ACADEMIC PRESS

Biochemical and Biophysical Research Communications 293 (2002) 225–230

BBRC

www.academicpress.com

Isolation of Chinese hamster ovary cell *pex* mutants: two *PEX7*-defective mutants☆

Eiko Yanago,[a] Takahide Hiromasa,[a] Tsuyoshi Matsumura,[a] Naohiko Kinoshita,[a] and Yukio Fujiki[a,b,*]

[a] *Department of Biology, Faculty of Sciences, Kyushu University Graduate School, Fukuoka 812-8581, Japan*
[b] *SORST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan*

Received 14 March 2002

Abstract

We searched for novel Chinese hamster ovary (CHO) cell mutants defective in peroxisome biogenesis by an improved method using peroxisome targeting signal 2 (PTS2)-tagged enhanced green fluorescent protein (EGFP). From mutagenized TKaEG2 cells, the wild-type CHO-K1 stably expressing rat Pex2p and PTS2-EGFP, cell colonies resistant to the 9-(1′-pyrene)nonanol/ultraviolet treatment were examined for intracellular location of PTS2-EGFP. Of six mutant cell clones two, ZPEG227 and ZPEG231, showed cytosolic PTS2-EGFP, indicative of impaired PTS2 import, and numerous PTS1-positive particles. *PEX7* expression restored the impaired PTS2 import in both mutants. Cell fusion with fibroblasts from a patient with *PEX7*-defective rhizomelic chondrodysplasia punctata did not complement PTS2 import defect of ZPEG227 and ZPEG231, confirming that these two are *pex7* mutants. Mutation analysis of *PEX7* by reverse transriptase (RT)-PCR indicated that ZPEG227-allele carried an inactivating nonsense mutation, Trp158Ter. Therefore, ZPEG227 is a *pex7* mutant possessing a newly identified mutation in mammalian *pex7* cell lines.

Keywords: CHO cell mutants; Peroxisome biogenesis; Complementation groups, peroxin; *PEX7*, peroxisome biogenesis disorders; Rhizomelic chondrodysplasia punctata; Green fluorescent protein

Peroxisomal matrix and membrane proteins are synthesized on free polyribosomes in the cytoplasm and post-translationally imported into peroxisomes [1]. Two distinct topogenic signals, peroxisomal targeting signal types 1 (PTS1) and PTS2 [2,3], direct newly synthesized proteins to the peroxisomal matrix. PTS1 is the C-terminal tripeptide SKL motif [4,5] and PTS2 comprises N-terminal cleavable presequence containing a nonapeptide with the conserved sequence, (R/K)(L/V/I)X_5(H/Q)(L/A) [6,7]. A number of *PEX* genes encoding peroxins are essential for peroxisome biogenesis [3,8,9]. *PEX5* and *PEX7* encode the cytoplasmic receptors for PTS1 and PTS2, respectively.

Peroxisomal functions are highlighted by human fatal genetic, peroxisome biogenesis disorders (PBDs) in which various metabolic pathways, including β-oxidation of very long fatty acids, are impaired. PBD is autosomal recessive and includes 12 different known genotypes [8–10], with four distinct phenotypes, Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum's disease, and rhizomelic chondrodysplasia punctata (RCDP) [11]. We previously isolated 13 complementation groups (CGs) of peroxisome biogenesis-defective CHO cell mutants, including Z24/ZP107, Z65, ZP92, ZP105/ZP139, ZP109, ZP110, ZP114, ZP119, ZP124, ZP126, ZP128, ZPG207, and ZPG208 [8], mostly by the 9-(1′-pyrene)nonanol (P9OH)/UV selection method [12]. ZPG207 and ZPG208 were isolated by using PTS2-GFP as a readily monitoring marker protein [8]. All CHO mutants resembled fibroblasts from PBD patients with regard to the defects in

☆ *Abbreviations:* AOx, acyl-CoA oxidase; CG, complementation group; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; PBD, peroxisome biogenesis disorders; PTS1 and PTS2, peroxisome targeting signal types 1 and 2; RCDP, rhizomelic chondrodysplasia punctata; RT, reverse transcriptase; thiolase, peroxisomal 3-ketoacyl-CoA thiolase.

* Corresponding author. Fax: +81-092-642-4214.
E-mail address: yfujiscb@mbox.nc.kyushu-u.ac.jp (Y. Fujiki).

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00219-X

peroxisome assembly despite normal synthesis of peroxisomal proteins. CHO cell mutants, Z24/ZP107, Z65, ZP92, ZP105/ZP139, ZP109, ZP119, ZP124, ZP128, ZPG207, and ZPG208, were classified into 10 CGs of 12 human CGs [8,13]; others such as ZP110, ZP114, and ZP126 represent 3 CGs distinct from 12 human CGs. Thereby, mammalian peroxisome assembly apparently requires at least 15 genes or their products.

We have so far isolated or identified nine peroxin cDNAs, including *PEX1*, *PEX2*, *PEX3*, *PEX5*, *PEX6*, *PEX7*, *PEX12*, *PEX14*, and *PEX19*, by genetic strategies such as functional phenotype-complementation assays using CHO cell mutants, ZP107, Z65, ZPG208, ZP105/ZP139, ZP92, ZPG207, ZP109, ZP110, and ZP119, respectively [8,14]. We also showed that these *PEX*s are pathogenic genes responsible for human PBDs [8,10,13,14]. Thus, peroxisome assembly-defective CHO cell mutants are indeed proven to be useful for investigating molecular and cellular mechanisms involved in peroxisome biogenesis and for delineation of the primary defects of PBD. Meanwhile, more CGs are very likely to be present in mammals, because over 20 *PEX* genes have been identified in yeast [3,15].

In the present study, we attempted to isolate more CGs of peroxisome biogenesis-defective animal cell mutants, by a modified method using "enhanced" green fluorescent protein (EGFP). We report here a *pex7* mutant ZPEG227 second isolated but with a novel mutation.

Materials and methods

Cell lines. CHO cells were cultured as described [16]. Skin fibroblast cell lines from patients, including fibroblasts from patients with RCDP [14] and CG1 Zellweger syndrome [17], were cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM) supplemented with 10% fetal bovine serum, as described [14].

Plasmid expressing PTS2-EGFP. Expression plasmid, pEGFP, coding for EGFP was purchased from Clontech (Clontech, Tokyo, Japan). Expression plasmid of peroxisomal targeting signal type 2 (PTS2)-tagged EGFP was constructed in a mammalian expression vector pUcD2Hyg, essentially as described for pUcD2HygPTS2-GFP [18].

EGFP-transformant of CHO cell. TKa cells, wild-type CHO-K1 transformed with rat *PEX2* cDNA [19,20], were transfected with pUcD2HygPTS2-EGFP. Stable transformant, termed TKaEG2, was selected in the presence of 200 μg/ml of hygromycin B, and transfectants highly expressing EGFP were cloned by the limiting dilution method.

Selection of peroxisome-deficient CHO cell mutants. TKaEG2 cells were mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Nacalai Tesque, Kyoto, Japan) and selected for cell mutants resistant to the P9OH/UV treatment, as described [18,20].

Morphological analysis. PTS2-EGFP in cells grown a cover glass was observed without cell fixation, under a Carl Zeiss Axioskop FL microscope using a No.17 filter (Oberkochen, Germany). Peroxisomes in CHO cells were also visualized by indirect immunofluorescence light microscopy, using rabbit antibodies to 3-ketoacyl-CoA thiolase [16], PTS1 peptide [21], and catalase [16]. Antigen–antibody complexes were detected by Texas Red-labeled goat anti-rabbit immunoglobulin G antibody (Leinco Technologies, Ballwin, MO) under a Carl Zeiss Axioskop FL microscope.

DNA transfection and cell fusion. Transfection of *PEX* cDNAs to CHO mutant cells was done by lipofection as described [22]. For cell fusion, parent CHO mutant cells and cells, including RCDP patient-derived fibroblasts, to be examined were cocultured for 24 h, then fused using polyethylene glycol, as described [16,23]. Complementation of cell mutants was assessed by peroxisomal punctate localization of PTS2-EGFP.

Identification of mutation. Cloning of *PEX7* from ZPEG227 was performed by reverse transcriptase (RT)-PCR, essentially as described [14]. RT-PCR was done to amplify the full-length *PEX7* open reading frame, with Superscript RT (Gibco BRL) and 10 μg of total RNA was obtained from ZPEG227, using primers: a forward primer ClPEX 7FFSalI, 5′-TCGACGCGGACGGGATGAGCGCGGCGCGGAC G-3′ (initiation codon, *underlined*) and a reverse primer ClPEX7FR-NotI, 5′-CGCCGGCAGCAGCAGCATATCTTAACCAGGAAC-3′ (termination codon, *underlined*). Amplified DNA fragments were subcloned into the pGEM-T Easy vector (Promega) and sequenced by the dideoxy chain termination method. ZPEG227-allele derived *PEX7* cDNA was cloned into pUcD2SRαMCSHyg. RT-PCR was likewise done using 6 μg total RNA each from ZPEG231 and CHO-K1.

Other method. Western blot analysis on polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) was performed using horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody and ECL Western blotting detection reagent (Amersham Biosciences, Tokyo, Japan).

Results and discussion

Isolation and morphological analysis of CHO cell mutants

In the present work, we investigated the isolation of peroxisome-deficient CHO cell mutants distinct from the previously isolated cell clones, by using as a parent cell line *PEX2*-transformed CHO-K1 cells, TKaEG2, that ectopically and stably expressed PTS2-EGFP and the P9OH/UV selection method. New aspect of the present study included the introduction of PTS2-tagged EGFP that would make its intracellular location more readily visible than using GFP [18] as a reporter under fluorescent microscope without cell fixation thereby highly accelerating the mutant screening step. Numerous punctate structures were seen in TKaEG2 (Fig. 1a), apparently brighter than those in TKaG2 expressing PTS2-GFP [18], which were superimposable on those stained with anti-catalase antibody in respective cells (data not shown), indicative of localization of PTS2-EGFP in peroxisomes. TKaEG2 cells were mutagenized and those cells resistant to P9OH/UV treatment were selected. Viable cell colonies were examined for peroxisome morphology by virtue of localization of PTS2-EGFP. Six P9OH/UV-resistant mutant cell clones, ZPEG224, ZPEG227, ZPEG228, ZPEG229, ZPEG230, and ZPEG231, were isolated in three separate cycles of mutant isolation. All cell mutants except for ZPEG224 showed a diffuse pattern of PTS2-EGFP in the cytoplasm, indicative of a defect in peroxisomal PTS2

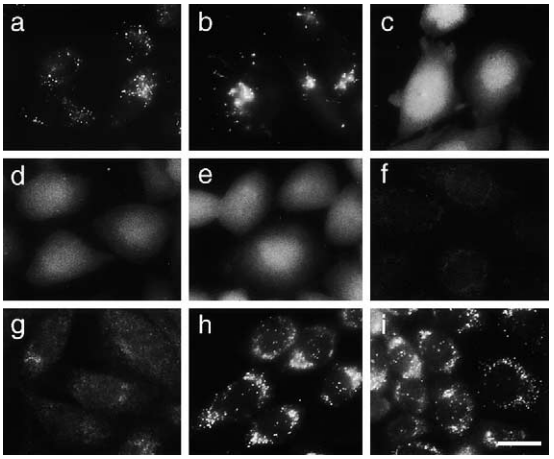

Fig. 1. Fluorescence microscopy of PTS2-EGFP-expressing wild-type CHO cells and peroxisome biogenesis-defective mutants. Cell mutants defective in peroxisome biogenesis were isolated from TKaEG2, rat *PEX2*-transformed wild-type CHO-K1 stably expressing PTS2-tagged EGFP. Unfixed cells were monitored by fluorescence microscopy. Cells: TKaEG2 (a); CHO cell mutants, ZPEG224, ZPEG227, ZPEG228, ZPEG231, and ZPG207 (b–f); ZPEG227 cells were stained with antibodies to 3-ketoacyl-CoA thiolase (g), PTS1 (h), and catalase (i). Scale, 10 μm.

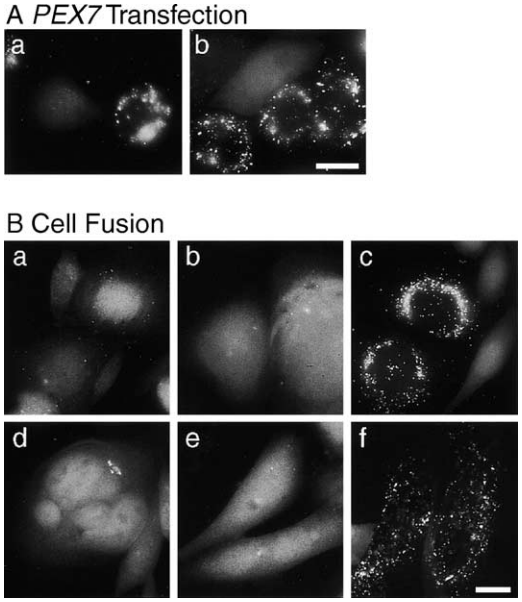

Fig. 2. Complementation group analysis. (A) ZPEG227 and ZPEG231 were transfected with Chinese hamster *PEX7* (a and b). (B) Cell mutants were pairwise fused: ZPEG227 fused with a *pex7* ZPG207 (a); ZPEG231 with ZPG207 (b); ZPEG227 with a *pex1* ZP107 (c); ZPEG227 with ZPEG231 (d); ZPEG227 cells fused with fibroblasts from PBD patients each with *PEX7*-impaired RCDP (e) and *PEX1*-defective CG1 Zellweger syndrome (f), respectively. Complementation was assessed by the punctate appearance of PTS2-EGFP fluorescence. Scale, 10 μm.

import (Fig. 1c–e). ZPEG224 showed PTS2-EGFP-positive, aberrant structures (Fig. 1b), similar to those isolated earlier [24,25]. Of note, the cytoplasmically localized PTS2-EGFPs in ZPEG227, ZPEG228, and ZPEG231 were more distinct in fluorescence intensity than PTS2-GFP seen in ZPG207 (Fig. 1f), hence making more readily detectable in screening for PTS2 import defective cell mutants.

Next, to assess the deficiency of peroxisome biogenesis, cell mutants were stained with antibodies to several peroxisomal matrix proteins. Peroxisomal 3-ketoacyl-CoA thiolase was found in the cytosol in ZPEG227 (Fig. 1g) and ZPEG231 (not shown), confirming the defect of PTS2 import. Cell staining using antibodies to PTS1 and catalase showed many particles in both ZPEG227 (Fig. 1h and i) and ZPEG231 (data not shown), indicating normal imports of PTS1 and catalase. In contrast, ZPEG228, ZPEG229, and ZPEG230 showed a cytosolic staining pattern of both PTS1 and catalase (data not shown), suggesting a defect of PTS1 and catalase imports as well.

Complementation group analysis

CG analysis of CHO mutants was performed by *PEX* cDNA transfection and cell fusion. Thirteen different *PEX* cDNAs, including *PEX1*, *PEX2*, *PEX3*, *PEX5*, *PEX6*, *PEX7*, *PEX10*, *PEX11* (α and β), *PEX12*, *PEX13*, *PEX14*, *PEX16*, and *PEX19*, were separately transfected into ZPEG227. PTS2-EGFP import was restored by expression of only *PEX7* (Fig. 2A, a), not others (Table 1), strongly suggesting that ZPEG227 is a *pex7* mutant. ZPEG231 was also complemented by transfection of *PEX7* (Fig. 2A, b). Transfection of *PEX1* restored import of not only PTS2-EGFP catalase in ZPEG228, ZPEG229, and ZPEG230 (Table 1), but also PTS1 and catalase (data not shown), suggesting that these cell mutants are *PEX1*-defective. It is possible that these three *pex1* mutants were derived from a single clone, since these were isolated from the same cell-culture plate. Expression of other *PEX* cDNAs did not complement these three mutants (data not shown).

Table 1
Complementation group analysis of CHO cell mutants

Mutant	Transfection		Cell fusion		
	PEX1	*PEX7*	*pex7* ZPG207	*pex1* ZP107	RCDP[a]
ZPEG224[b]	–	–	+	+	ND
ZPEG227	–	+	–	+	–
ZPEG228	+	–	ND	ND	ND
ZPEG229	+	–	ND	ND	ND
ZPEG230	+	–	ND	ND	ND
ZPEG231	–	+	–	+	–

+, PTS2-EGFP import was restored; –, not restored; ND, not done.

[a] Fibroblasts from a patient with RCDP.

[b] ZPEG224 showed aberrant peroxisome morphology (see text).

Next, cell fusion of ZPEG227 and ZPEG231 each with ZPG207, the currently available, a single *pex7* CHO mutant isolated earlier [18], produced no EGFP-positive punctate structures (Fig. 2B, a and b), indicating that both mutants belong to the same CG as ZPG207. In contrast, numerous PTS2-EGFP-positive particles were discernible in the fused cells between ZPEG227 and a *pex1* ZP107 (Fig. 2B, c) and those between ZPEG231 and each of *pex1* ZP107 (Table 1) as well as *pex2* Z65, *pex5* ZP105, and *pex14* ZP161 (data not shown). Furthermore, fusion of ZPEG227 with ZPEG231 resulted in no complementation of peroxisomes, demonstrating that these two were in the same CG (Fig. 2B, d). Fused cells of ZPEG227 and ZPG231 each with the wild-type CHO-K1 showed EGFP-positive particles as numerous as in the wild-type TKaEG2 (data not shown), thereby suggesting that lesions of allele(s) in the mutants were recessive. Furthermore, to confirm that ZPEG227 and ZPEG231 belong to *PEX7*-deficient human CG11, cell fusion was carried out using human fibroblasts from PBD patients. Peroxisomes were not re-established in fused cells of ZPEG227 with CG11 RCDP fibroblasts (Fig. 2B, e), whereas numerous punctate EGFP-containing structures, peroxisomes, were noted in the hybrid cells with *PEX1*-defective CG1 PBD fibroblasts (Fig. 2B, f). Essentially the same results were obtained for ZPEG231. Therefore, ZPEG227 and ZPEG231 belong to the same CG as *pex7* CG11 RCDP. Together with the findings by *PEX* cDNA transfection, ZPEG227 and ZPEG231 are the newly isolated, second and third cell lines of CHO *pex7* mutants. PTS2-EGFP may be an efficient marker cargo protein for screening of somatic cell mutants more readily with the defect of PTS2 import.

Properties of CHO cell mutants

We studied the biogenesis of peroxisomal enzymes in ZPEG227 and ZPEG231 by immunoblot. AOx, a PTS1 protein, consists of 75 kDa A, 53 kDa B, and 22 kDa C polypeptide components [16]; B and C are derived from A [16]. All three polypeptide components were evident in the wild-type CHO-K1 and *pex7* mutants ZPEG227 and ZPEG231 (Fig. 3, lanes 1–3), indicative of normal biogenesis of AOx and consistent with the morphological observation. Peroxisomal thiolase, a PTS2 protein, is synthesized as a larger precursor of 44 kDa, then processed to a 41 kDa mature form [16,20]. Only a 44 kDa thiolase precursor was detectable in ZPEG227 and ZPEG231 (Fig. 3, lanes 5 and 6), as seen in earlier mutants [16,20,23], whilst 41 kDa mature protein was apparent in CHO-K1 (Fig. 3, lane 4). Thus, normal import of PTS1 but impaired transport of thiolase precursor was evident in ZPEG227 and ZPEG231, in good agreement with morphological observation.

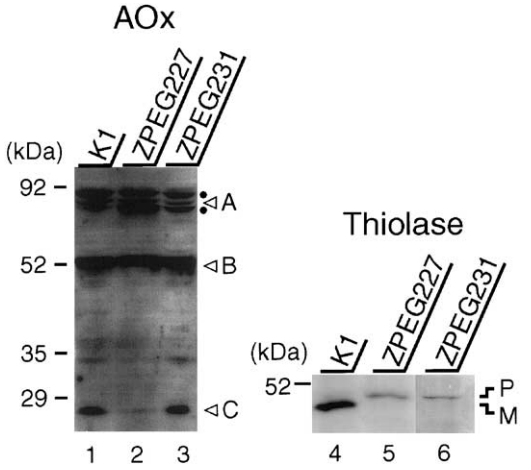

Fig. 3. Biogenesis of peroxisomal proteins. Cell lysates (5×10^5 cells) were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membrane. Immunoblot analysis was done using rabbit antibodies to AOx (left) and thiolase (right). Lanes: 1 and 4, CHO-K1; 2 and 5, ZPEG227; 3 and 6, ZPEG231. Open arrowheads show AOx components, A, B, and C; P and M indicate a larger precursor and mature protein of thiolase, respectively. Dots indicate nonspecific bands [14].

Dysfunction of Pex7p in CHO mutants

To investigate dysfunction of Pex7p in ZPEG227 and ZPEG231, we performed RT-PCR using total RNA and *PEX7*-specific primers. Resultant PCR products were detectable in a single, 1.0 kb band from CHO-K1 and in two, 1.0 and 0.7 kb bands from ZPEG227 (Fig. 4A, upper left). No apparent band was discernible from ZPEG231, implying a failure of *PEX7* transcription or degradation of *PEX7* mRNA. Northern blot analysis of RNA from ZPEG231 confirmed this finding (Fig. 4A, lower left). Subsequent sequencing of ZPEG227-derived, six independent 1.0 kb cDNA clones indicated a mutation of nucleotide G to A at position 474 (the A of the initiation codon ATG being 1) in a codon (TGG) for Trp^{158}, resulting in a termination codon (TGA) (Fig. 4A, right), named *PEX7W158Ter*. Of six independent 0.7 kb cDNA clones four indicated a deletion of the sequence corresponding to human exon 3 [26]; one likewise showed an apparent truncation of exons 4 and 5 and one lacking exons 4–6 (data not shown). Next, to assess the impaired function of Pex7p, *PEX7W158Ter* was transfected back to ZPEG227. PTS2-EGFP remained in the cytosol in the transfected cells (Fig. 4B, a). No restoration of PTS2 import was observed in *PEX7W158Ter*-transfected ZPG207 (Fig. 4B, c) and ZPEG231 (data not shown), whereas GFP-positive particles, peroxisomes, were discernible in wild-type *PEX7*-transfected ZPG207 cells (Fig. 4B, b) thereby suggesting that *PEX7W158Ter* protein was biologically inactive. Collectively, we conclude that dysfunction of Pex7p caused by W158Ter-mutation is likely to be responsible for the defect of

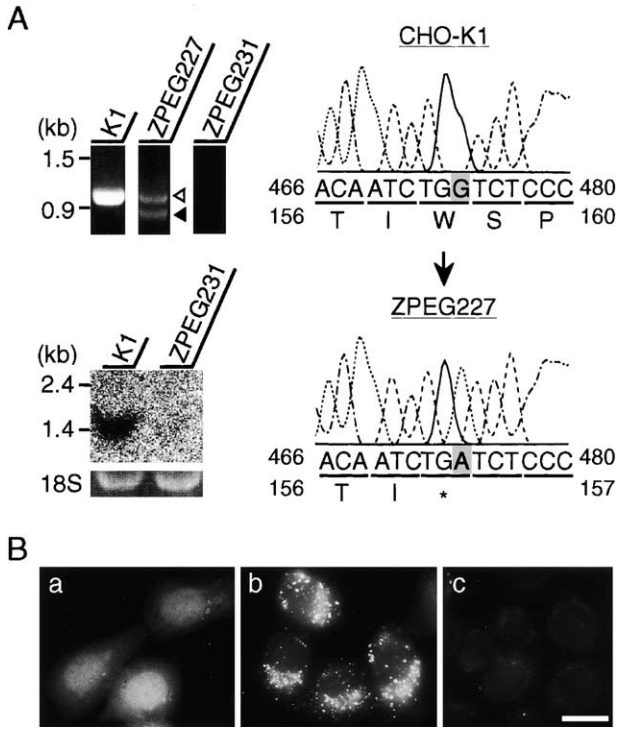

Fig. 4. Mutation analysis of *PEX7* in *pex7* mutants ZPEG227 and ZPEG231. (A) Upper left panel, RT-PCR products from CHO-K1, ZPEG227, and ZPEG231 cells were separated by 1%-agarose gel electrophoresis. DNA size markers are on the left. Open and solid arrowheads indicate ZPEG227-derived 1.0 and 0.7 kb PCR products, respectively. Lower left, Northern blot analysis of total RNA (20 μg) from CHO-K1 and ZPEG231. RNA blot was hybridized with ^{32}P-labeled *PEX7*. Ribosomal RNA (18S) was used to check the amount of RNA loaded. Exposure: 23 h. Right panel, partial and deduced amino acid sequences of *PEX7* cDNA isolated from CHO-K1 and ZPEG227-allele are shown. A point mutation at nucleotide residue 474 changes a codon for Trp^{158} to a termination codon. The mutant *PEX7W158Ter* sequence was deposited to the GenBank database accession number AB042621 for Chinese hamster *PEX7* [14]. (B) Transfection of *PEX7* and mutant *PEX7* to PTS2 import-defective mutant cells. Cells: ZPEG227 (a); ZPG207 (b and c). Transfection was done with *PEX7W158Ter* (a and c) and wild-type *PEX7* (b). Import of PTS2-EGFP in ZPEG227 and PTS2-GFP in ZPG207 was verified by fluorescence microscopy. Note that peroxisomal PTS2-protein import was restored in (b), but not in (a and c). Scale, 10 μm.

PTS2 import of ZPEG227. It is equally possible that the *PEX7* variants lacking exon 3, exons 4 and 5, and/or exons 4–6 are likewise responsible for the phenotype of ZPEG227, as deduced from the findings on alternative transcripts of human *PEX7* [26] and truncation mutants of CHO *PEX7* [14].

Four different site mutations in *PEX7* have been identified in mammals, including humans [14,27–29]. The most frequently occurring *PEX7* mutations in patients with RCDP are at L292Ter in the 6th Trp-Asp (WD) motif and less frequently at A218V and G217R [27,28]. Very recently, we identified a homozygous nonsense mutation, W221Ter, in ZPG207 alleles responsible for the PTS2 import defect [14]. Hence, we have here identified a novel inactivating mutation W158Ter that occurred in the 3rd WD motif in *PEX7*. Furthermore, we have isolated a novel type of *pex7* mutant ZPEG231 with no detectable *PEX7* mRNA. Such a mutant has not been reported in *PEX7*-defective cell mutants in mammals, including fibroblasts from RCDP patients.

In this study, totally six cell mutants were isolated, out of which two clones were classified into *pex7* CG11 and three mutants were in *pex1* CG1. Therefore, the method modified in the present work was indeed proven to be highly efficient and useful for isolating peroxisome biogenesis-defective mutant cells. In addition to a single, currently used *pex7* mutant ZPG207 expressing PTS2-GFP, ZPEG227 and ZPEG231 may serve as a model mammalian cell system where a cargo PTS2-EGFP is more readily detectable. Moreover, these *pex7* mutants also will be useful to study the function and dysfunction of Pex7p, the PTS2 receptor at molecular and cellular levels.

Acknowledgments

We thank K. Ghaedi and S. Mukai for the advice, and the other members of our laboratory for the helpful comments. The work was supported in part by CREST and SORST grants (to Y.F.) from the Japan Science and Technology Corporation, Grants-in-Aid for Scientific Research (09044094, 12308033, 12557017, 12206069, and 13206060 to Y.F.) from The Ministry of Education, Science, Sports, and Culture, and a grant from the Uehara Memorial Foundation.

References

[1] P.B. Lazarow, Y. Fujiki, Biogenesis of peroxisomes, Annu. Rev. Cell Biol. 1 (1985) 489–530.
[2] Y. Fujiki, K. Okumoto, H. Otera, S. Tamura, Peroxisome biogenesis and molecular defects in peroxisome assembly disorders, Cell Biochem. Biophys. 32 (2000) 155–164.
[3] S. Subramani, A. Koller, W.B. Snyder, Import of peroxisomal matrix and membrane proteins, Annu. Rev. Biochem. 69 (2000) 399–418.
[4] S.J. Gould, G.-A. Keller, N. Hosken, J. Wilkinson, S. Subramani, A conserved tripeptide sorts proteins to peroxisomes, J. Cell Biol. 108 (1989) 1657–1664.
[5] S. Miura, I. Kasuya-Arai, H. Mori, S. Miyazawa, T. Osumi, T. Hashimoto, Y. Fujiki, Carboxyl-terminal consensus Ser–Lys–Leu-related tripeptide of peroxisomal proteins functions in vitro as a minimal peroxisome-targeting signal, J. Biol. Chem. 267 (1992) 14405–14411.
[6] T. Osumi, T. Tsukamoto, S. Hata, S. Yokota, S. Miura, Y. Fujiki, M. Hijikata, S. Miyazawa, T. Hashimoto, Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting, Biochem. Biophys. Res. Commun. 181 (1991) 947–954.
[7] B.W. Swinkels, S.J. Gould, A.G. Bodnar, R.A. Rachubinski, S. Subramani, A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase, EMBO J. 10 (1991) 3255–3262.
[8] Y. Fujiki, Peroxisome biogenesis and peroxisome biogenesis disorders, FEBS Lett. 476 (2000) 42–46.

[9] S.J. Gould, D. Valle, Peroxisome biogenesis disorders: genetics and cell biology, Trends Genet. 16 (2000) 340–345.
[10] N. Matsumoto, S. Tamura, A. Moser, H.W. Moser, N. Braverman, Y. Suzuki, N. Shimozawa, N. Kondo, Y. Fujiki, The peroxin Pex6p gene is impaired in peroxisome biogenesis disorders of complementation group, J. Hum. Genet. 46 (2001) 273–277.
[11] P.B. Lazarow, H.W. Moser, Disorders of peroxisome biogenesis, in: C.R. Scriver, A.I. Beaudet, W.S. Sly, D. Valle (Eds.), The metabolic basis of inherited disease, McGraw-Hill, New York, 1995, pp. 2287–2324.
[12] O.H. Morand, L.-A.H. Allen, R.A. Zoeller, C.R.H. Raetz, A rapid selection for animal cell mutants with defective peroxisomes, Biochim. Biophys. Acta 1034 (1990) 132–141.
[13] K. Ghaedi, M. Honsho, N. Shimozawa, Y. Suzuki, N. Kondo, Y. Fujiki, *PEX3* is the causal gene responsible for peroxisome membrane assembly-defective Zellweger syndrome of complementation group G, Am. J. Hum. Genet. 67 (2000) 976–981.
[14] S. Mukai, K. Ghaedi, Y. Fujiki, Intracellular localization, function, and dysfunction of the peroxisome-targeting signal type 2 receptor, Pex7p, in mammalian cells, J. Biol. Chem. 277 (2002) 9548–9561.
[15] V.I. Titorenko, R.A. Rachubinski, The life cycle of the peroxisome, Nat. Rev. Mol. Cell Biol. 2 (2001) 357–368.
[16] T. Tsukamoto, S. Yokota, Y. Fujiki, Isolation and characterization of Chinese hamster ovary cell mutants defective in assembly of peroxisomes, J. Cell Biol. 110 (1990) 651–660.
[17] S. Tamura, K. Okumoto, R. Toyama, N. Shimozawa, T. Tsukamoto, Y. Suzuki, T. Osumi, N. Kondo, Y. Fujiki, Human *PEX1* cloned by functional complementation on a CHO cell mutant is responsible for peroxisome-deficient Zellweger syndrome of complementation group I, Proc. Natl. Acad. Sci. USA 95 (1998) 4350–4355.
[18] K. Ghaedi, A. Kawai, K. Okumoto, S. Tamura, N. Shimozawa, Y. Suzuki, N. Kondo, Y. Fujiki, Isolation and characterization of novel peroxisome biogenesis-defective Chinese hamster ovary cell mutants using green fluorescent protein, Exp. Cell Res. 248 (1999) 489–497.
[19] T. Tsukamoto, A. Bogaki, K. Okumoto, K. Tateishi, Y. Fujiki, N. Shimozawa, Y. Suzuki, N. Kondo, T. Osumi, Isolation of a new peroxisome deficient CHO cell mutant defective in peroxisome targeting signal-1 receptor, Biochem. Biophys. Res. Commun. 230 (1997) 402–406.
[20] K. Okumoto, A. Bogaki, K. Tateishi, T. Tsukamoto, T. Osumi, N. Shimozawa, Y. Suzuki, T. Orii, Y. Fujiki, Isolation and characterization of peroxisome-deficient Chinese hamster ovary cell mutants representing human complementation group III, Exp. Cell Res. 233 (1997) 11–20.
[21] H. Otera, K. Tateishi, K. Okumoto, Y. Ikoma, E. Matsuda, M. Nishimura, T. Tsukamoto, T. Osumi, K. Ohashi, O. Higuchi, Y. Fujiki, Peroxisome targeting signal type 1 (PTS1) receptor is involved in import of both PTS1 and PTS2: studies with *PEX5*-defective CHO cell mutants, Mol. Cell. Biol. 18 (1998) 388–399.
[22] K. Okumoto, N. Shimozawa, A. Kawai, S. Tamura, T. Tsukamoto, T. Osumi, H. Moser, R.J.A. Wanders, Y. Suzuki, N. Kondo, Y. Fujiki, *PEX12*, the pathogenic gene of group III Zellweger syndrome: cDNA cloning by functional complementation on a CHO cell mutant, patient analysis, and characterization of Pex12p, Mol. Cell. Biol. 18 (1998) 4324–4336.
[23] N. Shimozawa, T. Tsukamoto, Y. Suzuki, T. Orii, Y. Fujiki, Animal cell mutants represent two complementation groups of peroxisome-defective Zellweger syndrome, J. Clin. Invest. 90 (1992) 1864–1870.
[24] R. Toyama, S. Mukai, A. Itagaki, S. Tamura, N. Shimozawa, Y. Suzuki, N. Kondo, R.J.A. Wanders, Y. Fujiki, Isolation, characterization, and mutation analysis of *PEX13*-defective Chinese hamster ovary cell mutants, Hum. Mol. Genet. 8 (1999) 1673–1681.
[25] K. Ghaedi, A. Itagaki, R. Toyama, S. Tamura, T. Matsumura, A. Kawai, N. Shimozawa, Y. Suzuki, N. Kondo, Y. Fujiki, Newly identified Chinese hamster ovary cell mutants defective in peroxisome assembly represent complementation group A of human peroxisome biogenesis disorders and one novel group in mammals, Exp. Cell Res. 248 (1999) 482–488.
[26] N. Braverman, G. Steel, P. Lin, A. Moser, H. Moser, D. Valle, *PEX7* gene structure, alternative transcripts, and evidence for a founder haplotype for the frequent RCDP allele, L292ter, Genomics 63 (2000) 181–192.
[27] N. Braverman, G. Steel, C. Obie, A. Moser, H. Moser, S.J. Gould, D. Valle, Human *PEX7* encodes the peroxisomal PTS2 receptor and is responsible for rhizomelic chondrodysplasia punctata, Nat. Genet. 15 (1997) 369–376.
[28] A.M. Motley, E.H. Hettema, E.M. Hogenhout, P. Brites, A.L.M.A. ten Asbroek, F.A. Wijburg, F. Baas, H.S. Heijmans, H.F. Tabak, R.J.A. Wanders, B. Distel, Rhizomelic chondrodysplasia punctata is a peroxisomal protein targeting disease caused by a non-functional PTS2 receptor, Nat. Genet. 15 (1997) 377–380.
[29] P.E. Purdue, J.W. Zhang, M. Skoneczny, P.B. Lazarow, Rhizomelic chondrodysplasia punctata is caused by deficiency of human *PEX7*, a homologue of the yeast PTS2 receptor, Nat. Genet. 15 (1997) 381–384.