Disruption of the Interaction of the Longer Isoform of Pex5p, Pex5pL, with Pex7p Abolishes Peroxisome Targeting Signal Type 2 Protein Import in Mammals

STUDY WITH A NOVEL PEX5-IMPAIRED CHINESE HAMSTER OVARY CELL MUTANT

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We isolated peroxisome biogenesis-defective Chinese hamster ovary cell mutants from TK-aG2 cells, wild-type CHO-K1 cells transformed with two cDNAs encoding rat Pex2p and peroxisome targeting signal (PTS) type 2-tagged green fluorescent protein, by the 9-(1'-pyrene)nonanol/UV selection method. Ten mutant clones showed cytosolic PTS2-green fluorescent protein, indicative of a defect in PTS2 import, and were classified in five complementation groups, i.e. pex1, pex2, pex5, pex14, and group A. One PEX5-deficient mutant, ZPG231, showed a novel phenotype: PTS2 proteins in the cytosol, but PTS1 proteins and catalase in peroxisomes. In ZPG231, two isoforms of the PTS1 receptor Pex5p, a shorter Pex5pS and a longer Pex5pL, were expressed as in wild-type cells, but possessed the missense point mutation S214F in both Pex5p isoforms, termed Pex5pS-S214F and Pex5pL-S214F, respectively. The S214F mutation was located only one amino acid upstream of the Pex5-specific 37-amino acid insertion site. Pex5pS-S214F and Pex5pL-S214F interacted with peroxisomal proteins, including PTS1 protein, catalase, and Pex14p, as efficiently as normal Pex5p. In contrast, the S214F mutation severely affected the binding of Pex5pL to the PTS2 receptor Pex7p. Expression of Pex5pL-S214F in pex5 cell mutants defective in PTS1 and PTS2 transport restored peroxisomal import of PTS1, but not PTS2. Together, the results indicate that ZPG231 is the first cell mutant containing evidence that disruption of the Pex5pL-Pex7p interaction completely abolishes PTS2 import in mammals.

Mechanisms of peroxisome assembly, including peroxisomal import of newly synthesized proteins, are one of the major foci in peroxisome research (1). To address such issues, we earlier showed a potential approach system using mammalian somatic cell mutants (2). We previously isolated 13 different complementation groups (CGs) of peroxisome-deficient Chinese hamster ovary (CHO) cell mutants by colony autoradiographic screening (3) and the 9-(1'-pyrene)nonanol (P90H1/UV selection method) (4). All CHO mutants resembled fibroblasts from patients with peroxisome biogenesis disorders (PBDs) such as Zellweger syndrome in the defects in peroxisome assembly despite normal synthesis of peroxisomal proteins. Nine distinct CG mutants represented human CGs (5, 6). CHO mutants ZP105 and ZP139 represent pex5 CG2, and ZPG207 is in a PEX7-defective CG-R (CG11), the same group as fibroblasts from patients with rhizomelic chondrodysplasia punctata (RCDP). We have so far cloned eight peroxin cDNAs, PEX1, PEX2 (formerly peroxisome assembly factor-1 (PAF-1)), PEX5, PEX6, PEX12, PEX13, PEX14, and PEX19, by a functional phenotype complementation assay of CHO cell mutants (7–15). We also showed these PEX cDNAs, except for PEX14, to be responsible for human PBDs (7, 10–13, 15–18). Thus, peroxisome assembly-defective CHO cell mutants have indeed been proven to be useful for investigating the molecular and cellular mechanisms involved in peroxisome biogenesis and for elucidation of the primary defects of PBDs. However, mechanisms by which most of the peroxins function in peroxisome biogenesis have been little elucidated at the molecular level.

Pex5p and Pex7p, the receptors for PTS1 and PTS2, respectively, are relatively well characterized peroxins with respect to cellular and biochemical functions. Pex5p translocates PTS1 proteins to peroxisomes, and Pex7p likewise acts for PTS2 proteins. Two types of phenotypes of PEX5 deficiency in CHO cells (10) and PBD patient fibroblasts (19, 20) have been reported: a defect in PTS1 import and a defect in both PTS1 and PTS2 import. The former can be due to a dysfunction of the PTS1 receptor as seen in yeast pex5 mutants, but the latter cannot be eluted from the impaired activity of the PTS1 transporter. In mammals, two isoforms of Pex5p were identified: a shorter form termed Pex5pS serves as a PTS1 receptor, and the longer Pex5pL (with a 37-amino acid internal insertion) functions in both PTS1 and PTS2 import pathways (10, 20). Chinese hamster (Cl) PEX5S and PEX5L encode 595- and 632-amino acid long proteins, respectively, of a tetratricopeptide repeat protein family (10). Very recently, we found that Pex5pL acts in PTS2 translocation by directly binding to Pex7p carrying the cargo PTS2 protein (21). It is not conclusively understood, however, to what extent PTS2 import relies on Pex5pL in

* This work was supported in part by a Core Research for Evolutional Science and Technology grant from the Japan Science and Technology Corp. (to Y. F.) and by Grants-in-aid for Scientific Research 08557011 and 09044094 from the Ministry of Education, Science, Sports, and Culture (to Y. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: CG, complementation group; CHO, Chinese hamster ovary; P90H1, 9-(1'-pyrene)nonanol; P12, 12-(1'-pyrene)dodecanonic acid; PBD, peroxisome biogenesis disorder; RCDP, rhizomelic chondrodysplasia punctata; PTS1 and PTS2, peroxisome targeting signal types 1 and 2, respectively; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.
vivo. Moreover, neither a CHO cell pex5 mutant nor a PEX5-defective PBD patient with a defect only in PTS2 import has been identified. But there is no reason not to anticipate such a phenotypic cell mutant.

Therefore, in this study, we have searched for a CHO cell mutant showing such a pex5 phenotype with PTS2 import deficiency using the improved mutant isolation method making use of green fluorescent protein (GFP) tagged with PTS2 (22). We isolated 10 CHO cell mutant clones by the P90OH/UV method using PTS2-GFP (22) and classified them in five previously identified CGs. To our surprise, a mutant cell line (ZPG231) of CG2 showed a novel phenotype, PEX5-defective, but impaired solely in PTS2 import. A missense point mutation in the PEX5 gene disrupted the interaction of Pex5p with Pex7p, resulting in complete elimination of the PTS2 import pathway. Therefore, it is evident that Pex5p plays an exclusively pivotal role in PTS2 transport in vivo.

EXPERIMENTAL PROCEDURES

Cell Lines—Skin fibroblast cell lines from patients, including fibroblasts from CG2 Zellweger syndrome and CG11 RCDP patients, were cultured in Dulbecco modified Eagle’s medium supplemented with 10% fetal calf serum as described (12, 17). CHO cells, including wild-type CHO-K1, pex5 mutant ZP105 and ZP139 (10), and pex7 ZPG207 (22) cells, were cultured as described (8, 23).

GFP-transformed CHO Cells and Isolation of Peroxisome Biogenesis-defective Mutants—TKaG2 cells (22), rat PEX2-transformed CHO-K1 cells stably expressing N-terminally PEX2-tagged GFP, and selection of peroxisome biogenesis mutants (22, 24) were as described.

Morphological Analysis—PTS2-GFP in cells grown on a coverslip was observed without cell fixation under a Carl Zeiss Axioskop FL microscope using a no. 17 filter. Peroxisomes in CHO cells were also visualized by indirect immunofluorescence microscopy using rabbit antibodies to PTS1 peptide (10), 3-ketoacyl-CoA thiolase (referred to hereafter as thiolase) (23), and catalase (25). Antibody-antigen complexes were detected by fluorescein isothiocyanate-labeled sheep anti-rabbit IgG antibody (Cappel) or Texas Red-labeled goat anti-rabbit IgG antibody (Leinco Technologies) in the case of GFP-expressing cells under a Carl Zeiss Axioskop FL microscope.

PEX Transfection and Cell Fusion—Transfection of PEX cDNAs into CHO mutant cells was done by lipofection as described (12). For cell fusion, parental CHO mutant cells and cells to be examined were co-cultured for 24 h and then fused using polyethylene glycol as described (25). Supplementation of cell mutants was assessed by peroxisomal punctate localization of PTS2-GFP. Fused cells such as CHO mutants with patient fibroblasts were also stained using rabbit antibody against catalase, a peroxisomal membrane protein (Ref. 23; data not shown), immunoblotting with specific antibodies to PTS1 peptide (10), 3-ketoacyl-CoA thiolase (referred to hereafter as thiolase) (23), and catalase (25).

Reverse Transcription-Polymerase Chain Reaction—RNA was isolated from mutant ZPG231 as described (12). Reverse transcription-polymerase chain reaction was done with Superscript II RT (Life Technologies, Inc.) and 5 μg of total RNA as described (10). The primers used to cover the entire open reading frame of PEX5 were a sense primer (635, 5’-GCTGGTGCACCAATCGGCAA-3’, nucleotide sequence at positions 63–79 in CIPEX5L cDNA, where the initiation codon starts at position 86 (10)) and an antisense primer (1928R, 5’-GGTGGTCACCATGGCAA-3’, nucleotide sequence at positions 1928 to 1912). Amplified RNA products were analyzed by agarose gel electrophoresis and cloned into the pGEM-T vector (Promega). Several independent plasmids were isolated from Escherichia coli colonies, and their cDNA inserts were verified using agarose gel electrophoresis. Plasmids with two different sizes were sequenced and found to be corresponding to CIPEX5L and CIPEX5S (10), except for point mutations termed CIPEX5L-S214F and CIPEX5S-S214F, respectively (see “Results” and Fig. 4B). For transfection of ZPG231-derived PEX5, the Sse83871-NspV fragment from pGEM-CIPEX5L-S214F replaced the Sse83871-NspV site of pUcd2Hyp-CIPEX5SS (previously called P31 receptor cDNA) (10). pUcd2Hyp-CIPEX5SS was constructed by substituting the Sse83871-NspV fragment from pGEM-CIPEX5SS-S214F for the Sse83871-NspV site of pUcd2Hyp-CIPEX5L-S214F.

Expression of Fusion Protein—Glutathione S-transferase (GST) fusion protein with CIPEX5 was constructed by the E. coli expression vector pGEX6P-1 (Amersham Pharmacia Biotech, Tokyo, Japan) essentially as described (21). Briefly, the NcoI-BglII fragment from pBS-CIPEX5S (10) was ligated into the NcoI-BglII site of pGEX6P-1; the resulting construct was named pGEX6P-1-CIPEX5S. The Sse83871-KpnI fragment from ZPG231-derived pGEM-CPEX5L-S214F replaced the Sse83871-KpnI site of pGEX6P-1-CIPEX5S, resulting in a product termed pGEX6P-1-CIPEX5L-S214F. pGEX6P-1-CIPEX5L-S214F was constructed by replacing the Sse83871-KpnI fragment from pGEX6P-1-CIPEX5L-S214F with the Sse83871-KpnI fragment from pGEM-CIPEX5S-S214F. All plasmid constructs were assessed by nucleotide sequence analysis. GST fusion proteins were expressed in E. coli and purified using glutathione-Sepharose (Amersham Pharmacia Biotech) as described (14). Purity of the fusion proteins was verified by SDS-PAGE (data not shown).

In Vitro Binding Assay—In vitro binding assay was performed using lysates of 20T7F cells, human PEX7-transformed CHO pex7 mutant ZPG207 (21, 22). Cells (2 × 10⁶) were lysed for 1 h on ice with binding buffer (2.4 ml) containing 50 mM Hepes-KOH, pH 7.4, 150 mM NaCl, 0.2% Nonidet P-40, 10% glycerol, 25 μg/ml each leupeptin and antipain, 1 mM EDTA, and 1 mM diithiothreitol and centrifuged at 16,800 × g for 10 min at 4 °C. The supernatant fraction (typically a 400-μl aliquot) was incubated with GST or GST-Pex5p fusion protein (~5 μg each)-bound glutathione-Sepharose (30 μl; Amersham Pharmacia Biotech) in 1 ml by rotating for 2 h at 4 °C. Sepharose beads were washed three times with binding buffer. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

Results

Isolation and Morphological Analysis of CHO Cell Mutants

In this work, we isolated 10 peroxisome-deficient CHO cell mutants from PEX-GFP-transformed TKaG2 cells by eight cycles of the improved mutant isolation procedure (22). The intracellular location of PTS2-GFP was readily visible without cell fixation; therefore, the mutant screening, morphologically verifying step was accelerated as described (22). ZPG217 and ZPG231 (Fig. 1A, panels B and C) as well as ZPG213, ZPG219, ZPG221, ZPG226, ZPG230, ZPG232, ZPG233, and ZPG234 (Table I; data not shown) showed PTS2-GFP in a diffuse pattern in the cytoplasm, in contrast to its peroxisomal localization in TKaG2 cells (panel a). Next, to assess the deficiency of peroxisome biogenesis, ZPG231 was stained with several different antibodies raised against peroxisomal proteins. Peroxisomal 3-ketoacyl-CoA thiolase, a PTS2 protein, was in the cytosol, confirming the defect in PTS2 import (Fig. 1B, panel a). When ZPG231 was separately stained with antibodies to PTS1 or catalase, a punctate staining pattern was seen in both cases, indicating that the import of PTS1 is normal (panel e). The import of catalase could also be discerned, but did not appear to be complete (panel i). Numerous punctate spots were also seen in as TKaG2 cells when ZPG231 was stained with antiseraum against 70-kDa peroxisomal membrane protein (Ref. 23; data not shown), implying normal import of peroxisomal membrane protein. In contrast, the other mutants, including ZPG217, showed a cytosolic PTS1 staining pattern (data not shown), suggesting a defect in PTS1 import as well. Together, the results indicate that ZPG231 is apparently a cell mutant defective only in PTS2 import.

Complementation Group Analysis

Transfection of CIPEX5L encoding Chinese hamster Pex5pL (10) into ZPG231 restored the import of PTS2-GFP, whereas
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PEX7 expression did not, therefore, demonstrating ZPG231 to be in PEX5-defective CG2 (Fig. 2A, panels a and c). However, CIPEX5S did not complement the impaired PTS2 import in ZPG231 (panel b), similar to findings in the case of pex5 mutant ZP105 (Ref. 10; see below). CG analysis of ZPG231 was also performed by cell fusion. We previously isolated two types of pex5 mutants, represented by ZP105 and ZP139 (10). ZP105 shows an defect in the import of both PTS1 and PTS2 represented by thiolase (Fig. 1, A, panels b and c), whereas ZP139 is impaired in the import of PTS1, but not PTS2 (panels c and g). Catalase was in the cytoplasm in both mutants (panels j and k). The pex7 mutant ZPG207 is deficient only in PTS2 import (panels d, h, and l) (22). When ZPG231 was fused with ZP105, PTS2 thiolase remained in the cytosol, as assessed by immunostaining with antibody to thiolase (Fig. 2B, panel a), indicative of the same CG and consistent with the results obtained by PEX5L transfection in CG analysis. Likewise, PTS2 import was not complemented in fused cells of ZPG231 with fibroblasts from a PEX5-impaired patient with CG2 PBD (panel d), confirming the results obtained using ZP105. In contrast, numerous GFP-positive particles, peroxisomes, were seen in cell hybrids of ZPG231 with pex7 ZPG207 as well as RCDP fibroblasts (panels b and e), indicating that ZPG231 is distinct from the pex7 mutant. Moreover, fused cells of ZPG231 with wild-type CHO-K1 and human control fibroblasts showed GFP-positive particles (panels c and f), as numerous as in wild-type TKaG2 cells, thus suggesting that lesion of allele(s) in the mutant is recessive. Taken together, these observations strongly suggest that ZPG231 belongs to pex5 CG2 with a defect only in PTS2 import, distinct from the CHO mutants and patient fibroblasts of CG2 so far identified.

CG analysis of the other cell mutant clones was likewise done by PEX cDNA transfection and cell fusion (Tables I and II). Three pex1, four pex2, and one pex14 mutant clones were identified; mutant ZPG217 was not in any of these mutant CGs (Table I). TKaG2 cells were used to avoid possible isolation of pex2 mutants, but several PEX2-defective mutants were isolated as in our earlier studies (6, 22, 27). The integrated rat PEX2 cDNA appears to be occasionally disintegrated. ZPG217 was not complemented by fusion with the recently isolated mutant ZP124 of CG-A (CG8) (6), whereas PTS2-GFP was imported into peroxisomes in fused cells with other CG mutants, including PEX14-deficient ZP110 (14), ZP114 (5), and ZP126 (6) and PEX13-defective ZP128 (13) (Table II). Collectively, five CGs of CHO mutant cell lines were isolated in this study. ZPG217 will be useful for searching for a complementing PEX gene for CG-A.

**Properties of CHO Cell Mutants**

The intracellular location of catalase was also examined by the digitonin assay (23, 25). At 100 μg/ml digitonin, ~40% of the activity of catalase was latent in ZPG231, whereas nearly full activity was detected in the ZPG217 and pex5 ZP139 mutants (Fig. 3A). In TKaG2 cells, ~70% of catalase activity was latent, consistent with our earlier observation (22), as in CHO-K1 cells (23, 24). It is noteworthy that pex7 ZPG207 showed a catalase latency of ~50% (Table III) (22). Treatment with 300 μg/ml digitonin released the full catalase activity of ZPG231 and TKaG2 cells. Therefore, these results suggest that catalase is mostly, if not completely, in peroxisomes in ZPG231. The subcellular localization of catalase was also determined by immunoblotting. A catalase band was detected predominantly in a membrane fraction and in a small amount in a soluble fraction in wild-type CHO-K1 and TKaG2 cells, whereas nearly all catalase was soluble in ZP139 (Fig. 3B) and ZP105 (data not shown), similar to observations in our earlier work using other CG mutants (23). Contrary to such pex5 mutants, catalase was more abundant in the organelle pellet and less in the soluble fraction in ZPG231 (Fig. 3B), consistent with the results from the digitonin assay shown in Fig. 3A. Collectively, these results were interpreted to mean that catalase is largely localized in peroxisomes in ZPG231, but not in other mutants. Reduced latency of catalase in ZPG231 and ZPG207 may be due to the defect in the import of PTS2 proteins, causing an alteration of biochemical properties of peroxisome membranes.

We studied the biogenesis of peroxisomal enzymes by immunoblotting. Acyl-CoA oxidase comprises 75-kDa A, 53-kDa B, and 22-kDa C polypeptide components (23); B and C are derived from A (23, 28). The A, B, and C components of acyl-CoA oxidase were evident in ZPG231, as in CHO-K1 and TKaG2 cells, indicative of normal biogenesis of acyl-CoA oxidase and consistent with the morphological observation (Fig. 1B, panel e), whereas only the 75-kDa acyl-CoA oxidase A form was detected in two pex5 cell lines, ZP105 and ZP139 (10) (Fig. 3C, lanes 1–5). Peroxisomal thiolase is synthesized as a larger precursor of 44 kDa and then matures to a 41-kDa form by PTS2 processing (23, 24). Only a 44-kDa thiolase precursor was detectable in ZPG231, as in ZP105 and ZP139 (10) (lanes 8–10), whereas the 41-kDa mature protein was apparent in wild-type CHO-K1 and TKaG2 cells (lanes 6 and 7). It is noteworthy that the thiolase precursor was found inside the peroxisomes in ZP139 (10). Thus, peroxisomal proteins are synthesized in these mutants. Normal import of PTS1 but impaired PTS2 transport in ZPG231 resembles the phenotype of a CHO pex7 mutant, ZPG207 (22), and fibroblasts from RCDP patients defective in the import of only PTS2 proteins.

P9OH is incorporated into plasmalogens at an early step of...
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TABLE I
Complementation Group Analysis: PEX cDNA transfection assay

CHO cell mutants were separately transfected with human PEX1 (7), rat PEX2 (8), Chinese hamster PEX5L (10), rat PEX6 (9), rat PEX12 (12), and rat PEX14 (14). Cells were assessed for PTS2-GFP import by fluorescence microscopy. +, peroxisomes were complemented; −, peroxisomes were not complemented.

| CHO mutant | PEX1 | PEX2 | PEX5L | PEX6 | PEX12 | PEX14 |
|------------|------|------|-------|------|-------|-------|
| ZPG213     | −    | −    | +     | −    | −     | −     |
| ZPG217     | −    | −    | +     | −    | −     | −     |
| ZPG219     | −    | −    | +     | −    | −     | −     |
| ZPG221     | −    | −    | +     | −    | −     | −     |
| ZPG224     | −    | −    | +     | −    | −     | −     |
| ZPG230     | −    | −    | +     | −    | −     | −     |
| ZPG231     | −    | −    | +     | −    | −     | −     |
| ZPG232     | −    | −    | +     | −    | −     | −     |
| ZPG233     | −    | −    | +     | −    | −     | −     |
| ZPG234     | −    | −    | +     | −    | −     | −     |

Fig. 2. Complementation group analysis. A, ZPG231 was transfected with each of two Chinese hamster PEX5 isoforms, PEX5L (panel a) and PEX5S (panel b) (10), or with human PEX7 (panel c). Note that the import of PTS2-GFP was restored only with Pex5pL. B, ZPG231 cells were pairwise fused with pex5 ZP105 (panel a), pex7 ZPG207 (panel b), CHO-K1 cells (panel e), and fibroblasts from a CG2 Zellweger syndrome patient (panel d), a RCDP patient (panel e), and a normal control (panel f). Complementation was assessed by GFP fluorescence, except that cells in panels a and d were stained with anti-thiolase antibody. Bar = 20 μm.

TABLE II
Complementation Group Analysis: Cell fusion assay

After cell fusion, complementation was determined as described in the legend to Table I. +, complemented; −, not complemented.

| CHO mutant | ZP110 (pex14) | ZP114 | ZP124 | ZP126 | ZP128 (pex13) |
|------------|----------------|------|-------|-------|---------------|
| ZPG217     | +              | +    | −     | +     | +             |

synthesis and produces active oxygen by UV irradiation (4). Cell culture in the presence of P9OH, followed by a short exposure to UV, kills wild-type CHO cells, but not peroxisome-defective mutants. Upon this treatment, >95% of mutants ZPG217 and ZPG231 survived, similar to pex7 ZPG207 (22), whereas TkaG2 cells were not viable (Table III). Conversely, P12/UV treatment specifically kills peroxisome-defective cells, due to the lack of synthesis of plasmalogen, an oxygen radical scavenger (29). ZPG217 and ZPG231 were highly sensitive to the P12/UV treatment, as noted for ZPG207 (22), but nearly 70% of TkaG2 cells were resistant (Table III). Taken together, ZPG231 showed a typical, but partly specific, phenotype of peroxisome biogenesis mutants with respect to the biochemical properties.

Dysfunction of Pex5p in ZPG231

Expression Level of Pex5p—As a step toward investigating the dysfunction of Pex5p in ZPG231, the expression level of Pex5p was determined by immunoblotting. Pex5p was detected predominantly in the cytosolic fractions of wild-type CHO-K1, TkaG2, and ZPG231 cells in two bands clearly separated during a prolonged SDS-PAGE (Fig. 4A). The lower and upper bands are Pex5pL and Pex5pS, respectively (21). Pex5p in ZPG231 was apparently less in amount, but indistinguishable in size compared with that in wild-type cells, suggesting that Pex5p was not impaired by a premature termination or distinct truncation in ZPG231. The reason for a lower level of Pex5p in ZPG231 is presently unknown.

Mutation of PEX5—Next, we isolated PEX5 cDNA from ZPG231 by reverse transcription-polymerase chain reaction. Subsequent sequencing of all nine cDNA clones for each PEX5S and PEX5L indicated a point mutation, thereby apparently in a homozogotic form: a mutation of nucleotide C to T at position 641 (the A of the initiating ATG being position 1) in a codon for Ser214, resulting in a codon for Phe214 in both Pex5pS and Pex5pL (Fig. 4B). It is noteworthy that the mutation site is located one amino acid upstream of the 37-amino acid insertion in Pex5pL (10). Thus, dysfunction of Pex5p caused by a missense point mutation is most likely the primary defect in ZPG231. ZPG231-derived PEX5 was named ClPEX5-S214F.

To assess the impaired function of Pex5p in ZPG231, ClPEX5L-S214F was transfected back to ZPG231. Pts2-GFP remained in the cytosol in ClPEX5L-S214F-transfected ZPG231 (Fig. 4C, panel a), suggesting that ClPEX5L-S214F protein is biologically inactive in Pts2 import. The same results were obtained using ClPEX5S-S214F (data not shown). When ClPEX5L-S214F was transfected into PEX5-impaired ZP105 (10), PTS2 thiolase was present in the cytosol (panel b), confirming the results obtained using ZPG231. In contrast, a number of PTS1-positive particles were found in the transfectants (panel c), demonstrating that Pex5pL-S214F is functional in PTS1 transport. ClPEX5S-S214F expression restored PTS1 (but not PTS2) import upon transfection into ZP105 (data not shown). Expression of ClPEX5S-S214F and ClPEX5L-S214F also complemented PTS1 import in ZPG139 (data not shown). Catalase was located in numerous particles (peroxisomes) in ClPEX5L-S214F-transfected ZP139 (panel d) and ZP105 (data not shown) as well as in ClPEX5S-S214F-transfected ZP105 and ZP139 (data not shown). These morphological phenotypes...
Biogenesis of peroxisomal proteins. A, latency of catalase in wild-type and mutant cells was determined as described (23). ○, wild-type TKaG2 cells; ●, ZPG231; □, ZPG217; △, pex5 ZP139 (10). The results are representative of the average of duplicate assays. B, shown is the intracellular localization of catalase. Subcellular fractions from CHO cells (3 × 10⁶ each) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. Immunoblot analysis was performed with anti-catalase antibody. Cell types are indicated at the top. S and P, cytosolic and organellar fractions derived from the post-nuclear supernatant fraction, respectively. C, shown are the results from the biosynthesis of peroxisomal enzymes. Lysates from 3 × 10⁶ cells were subjected to SDS-PAGE. Immunoblot analysis was done as described for B using rabbit antibodies to peroxisomal acyl-CoA oxidase (AOx) and thiolase. Lanes 1 and 6, CHO-K1 cells; lanes 2 and 7, TKaG2 cells; lanes 3 and 8, ZP105; lanes 4 and 9, ZP139; lanes 5 and 10, ZPG231. Arrows show acyl-CoA oxidase components A, B, and C; arrowheads P and M indicate a larger precursor and mature protein of thiolase, respectively. Dots indicate nonspecific bands (7, 23).

noted in the pex5 mutants transfected with ZPG231-derived PEX5 are exactly the same as noted in ZPG231 per se. Collectively, we conclude that the dysfunction of Pex5p caused by the S214F missense mutation is responsible for the impaired peroxisome biogenesis in ZPG231.

Interaction of ZPG231-derived Pex5p with Peroxinsomal Proteins

To delineate the impaired function of Pex5p-S214F at the molecular level, a protein binding assay was performed using GST fusion proteins: normal-type GST-Pex5pS and GST-Pex5pL and ZPG231-type GST-Pex5pS-S214F and GST-Pex5pL-S214F. Lysates of 207P7 cells, PEX7 transfectants of pex7 ZPG207 (22), were incubated with each type of GST-Pex5p fusion protein. Bound protein fractions were analyzed by SDS-PAGE and immunoblotting using specific antibodies. Pex7p was detected only in the fraction bound to GST-Pex5pL, but not in those bound to GST-Pex5pL-S214F, GST-Pex5pS-S214F, GST-Pex5pS, and GST (Fig. 5). Contrary to Pex7p, Pex14p, a Pex5p-docking factor, interacted with all types of GST-Pex5p fusion proteins, but not with GST, strongly suggesting that both isoforms of ZPG231-derived Pex5p function in binding to Pex14p. Pex1 protein acyl-CoA oxidase apparently bound to both forms of the wild type as well as ZPG231-derived Pex5pS and Pex5pL, but not to GST. Binding to an oligomeric acyl-CoA oxidase, assessed by acyl-CoA oxidases A and B (28), was in good agreement with the notion using wild-type Pex5p by Otera et al. (21). Furthermore, catalase possessing a PTS1-like C-terminal sequence, KNAL (30, 31), also bound to wild-type and ZPG231-type GST-Pex5pS and GST-Pex5pL. Thus, ZPG231-derived Pex5p was indistinguishable from the wild type in the interaction with peroxisomal proteins such as Pex1, catalase, and Pex14p, except for Pex7p. Taken together, it is evident the S214F mutation affected the binding of Pex5pL to Pex7p, causing a phenotype, the defect in PTS2 import. Pex5pL plays an indispensable role in transport of PTS2 proteins.

DISCUSSION

Deficiency in PTS2 transport is a characteristic of pex7 mutants caused by dysfunction of Pex7p (2, 32) not only in yeast (33, 34), but also in mammalian cells such as fibroblasts from patients with RCDP (35–37). In one of two types of pex5 mammalian cells, including CHO ZP105-type mutants (10) and fibroblasts from a CG2 Zellweger syndrome patient (19), the import of not only PTS1 (as in yeast) but also PTS2 is impaired. Only Pex5pL could restore the defect in PTS2 import to these Pex5 cells, implying that Pex5pL plays a pivotal role in PTS2 import (10, 20). Pex5pL indeed interacts with PTS2 protein in a Pex7p-mediated manner in vitro as well as in vivo, indicating the dual function of Pex5pL (21). Meanwhile, we recently demonstrated using an in vitro system that mammalian Pex14p binds to both Pex5pS and Pex5pL carrying their cargo (PTS1 protein) and Pex7p with PTS2 protein, but with less efficiency (14). Therefore, how physiologically relevant such findings are remain partly inconclusive.

In this investigation working toward further isolation of novel CHO mutants, we serendipitously cloned for the first time the pex5 mutant cell line ZPG231 with a defect only in PTS2 protein import. ZPG231-derived Pex5pL with a point mutation of S214F no longer bound to Pex7p, whereas both mutant forms of Pex5p, Pex5pS-S214F and Pex5pL-S214F,

### Table III

Properties of wild-type TKaG2, ZPG217, and ZPG231 cells

| Cells   | Peroxisome PTS2 import | Catalase latency | P9OH/UV | P12/UV |
|---------|------------------------|-----------------|---------|--------|
| TKaG2   | +                      | +               | 67      | 0.02   | 68     |
| ZPG217  | −                      | −               | 0       | 95     | 0      |
| ZPG231  | +                      | −               | 38      | 98     | 0.04   |
| ZPG207* | +                      | −               | 48      | 84     | 0.8    |

* From Ghaedi et al. (22).
recognized PTS1, evidently reflecting the phenotype of ZPG231. It is noteworthy that the S214F mutation is located immediately upstream, but interestingly not inside, of the Pex5pL-specific 37-amino acid insertion sequence. It is possible that residue 214 is within the sequence directly interacting with Pex7p. Alternatively, such a mutation from a hydrophilic to a hydrophobic residue may alter, directly or indirectly, the structure of the region interacting with Pex7p, possibly including the 37-amino acid insertion part. We must await further investigation to delineate the regions and their structures specifically involved in Pex5pL-Pex7p interaction. Given the fact that Pex5pL impaired in binding to Pex7p eliminates PTS2 import in ZPG231, it is apparent that one of the two potential translocation pathways, namely Pex14p-Pex5pL-Pex7p-PTS2 protein, is exclusively utilized for PTS2 import in vivo rather than another import route, Pex14p-Pex7p-PTS2 protein. The receptor and cargo complexes, including Pex5pPTS1 proteins and Pex5pL-Pex7p-PTS2 proteins, are formed in the cytosol and then traverse to peroxisomes and finally dock Pex14p in the potential translocation machinery (14, 21). Taken together, Pex5pL is indispensable for PTS2 import in mammals, as distinct from yeast.

From an evolutional viewpoint, it is tempting to speculate that the PEX5L gene may have evolved from PEX5S by exon shuffling, causing divergence from PEX5 in lower eukaryotes such as yeast. Pex5pL then started to play an additional role substituting for Pex7p in targeting the Pex7pPTS2 protein complex to Pex14p. It is presently unknown, however, whether

Fig. 4. Expression of Pex5p in ZPG231. A, the expression level of Pex5p in wild-type CHO-K1, TKaG2, and pex5 mutant ZPG231 cells was determined by immunoblotting using anti-Pex5p antibody. Subcellular fractions from CHO-K1, TKaG2, and ZPG231 cells were subjected to prolonged SDS-PAGE to better separate Pex5pS and Pex5pL. PNS, post-nuclear supernatant fraction (20 μg); P and S, organellar and cytosolic fractions derived from 20 μg of post-nuclear supernatant, respectively. Open and closed arrowheads indicate Pex5pS and Pex5pL, respectively. Dots indicate an unknown protein band. B, shown are the results from mutation analysis of PEX5 derived from ZPG231. The nucleotide sequences at residues 634–648 of PEX5L (left panels) and PEX5S (right panels) from wild-type (10) (upper panels) and ZPG231 (lower panels) cells are shown. A point mutation at nucleotide 641 changes a codon (TCC) for Ser214 to a codon (TTC) for Phe 214 in both Pex5pL and Pex5pS. A schematic representation of Pex5p and the S214F mutation site are shown below.

C, shown are the results from the transfection of mutated Chinese hamster PEX5. ZPG231-derived PEX5L (CIPEX5L-S214F) was transfected into ZPG231 (panel a) and PEX5-defective ZP105 (panels b and c) and ZP139 (panel d). ZPG231 was assessed for import of PTS2-GFP (panel a); ZP105 was stained with antibodies to thiolase (panel b) and PTS1 (panel c). ZP139 was stained with anti-catalase antibody (panel d). Magnification × 630; bar = 20 μm.

Fig. 5. Binding of Pex5p to potential Pex5p-interacting peroxins and PTS1-related proteins. An in vitro binding assay was performed using two isoforms of Pex5p from the wild type and pex5 mutant ZPG231. GST and fusion proteins GST-Pex5pL, GST-Pex5pS, GST-Pex5pL-S214F, and GST-Pex5pS-S214F were separately incubated with lysates of 207P7 cells (8 × 10^6 each). After thorough washing, proteins bound to glutathione-Sepharose were analyzed by SDS-PAGE. Input represents the lysate from 4 × 10^6 cells. Pex7p, Pex14p, acyl-CoA oxidase (AOx), and catalase were determined by immunoblotting using antibodies to the respective proteins. Two bands immunoreactive to anti-Pex7p antibody were discernible; the one with a higher mobility is presumably the product translated from the second Met located 10 amino acids downstream of the initiator (21).
Pex5pL distinguishes Pex7p carrying cargo from the unloaded one. Other features of Pex5p function such as mechanisms involved in recognizing and binding to cargoes including PTS1 and Pex7p, transporting the cargoes, anchoring to the docking site, and subsequently translocating to other members of the peroxisomal translocon remain to be addressed at the molecular level. Investigation using three mutually distinct pex5 mutants (ZPG231, ZP105, and ZP139) together with PEX5 expression products that are derived from these cell mutants will shed light on such issues.

From a diagnostic viewpoint, a CG test that stains only PBD patient-derived specimens with antibodies such as those to catalase, PTS1, and PTS2 is not sufficient. Two types of CGs, pex5 CG2 and pex7 CG11, showing the same morphological cell phenotype are indistinguishable, requiring transfection of PEX5L, as clearly seen in the CHO mutants ZPG231 and ZPG207 in this report. Future work will therefore examine CG2 patients for whom only the PTS2 import role of Pex5pL is impaired.

Acknowledgments—We thank the other members of the Fujiki laboratory for helpful comments and R. J. A. Wanders for fibroblasts from a RCDP patient. We also thank N. Thomas for comments.

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J. Biol. Chem. 2000, 275:21715-21721.
doi: 10.1074/jbc.M000721200 originally published online April 14, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000721200

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