Catalase-less Peroxisomes

IMPLICATION IN THE Milder FORMS OF Peroxisome Biogenesis Disorder*

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We established a Chinese hamster ovary cell line having a temperature-sensitive phenotype in peroxisome biogenesis.
This mutant (65TS) was produced by transforming a PEX2-defective mutant, Z65, with a mutant
PEX2 gene, PEX2E55K, derived from a patient with infantile Refsum disease, a milder form of peroxisome biogenesis disorder. In 65TS, catalase was found in the cytosol at a nonpermissive temperature (39 °C), but upon the shift to a permissive temperature (33 °C), catalase gradually localized to the structures containing a 70-kDa peroxisomal membrane protein, PMP70. In contrast to catalase, other matrix proteins containing typical peroxisome targeting signals, acyl-CoA oxidase and peroxisomal 3-ketoacyl-CoA thiolase, were co-localized with PMP70 in most cells, even at 39 °C. We found that these structures are partially functional peroxisomes and named them “catalase-less peroxisomes.” Catalase-less peroxisomes were also observed in human fibroblasts from patients with milder forms of peroxisome biogenesis disorder, including the one from which the mutant PEX2 gene was derived. We suggest that these structures are the causes of the milder phenotypes of the patients. Temperature-dependent restoration of the peroxisomes in 65TS occurred even in the presence of cycloheximide, a protein synthesis inhibitor. Thus, we conclude that in 65TS, catalase-less peroxisomes are the direct precursors of peroxisomes.

Peroxisomes are present in a wide variety of eukaryotic cells, from yeast to human (1). Peroxisomes commonly contain catalase and at least one enzyme that generates hydrogen peroxide. The roles of mammalian peroxisomes include oxidative processes involving H₂O₂. The oxidation of fatty acids, and biosynthesis of plasmalogens. More than 20 genes named PEX (2) have been shown to be involved in peroxisome assembly processes, based on the functional complementation of genetically peroxisome-deficient mutants of mammalian cells and yeasts (3). Peroxisomes have been thought to be formed by the division of preexisting peroxisomes following the import of newly synthesized peroxisomal proteins (4), but recent observations suggest that endoplasmic reticulum may be involved in the peroxisomal membrane biogenesis at least in certain yeast species (5, 6), for review, see Ref. 7. Thus, questions are again being raised about the intracellular origin of peroxisomes.

Peroxisome biogenesis disorders (PBDs) are caused by abnormalities in the assembly processes of peroxisomes. In many PEX mutant cells, including the fibroblasts from PBD patients, abnormal membrane structures called peroxisomal ghosts are observed (8, 9). These structures contain a 70-kDa peroxisomal matrix protein (PMP70) but lack the peroxisomal matrix proteins. PBDs are genetically classified into at least 12 complementation groups (CGs) (10), and each CG contains various clinical phenotypes, e.g. Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD). ZS represents the severest form of PBD, NALD is the next severest, and IRD is the mildest (11). These diseases differ markedly in clinical features, such as age of death and severity of the neurological abnormalities.

In the fibroblasts of PBD patients with milder forms, temperature sensitivity was observed in peroxisome assembly, and heterozygous mutations (E55K/R119Stop) were identified in the PEX2 genes of an IRD fibroblast line of CG-F (12). Temperature-sensitive (TS) mutations were further identified in PEX1 (13) as well as PEX13 (14). The TS mutant cells seem to be useful tools for investigating the mechanisms of peroxisome biogenesis, because the peroxisome recovery process can be turned on simply by shifting the culture temperature. Accordingly, we established a stable Chinese hamster ovary (CHO) cell transformant with PEX2E55K (65TS) on the background of a PEX2-defective mutant, Z65 (15). In 65TS, peroxisomes formed temperature-dependently. We now report that in 65TS the peroxisomal ghosts indeed are partially functional “catalase-less peroxisomes.” Similar structures were also observed in the fibroblasts from several patients with milder forms of PBD, including the original IRD fibroblast line of CG-F. We suggest that catalase-less peroxisomes are the cause of the milder phenotypes of these patients. In addition, we present evidence that peroxisomes are formed from the catalase-less peroxisomes in 65TS upon temperature shift-down.

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1 The abbreviations used are: PBD, peroxisome biogenesis disorder;
PMP70, 70-kDa peroxisomal membrane protein; CG, complementation
group; ZS, Zellweger syndrome; NALD, neonatal adrenoleukodystro-
phy; IRD, infantile Refsum disease; TS, temperature-sensitive; CHO,
Chinese hamster ovary; GFP, green fluorescent protein; AOX, acyl-CoA
oxidase; PTS, peroxisome targeting signal; PT, peroxisomal 3-ketoacyl-
CoA thiolase; VLCFA, very long chain fatty acid; DHAP-AT, peroxiso-
mal dihydroxyacetone phosphate acyltransferase.
Establishment of the TS CHO Mutant—The human PEX2<sup>EX55K</sup>-defective CHO cell mutant, Z65, by the calcium phosphate method. Stable clones were selected by limiting dilution, by culturing the transfectants for 6 days in the presence of 400 μg/ml G418. One of the stable transformants revealed a punctate distribution of catalase after 24 h of incubation at 30 °C but no catalase-positive granules at 37 °C, like Z65. We named this clone 65TS. To visualize the peroxisomes in living 65TS cells, we produced a transformant stably expressing green fluorescent protein (GFP)-SKL. This fusion protein has a carboxy-terminal 25 residues of rat acyl-CoA oxidase (AOX) containing the Ser-Lys-Leu-COOH tripeptide (a typical peroxisome targeting signal (PTS)-1) motif (17, 18) at the carboxyl terminus of GFP(105). GFP(105) is a variant green fluorescent protein having much improved fluorescence at higher temperatures (19). An expression vector of GFP-SKL, pGFP-SKL, and pMiwph, a vector carrying a marker gene conferring hygromycin resistance, were co-transfected to 65TS by the calcium phosphate method. Selection was carried out by limiting dilution, by culturing the transfectants for 9 days in the presence of 400 units/ml hygromycin B, and then by culturing for 5 days with both G418 and hygromycin B. A stable clone with a punctate distribution of GFP at 30 °C was isolated and named 65TS-TSKL.

Cell Culture—Cells were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum. The fibroblasts from PBD patients maintained at 37 °C in 5% CO<sub>2</sub> and TS CHO mutant cells were maintained at 39 °C in 5% CO<sub>2</sub>. For the analysis of temperature-dependent peroxisome restoration, temperature pairs of 37/30 °C and 39/33 °C were used for human fibroblasts and CHO cells, respectively. Higher temperatures were used for the CHO-derived cells, because they grew much faster at 33 °C than 30 °C and exhibited clearer TS phenotypes at 39 °C than at 37 °C.

Immunofluorescence Studies—Peroxisomes of CHO cells and human fibroblasts were visualized by indirect immunofluorescence staining, as described (20). The first antibodies used were rabbit antibodies to rat catalase (from Dr. N. Usuda), rat AOX, rat peroxisomal 3-ketoacyl-CoA thiolase (PT), and human catalase (each from Dr. T. Hashimoto), and rat PMP70 or guinea pig antibodies to rat PMP70 and catalase. The anti-PMP70 antibodies were prepared using the carboxy-terminal 15-residue peptide of the protein as an antigen (21). The antigen-antibody complexes were detected with fluorescein isothiocyanate- or Cy3-labeled goat antibody to rabbit IgG or Cy3-labeled donkey antibody to guinea pig IgG.

Radio-labeling of Cells—Cells were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum for 2 days at selected temperatures. The medium was changed to Dulbecco’s modified Eagle’s medium, the drug was added at varying concentrations to the medium 1 h before the addition of radioactive amino acids, and the radioisotope was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, as described (22). For the experiment on the effect of cycloheximide, the drug was added at varying concentrations to the medium 1 h before the addition of radioactive amino acids, and the radioisotope was allowed to be incorporated for 24 h at 33 °C.

RESULTS

Isolation and Characterization of the TS CHO Mutant—We previously showed that an IRD patient with CG-F PBD carried heterozygous mutations in the PEX2 gene, E55K and R119Stop (12) (Fig. 1A), and the former was responsible for the TS phenotype of the patient’s cells in peroxisome assembly. We isolated a temperature-sensitive CHO mutant, 65TS, by introducing the human PEX2 gene into a PEX2-defective mutant, Z65 (15). Z65 itself exhibited a punctate distribution of catalase at neither 33 °C (Fig. 1B, a) nor 39 °C (data not shown), although PMP70 exhibited a punctate staining pattern, representing the ghosts (Fig. 1B, b). In 65TS, catalase was found in the cytosol at 39 °C (Fig. 1B, c), whereas PMP70 had a particular distribution (Fig. 1B, d). When 65TS was incubated at 33 °C for 48 h, however, catalase was co-localized with PMP70 in almost all the cells (Fig. 1B, e and f). We pursued the time course of peroxisome recovery in 65TS (Fig. 2). After 3 h of incubation at 33 °C, we observed some cells containing a few punctates of catalase. After 6 h, we could identify in many cells catalase-positive granules that were also positive for PMP70 (data not shown). Most cells contained catalase-positive granules after 24 h of incubation at 33 °C, and virtually all cells had peroxisomes by 48 h. Thus, it took at least 3 to 6 h for the recovery of peroxisomes in 65TS, and the process was completed within 48 h.

With respect to the peroxisome assembly, 65TS shuttled between the phenotypes similar to the wild-type CHO and Z65, depending on the temperatures. Fibroblasts from a TS patient belonging to CG-F (F-05) lack the peroxisomes, like the fibroblasts from ZS patients at 37 °C, but when incubated at 30 °C, the F-05 fibroblasts regain the peroxisomes to the same level as those of the normal fibroblasts (12). Thus, the phenotypes of 65TS seemed similar to those of the fibroblasts from the TS patient and would be useful for studying the mechanism of peroxisome biogenesis and molecular defects of TS PBDs.

Proteins Carrying Typical PTSs Are Localized to Peroxisome-like Structures at the Nonpermissive Temperature—Using...
GFP-SKL carrying a typical PTS-1 at the carboxyl end, we tried to visualize the peroxisomes in living 65TS cells. Interestingly, the stable transformant expressing GFP-SKL, named 65TS-TSKL, revealed a punctate GFP distribution consistent with that of PMP70 at 39 °C (Fig. 3, a and b) as well as at 33 °C (Fig. 3, c and d). This was in contrast to the cytosolic distribution of catalase at 39 °C (Fig. 3, e and f). Catalase exhibited punctate staining superimposable with the GFP-SKL distribution in 65TS-TSKL at 33 °C (Fig. 3, g and h). Even at the permissive temperature, however, the punctate distribution of catalase and expression of GFP-SKL were complementary to each other, i.e. catalase-positive particles were only observed in the cells weakly expressing GFP-SKL (see Fig. 3 legend). On the other hand, we observed that AOX and PT, having a typical PTS-1 and PTS-2, respectively, were colocalized with GFP-SKL to particulate structures at 39 °C (data not shown). Catalase has a variant PTS-1, KANL, at the carboxyl terminus. Thus, we reasoned that the proteins carrying typical PTSs would be translocated to the peroxisome-like structures more easily than catalase. Typical PTS proteins but not catalase would be translocated at 39 °C, and the former would be more efficiently imported to peroxisomes than the latter at 33 °C.

Catalase-less Peroxisomes in 65TS Cells at the Nonpermissive Temperature—We next examined the localization of the peroxisomal matrix proteins in the original 65TS cells instead of 65TS-TSKL (Fig. 4A). When 65TS cells were stained with anti-AOX or anti-PT antibody, punctate staining overlapping with that of PMP70 was observed, even at 39 °C. In a parallel experiment, we confirmed that catalase was diffusely distributed in the cytosol at this temperature (data not shown). Thus, the proteins having typical PTSs were indeed selectively translocated to peroxisome-like structures at the nonpermissive temperature. Catalase, on the other hand, was hardly imported to these structures under the same conditions. This seems rather surprising, because catalase has been used as a representative marker of peroxisomes.

We examined whether these peroxisome-like structures had normal biochemical functions of peroxisomes, based on several criteria. We first inspected whether AOX was correctly processed in these structures by a radiolabeling experiment, as described (22). For 65TS incubated at 33 °C, processing of AOX (proteolytic conversion of the component A to components B and C (18), although component C was hardly detected under these conditions) was observed to the same extent as in the wild-type CHO (Fig. 4B). Comparable amounts of the enzyme (the sum of components A and B) were recovered from 65TS and the wild-type CHO; these amounts were significantly larger than that from Z65, reflecting the stabilization of the enzyme by segregation into peroxisomes. It was indeed shown (15) that AOX is highly unstable in Z65 and stabilized upon complementation by the normal PEX2 gene. These results in-

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**Fig. 2.** Time course of peroxisome recovery in 65TS. The cells were first cultured at 39 °C and then kept for the period indicated at 33 °C. Although only the immunostaining patterns for catalase are shown, FMIP70 staining was also carried out to confirm the co-localization. Bar, 20 μm.

**Fig. 3.** GFP-SKL is accumulated in peroxisome-like structures in the TS cells even at 39 °C. Cells of the stable transformant of 65TS with GFP-SKL, 65TS-TSKL, were cultured at 39 °C (a, b, e, and f) or 33 °C (c, d, g, and h). The green fluorescence of GFP-SKL (a, c, e, and g) was observed on the same field as that for the immunostaining of PMP70 (b and d) and catalase (f and h), with the Cy3-labeled second antibody. Pairs of pictures of the same fields are presented side by side. Bar, 10 μm. Note that all GFP-SKL transformant cells did not express GFP-SKL efficiently, as particularly emphasized in e and g. We usually observe such variable expression of stably integrated ectopic genes among the cells derived from a single clone, even when the cells are maintained in selection media. This phenomenon is probably related to the stochastic nature of the actions of gene enhancers to increase the probability but not the level of gene expression (44). Thus, a substantial number of the cells in a given population would not express the gene under consideration, by chance.
Translocation of AOX and PT to peroxisome-like structures in 65TS at 39 °C. A, colocalization of AOX and PT with PMP70, PMP70 (b and d), and PT (c). Pairs of pictures of the same fields are presented side by side. Bar, 10 μm. B, radiolabeling of AOX. Wild-type CHO (lanes 1 and 4), Z65 (lanes 2 and 5), and 65TS (lanes 3 and 6) were radiolabeled with [35S]methionine/cysteine for 24 h at 39 °C (lanes 1–3) or 33 °C (lanes 4–6). Radiolabeled AOX was immunoprecipitated, separated by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. The full-length polypeptide A and processing product B are indicated by arrowheads. The smaller processing product C was hardly detectable. A nonspecific faint band usually appears at the same position as that of band B (15).

Table I

|                                | 37 °C | 30 °C |
|--------------------------------|------|------|
| C24/C16                        | 5.53 | 11.5 |
| Z65                           | 0.68 | 0.78 |
| 65TS                           | 10.9 | 11.7 |
| DHAP-AT                        |      |      |
| CHO                            | 1.06 | 1.45 |
| Z65                           | 0.27 | 0.25 |
| 65TS                           | 1.24 | 0.91 |

lower processing activity of AOX. We named the structures catalase-less peroxisomes.

Catalase-less Peroxisomes in Human PBD Fibroblasts—Catalase-less peroxisomes were also found in the IRD fibroblast line of CG-F that originally carried the PEX2535K mutation (Fig. 5). Although catalase exhibited only diffuse cytosolic staining, both AOX and PT did exhibit a punctate distribution in many cells at 37 °C. The punctate pattern was more prominent and found in a higher percentage of cells (more than 70% of the total) with anti-PT antibody than with anti-AOX antibody, indicating an efficient translocation of PT in this IRD cell line. AOX, PT, and catalase were all cytosolic in the ZS cells of CG-E (F-01) (data not shown). It should be noted that the VLCFA β-oxidation and DHAP-AT activities of the same CG-F TS human fibroblasts as used in this study (F-05) were considerably higher than those of the CG-F ZS fibroblasts, even at 37 °C (24). Thus, the milder phenotypes of this IRD patient are likely to have resulted from these catalase-less peroxisomes, having partial biochemical functions at 37 °C. It should be pointed out, however, that the functions of the catalase-less peroxisomes of human fibroblasts were significantly reduced compared with those of 65TS, with respect to these biochemical parameters.

Catalase has usually been used as the definitive marker to discriminate normal peroxisomes from nonfunctional peroxisomal ghosts. The presence of catalase-less peroxisomes in an IRD patient of CG-F (F-05) raised the question whether patients with milder forms of PBD generally have hitherto overlooked similar structures. Accordingly, we examined this possibility with the fibroblasts from an NALD patient of CG-C (C-11) and an NALD and IRD patient of CG-E (E-13 and E-24, respectively; see Ref. 13). The responsible genes of CG-C and CG-E are PEX6 (16, 25, 26) and PEX1 (27–29), respectively, both coding for the members of the AAA-ATPase family. Interaction between these two peroxins was shown (30–32), and hence it would seem reasonable that a defect in either of them would result in similar phenotypes. In addition, a TS mutation (G843D) has been identified in the PEX1 gene of patient E-24 (13). When these fibroblasts were cultured at 37 °C, catalase was found in the cytosol, without a punctate pattern of distribution (Fig. 6). On the other hand, AOX and PT exhibited punctate staining in around 80% of cells (Fig. 6), although the signals were weaker, and the number of particles in each cell was smaller than in normal cells (data not shown). These particles were mostly superimposable with those of PMP70 and hence regarded as peroxisome-related structures, although
some particles seemed to be irregular protein aggregates not overlapping with PMP70 signals. Heavy abnormal aggregations of AOX and PT were observed in a considerable number of cells, especially for C-11 and E-13 (data not shown). All cells contained many PMP70-positive particles. A considerable number of these particles were negative or below the detection limit for the staining of AOX or PT. Thus, the import of AOX and PT in these mutant cells seemed less efficient than that in normal cells. We observed no such particulate staining of AOX in ZS cells of the same CGs, although weak particulate signals of PT were found in a small number of cells (data not shown). Hence, we conclude that the cells of milder PBD phenotypes contain catalase-less peroxisomes for at least CGs C, E, and F, albeit at lower levels of matrix protein accumulation in these structures.

**Peroxisomes Are Formed from the Catalase-less Peroxisomes in 65TS Cells**—In 65TS cells, peroxisomes were formed temperature-dependently. To examine whether peroxisomes were restored from the preexisting catalase-less peroxisomes by the import of catalase from the cytosol, we investigated whether the recovery process was inhibited by cycloheximide, a protein synthesis inhibitor. 65TS cells cultured at 39 °C were further cultured for 24 h at 33 °C in the presence or absence of 10 μg/ml cycloheximide. Immunostaining of catalase showed that the enzyme was colocalized with PMP70 even in the presence of the drug (Fig. 7A, c and d), although the PMP70-containing structures seemed to be large in size and irregular in shape as compared with those of the cells cultured without the drug (Fig. 7A, a and b). By radiolabeling the cells, we confirmed that this concentration of cycloheximide completely inhibited the synthesis of catalase and PMP70 (Fig. 7D). Total protein synthesis also virtually ceased at this concentration of the drug (Fig. 7D), and the incorporation of radioactivity into trichloroacetic acid-insoluble materials decreased by 97% under these conditions (data not shown).

These results suggest that the cytosolically distributed catalase became colocalized with the pre-accumulated PMP70 upon temperature shift, without requiring de novo protein synthesis. This also means that pre-accumulated mutant Pex2p is converted from a less active to an active form upon temperature shift, which in turn supports the translocation of catalase. Hence, we conclude that peroxisomes are derived from the catalase-less peroxisomes in 65TS. These results well conform to the notion that peroxisomes are formed from the ghosts by functional complementation of the peroxisome-deficient mutant cells (33). In addition, these results support the hypothesis that peroxisomes can develop by incorporating the newly synthesized peroxisomal proteins from the cytosol.

**DISCUSSION**

The present results indicate that transfer of the **PEX2**$^{E55K}$ gene into the **PEX2**-defective CHO mutant, Z65, transmitted the TS phenotype observed in the F-05 IRD fibroblasts. In the resulting CHO line, 65TS, catalase was found in the cytosol at 39 °C, but its distribution became peroxisomal upon shift-down to 33 °C. On the other hand, AOX and PT carrying a typical PTS-1 and PTS-2, respectively, exhibited a peroxisomal distribution in many cells, even at 39 °C. Although catalase seems to be translocated by the PTS-1 pathway, its KANL peptide sequence at the carboxyl terminus deviates from the consensus (34). Therefore, the complex of catalase and Pex5p, the PTS-1 receptor, is possibly formed with lower efficiency than complexes of typical PTS-1 proteins. This would make the steady state concentration of catalase-Pex5p too low to be captured by the peroxisomal import machinery containing the TS Pex2p, at the nonpermissive temperature. The effect of poorer recognition by the receptor is accentuated when catalase must compete with an excess amount of a typical PTS-1 protein (Fig. 3, g and h). In this situation, catalase is not translocated to peroxisomes to a significant extent even at 33 °C. Proteins carrying typical PTSs, however, form high steady state concentrations of the complexes with the receptors, high enough to be recognized even by the less active import machinery at the nonpermissive temperature.

The restoration of catalase import at the permissive temperature clearly depends on the transition of the mutant Pex2p from the less active (or inactive) state to the (more) active state, because the process was not inhibited by cycloheximide. The mutant Pex2p would be integrated into the membrane of the catalase-less peroxisomes normally even at the nonpermissive temperature, thus supporting the import of typical PTS proteins and, upon temperature shift-down, the import of catalase. It is reasonable to suspect that the mutant Pex2p is in an equilibrium between the two states at any temperature. Thus, at 39 °C, the total activity of the import machinery involving the mutant Pex2p would be insufficient to support the translocation of catalase but nearly sufficient to take up typical PTS proteins, whereas the activity would reach a level high enough to allow the catalase import at 33 °C. To verify this notion, we expressed the **PEX2**$^{E55K}$ gene at a lower level by putting the gene under the control of the basal promoter of the herpes simplex virus thymidine kinase gene. This resulted in incomplete import of AOX and PT at 39 °C and poor catalase import even at 33 °C (data not shown). Thus, the import efficiencies of individual peroxisomal matrix proteins depend on a delicate balance between the intrinsic intensities of the individual import signals and the net activity of the peroxisomal import machinery.

The peroxisome-like structures observed in 65TS cells at 39 °C, which contain AOX and PT but not catalase, indeed are nearly functional catalase-less peroxisomes (Table I and Fig. 4B). AOX was equally stable at 39 °C as in the wild-type CHO cells, probably reflecting the effective segregation into the peroxisomal import machinery.
and fibroblasts of CG-E (E-24; m–r) were cultured at 39 °C and then for 24 h in the absence of cycloheximide. [35S]methionine/[35S]cysteine and cycloheximide at the concentrations respectively. 65TS cells were incubated at 33 °C in the presence of [35S]methionine/[35S]cysteine and cycloheximide at the concentrations respectively. 65TS cells were incubated at 33 °C in the presence of cycloheximide at 33 °C. Thus, the patient fibroblasts of CG-F and the 65TS model CHO cells had similar TS phenotypes, but the functional defect at the nonpermissive temperature was much more prominent in the former. This was probably due to the weaker expression of the endogenous PEX2 gene in the human fibroblasts than of the ectopic PEX2 gene under the control of the viral expression system, which was carried by 65TS. The present observations suggest that the partially functional catalase-less peroxisomes would support the milder phenotypes of NALD and IRD patients, at least in the cases studied. Evaluation of peroxisomes of PBD patients based on immunofluorescent microscopy should be done not only for catalase but also for typical PTS proteins such as β-oxidation enzymes.

Import of catalase to the catalase-less peroxisomes at the permissive temperature was not inhibited by cycloheximide, a protein synthesis inhibitor. The most feasible interpretation of these results would be that the catalase pre-accumulated in the cytosol directly enters the preexisting catalase-less peroxisomes. The involvement of a vesicle fusion process in peroxisome biogenesis was proposed, based on the results of biochemical studies (36). It was also suggested that catalase import occurs only to immature peroxisomes (or precursor vesicles), not mature peroxisomes. Our results would be compatible with this model if we suppose the involvement of recycling membrane vesicles in the translocation of pre-accumulated catalase to the catalase-less peroxisomes.

The present results are consistent with the result for the PEX5 mutant CHO cells (33) that the restoration of peroxisomes by the microinjection of Pex5p can occur in the presence of cycloheximide and hence involves the translocation of the cytosolically accumulated PTS proteins into the peroxisomal ghosts. Catalase-less peroxisomes as well as peroxisomal ghosts are the structures that form on the blockade of the normal course of peroxisome biogenesis because of a deficiency of one of the essential PEX gene products. Thus, these structures reflect the processes of peroxisome biogenesis, even if they themselves do not represent the biogenesis intermediates. They are ready to accept the PTS proteins if the correct PEX gene products are supplied. The shapes and biochemical compositions, however, may differ depending on the defective gene as well as the severity of deficiency. The recovery processes also probably differ depending on the defective genes. Moreover, net formation of the peroxisomal membrane from a certain membrane seems essential in the mutants defective in one of the three PEX genes, PEX3, PEX16, and PEX19, because these

FIG. 6. Catalase-less peroxisomes of the CG-C and CG-E fibroblasts. NALD fibroblasts of CG-C (C-11; a–f) and CG-E (E-13; g–l) and IRD fibroblasts of CG-E (E-24; m–r) were cultured at 37 °C and immunostained for catalase (a, g, and m), AOX (c, i, and o), PT (e, k, and q), and PMP70 (b, d, f, h, j, l, n, p, and r). Pairs of pictures presented side by side in the two rows on the left, middle, and right represent the same fields. Bar, 40 μm.

FIG. 7. TS restoration of peroxisomes is not blocked by cycloheximide. A, translocation of catalase to catalase-less peroxisomes. 65TS cells were cultured first at 39 °C and then for 24 h in the absence (a and b) or presence (c and d) of 10 μg/ml cycloheximide at 33 °C. Immunostaining was performed for catalase (a and c) and PMP70 (b and d). Pictures of the same fields are presented side by side. Bar, 10 μm. B, C, and D, radiolabeling of PMP70, catalase, and total proteins, respectively. 65TS cells were incubated at 33 °C in the presence of [35S]methionine/[35S]cysteine and cycloheximide at the concentrations indicated (in μg/ml). Cells were lysed with an equal amount of lysis solution, and equal amounts of lysates were subjected to immunoprecipitation (B and C) or analyzed directly by SDS-polyacrylamide gel electrophoresis (D). Oxissomal lumen. The processing of AOX, however, was less efficient, possibly because of poorer assembly of the processing system. Catalase-less peroxisomes were also observed in the fibroblasts of PBD patients with milder phenotypes, belonging to three CGs: C, E, and F. The CG-F patient was the original carrier of the PEX2E55K mutant gene. In these cells, AOX and PT exhibited punctate patterns of distribution overlapping with that of PMP70 at 37 °C, although the staining intensities were weaker than those in 65TS. In the CG-F cells, the VLCFA oxidation and DHAP-AT activities were higher than those of ZS cells of the same CG, although much lower than the wild-type activities (24, 35). Thus, the patient fibroblasts of CG-F and the 65TS model CHO cells had similar TS phenotypes, but the functional defect at the nonpermissive temperature was much more prominent in the former. This was probably due to the weaker expression of the endogenous PEX2 gene in the human fibroblasts than of the ectopic PEX2 gene under the control of the viral expression system, which was carried by 65TS. The present observations suggest that the partially functional catalase-less peroxisomes would support the milder phenotypes of NALD and IRD patients, at least in the cases studied. Evaluation of peroxisomes of PBD patients based on immunofluorescent microscopy should be done not only for catalase but also for typical PTS proteins such as β-oxidation enzymes.

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The present results are consistent with the result for the PEX5 mutant CHO cells (33) that the restoration of peroxisomes by the microinjection of Pex5p can occur in the presence of cycloheximide and hence involves the translocation of the cytosolically accumulated PTS proteins into the peroxisomal ghosts. Catalase-less peroxisomes as well as peroxisomal ghosts are the structures that form on the blockade of the normal course of peroxisome biogenesis because of a deficiency of one of the essential PEX gene products. Thus, these structures reflect the processes of peroxisome biogenesis, even if they themselves do not represent the biogenesis intermediates. They are ready to accept the PTS proteins if the correct PEX gene products are supplied. The shapes and biochemical compositions, however, may differ depending on the defective gene as well as the severity of deficiency. The recovery processes also probably differ depending on the defective genes. Moreover, net formation of the peroxisomal membrane from a certain membrane seems essential in the mutants defective in one of the three PEX genes, PEX3, PEX16, and PEX19, because these
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mutants lack peroxisomal ghosts but nevertheless are complemented for peroxisome biogenesis by the transfer of respective PEX mutants. Clues to the biochemical mechanism of peroxisome biogenesis will be obtained by characterizing the processes of genetic complementation of the respective PEX mutants.

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REFERENCES

1. Lazarow, P. B., and Fujiki, Y. (1985) Annu. Rev. Cell Biol. 1, 489–530
2. Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Miyazawa, S., and Tabak, H. F. (1995) Science 268, 1–17
3. Miyazawa, S., Hayashi, H., Hikijaka, M., Ishii, N., Furuta, S., Matsuishi, T., and Fujiki, Y. (1987) J. Biol. Chem. 262, 8391–8395
4. Yamasaki, M., Hashiguchi, N., Tsukamoto, T., and Osumi, T. (1998) Bioimaging 6, 1–7
5. Tange, T., Shibata, T., and Fujiki, Y. (1997) Biochem. Biophys. Res. Commun. 241, 1232–1237
6. Shimozawa, N., Suzuki, Y., Zhang, Z., Imamura, A., Kondo, N., Kinoshita, N., Miyazawa, S., and Hashimoto, T. (1996) J. Biol. Chem. 271, 3706–3713
7. Lazarow, P. B., Robbi, M., Fujiki, Y., and Gould, S. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4350–4355
8. Shimozawa, N., Suzuki, Y., Zhang, Z., Imamura, A., Toyama, R., Mukai, S., Orii, T., Osumi, T., and Fujiki, Y. (1998) Trends Cell Biol. 8, 223–233
9. Elgersma, Y., Kwast, L., van den Berg, M., Snyder, W. B., Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Miyazawa, S., and Tabak, H. F. (1995) Science 268, 1–17
10. Shimozawa, N., Suzuki, Y., Zhang, Z., Imamura, A., Kondo, N., Hikijaka, M., Miyazawa, S., and Hashimoto, T. (1996) Biochem. Biophys. Res. Commun. 181, 947–954
11. Inouye, H., Tsukamoto, T., and Fujiki, Y. (1997) J. Biol. Chem. 272, 1539–1543
12. Lazarow, P. B., Robbi, M., Fujiki, Y., and Gould, S. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 883–886
13. Tange, T., Shibata, T., and Fujiki, Y. (1997) Biochem. Biophys. Res. Commun. 241, 1232–1237
14. Shimozawa, N., Suzuki, Y., Zhang, Z., Tomatsu, S., Tsukamoto, T., Hashiguchi, N., Orii, T., Masuno, M., Inai, K., Kuruji, Y., Orii, T., and Kondo, N. (1996) Am. J. Hum. Genet. 59, 1210–1220
15. Tabak, H. F., Braakman, I., and Distel, B. (1996) Annu. Rev. Cell Biol. 12, 447–453
16. Lazarow, P. B., and Fujiki, Y. (1985) Annu. Rev. Cell Biol. 1, 489–530
17. Yamasaki, M., Hashiguchi, N., Tsukamoto, T., Orii, T., and Fujiki, Y. (1998) Biochem. Biophys. Res. Commun. 255, 1–7
18. Lazarow, P. B., Robbi, M., Fujiki, Y., and Gould, S. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4350–4355
19. Takah, H. F., Braakman, I., and Distel, B. (1999) Trends Cell Biol. 9, 447–453
20. Lazarow, P. B., Fujiki, Y., and Gould, S. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4350–4355
21. Lazarow, P. B., and Fujiki, Y. (1985) Annu. Rev. Cell Biol. 1, 489–530
22. Tabak, H. F., Braakman, I., and Distel, B. (1999) Trends Cell Biol. 9, 447–453
23. Lazarow, P. B., and Fujiki, Y. (1985) Annu. Rev. Cell Biol. 1, 489–530
24. Shimozawa, N., Suzuki, Y., Zhang, Z., Imamura, A., Kondo, N., Kinoshita, N., Fujiki, Y., Tsukamoto, T., Orii, T., Osumi, T., Kondo, N., and Fujiki, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4350–4355
25. Miyazawa, S., Hayashi, H., Hikijaka, M., Ishii, N., Furuta, S., Matsuishi, T., and Fujiki, Y. (1987) J. Biol. Chem. 262, 8313–8317