p acts as a docking factor for Pex19p it could be predicted that is required for Pex16p peroxisomal import. However, there is evidence suggesting Pex16p functions upstream of Pex3p in peroxisomal import, as cells overexpressing either Pex16p or a carboxyterminal segment of Pex16p mislocalize Pex3p to ER or mitochondria, respectively [Kim et al., 2006]. Our results are more congruent with this possibility. Pex16p-GFP expressed by transfection in GM6231 cells distributed in the ER and in vesicles that did not contain PMPs, as described [Kim et al., 2006]. These vesicles could correspond to peroxisome precursors or perhaps ER "peroxisome exit sites". In MR fibroblasts lacking Pex3p, Pex16p-GFP remained arrested in the ER, mimicking PEX19 mutant cells [Kim et al., 2006]. These results indicate that Pex3p is not required for Pex16p association with the ER, but is required for its exit from the ER (perhaps like a "pre-peroxisome") and promotes further steps in peroxisome biogenesis. In contrast,

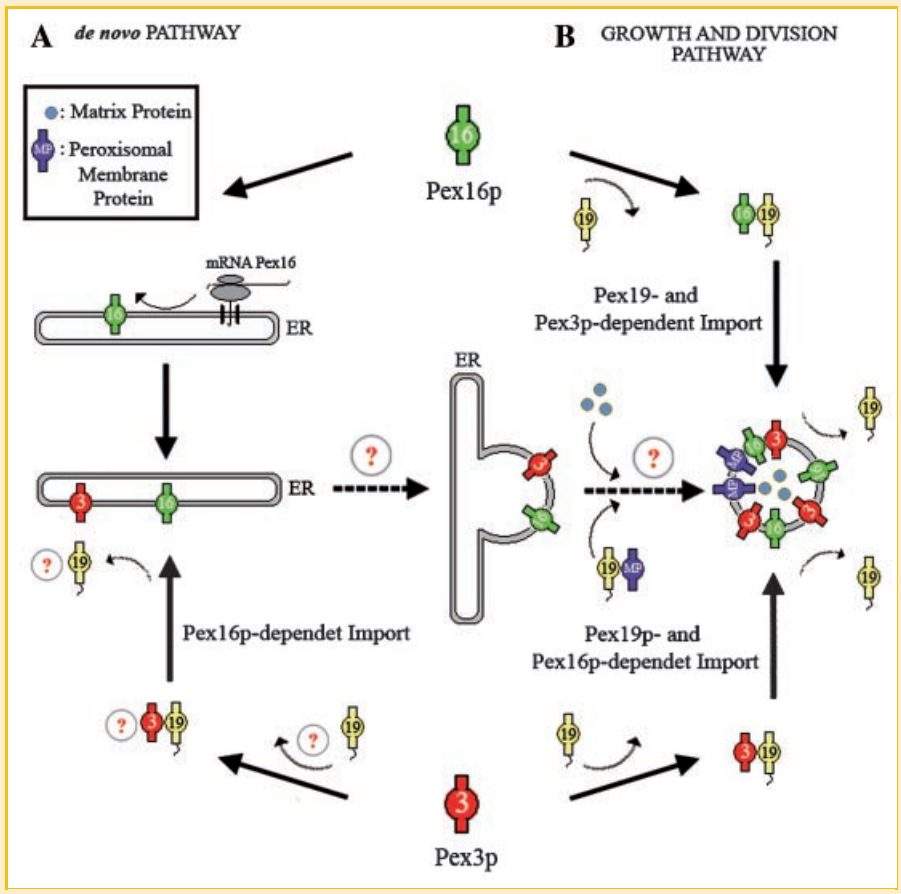

Fig. 8. Model of Pex16p and Pex3p targeting in mammalian cells. Both peroxins can be imported to the ER and peroxisomes. The Pex16p cotranslational insertion into ER membranes previously reported [Kim et al., 2006] may be independent of Pex3p, as shown in this study. This contrasts with the direct targeting of Pex16p into peroxisomes, which has been reported to be both Pex19p- and Pex3p-dependent [Fang et al., 2004; Jones et al., 2004]. ER destination of Pex3p is Pex16-dependent (this study), and presumably also needs Pex19p. Direct peroxisomal targeting of Pex3p is also Pex19- and Pex16p-dependent, as recently reported [Matsuzaki and Fujiki, 2008].

Pex3p showed a mitochondrial distribution in PEX16 mutant cells indicating that it does require Pex16p for targeting the ER. Therefore, as previously proposed [Honsho et al., 2002; Kim et al., 2006], Pex16p seem to act at earlier stages than Pex3p in the ER-dependent peroxisomal membrane biogenesis in mammalian cells.

Because the kinetics of *PEX3*-mediated peroxisome synthesis during the complementation of *pex3*-null mutants is one-to-two orders of magnitude slower than the kinetics of PEX3 import into peroxisomes of WT cells, it has been argued that most PEX3 is imported into preexisting peroxisomes long before it could mediate "de novo" peroxisome synthesis[South et al., 2000]. A recent study in peroxisomes in vitro suggests that these organelles possess the machinery for direct import of Pex3, in a Pex19p- and Pex16p-dependent way [Matsuzaki and Fujiki, 2008]. Pex16p belongs to the Class I PMP [Fang et al., 2004; Jones et al., 2004], and its peroxisomal targeting is dependent on Pex19p and Pex3p. Interestingly, Pex16p can reach the ER membrane in the absence of Pex3p [Kim et al., 2006; our results], but cannot leave this organelle (our findings). Taken all together, we speculate that the ER targeting of Pex16p conforms the platform for de novo peroxisome biogenesis. Pex16p can provide a docking site for Pex3p as it does at the peroxisomal membrane (Fig. 8). Our model resolves the "chicken-and-egg" issue implicit in the "mutual-dependent targeting" of Pex3p and Pex16p, recently proposed [Matsuzaki and Fujiki, 2008]. Both peroxins could be targeted to peroxisomal membrane in a "mutual-dependent targeting", and then follow the classical "Growth and Division" model of peroxisome biogenesis. Because Pex16p is targeted into ER membrane before Pex3p, it would begin the de novo formation of peroxisomes from the ER, generating pre-peroxisomes that may mature toward complete and functional entities. Thus, two essentially distinct mechanisms of peroxisomal biogenesis can coexist in the cell. One ER-dependent, could be responsible for de novo renewal of peroxisomal membranes, while the other corresponding to the Lazarow and Fujiki's autonomous "Growth and Division" model, could exert rapid and direct protein import into preexisting peroxisomes.

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