Peroxisomes Are Formed from Complex Membrane Structures in *PEX6*-deficient CHO Cells upon Genetic Complementation

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Pex6p belongs to the AAA family of ATPases. Its CHO mutant, ZP92, lacks normal peroxisomes but contains peroxisomal membrane remnants, so called peroxisomal ghosts, which are detected with anti-70-kDa peroxisomal membrane protein (PMP70) antibody. No peroxisomal matrix proteins were detected inside the ghosts, but exogenously expressed green fluorescent protein (GFP) fused to peroxisome targeting signal-1 (PTS-1) accumulated in the areas adjacent to the ghosts. Electron microscopic examination revealed that PMP70-positive ghosts in ZP92 were complex membrane structures, rather than peroxisomes with reduced matrix protein import ability. In a typical case, a set of one central spherical body and two layers of double-membraned loops were observed, with endoplasmic reticulum present alongside the outer loop. In the early stage of complementation by *PEX6* cDNA, catalase and acyl-CoA oxidase accumulated in the lumen of the double-membraned loops. Biochemical analysis revealed that almost all the peroxisomal ghosts were converted into peroxisomes upon complementation. Our results indicate that 1) Peroxisomal ghosts are complex membrane structures; and 2) The complex membrane structures become import competent and are converted into peroxisomes upon complementation with *PEX6*.

INTRODUCTION

Peroxisomes are ubiquitous organelles in eukaryotic cells and surrounded by a single membrane (Lazarow and Fujiki, 1985). They are 0.3–1.0 μm in diameter in liver but smaller in other tissues or cultured cells including CHO. Mammalian

peroxisomes have many important metabolic functions including β -oxidation of fatty acids, especially long-chain fatty acids of more than 22 carbon atoms, and synthesis of ether phospholipid (Subramani, 1993). Several peroxisome biogenesis disorders have been discovered and studied. By genetic complementation using peroxisome-deficient mutants of yeast and mammalian cells, at least 23 *PEX* genes essential for peroxisome assembly have been isolated (for reviews, see Distel *et al.*, 1996; Subramani, 1998; Tabak *et al.*, 1999). *PEX5* and *PEX7* encode peroxisome targeting signal (PTS)-1 and PTS-2 receptors, respectively. *PEX13* and *PEX14* are thought to encode the receptor docking proteins on the peroxisomal membrane, based on their ability to bind PTS receptors. *PEX3*, *PEX19*, and *PEX16* are proposed to be essential for the proper localization and stability of peroxisomal membrane proteins (Honsho *et al.*, 1998; South and Gould, 1999; Hettema *et al.*, 2000). However, the functions and functional mechanisms of most *PEX* gene products, especially for membrane dynamics, are still unknown.

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Abbreviations used: AAA, ATPases associated with diverse cellular activities; LDH, lactate dehydrogenase; LGP85, 85-kDa lysosomal sialoglycoprotein; PDI, protein disulfide isomerase; PMP, peroxisomal membrane protein; PNS, postnuclear supernatant; PTS, peroxisome targeting signal; Px, peroxisomal pellet; SKL, Ser-Lys-Leu-COOH tripeptide.

PEX6 and *PEX1* encode AAA family ATPases (Kunau *et al.*, 1993). Members of the AAA family such as NSF (Haas and Wickner, 1996) and VCP (Acharya *et al.*, 1995; Rabouille *et al.*, 1995) were shown to be involved in the membrane dynamics. Recently, it was reported that Pex6p and Pex1p are required for membrane fusion in the early step of peroxisome biogenesis in the yeast *Yarrowia lipolytica*, and the fusion was reconstituted in vitro (Titorenko *et al.*, 2000; Titorenko and Rachubinski, 2000). In contrast, genetic analysis using *Pichia pastoris* suggested that Pex6p and Pex1p are involved in the late steps of peroxisome biogenesis (Collins *et al.*, 2000). Thus, the role of *PEX6* and *PEX1* in peroxisome biogenesis is still unclear.

Santos *et al.* (1988a, 1988b) reported that fibroblasts (GM 4340) derived from a *PEX6*-deficient patient contained membrane structures having peroxisomal membrane proteins (PMPs) including PMP70 but lacking peroxisomal matrix proteins. They hypothesized that the primary defects of *PEX* mutants are in the import of the matrix proteins across the peroxisomal membrane. Knowledge about the morphology of the peroxisomal remnant structures (peroxisomal ghosts) in *PEX6* or *PEX1* mutants is still limited, especially about their membrane morphology. Another point to be clarified is whether peroxisomes were assembled from the ghosts during the genetic complementation. We presented indirect evidence that the preexisting ghosts serve as precursors of peroxisomes at least in a *PEX5*-mutant, by showing that peroxisomes are restored upon microinjecting recombinant Pex5 protein in the absence of de novo protein synthesis (Yamasaki *et al.*, 1999). On the other hand, it was proposed that peroxisome can be synthesized in the absence of preexisting peroxisomes in *PEX16*-deficient human fibroblasts, which had no detectable peroxisomal ghosts (South and Gould, 1999). In this article, we characterized the ghosts of *PEX6*-mutant cells as highly complex membrane structures containing PMP70. We also obtained direct morphological and biochemical evidence that the complex membrane structures become import competent and are converted into peroxisomes upon complementation by *PEX6* gene.

MATERIALS AND METHODS

DNA Constructs

phGFP(105)-C1 (pGFP) was used as a GFP expression vector (Yamasaki *et al.*, 1998). pGFPSKL was constructed by insertion of a 108-base pair *HaeIII/KpnI* fragment of the rat acyl-CoA oxidase cDNA encoding a peptide containing a Ser-Lys-Leu-COOH tripeptide (SKL; Miyazawa *et al.*, 1987), between the blunt-ended *EcoRI* and *KpnI* sites of pGFP. The rat *PEX6* cDNA expression plasmid was pUCD2-92A (Tsukamoto *et al.*, 1995). Rat catalase cDNA was mutated to encode SKL at the C terminus (CatalaseSKL), compared with the wild-type sequence ANL, by PCR using oligonucleotide CCGGATCCTTACAGCTTAGATTTCCCTTGGCAGCTAT. GFP cDNA of pGFP was removed by digestion with *NcoI* and *BglII*, and the *NcoI-BamHI* fragment of CatalaseSKL cDNA was inserted, yielding pCatalaseSKL. pMiwhph, an expression vector conferring hygromycin resistance, was obtained from Dr. Higashi (Osaka University). pTRE16 β contained 16 repeats of the tetracycline response element and a β -globin gene-derived intron (Tsukamoto *et al.*, 2000). A regulatable expression vector of *PEX6*, pTRE16*PEX6*, was produced by trimolecular ligation involving the 5' and 3' halves of rat *PEX6* cDNA, a *SacII/KpnI* fragment and a *KpnI/XhoI* (partially filled-in with dTTP and dCTP) fragment, respectively, and pTRE16 β

cleaved with *SacII* and *BamHI* (partially filled-in with dGTP and dATP).

Cell Lines and Isolation of Stable Clones Expressing GFPSKL

CHO cells and peroxisome-deficient mutant cells (Z65, Tsukamoto *et al.*, 1990; ZP92, Shimozaawa *et al.*, 1992; ZP102, Tsukamoto *et al.*, 1997) were grown in F12 medium supplemented with 10% FBS. CHO, Z65, and ZP92 were transfected with pGFPSKL by the calcium phosphate precipitation method and subjected to the selection of transformants with 400 μ g/ml G418. Stable clones were isolated with cloning cylinders and purified by limiting dilution. Because ZP102 was originally G418 resistant, it was cotransfected with pGFP-SKL and pMiwhph and selected with 400 U/ml hygromycin B.

Immunofluorescence Microscopy

Cells were seeded onto glass bottom dishes (No. 1.5; MatTek, Ashland, MA) coated with mouse type IV collagen (Invitrogen, Carlsbad, CA) and incubated overnight in 5% CO₂ at 37°C. Cells were washed with PBS once and fixed with 4% formaldehyde prepared from paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 1 h at room temperature. After three washes with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and then washed with PBS three times. The cells were incubated with 1% BSA in PBS for 1 h and then with primary antibody in 1% BSA in PBS for 1 h at room temperature. Rabbit anti-rat catalase antibody (a gift from Dr. Usuda, Shinshu University), rabbit anti-rat PMP70 C-terminal peptide antibody (Imanaka *et al.*, 1996), rabbit anti-rat peroxisomal 3-ketoacyl-CoA thiolase antibody, rabbit anti-rat acyl-CoA oxidase antibody, and rabbit anti-rat mitochondrial 3-ketoacyl-CoA thiolase antibody (Miyazawa *et al.*, 1980; from Dr. Hashimoto, Shinshu University) were used as primary antibodies. After three washes with PBS, cells were kept in PBS overnight at 4°C. The cells were washed with PBS twice and incubated with Cy3-labeled goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS containing 1% BSA, for 1 h at room temperature. The second antibody was rinsed away with PBS five times for 5 min each. The cells were sequentially incubated for 10 min each with 20, 40, 60, and 80% glycerol in PBS and finally mounted with 90% glycerol in PBS containing 25 mg/ml 1,4-diazabicyclo-[2.2.2]octane.

For the lysosome stain, cells were incubated overnight in the growth medium containing 100 μ M leupeptin followed by 300 nM LysoTracker Red (Molecular Probes, Eugene, OR) for 1 h. After one wash with PBS, cells were fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 1 h at room temperature.

Microscope System

The details of the microscope system have been described previously (Hiraoka *et al.*, 1991) with some modifications (Haraguchi *et al.*, 1997). In brief, fluorescence microscopic images were obtained with an Olympus microscope (IMT-2) using an oil immersion objective lens (SPlanApo 60, NA = 1.4) and high-selectivity filters for GFP and Cy3 (Chroma Technology, Brattleboro, VT). Serial optical section data (15–30 focal planes at 0.25- μ m intervals) were collected on a Peltier-cooled charge-coupled device (Photometrics, Tucson, AZ), and computationally processed by three-dimensional deconvolution method (Agard *et al.*, 1989).

Transient Expression of *PEX6* cDNA

The cell suspension (200 μ l, 5×10^7 cells/ml) was mixed with 10 μ g of pUCD2-92A and electroporated (Model BT-600; BTX, San Diego, CA). A cuvette with electrodes 2 mm apart was used, and the settings were 110 V, 3100 μ F, and 72 Ω . Cells from two electroporation cuvettes were mixed and plated into four 60-mm dishes,

cultured for 1, 4, 8 and 24 h, and then used for EM. To intensify signals of catalase histochemistry, 5×10^7 cells/ml ZP92 were transfected with 30 μ g of pCatalaseSKL, 20 μ g of pTet-On (Clontech, Palo Alto, CA), and 2 μ g of pTRE16PEX6. Cells from three electroporation cuvettes were mixed and plated into six 60-mm dishes. Doxycycline was added at the concentration of 1 μ g/ml 16 h after electroporation, and cells were fixed at 1, 4, 8, and 24 h after doxycycline addition.

Western Blot and Pulse-chase Experiment

Proteins were separated on 12% or 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Immobilon-NC standard; Millipore, Bedford, MA). Blots were blocked in PBS containing 1% skimmed milk and incubated with primary antibodies diluted in PBS with 0.1% skimmed milk. The antibodies used were rabbit anti-PMP70, rabbit antimitochondrial thiolase, rabbit anti-85-kDa lysosomal sialoglycoprotein (LGP85; Okazaki *et al.*, 1992; a gift from Dr. Himeno, Kyushu University), rabbit antiprotein disulfide isomerase (PDI; StressGen Biotechnologies Corp, Victoria, British Columbia, Canada) or rabbit anti-lactate dehydrogenase (LDH; Suzuki *et al.*, 1992). After washing with PBS containing 0.05% NP-40 three times, the membrane was incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Amersham, Arlington Heights, IL). After the same wash as that for primary antibody, antibody-decorated bands were visualized with an ECL kit (Amersham). Images were taken and quantified by lumino image analyzer (LAS1000; Fuji Film, Tokyo, Japan). The pulse-chase experiment and immunoprecipitation were done as described previously (Tsukamoto *et al.*, 1990) except that [35 S]methionine and [35 S]cysteine were used instead of [35 S]methionine. Rabbit anti-GFP antibody was purchased from CLONTECH (Palo Alto, CA). Autoradiographic images were taken by an imaging plate and quantified by BAS 2000 or FLA 3000 (Fuji Film).

Electron Microscopic Cytochemistry

Cells were fixed for 1 h at room temperature with 2% glutaraldehyde in 0.1 M HEPES/NaOH (pH 7.4). Cells were postfixed with 1% reduced osmium tetroxide for 1 h at room temperature, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections on copper grids were briefly contrasted with uranyl acetate and lead citrate before examination. For cytochemistry of catalase, fixed cells were incubated for 2 h at 37°C in medium containing 2 mg/ml DAB, 0.1 M glycine buffer (pH 10.5), and 0.02% H_2O_2 before postfixation, and uranyl acetate staining was omitted.

Immunoelectron Microscopy

For postembedding immunoelectron microscopy (immuno-EM), cells were fixed for 1 h at room temperature with 4% formaldehyde and 0.25% glutaraldehyde in 0.1 M HEPES/NaOH (pH 7.4). After rapid dehydration in ethanol, they were embedded in L. R. White. Thin sections on nickel grids were preincubated on a drop of 0.5% BSA in PBS. The sections were incubated overnight in the 1/1000 diluted primary antibody. After being rinsed with PBS, the sections were incubated for 30 min on a drop of protein A-gold (15 nm) prepared as described (De Roe *et al.*, 1987). Sections were briefly contrasted with uranyl acetate and lead citrate before examination.

For preembedding immuno-EM using nanogold, cells were fixed for 1 h at room temperature with 2% formaldehyde and 0.025% glutaraldehyde in 0.15 M HEPES/NaOH (pH 7.4). After three washes with PBS containing 20 mM glycine, cells were blocked and permeabilized with 1% BSA and 1% saponin in PBS for 1 h. Anti-rat PMP70 antibody and anti-rat acyl-CoA oxidase antibody were added to the same solution at dilutions of 1/200 and 1/1000, respectively. After a 5-h incubation with the primary antibody at 4°C, cells were washed with PBS containing 1% saponin and incubated with nanogold-labeled secondary antibody (Nanoprobes, Yaphank,

NY) overnight. The secondary antibody was rinsed away and cells were fixed with 2% glutaraldehyde in 0.1 M HEPES/NaOH (pH 7.4) for 1 h at room temperature. Silver enhancement and gold toning was done as described (Sawada and Esaki, 2000). Cells were rinsed with PBS and postfixed with reduced osmium tetroxide for 1 h at room temperature, dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections on copper grids were briefly contrasted with Nanovan (Nanoprobes) before examination.

Subcellular Fractionation

Cells were harvested from a nearly confluent 100-mm dish with PBS containing 1 mM EDTA. After a wash with homogenization buffer (0.25 M sucrose, 3 mM imidazole, 1 mM EDTA, 0.1% ethanol, pH 7.2), cells were resuspended in 200 μ l of homogenization buffer containing protease inhibitors (10 μ g/ml each of aprotinin, leupeptin and pepstatin, and 1 mM PMSF). Cells were homogenized with a Teflon/glass Potter-Elvehjem homogenizer and a postnuclear supernatant (PNS) fraction was obtained by centrifugation at $1000 \times g$ for 10 min. The PNS fraction was divided into three portions. One part of the PNS was centrifuged for 20 min at $18,500 \times g$, resulting in the cytosolic (S) and organellar pellet (P) fractions. Another part of the PNS fraction was loaded on the top of 500 μ l of 30% Nycodenz (Sigma Chemical Co., St. Louis, MO) and centrifuged for 1 h at $130,000 \times g$. The supernatant was removed by aspiration and peroxisomal pellet (Px) was obtained.

For the complementation experiment, cells were seeded in 100-mm dishes and cultured for 16 h with complete medium containing 5 μ Ci/ml [35 S]methionine and [35 S]cysteine and were further incubated for 1 h without 35 S-labeled amino acids. Labeled ZP92 cells were harvested and transfected with 5 μ g of pCatalaseSKL and 10 μ g of pUcD2-92A by electroporation, plated in a 100-mm dish, and cultured for 24 h before cell fractionation. For immunofluorescence staining with anticatalase antibody or EM cytochemistry, an experiment without 35 S labeling was performed in parallel. Catalase activity was measured as described (Tsukamoto *et al.*, 1990).

RESULTS

Peroxisomal Ghosts in PEX6-deficient CHO Mutant

Immunostaining for PMP70 showed punctate patterns in the PEX6-deficient ZP92 mutant cells (Figure 1A). In contrast, immunostaining for catalase, acyl-CoA oxidase or peroxisomal 3-ketoacyl-CoA thiolase indicated their cytosolic distribution (Figure 1, B–D). This is consistent with previous observations of peroxisomal remnants, so called peroxisomal ghosts, containing peroxisomal membrane proteins but not matrix proteins (Santos *et al.*, 1988a, 1988b).

In wild-type CHO cells, GFP fused with the C-terminal 25 amino acids of rat acyl-CoA oxidase, containing a SKL-COOH tripeptide as PTS-1 showed a punctate pattern of distribution (Figure 2A). This pattern coincided with that of PMP 70 (Figure 2, B and C), hereby indicating the import into peroxisomes, although the relative fluorescence intensities of individual dots of GFPSKL did not necessarily correlate with those of PMP70. In the GFPSKL transformant of ZP92 cells (ZP92/GFPSKL), to our surprise, around 20 GFPSKL-accumulating structures per cell were observed against a background of strong and uniform cytoplasmic and nuclear fluorescence (Figure 2D). In most cases, the GFPSKL-accumulating structures were adjacent to the PMP70-positive structures, but they did not overlap (Figure 2, E and F), and their shapes differed (Figure 2F, insets). Simple GFP was distributed throughout the cells in both wild-type CHO and ZP92, without accumulating to a par-

Figure 1. Peroxisomal ghosts in ZP92 cells. ZP92 cells were fixed and immunostained with anti-rat PMP70 antibody (A), anti-rat catalase antibody (B), anti-rat acyl-CoA oxidase (C) and anti-rat peroxisomal 3-ketoacyl-CoA thiolase (D), and then with Cy3-labeled anti-rabbit IgG secondary antibody. Images were taken by a simple CCD camera without a deconvolution system. Bar: 20 μ m.

ticular structure (our unpublished results). The pattern of GFPSKL accumulation was inconsistent with that of the immunofluorescence of mitochondrial 3-ketoacyl-CoA thiolase (Figure 3A) or distribution of LysoTracker Red stain for detecting the intracellular acidic compartments (Figure 3B). A pulse-chase experiment revealed that PMP70 was very stable in wild-type and ZP92 cells (Figure 4, top panel), and GFPSKL showed similar stability in both cells (Figure 4, bottom panel). Hence, the structures accumulating PMP70 and/or GFPSKL in ZP92/GFPSKL cells are not mitochondria or the intermediates of the autophagosome/lysosome pathway.

The closely neighboring but nonoverlapping localization of GFPSKL and PMP70 suggests that the peroxisomal ghosts in *PEX6*-deficient cells are not simply peroxisomal shells

Figure 3. GFPSKL accumulation in ZP92/GFPSKL is independent of mitochondria or lysosomes. ZP92/GFPSKL cells were fixed and stained with antimitochondrial thiolase antibody (A) or LysoTracker Red (B) and are shown as red. GFPSKL is shown as green. Bar: 10 μ m.

with reduced import ability. In contrast to ZP92, GFPSKL was distributed uniformly in the *PEX2*-deficient Z65 mutant cells (Figure 2, G–I) or in the *PEX5*-deficient ZP102 mutant cells (our unpublished results).

Electron Microscopic Analysis of ZP92 and ZP92/GFPSKL Cells

To characterize the peroxisomal ghosts, we searched for membrane structures present in ZP92 and ZP92/GFPSKL but not the wild-type cells, by EM. In the wild-type cells, peroxisomes positive for catalase in an alkaline DAB reaction were observed as structures bounded by a single membrane (Figure 5A). In ZP92 cells and ZP92/GFPSKL cells, membrane structures such as ER and mitochondria were found, but no alkaline DAB-positive peroxisomes were

Figure 2. GFPSKL accumulation in *PEX6*-deficient mutant cells. CHO/GFPSKL (A–C), ZP92/GFPSKL (D–F), and Z65/GFPSKL (G–I) were fixed and stained with anti-PMP70 antibody. (A, D, and G) GFP fluorescence; (B, E, and H) PMP70; (C, F, and I) merged images of GFP (green) and PMP70 (red). Inset in F is magnified images of the boxed area. Three-dimensional data were recorded by 0.25- μ m Z-axis scanning. To remove out-of-focus data, deconvolution was performed. The deconvoluted pictures of different Z-axis positions were projected into one figure, presenting all fluorescent signals in a single plane. Bar: 10 μ m.

Figure 4. Stability of PMP70 and GFPSKL in CHO or ZP92 cells. CHO, ZP92, CHO/GFPSKL, or ZP92/GFPSKL cells were pulse-labeled with [35 S]methionine and [35 S]-cysteine for 1 h, and chased for 0, 4 and 24 h. PMP70 (top panel) or GFPSKL (bottom panel) was immunoprecipitated with antibodies and separated by SDS-PAGE. Images were taken by a BAS 2000 Image Analyzer (Fuji Film).

present (our unpublished results). Instead, ring-shaped structures enclosed by double membranes were observed (Figure 5B). They had no segmentation, and the space between the two membrane layers was smaller than that in ER. They probably corresponded to the structures reported as “double-membraned loops” in the hepatocytes of the rat treated with an inhibitor of cholesterol biosynthesis

(BM15766; Baumgart *et al.*, 1989) or in the hepatocytes of the *PEX5* knockout mouse (Baes *et al.*, 1997). Almost always, the double-membraned loops of ZP92 and ZP92/GFPSKL cells were accompanied by another type of structures (Figure 5B, open triangles). These structures were judged to be ER, because ribosomes were seen at their cytosolic face on the side opposite to the double-membraned loop (Figure 5B). On the other hand, the double-membraned loops did not have ribosome particles.

The double-membraned loops were often contained in more complicated membrane structures. As shown in Figure 5, C and D, spherical bodies (asterisks) containing electron-dense materials were found in the invagination of double-membraned loops. They had a single membrane and directly faced the cytosol (Figure 5D, arrow). In Figure 5D, another spherical body (closed circle) was present inside the double-membraned loop. Such complicated structures were also observed as nested circular structures composed of several layers of membrane (ER, outer and inner double-layered membranes and a central sphere) in other sections (e.g., Figure 5, E and F). Association of ER was observed in most of such structures (26 of 30 randomly selected ones). In some

Figure 5. Complex membrane structures in *PEX6*-deficient cells. (A) A normal peroxisome in wild-type CHO cells. (B) Double-membraned loop in ZP92/GFPSKL cells. (C and E) Complex membrane structures in ZP92 and (D and F) ZP92/GFPSKL. Open triangles indicate ER. In B, ribosomes were seen around open triangles, and their sizes and electron densities were similar to those of rough ER in A. In C and D, a spherical body (asterisk) is invading into the double-membraned loop. Another spherical body (●) is observed inside the double-membraned loop. Arrowhead in E indicates a possible discontinuity of the outer double-membraned loop. Catalase cytochemistry was done only in A. All samples were postfixed with reduced osmium. (G) Schematic model of the complex membrane structure observed in ZP92 cells. Areas 1–7 are as discussed in the text. Bar: 200 nm.

cases, the outer double-membraned loop seemed to be discontinuous (Figure 5E, arrowhead), but, in most cases, the inner and outer double-membraned loops were each in continuity (Figure 5F). The number of these complicated membrane structures, hereafter referred to as “complex membrane structures,” was comparable to that of the structures containing PMP70 and GFPSKL observed by fluorescent microscopy. This estimation is based on the number of complex membrane structures relative to that of mitochondria in fluorescent microscopy and EM. A schematic model of the complex membrane structure is shown as Figure 5G. Complex membrane structures are probably exaggerated structures derived from normal peroxisome biogenesis intermediates due to the cessation of biogenesis pathway at a restricted step. In fact, such complex membrane structures were not observed in wild-type CHO cells. Equivalent membrane structures might be smaller and/or present temporarily in normal peroxisome biogenesis.

To identify the peroxisome-related membrane structures, immuno-EM was performed on ZP92/GFPSKL cells. By the postembedding protein A gold labeling, anti-PMP70 antibody gave specific signals on the membranes of structures, probably corresponding to the complex membrane structure (Figure 6A). We applied the preembedding method using nanogold-labeled antibody (Tanner *et al.*, 1996) with some modifications. This method gives excellent membrane images with high sensitivity for antigen detection. High saponin concentration (1% and prolonged incubation ensured good penetration of antibody into peroxisomes (our unpublished results). The outer and inner double-membraned loops were PMP70 positive, but the central sphere and ER seemed to be PMP70 negative (Figure 6, A–C). To identify the site of GFPSKL accumulation, immunoelectron microscopic examination with anti-GFP antibody was attempted by both methods. Although many gold particles were present in the peroxisomes of CHO/GFPSKL cells, no specific labeling was observed in any region of ZP92/GFPSKL cells, probably due to the lower level of GFPSKL accumulation (our unpublished results).

Complementation by PEX6 cDNA

Next, we analyzed whether the complex membrane structures become import competent upon complementation by PEX6 cDNA. Cells were cultured for different periods after transfection of PEX6 cDNA and processed for cytochemical staining for catalase. One hour after transfection, there was no significant DAB signal. We could identify complex membrane structures having DAB-positive regions in the double-membraned loops at 4 or 8 h after transfection (Figure 7, A and B, arrowheads). Dilatation of the spaces between double membranes was evident at the catalase accumulation sites. In Figure 7A, three layers of double-membraned loops were seen, but a central sphere was not. In Figure 7B, there were several small membrane structures in the central sphere. Round peroxisomes were also found at 8 or 24 h after transfection (Figure 7C). To intensify the signal of the alkaline-DAB reaction, especially for the early stage of complementation, we engineered a mutant catalase cDNA encoding a typical PTS-1 (KSKL) instead of the original atypical PTS-1 signal (KANL) at the C terminus. This cDNA was cotransfected into ZP92 cells with an expression vector of rat PEX6 cDNA controlled by a tet-On system. To ensure accumula-

Figure 6. PMP70 exists on the complex membrane structure in ZP92/GFPSKL. (A) Sections of ZP92/GFPSKL cells were embedded in LR White and incubated with anti-PMP70 antibody and protein A gold. The membranes appear as negative images. Complex membrane structure is labeled with gold particles representing the antigenic sites for PMP70. (B and C) PMP70 was detected by nanogold-labeled secondary antibody. Complex membrane structures are labeled with gold particles representing the antigenic sites for PMP70. Bar: 200 nm.

tion of catalaseSKL in the transfected cells, doxycycline was added 16 h after electroporation. Strong DAB-positive regions in the double-membraned loops confirmed that catalase accumulated in the complex membrane structures upon complementation (Figure 7, D–G). The dilatation at the accumulation sites was even more significant than those in Figure 7, A and B, probably because of a higher accumulation

Figure 7. Complex membrane structures become import-competent in ZP92 cells upon complementation. (A–C and J) ZP92 cells were transfected with pUcD2-92A. (D–H) ZP92 cells were cotransfected with pCatalaseSKL and rat *PEX6* cDNA under the control of a tet-ON system. (I) ZP92 cells were transfected only with pCatalaseSKL and cultured without doxycycline. (J) ZP92 cells were transfected with pUcD2-92A and processed for immuno-EM with rabbit anti-rat acyl-CoA oxidase, as in Figure 6, B and C. Catalase cytochemistry was performed on all samples except for J. The numbers indicate the time (h) after transfection (A–C, I, and J), or after doxycycline induction (D–H). Arrowheads in A and B indicate DAB signals in the double-membraned loops. The arrow in G indicates a possible connection between the inner and outer lumens of double-membraned loops. Arrowhead in J indicates nanogold signals in the lumen. Bar: 200 nm.

level. Figure 7D shows a typical complex membrane structure DAB-positive in the lumen. Figure 7, E–G, clearly show that DAB signal was present in the outer but not in the inner lumen. A narrow connection between the outer and inner lumens was seen (Figure 7G, arrow). It is noteworthy that no DAB reaction was observed in the spherical body, and catalase was found in a restricted area within the lumen of the double-membraned loop. Catalase accumulation was dependent on *PEX6*, because control transfection of CatalaseSKL cDNA without *PEX6* cDNA resulted in no DAB signals (Figure 7I). We examined whether other peroxisomal matrix proteins also accumulated in the double-membraned loops upon complementation. Preembedding immuno-EM was performed. Complex membrane structures labeled with antiacyl-CoA oxidase antibody were found 8 h after *PEX6* cDNA transfection (Figure 7J, arrowhead). An antibody to peroxisomal thiolase gave numerous cytoplasmic signals (our unpublished results). Hence, unfortunately, we failed to determine whether this enzyme accumulated in the complex membrane structures upon complementation.

These data indicate that in the early stage of *PEX6* complementation, complex membrane structures become important for at least two peroxisome matrix proteins. Because the expression level of Pex6p differed among cells, Figure 7, D–H, does not necessarily represent the temporal sequence of the structural change from the complex membrane structures to mature peroxisomes. However, it was obvious that the number of mature round-shaped peroxisomes with a single membrane (Figure 7H) increased with time, whereas that of DAB-positive complex membrane structure decreased after prolonged incubation.

To elucidate the three-dimensional morphology of the complex membrane structures, we performed serial sectioning. Analysis of the sample at 8 h revealed that the central spherical structure was indeed a closed sphere (Figure 8A, 1–8). The complex membrane structure in these sections lacked a DAB signal, probably because the observed cell did not incorporate the *PEX6* cDNA by chance. In all sections, ER attached to the outer layer of the double-membraned loop, strongly suggesting that ER is a bona fide component of the complex membrane structure, not simply a neighbor of the double-membraned loop. In the complemented cells, three transitional membrane structures from double-membraned loops to peroxisomes were observed (Figure 8B, 1–7). The structure marked by an arrow seemed to be a peroxisome in section 2, but appeared as a double-membraned loop with a DAB signal in section 3. This particular structure was DAB-positive in sections 4–6, but not in section 7. At least three small spheres were distinguishable inside the double-membraned loop (Figure 8B, asterisks). The structure marked with a large arrowhead did not contain a central sphere. Another small double-membraned loop was seen in the 6th and 7th sections (small arrowhead).

Peroxisomes Are Formed from Peroxisomal Ghosts

To elucidate whether the peroxisomal ghosts are converted into peroxisomes, PMP70 was prelabeled with ^{35}S and pursued for the change in its intracellular distribution upon genetic complementation. First, we evaluated the fractionation method. Using wild-type CHO cells, 83% of catalase activity and 92% of PMP70 contained in PNS were recovered in a $18,500 \times g$ pellet containing light and heavy mitochon-

drial fractions (P; Figure 9A, CHO). In ZP92 cells, catalase was mostly recovered in the S fraction, whereas 87% of PMP70 was recovered in the P fraction indicating the presence of peroxisomal membrane remnants. A significant amount of PMP70 in ZP92 cells was reproducibly recovered in the S fraction (18% in Figure 9A and 19% in Figure 9B) together with a microsomal enzyme (Figure 9A, PDI). The conditions of centrifugation ($18,500 \times g$ for 20 min) were not strong enough to pellet all the small vesicles such as microsomes, and almost all PMP70 and PDI were indeed pelleted after $100,000 \times g$ for 1 h centrifugation (our unpublished results). Thus, PMP70 in ZP92 cells are mostly present in the membrane structures, although some PMP70 might exist in small vesicles. It is also probable that the complex membrane structures might be broken in part during homogenization.

To obtain the highly purified peroxisomes with minimum contamination of other organelles including the peroxisomal ghosts, preparation of a peroxisomal fraction (Px) was done by centrifugation through 30% Nycodenz (Ghosh and Hajra, 1986). This method was originally developed to isolate highly purified peroxisomes, although the recovery was low. It was reported that 17% of catalase and 33% of urate oxidase activity of rat liver homogenate were recovered into the Px fraction (Ghosh and Hajra, 1986). This method was most suitable for our purpose in spite of the lower recovery, because we needed to obtain highly purified peroxisomes devoid of the ghosts to show that PMP70 prelabeled with ^{35}S appeared in peroxisomes upon complementation.

With wild-type CHO cells, 40% of catalase activity and 32% of PMP70 contained in PNS were recovered in the peroxisomal fraction (Px; Figure 9A, CHO). In ZP92 cells, catalase was rarely found in the Px fraction. Although 87% of PMP70 was recovered in the P fraction, only 2% was recovered in the Px fraction (Figure 9A, ZP92). Western blotting using antibodies against organelle marker proteins (mitochondrial thiolase, LGP85 [lysosomes], PDI [ER], and LDH [cytosol]) gave no bands in the Px fraction (Figure 9A). Taken together, the Px fraction contained peroxisomes with minimum contamination of other organelles, including the peroxisomal ghosts.

Using this fractionation method, we examined whether the peroxisomal ghosts of ZP92 are converted into the peroxisomes after complementation. Cells were labeled with [^{35}S]cysteine and [^{35}S]methionine for 16 h and further cultured without ^{35}S label for 1 h before *PEX6* cDNA transfection. If peroxisomes are derived from the peroxisomal ghosts, the restored peroxisomes should contain ^{35}S -labeled PMP70. Western blotting using anti-PMP70 antibody revealed that 17% of PMP70 was recovered in the Px fraction 24 h after *PEX6* transfection, whereas <1% without *PEX6* transfection (Figure 9B, top panel). This 17% recovery is half of that in CHO cells (32%, Figure 9A) and is consistent with the complementation efficiency (53%, 65 cells in 123 cells counted), as judged by the immunofluorescence staining with anticatalase antibody on the cells transfected in parallel. Recovery of ^{35}S -labeled PMP70 in the Px fraction was assessed by immunoprecipitation and autoradiography. The ^{35}S -labeled PMP70 appeared in the Px fraction only for the *PEX6*-transfected cells at a significant level (14%, Figure 9B, bottom panel), and this value was similar to the total recovery of PMP70 in the Px fraction (17%, see above). The recov-

Figure 8. Serial sections of complex membrane structures. Serial ultrathin sections were made from the 8-h sample of Figure 7, F and G. Two series, A and B, are shown. The numbers indicate the order of sections. The 5th section of series A was not analyzed because of shrinking. Arrows indicate complex membrane structures with spherical bodies. Asterisks in series B indicate the spheres, the surrounding membrane not being clear in the 4th sections, probably due to oblique sectioning. Large and small arrowheads indicate double-membraned loops positive for DAB. Bar: 200 nm.

Figure 9. Peroxisomal ghosts are converted into peroxisomes upon complementation. After subcellular fractionation, equivalent amounts of the $1000 \times g$ postnuclear supernatant (PNS), $18,500 \times g$ supernatant (S), $18,500 \times g$ pellet (P), and $130,000 \times g$ Nycodenz pellet (Px) were analyzed by Western blotting. Percent recoveries of PMP70 and catalase activity from PNS are shown. (A) Subcellular fractions of CHO and ZP92 cells were analyzed by Western blotting with antibodies specific for PMP70, mitochondrial thiolase, LGP85 (lysosome), PDI (ER), and LDH (cytosol). (B) Recovery of PMP70 after transfection with pCatalaseSKL (left) or pUcD2-92A (PEX6) and pCatalaseSKL (right). Parallel experiments without (top) or with (bottom) ^{35}S labeling were done. Recoveries of PMP70 (Western) and ^{35}S -labeled PMP70 (immunoprecipitation) were determined. n.d., not detectable.

ery of PMP70 into the Px fraction was 32% in the wild-type cells (Figure 9A), and the efficiency of complementation was 53%. Thus, 14% recovery of labeled PMP70 into the Px fraction means that 82% ($14\%/0.32/0.53$) of labeled PMP70 is now present in the peroxisomes in the complemented cells. Reduction of PMP70 in the S fraction upon complementation (from 19 down to 10% by Western blotting, from 23% down to 11% by immunoprecipitation) can be explained as an average of the values of uncomplemented and complemented cells and is too small to match the recovery of ^{35}S -labeled PMP70 in the Px fraction. These results indicate that almost all of the PMP70 in peroxisomal ghosts became present in the peroxisomes upon complementation. EM analysis revealed that 93% (67/72) of the DAB-positive structures were round peroxisomes with single membrane at this time point, assuring that PMP70 in the Px fraction is

present in the peroxisomes, not in the complex membrane structures containing catalase. Although we could not exclude the possibility that peroxisomes were also formed *de novo*, our results clearly indicate that almost all the peroxisomal ghosts were converted into peroxisomes upon complementation.

DISCUSSION

Peroxisomal Ghosts in the PEX6-Deficient Mutant Are Complex Membrane Structures

The peroxisomal membrane ghosts were identified as empty membrane structures larger than normal peroxisomes and independent of lysosomes (Santos *et al.*, 1988b). In contrast, it was reported that most of "peroxisomal ghosts" in human fibroblasts from patients with inherited peroxisome biogenesis disorder contained lysosomal enzymes (Heikoop *et al.*, 1992). Low level import of matrix proteins into peroxisomes has been reported in the fibroblasts of peroxisome-deficient patients by immunofluorescence microscopic study (Slawski *et al.*, 1995). In our study, no accumulation of endogenous matrix proteins was found in the peroxisomal ghosts. However, GFP containing typical PTS-1 at its C terminus accumulated adjacent to the peroxisomal ghosts. Our observations suggest that the peroxisomal ghosts are not simple vesicles surrounded by PMP-containing membranes with reduced import ability. They are independent of other organelles such as mitochondria and lysosomes.

EM analysis revealed that complex membrane structures were present in ZP92 or in ZP92/GFPSKL cells. In typical cases, we could distinguish seven areas in this complex membrane structure (Figure 5G). Area 1 (central sphere) was always separated from the cytosol by a single membrane and the electron density was higher than that of the cytosol. PMP70 was not present on the membrane of the spherical body (Figure 6, B and C). An alkaline DAB reaction was not detected in the central sphere in ZP92 cells transfected with CatalaseSKL cDNA (Figure 7I). No DAB staining was observed in this region even upon the complementation by PEX6 cDNA, although it resembled an empty peroxisome morphologically (Figure 7, D and G). Central sphere was not observed in the double-membraned loops of some sections (Figures 5B and 7A). We cannot tell whether the loop structures lacking the central sphere were real or simply appeared so because the section plane did not cross the sphere. In addition, a simple double-membraned loop (Figure 5B) may have resulted from sectioning a part of the complex membrane structure. The entity of this spherical body is unknown and should be characterized by future studies.

Areas 2 and 6 are clearly connected to the cytosol. Lumens of the inner (area 3) and outer (area 5) double-membraned loops are connected to each other. Invasion of the central spherical body into a large double-membraned loop results in an outer and inner double-membraned loops. During the complementation, catalase accumulated in the lumens of double-membraned loops, especially in the outer lumen (area 5). Serial section analysis revealed the presence of apparent "peroxisomes" physically continuous to the double-membraned loop. Such a structural transition has been reported in the hepatocytes of rats treated with a peroxisome proliferator (BM 15766; Baumgart *et al.*, 1989). Area 4, the space between two double membranes, had an electron den-

sity similar to that of the cytosol, and sometimes a single-membraned structure was found inside (Figure 5D, closed circle). A possible connection between area 4 and the cytosol is seen in Figure 5E. However, this opening is so small that we cannot exclude the possibility of oblique sectioning of a continuous membrane. Area 7 is a part of ER because it is sometimes attached with ribosomes, and its membrane does not contain PMP70. Close association between ER and peroxisomes was reported in the early morphological studies (Novikoff and Novikoff, 1982). We did not obtain direct evidence for the localization of GFPSKL in the complex membrane structure by immuno-EM. Area 1 is the only membrane-bounded space similar in size to the area of GFPSKL accumulation. Because DAB reaction was observed in the lumen of the double-membraned loops, area 3 or 5 is also a candidate for the place of GFPSKL accumulation. However, we did not observe dilation in these areas in ZP92/GFPSKL cells.

Our observations were different from those for the original peroxisomal ghosts described by Santos *et al.* (1988a, 1988b). They used fibroblasts (GM 4340) from a peroxisome-deficient patient of complementation group C (group 4 at the Kennedy Krieger Institute), and hence the pathogenic gene was *PEX6* (Fukuda *et al.*, 1996). They mentioned that the ghost was a roughly spherical structure, larger than the normal peroxisome, and contained little material (Santos *et al.*, 1988a). In *PEX1* and *PEX6* mutants of *Y. lipolytica*, the accumulation of an extensive network of ER membranes as well as a significant reduction in the size and number of peroxisomes was shown by an EM analysis (Titorenko and Rachubinski, 1998). However, we did not find such development of ER. At present, we do not know the reason for the discrepancy between our results and those of previous reports.

How Does PEX6 Deficiency Affect the Matrix Protein Import?

There is no doubt that the DAB-negative complex membrane structures were converted to the DAB-positive structures because of their morphological identity. Our experiment using ³⁵S-labeled PMP70 indicates that almost all the peroxisomal ghosts were converted into peroxisomes upon complementation. *PEX6* dysfunction seems to cause complex membrane structures, rather than peroxisomes with reduced matrix protein import ability. It is then important to understand how the *PEX6* mutation causes and how the forced expression of Pex6p corrects the defect of matrix protein import into peroxisomes. Recently, Pex6p and Pex1p of the yeast, *P. pastoris*, were suggested to be present in different small vesicles distinct from peroxisomes, based on biochemical evidence (Faber *et al.*, 1998). Using another yeast species, *Y. lipolytica*, the presence of five distinct subpopulations of small peroxisomal vesicles (P1–P5) was shown, and Pex6p and Pex1p are required for the fusion of P1 and P2 (Titorenko *et al.*, 2000; Titorenko and Rachubinski, 2000). Although there is no direct evidence that the central sphere and double-membraned loop correspond to P1 and P2, it is an attractive hypothesis that the central sphere and the double-membraned loop have different sets of Pex proteins and that assembly of all Pexps on the same membrane structure (probably the double-membraned loop) is attained by fusion involving Pex6p and Pex1p, hereby resulting in an

efficient import. Analysis of the localization of individual Pex proteins in the complex membrane structures in the mutants and wild-type cells at the ultrastructural level will facilitate understanding the origin and role of each membranous component and finally the mechanism of peroxisome biogenesis.

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