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Temperature-Sensitive Mutation of *PEX6* in Peroxisome Biogenesis Disorders in Complementation Group C (CG-C): Comparative Study of *PEX6* and *PEX1*

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ABSTRACT

Peroxisome biogenesis disorders (PBD), including Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease, are a group of genetically heterogeneous autosomal-recessive diseases caused by mutations in *PEX* genes that encode peroxins, proteins required for peroxisome biogenesis. Zellweger syndrome patients present the most severe phenotype, whereas neonatal adrenoleukodystrophy patients are intermediate and infantile Refsum disease patients have the mildest features. *PEX6* is a causative gene for PBD of complementation group C (CG-C) and encodes the peroxin Pex6p, one of the ATPases associated with diverse cellular activities and a member of the same family of proteins as Pex1p, a causative protein for PBD of CG-E (CG1). Here, we identified the temperature sensitivity of peroxisomes in the fibroblasts of a patient with neonatal adrenoleukodystrophy in CG-C. Peroxisomes were morphologically and biochemically formed at 30°C but not at 37°C. This patient was homozygous for a missense mutation, T→C at nucleotide 170 resulting in a change from leucine to proline at amino acid 57 (L57P) in Pex6p. CG-C cell mutants (ZP92) in the Chinese hamster ovary transfected with L57P in *HsPEX6* revealed the same temperature-sensitive phenotype. However, *PEX1*-deficient Chinese hamster ovary cell mutants (ZP101) transfected with L111P in *PEX1*, the counterpart to L57P in *PEX6*, showed no temperature sensitivity. In addition, ZP92 transfected with G708D in *PEX6*, the counterpart to the temperature-sensitive mutation G843D in *PEX1*, revealed no temperature-sensitive phenotype. These results indicate that L57P in Pex6p is a temperature-sensitive mutation causing the milder phenotype in a patient with PBD in CG-C. They also indicate that the amino acid residues responsible for temperature sensitivity do not seem to be conserved between Pex6p and Pex1p. **(*Pediatr Res* 48: 541–545, 2000)**

Abbreviations

ts, temperature sensitive
AAA, ATPases associated with diverse cellular activities
CG, complementation group
PBD, peroxisome biogenesis disorders
ZS, Zellweger syndrome
NALD, neonatal adrenoleukodystrophy
IRD, infantile Refsum disease
RT-PCR, reverse transcription PCR
DHAP-AT, dihydroxyacetone phosphate acyltransferase
CHO, Chinese hamster ovary
VLCFA, very-long-chain fatty acids
PMP70, 70-kD peroxisomal membrane protein

Peroxisomes are ubiquitous organelles involved in numerous metabolic functions including the synthesis of plasmalogens, cholesterol, and bile acids and the oxidative processes involving H_2O_2, namely, the beta oxidation of VLCFA (1). In humans, defective peroxisomal metabolic functions result in ZS, NALD, and IRD, collectively referred to as PBD, which are genetically classified into at least 12 CG (2). Ten causative genes (*PEX*) have been cloned: *PEX1*, *PEX2*, *PEX5*, *PEX6*, *PEX7*, *PEX10*, *PEX12*, *PEX13*, *PEX16*, and *PEX19*, corresponding with CG-E (CG1), CG-F (CG10), CG2, CG-C

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(CG4), CG11, CG-B (CG7), CG3, CG-H, CG-D (CG9), and CG-J in humans, respectively (3–22). ZS patients have profound dysmorphic features, skeletal abnormalities, renal cysts, and severe progressive neurologic and hepatic disease and, therefore, rarely survive early infancy. The NALD and IRD phenotypes are similar but manifest milder clinical signs than ZS. Patients with IRD do not have significant abnormalities of nervous tissue. In previous studies, we revealed the restoration of peroxisomes in a temperature-dependent manner in the fibroblasts of patients with milder types of PBD, those with all types of IRD, and some with the NALD phenotype in CG-E (CG1), CG-A (CG8), CG-F (CG10), and CG-H. In addition, we demonstrated ts mutations that cause this phenomenon in IRD and NALD patients with CG-E (CG1), CG-F (CG10), and CG-H; G843D in *PEX1*, E55K in *PEX2*, and I326T in *PEX13*, respectively (18, 23, 24). We have now identified a missense mutation in *PEX6* from a NALD patient with CG-C (CG4) whose fibroblasts revealed the same ts phenotype. The mutation leads to the ts assembly of peroxisomes. In the present study, we discuss the relationship between the ts phenotype and the clinical signs in the NALD patient with CG-C (CG4) of PBD and the comparative function of temperature sensitivity with each corresponding ts mutation between *PEX6* and *PEX1* belonging to the genes encoding the same protein family.

METHODS

Cell lines and culture conditions. The patient was the only girl of healthy unrelated parents and was born after cesarean section. There was retinitis pigmentosa, sensorineural hearing loss, hypotonia, and mental and growth retardation with slight facial dysmorphia different from ZS. Skin fibroblasts of the patient, obtained with the agreement of her parents, showed a very reduced number of peroxisomes. Because of these clinical features and the cytologic appearance of fibroblasts, she was diagnosed as NALD. The cell line was classified as CG-C (CG4) of PBD and belonged to the same CG as CHO mutant ZP92, which was defined by somatic cell fusion analysis. Skin fibroblasts and CHO cells were cultured in Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% FCS, respectively. This study was approved by the Institutional Review Board of Gifu University.

Immunofluorescence study. Cultured cell lines were fixed with 4% paraformaldehyde/0.1 M potassium phosphate, pH 7.4, permeabilized with 0.1% Triton X-100/PBS, and blocked with 4% FCS/0.1% Triton X-100/PBS. In these fixed fibroblasts and CHO cells, peroxisomes were immunohistochemically stained with rabbit anti-human catalase and anti-rat catalase antibodies, respectively, as previously described (25).

Mutation analysis. The mRNA and genomic DNA were obtained from cultured fibroblasts by use of the QuickPrep mRNA purification kits (Pharmacia, Tokyo, Japan) and SepaGene kits (Sanko Jun-Yaku, Tokyo, Japan), respectively. The pathologic mutation in *PEX6* was identified in the cDNA prepared by RT-PCR from mRNA and genomic DNA as previously described (9, 26).

Site-directed mutagenesis of the mutation. The *Bam*HI and *Eco*RI fragment (nucleotide number 1–880) of the normal *HsPEX6* cDNA was replaced with a cassette with a corresponding fragment harboring the mutation (L57P) identified in the patient. The G708D and L111P mutation was constructed artificially by use of the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) in *HsPEX6* and *HsPEX1* cDNA, respectively. These mutations were confirmed by the sequence.

Transfection of cells. Transfection of plasmids pUcD2*HsPEX6*, pUcD2*HsPEX6L57P*, or pUcD2*HsPEX6G708D* into CG-C CHO mutant ZP92 cells (8×10^5) was performed using a Gene PulserII electroporator (Bio-Rad, Hercules, CA, U.S.A.) at 300 V and 400 μF. Stable transfectants were obtained after 400 μg/mL of G418 selection. Cotransfection of plasmid pUcD2Hyg and either pCMVSPORT*HsPEX1* or pCMVSPORT*HsPEX1L111P* into CG1 CHO mutant ZP101 was performed by electroporation under the same conditions, and stable transformants were isolated by selective growth in the presence of hygromycin (400 IU/mL).

Other methods. Lignoceric acid (C24:0) oxidation (27) and DHAP-AT activities were measured using ^{14}C-labeled substrates as described previously (28).

RESULTS

Immunofluorescence study with the fibroblasts of the NALD patient. The fibroblasts of a patient with NALD belonging to CG-C (C-11) were incubated for 72 h at 37 and 30°C and subjected to immunofluorescence staining with the anti-human catalase antibody. The punctate staining pattern of catalase typical for peroxisomes was observed at 37°C in only 5% of the patient's fibroblasts. The number of catalase-positive cells increased to 40% after the fibroblasts incubated at 30°C for 3 d (Fig. 1*A*). Catalase and the PMP70 were colocalized in the cells after 72 h incubation at 30°C (data not shown), confirming the identity of these catalase-positive granules as peroxisomes.

***Mutation analysis of* PEX6.** To determine the dysfunction of *PEX6* in the patient, we examined *PEX6* cDNA from fibroblasts by means of RT-PCR. We detected a missense mutation in all 10 cDNA clones isolated: T→C at nucleotide 170, producing an amino acid substitution at position 57 (Leu→Pro; L57P). Genomic PCR between nucleotide 1 and 882 in the *PEX6* open reading frame (exon 1 of *PEX6*) resulted in a single type of PCR product containing nucleotide 170C, indicating that the patient was probably homozygous for the L57P mutation (Fig. 1*B*).

***Identification of the ts mutation in* PEX6.** A CG-C (CG4) CHO cell mutant, ZP92, transfected with normal *HsPEX6* revealed restoration of peroxisomes under incubation at both 37 and 30°C (Fig. 2, *A* and *B*). To determine whether the L57P in *PEX6* was a ts mutation, ZP92 was stably transfected with *HsPEX6L57P*. The transfectants with a punctate distribution of catalase increased drastically to 40% of cells after 72 h of incubation at 30°C, whereas they appeared in 5% of cells incubated at 37°C (Fig. 2, *C* and *D*). The immunohistochemical appearance of ZP92 transfected with *HsPEX6L57P* was similar to that of the fibroblasts of the patient with NALD (C-11)

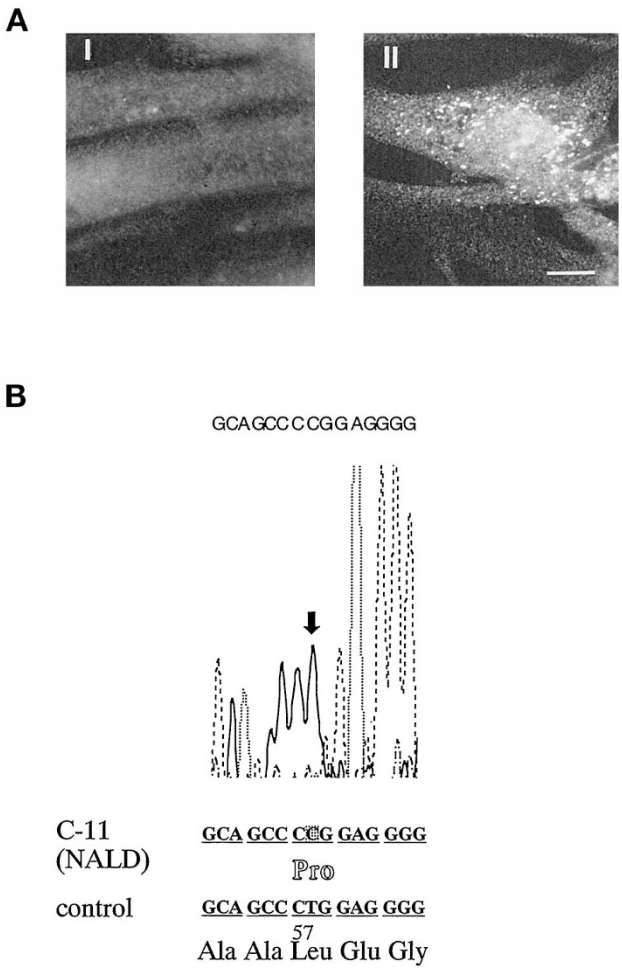

Figure 1. (*A*) Immunofluorescence study of peroxisomes in fibroblasts of the NALD patient (C-11) cultured for 72 h at either 37 (*panel I*) or 30°C (*panel II*). Specimens were stained with the anti-human catalase rabbit antibody. *Bar* = 10 μ m. (*B*) Mutation analysis of *PEX6* from the NALD patient (C-11) by use of an automated DNA sequencer. The partial nucleotide sequence and deduced amino acid sequence of *PEX6* cDNA isolated from the patient (*upper*) and a normal control (*lower*) are indicated. A T→C mutation at position 170 in codon 57 (Leu) results in the creation of a Pro codon (*arrow* and *shadow*).

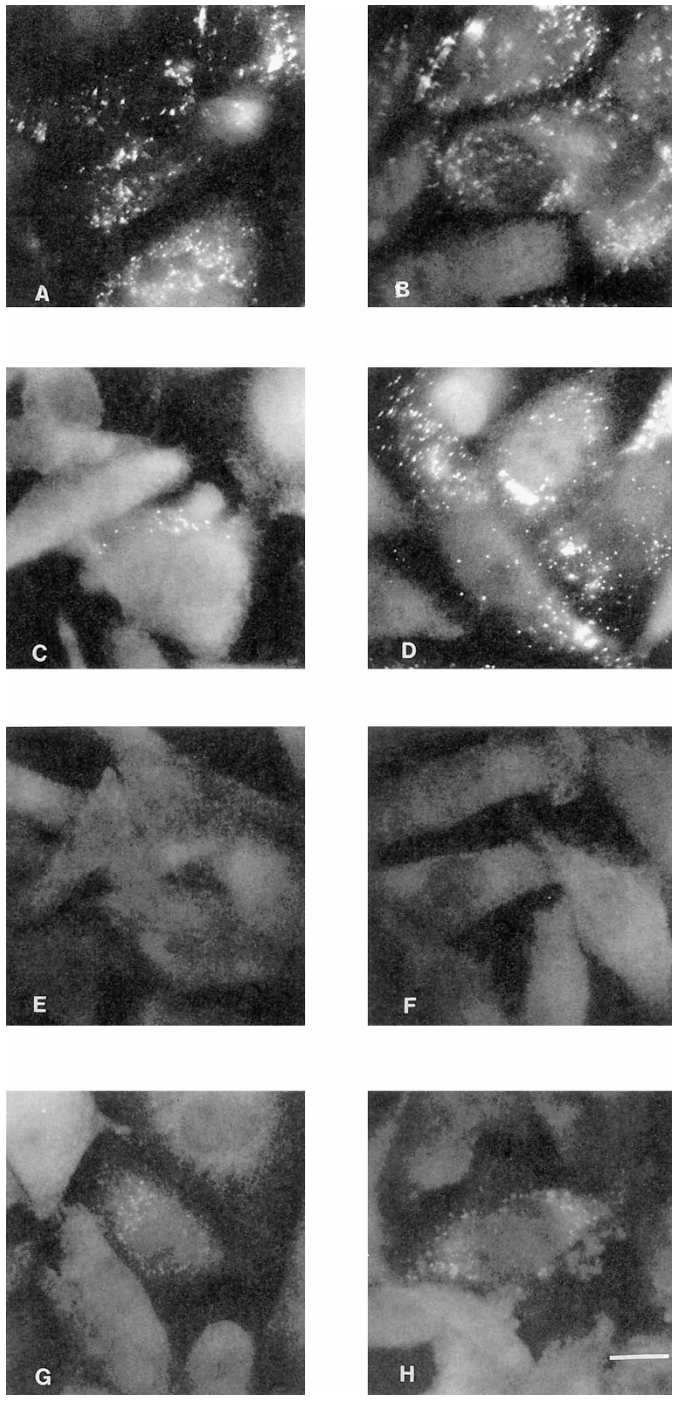

Figure 2. Immunofluorescence study of peroxisomes in CHO transfectants cultured for 72 h at either 37°C (*panels A, C, E*, and *G*) or 30°C (*panels B, D, F*, and *H*) and stained with anti-rat catalase. ZP92 cells were transfected, and stable transformants with *HsPEX6* (*panels A* and *B*), *HsPEX6L57P* (*panels C* and *D*), and *HsPEX6G708D* (*panels E* and *F*) were obtained. In the same way, stable transformants of ZP101 cells with *HsPEX1* (data not shown, but they had the same punctate appearance as in panels A and B) and *HsPEX1L111P* (*G* and *H*) were isolated. *Bar* = 10 μm.

incubated at either 37 or 30°C. These results demonstrated that L57P was a ts mutation in *PEX6*.

Biochemical assay of peroxisome in the ts cells. The peroxisomal beta-oxidation activity of lignoceric acid (C24:0) and the activity of DHAP-AT, the first enzyme of plasmalogen biosynthesis, were markedly elevated in both the fibroblasts of the patient and in the ZP92 cells transfected with *HsPEX6L57P* cultured at 30°C. These activities were lower in the cells cultured at 37°C (Table 1). These results suggested that the biochemical function and morphologic complementation of peroxisomes were improved in these cells in a temperature-dependent manner.

***Comparative study for peroxisome assembly of counterparts to ts mutations between* PEX6 *and* PEX1.** ZP92 transfected with *HsPEX6G708D*, which appears to be the counterpart of the ts mutation G843D in Pex1p (29), revealed no peroxisome under incubation at either 37 or 30°C (Fig. 2, *E* and *F*). ZP101 transfected *HsPEX1L111P*, the counterpart of the L57P amino acid substitution in Pex6p (29), showed 5% catalase-positive cells in stable transfectants under these conditions and no increase in the number of catalase-positive cells after incubation at 30°C (Fig. 2, *G* and *H*).

Table 1. *Temperature sensitivity of peroxisome biogenesis in the fibroblasts and CHO mutant belonging to CG-C (CG4)*

	Peroxisome-positive cells (%)*		C24:0¶		DHAP-AT‖	
	37°C	30°C	37°C	30°C	37°C	30°C
NALD patient (C-11)	5	40	42.7	171	0.208	0.672
Control	100	100	294	260	1.73	1.04
ZP92 with HsPEX6L57P	10	80	543	1040	0.508	0.920
Control (CHO-K1)	100	100	2110	1060	1.85	1.55

Cells were cultured for 72 h at either 37 or 30°C and then assayed for beta-oxidation activity of lignoceric acid and DHAP-AT activity at 37°C.

* The no. of immunofluorescent catalase-positive cells among 20 cells were counted in each of five view fields at ×1000, and data are averages of these indicated as %.

¶ The VLCFA oxidation capacity is expressed as lignoceric acid (C24:0) oxidation activity. Values are in pmol/h/mg protein.

‖ DHAP-AT, the first enzyme in the pathway leading to plasmalogen biosynthesis. Values are in nmol/120 min/mg protein.

DISCUSSION

In the present study, we found a patient with NALD of CG-C (CG4) showing a ts phenotype and identified a new ts mutation, L57P in the Pex6p. Similarly, an IRD patient (E-06) from CG-E (CG1) was homozygous for a ts mutation in *PEX1*, leading to a G843D amino acid substitution (24). Interaction between *PEX6* and *PEX1* for peroxisome biogenesis has been reported in both yeast and humans (29–32). The two ATP/GTP-binding motifs are highly conserved between *PEX6* and *PEX1* (3–5). Although the L57P mutation is located at exon 1 in *PEX6*, having lower homology than the AAA domain with *PEX1*, the codon Leu57 in Pex6p is also conserved as codon Leu111 in Pex1p (29). However, the L111P mutation is not fulfilled as the ts mutation in Pex1p because ZP101, which are *PEX1*-deficient CHO mutant cells, transfected with *HsPEX1L111P* reveal no ts phenotype, whereas 5% peroxisomes are assembled under 37°C incubation in these cells. The fibroblasts of a NALD patient (C-11) and ZP92 transfected with *HsPEX6L57P* also show 5–10% peroxisomes at 37°C. Therefore, L57P and L111P mutations may cause a leak for peroxisome assembly in *PEX6* and in *PEX1*, respectively.

In addition, the G843D mutation in Pex1p is a ts mutation located at the beginning of the second AAA cassette of Pex1p. The amino acid is also conserved even in Pex6p, codon Gly708 (29). In the same way, a missense mutation was constructed G→A at residue position 2123 in *PEX6*, in a codon (G*G*T) for Gly708, and resulted in a codon (G*A*T) for Asp708 in Pex6p, termed *HsPEX6G708D*. It transfected *HsPEX6G708D* to ZP92, *PEX6*-deficient CHO mutant cells. The stable transfectants reveal neither the ts phenotype nor formation of new peroxisomes. The AAA domain may have an important function in importing the peroxisomal matrix protein in the presence of ATP; therefore, a G708D mutation in the AAA domain may destroy the function of Pex6p. These results suggested that the amino acid residues responsible for temperature sensitivity may not be conserved between Pex6p and Pex1p, both of which belong to the same AAA protein family.

In conclusion, we identified a novel missense ts mutation in *PEX6*. Growing numbers of ts mutations have been identified in patients with mild PBD including multiple CG. Ts is an important factor in determining the clinical features of PBD and in the assembly of peroxisomes.

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