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Identification of Pex5pM, and Retarded Maturation of 3-Ketoacyl-CoA Thiolase and Acyl-CoA Oxidase in CHO Cells Expressing Mutant Pex5p Isoforms

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Recently, we isolated CHO cells, termed SK32 cells, that express mutant Pex5p (G432R), and showed mislocalization of catalase in the cytosol, but peroxisomal localization of 3-ketoacyl-CoA thiolase (thiolase) in the mutant cells [Ito, R. *et al.* (2001) *Biochem. Biophys. Res. Commun.* 288, 321–327]. While analyzing the mutant cells, we found a novel Pex5p isoform (Pex5pM), which was shorter by seven amino acids than Pex5pL and longer by 30 amino acids than Pex5pS. Similar levels of mRNA syntheses for the PEX5 gene were observed in both the wild type and mutant cells, but the protein levels of Pex5p isoforms were markedly reduced in the mutant cells cultured at 37°C and only slightly discernible at 30°C, suggesting that they could be rapidly degraded. Furthermore, we characterized the peroxisomal localization of thiolase and acyl-CoA oxidase (Aox) in SK32 cells. The proteins in the organelle fraction were protected from proteinase K-digestion in the mutant cells, indicating that they were translocated inside peroxisomes. However, the conversion of Aox from component A to components B and C was completely prevented at both 30 and 37°C, and the precursor form of thiolase was partially processed to the mature one in a temperature-sensitive manner. Transformed SK32 cells stably expressing one of the wild type Pex5p isoforms were isolated, and then the maturation steps for thiolase and Aox were examined. Pex5pM and S restored the processing of the two enzymes, but Pex5pL did not. In addition, Pex5pL prevented the maturation of thiolase observed at 30°C. These results indicate that (i) the novel Pex5pM is functional and (ii) a seven amino acids-insertion, which is present in the L isoform but absent in the M isoform, plays some role in the process of maturation of thiolase and Aox.

Key words: acyl-CoA oxidase, mutant CHO cells, peroxisomal targeting, peroxisome, Pex5p, thiolase.

Abbreviations: Aox, acyl-CoA oxidase; GFP, green fluorescent protein; LM, heavy plus light mitochondrial; PAGE, polyacrylamide gel electrophoresis; PMP70, 70 kDa peroxisomal integral membrane protein; PMSF, phenylmethanesulfonyl fluoride; PN, post-nuclear; post-LM, post-heavy plus light mitochondrial; RT-PCR, reverse-transcription polymerase chain reaction; PTS, peroxisomal targeting signal; thiolase, 3-ketoacyl-CoA thiolase; TS, temperature-sensitive.

Peroxisomes are organelles that are distributed ubiquitously in eukaryotic cells, from yeast to man. The organelles are bounded by a single membrane and involved in many metabolic pathways, including hydrogen peroxide–based cellular respiration, β-oxidation of fatty acids and biosynthesis of plasmalogen (*1*). Peroxisomal proteins are nuclear-encoded and synthesized on membrane-free polyribosomes in the cytosol. Two types of peroxisomal targeting signal (PTS), PTS1 and PTS2, have been identified as amino acid sequences necessary for peroxisomal sorting (*2–5*). PTS1 consists of the triplet sequence (S/A/C)(K/R/H)(L/H) at the carboxyl terminus of the protein. PTS2 consists of the amino-terminal sequence (R/K)(L/V/I)X_5(H/Q)(L/A) and is cleaved in peroxisomes in mammalian cells. Peroxisomal proteins containing PTS1 and PTS2 are recognized by specific receptors, Pex5p (*6–8*) and Pex7p (*9–11*), respectively, and are delivered to the peroxisomal membrane. After binding of a targeting complex to a docking site composed of Pex13p and Pex14p (*12–14*), the cargo-proteins are translocated across the membrane *via* a translocation apparatus (translocon) into the matrix. The current model proposes that the receptor, Pex5p, will shuttle back to the cytosol after release of the cargo-proteins in peroxisomes (*15–17*).

The fundamental mechanism by which peroxisomal proteins are translocated into the matrix is principally similar in eukaryotic cells, from yeast to man. However, some points are distinct. Firstly, in yeast, Pex13p and Pex14p, each of which is able to bind with Pex5p, function as a

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docking site for a Pex5p/cargo-protein complex. On the other hand, in mammalian cells, Pex14p but not Pex13p binds with Pex5p, indicating that mammalian Pex14p is the initial docking site. After binding of a Pex5p/cargo-protein complex at the docking site on the peroxisomal membrane, Pex14p will form a complex with Pex13p and then act as a part of the translocon organized together with other peroxins. Analyses using mammalian mutant cells have suggested that the Pex14p and Pex13p complex shares the translocon with Pex2p (*18*), Pex10p (*19*, *20*), and Pex12p (*21–23*). Secondly, the yeast PEX5 gene encodes one Pex5p. On the other hand, mammalian cells produce two isoforms, Pex5pL and Pex5pS, which will be created through alternative splicing (*24*, *25*). Pex5pL contains an internal insertion and is longer by 37–amino acids than Pex5pS, which is a cytosolic receptor for the peroxisomal targeting of PTS1 proteins. Pex5pL plays a crucial role not only in the targeting of PTS1 proteins but also in that of PTS2 proteins by binding with Pex7p (*26*). Thus, Pex5pL is involved in the peroxisomal translocation of both the PTS1 and PTS2 proteins.

To investigate the mechanism implicated in protein translocation to peroxisomes, we have isolated CHO mutant cells, which are deficient in peroxisome biogenesis (*27*). The study showed that 3-ketoacyl-CoA thiolase (thiolase) (PTS2 protein) was translocated without cleavage of its amino-terminal extension sequence in the peroxisomal membrane of PEX2 mutant cells (*28*). This observation suggested that the translocation and maturation of thiolase occurred in part through separate processes in the peroxisomal membrane. In addition, mutant CHO cells, termed SK32 cells, that express mutant Pex5p also exhibited peroxisomal localization of thiolase without cleavage of the precursor form (*29*). This finding indicated that Pex5p was involved in the process of maturation of thiolase, as was Pex2p. Since the proposed model postulates that the Pex5p/the cargo-protein complex will pass through the translocon complex including Pex2p in the membrane, retardation of processing of the thiolase precursor gives rise to the question as to how the peptidase activity is depressed in peroxisomes of PEX2 or PEX5 mutant cells.

So far, the functions of Pex5p isoforms have been investigated regarding protein translocation in detail. On the other hand, few reports have demonstrated any implication in the process of maturation of peroxisomal proteins (*30*). Thus, we further characterized SK32 cells in order to elucidate the involvement of Pex5p in the maturation steps for the peroxisomal protein. In the process, we found a novel Pex5p isoform (Pex5pM), which was longer by 30–amino acids than the Pex5pS isoform (and shorter by 7–amino acids than the Pex5L isoform). In this study, we show that thiolase (PTS2 protein) and Aox (PTS1 protein) are translocated without maturation of their proteins into the peroxisomes of SK32 cells. In addition, we demonstrate that the 7–amino acids sequence in Pex5pL, which is absent in Pex5pM, plays a pivotal role in antagonization of the maturation process.

EXPERIMENTAL PROCEDURES

Cell Culture—CHO-K1 cells expressing a peroxisomal form of green fluorescent protein (GFP), which is referred to as CHO-K1/GFP-SKL, was used as a wild type cell line, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum and 0.4 mg/ml hygromycin B (*27*). SK32 cells, which harbor a missense mutation in the PEX5 gene, are derived from CHO-K1/GFP-SKL, as described elsewhere (*27*, *29*). Transformed SK32 cells stably expressing one of the wild type Pex5p isoforms were cultured in the medium containing 1 mg/ml geneticin. Human skin fibroblasts, wild type and PEX5-defective cells were cultured in DMEM containing 10% (v/v) fetal calf serum.

Plasmid Construction, Transfection and Transformation of Cells—Wild type PEX5 cDNA encoding each isoform was inserted between the *Xho*I and *Not*I sites in a eukaryotic expression vector, pCI-neo (Promega, Madison, WI). Each plasmid was then transfected into SK32 cells by means of a liposome-mediated procedure. The stable transformants expressing wild type Pex5pL, Pex5pM and Pex5pS, respectively, were isolated in the medium containing 1 mg/ml geneticin, and referred to as SK32/Pex5pL, SK32/Pex5pM, and SK32/Pex5pS, respectively.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)—To verify the appearance of the Pex5pM isoform, RT-PCR was carried out on total RNA derived from wild type and SK32 cells. For first strand DNA synthesis, total RNA was prepared from either cell line and used as the template with an oligo(dT)18 primer in the presence of Moloney murine leukemia virus reverse transcriptase (Takara, Kyoto, Japan) at 42°C. The single-stranded DNA synthesized was used as a template and the region corresponding nucleotide positions 488 to 1077 (positions in Pex5pL cDNA) was amplified with KOD-plus-polymerase (Toyobo, Osaka, Japan), using a sense primer appending *Eco*RI linker (5′-GGAATTCATCTGGAGCAGTCTGAGGAGAAGC-3′) and an antisense primer appending *Eco*RI linker (5′-GGAATTCAAAAAGCAGCACGGCATTGGGCAG-3′). The conditions were as follows: initial heating at 94°C for 2 min, then 30 cycles of 94°C for 15 s, 62°C for 30 s and 68°C for 60 s, followed by one cycle of 68°C for 3 min. The PCR product (about 600 bp) was digested with *Eco*RI and then subcloned into pBluescript II KS (+) to verify the nucleotide sequences of both strands with an ABI373A sequencer (Perkin-Elmer, Foster, CA). To examine expression of the genes encoding the Pex5p isoforms in SK32 cells, semi-quantitative RT-PCR was carried out using total RNA prepared from wild type and mutant cells. In the reaction, DNA fragments from nucleotide positions 488 to 1077 (positions in Pex5pL cDNA) were amplified as described above except that primers without the *Eco*RI linker (sense primer: 5′-ATCTGGAGCAGTCTGAGGAGAAGC-3′, antisense primer: 5′-AAAAAGCAGCACGGCATTGGGCAG-3′) were used.

For Pex5p isoforms in human skin fibroblasts, semi-quantitative RT-PCR was carried out using a pair of primers (sense primer: 5′-TCAGAGGAGAAGCTGTGGCT-3′, antisense primer: 5′-TGTCCCAGAAATCGACATCA-3′). The DNA fragments from nucleotide positions 513 to 771 (positions in human Pex5pS cDNA, accession number U19721) were amplified.

The PCR products were separated by 2% (w/v) agarose-gel electrophoresis using Certified LowRange Ultra agarose (Bio-Rad).

Morphological Analysis—CHO cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline for

30 min, washed three times and then treated with 0.5% (v/v) TritonX-100 for permeabilization. Peroxisomal proteins were then subjected to indirect immunocytochemical staining, using rabbit antibodies raised against rat thiolase, rat Aox, rat catalase, or the C-terminal 15 amino acid peptide of rat 70 kDa peroxisomal integral membrane protein (PMP70), and rhodamine-conjugated swine anti-rabbit immunoglobulin (DAKO, Kyoto, Japan) under a fluorescent microscope with a No. 15 filter (Zeiss, Axioplan, Oberkochen, Germany). Peroxisomal GFP was observed under the fluorescent microscope with a No. 10 filter.

Subcellular Fractionation—CHO cells were harvested in ice-cold STE (0.25 M sucrose, 20 mM Tris-HCl, pH8.0, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin A and 1 mM phenylmethanesulfonyl fluoride (PMSF)) and then homogenized gently using a Teflon glass homogenizer. After centrifugation at $600 \times g$ for 5 min to precipitate nuclei and unbroken cells, the resultant supernatant, the post-nuclear (PN) fraction, was recentrifuged to separate the heavy plus light mitochondrial (LM) fraction and the microsomal plus cytosolic fraction (post-LM fraction) at $20{,}000 \times g$ for 10 min in a TLA120.2 rotor (Beckman, Palo Alto, CA). The procedures for subcellular fractionation were performed at 4°C. The LM fraction contained mitochondria and peroxisomes. This observation was based on the preferential distributions of a mitochondrial enzyme, malate dehydrogenase, and a peroxisomal protein, PMP70 (data not shown).

Immunoblotting—The subcellular fractions were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer of the proteins to a nitrocellulose membrane, and the binding of rabbit antibodies against rat thiolase, rat Aox, the C-terminal 15 amino acid peptide of rat PMP70 or the C-terminal 19 amino acids peptide of CHO Pex5p (*29*). The immunoblotted membrane was incubated with ^{125}I-labeled protein A (Amersham IM144, Buckinghamshire, England) and the radioactive band was visualized with an image analyzer, Fuji Bas 2000.

Proteinase K-Treatment of the LM Fraction—The LM fraction was digested for 30 min in protease inhibitor-free STE buffer with a final concentration of 0–20 μg/ml of proteinase K on ice. The reaction was stopped by the addition of 2 mM PMSF (final concentration) and an equal volume of 120 mM Tris-HCl, pH 6.8, 4% (w/v) SDS and 10% (v/v) 2-mercaptoethanol, and then the mixture immediately boiled for 5 min, and subjected to SDS-PAGE, followed by immunoblotting.

RESULTS

Novel Pex5p Isoform—When we isolated cDNA clones encoding Pex5p by using a cDNA library of wild type CHO cells (Invitrogen, NV Leek, The Netherlands), we found one clone that encodes a novel isoform in addition to two clones encoding Pex5pL or Pex5pS. The novel isoform comprising 633 amino acid residues, referred to as Pex5pM (the middle isoform), lacks seven amino acid residues, positions 216 to 222, of Pex5pL (Fig. 1). To confirm the existence of Pex5pM mRNA, RT-PCR was carried out using mRNA derived from wild type and SK32 cells. The PCR products amplified from nucleotide positions 488 to 1077 (corresponding amino acid positions 163 to 359) were sequenced. The sequence analyses showed that the RT-PCR products derived from wild type cells were composed of eight clones for the L isoform, four for the M isoform and four for the S isoform, and those derived from SK32 cells comprised three clones for the L isoform, one for the M isoform and seven for the S isoform. The appearance of cDNA encoding Pex5pM, even though at variable frequency, indicated that the transcript for the novel isoform is synthesized in both the wild type and mutant cells. Alignment of the Pex5p isoforms demonstrated that the nucleotide sequence at the 3′-end of the intron creating the M or S isoform is AG in common, which is a consensus acceptor sequence for the splicing reaction (*31*) (Fig. 1). These data suggest that alternative splicing gives rise to the Pex5pM isoform in addition to Pex5pL and Pex5pS.

PEX5 Expression and the Protein Level in the Mutant Cells—To compare the mRNA levels for Pex5p isoforms in wild type and mutant cells, we carried out semi-quantitative RT-PCR with a pair of primers (488S and 1077R, see "EXPERIMENTAL PROCEDURES") using total RNA prepared from each cell line. PCR products amplified from PEX5L, M and S cDNAs comprised 590 bp, 569 bp and 479 bp, respectively. Figure 2A shows that the cDNAs encoding the L and S isoforms were slightly more than that for the M isoform in SK32 cells, in contrast, cDNA encoding the L isoform was more than that for the M and S

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                TTGGCTAACTCCGAGTTCCTGAAATTCGTGCGACAGATTGGCGAGGGGCAGGTGTCCCTGGAGTCTGCTGCAGGG
Pex5pL 211  LeuAlaAsnSerGluPheLeuLysPheValArgGlnIleGlyGlnGlyGlnValSerLeuGluSerAlaAlaGly 235
Pex5pM 211  LeuAlaAsnSerGlu  -  -  -  -  -  -  -  IleGlyGlnGlyGlnValSerLeuGluSerAlaAlaGly 226
Pex5pS 211  LeuAlaAsnSerGlu  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  215

                TCGGGCCGAGCTCAGGCAGAACAGTGGGCAGCAGAGTTTATACAGCAGCAGGGCACATCAGAGGCCTGGGTCGAT
Pex5pL 236  SerGlyArgAlaGlnAlaGluGlnTrpAlaAlaGluPheIleGlnGlnGlnGlyThrSerGluAlaTrpValAsp 260
Pex5pM 229  SerGlyArgAlaGlnAlaGluGlnTrpAlaAlaGluPheIleGlnGlnGlnGlyThrSerGluAlaTrpValAsp 253
Pex5pS 216   -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  -  GlyThrSerGluAlaTrpValAsp 223
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Fig. 1. **Amino acid sequence alignment of Chinese hamster Pex5p isoforms.** Amino acid positions 211 to 260 in the L isoform and the corresponding positions in the M and S isoforms are shown. Dashes indicate deletion of seven amino acids in the M isoform and of 37 residues in the S isoform. The nucleotide sequence is given in the upper line. The consensus sequence AG at the 3′-end of the intron, which creates the M and S isoforms, is underlined. The DDBJ data base accession number for the Chinese hamster Pex5pM isoform is AB098709.

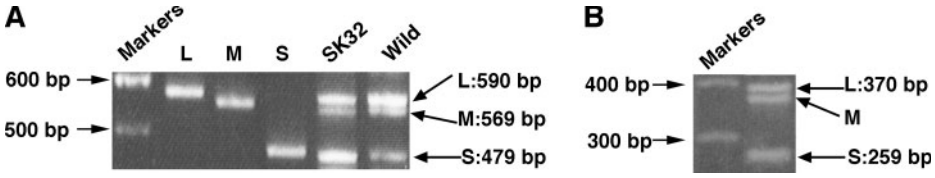

Fig. 2. **Pex5p isoform transcripts in CHO cells (wild type and SK32) and human fibroblasts.** Semi-quantitative RT-PCR was carried out for evaluating mRNA synthesis. Pex5 cDNAs were amplified with primer pairs as described under "EXPERIMENTAL PROCEDURES," using total RNA prepared from SK32 cells or wild type cells (A), and from human skin fibroblasts (B). The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. A: The plasmids encoding Pex5pL (lane L), Pex5pM (lane M) and Pex5pS (lane S) were amplified using the same primer pair as that used for RT-PCR. The amplified Pex5p cDNAs were 590 bp for L, 569 bp for M and 479 bp for S, using the primer pair. B: The amplified Pex5p cDNAs were 370 bp for L and 259 bp for S.

forms in wild type cells. These results were consistent with the frequencies of Pex5p isoforms in each cell type, as described above. Braverman *et al*. (*24*) have reported that a human PEX5 transcript undergoes alternative splicing to produce PEX5L and PEX5S. PEX5L differs from PEX5S in a 111 bp insert. To investigate the appearance of PEX5M form in humans, we attempted to detect PEX5M in human skin fibroblasts by RT-PCR as described above. Fig. 2B shows that there were three PCR fragments. The bands at the migration positions of 259 bp and 370 (259 + 111) bp correspond to PCR fragments derived from PEX5S and PEX5L, respectively. An additional band was detected at the position of about 340 bp, suggesting that Pex5pM might be expressed in the human cell line.

Then, we examined the protein levels of Pex5p isoforms produced in these cells (Fig. 3A). The immunoblot analysis showed that Pex5p isoforms were detected in wild type cells but not in SK32 mutant ones (Fig. 3A, lanes 2–4). However, the experiment with longer period exposure showed the production of Pex5p isoforms in the mutant cells (Fig. 3B). The protein levels in the mutant cells at 30°C could be roughly estimated to be less than one-tenth of those in wild type cells at 37°C, and much less at 37°C. Considering that mRNA synthesis was normal in SK32 mutant cells (Fig. 2), the Pex5p isoforms would be rapidly degraded in the mutant cells. On the other hand, the abundant production of each Pex5p isoform in the transformed SK32 cells indicated that wild type isoforms were stable in the mutant (G432R) cells (Fig. 3A, lanes 5–7). The transformant cells also showed that the M isoform moved more slowly on SDS-PAGE than the L and S isoforms did, inconsistent with their molecular masses (Fig. 3A, lanes 5–7). Disagreement between the molecular mass and mobility was previously noted for mammalian Pex5pL and S isoforms (*32*). Nevertheless, under our experimental conditions, the L and S isoforms migrated with similar mobility.

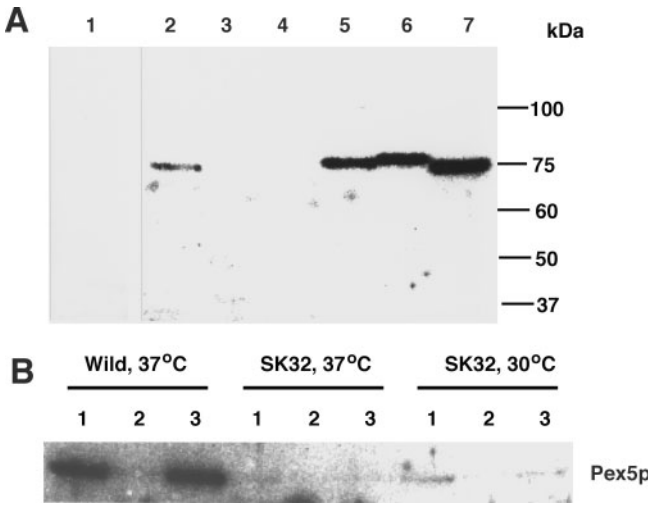

Fig. 3. **Immunoblot analysis of Pex5p in wild type, SK32 and transformed SK32 cells.** (A) Wild type cells (lanes 1 and 2), SK32 cells (lanes 3 and 4), and the transformed SK32 cells stably expressing wild type Pex5pL (lane 5), wild type Pex5pM (lane 6), and wild type Pex5pS (lane 7) were cultured at 37°C (lanes 1, 2 and 4–7) or at 30°C (lane 3). Cell lysates (6×10^5 cells) prepared immediately after harvesting were subjected to SDS-PAGE, blotted and detected with anti-Pex5p antiserum (lanes 2–7) or preimmune serum (lane 1), followed by binding with ^{125}I-protein A. Molecular markers are indicated on the right. (B) Cells were cultured at the indicated temperature, harvested and then homogenized in STE buffer. PN (1), LM (2) and post-LM (3) fractions were separated by centrifugation. The PN fraction (75 μg protein) and equivalent amounts of the LM and post-LM fractions were examined using anti-Pex5p antiserum as in (A) except that the image plate was exposed to the isotope for a longer period (120 h) in (B) than the usual period (20 h) in (A).

Mutant Phenotypes of SK32 Cells—Since Pex5pL and S isoforms are known to act as receptors in the process of the peroxisomal translocation of PTS1 and/or PTS2 proteins (*24*, *25*), SK32 cells are supposed to exhibit defective phenotypes in these processes. Immunocytochemical observations showed that catalase (PTS1 protein) was mislocalized in the cytosol and a recombinant GFP-appending SKL sequence at the carboxyl-terminus was mislocalized in a temperature-sensitive (TS) manner, as described previously (*29*). However, thiolase (PTS2 protein) and Aox (PTS1 protein) are localized in peroxisomes, as was PMP70 (Fig. 4). It is well known that thiolase is processed from the precursor form to the mature one (*33*, *34*), and that Aox is converted from component A to components B and C (*35*) in peroxisomes. Accordingly, these processing events were investigated using PN fraction prepared from the wild type and mutant cells (Fig. 5A). In wild type cells, thiolase was processed to the mature form (41 kDa), and Aox was almost completely converted from component A into component B. Herein, component C was hardly detectable under the conditions used, suggesting that this component might be degraded in CHO cells, as described by Fujiwara *et al*. (*36*). On the other hand, SK32 cells showed that the processing of thiolase was completely prevented at 37°C and partially restored at 30°C. The conversion of

Aox was completely prevented at both temperatures, resulting in the single appearance of component A in the mutant cells. The distinctive phenotypes on maturation suggest that different processing enzymes could be involved in the respective steps for thiolase and Aox.

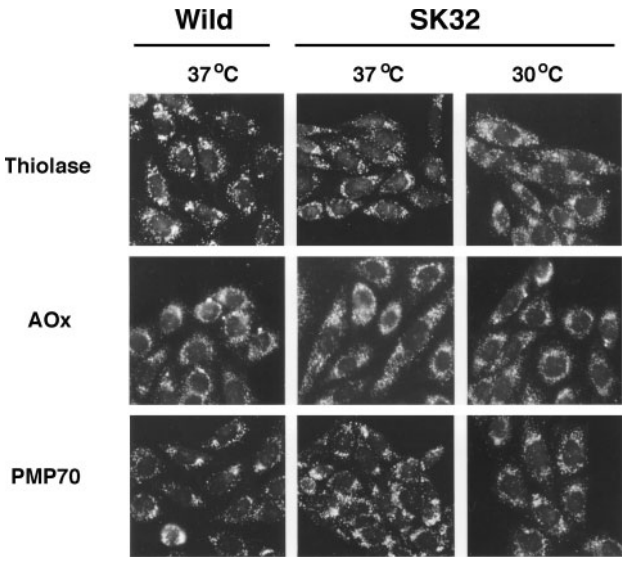

Fig. 4. **Peroxisomal assembly in SK32 cells.** Wild type and SK32 cells were cultured for 7 days at the indicated temperature, and then observed as to immunofluorescence after staining with anti-thiolase, anti-Aox, or anti-PMP70 antibodies. The fluorescent patterns were similar at 37°C and 30°C in wild type cells (data not shown).

To confirm the peroxisomal translocation of thiolase and Aox, we assessed the translocation by means of subcellular fractionation. Subcellular fractions, PN, LM and post-LM, were prepared from wild type and SK32 cells, and then subjected to immunoblot analysis (Fig. 5B). Thiolase (mature form), Aox (components A and B), and PMP70 were recovered in the LM fraction of wild type cells. Similarly, the precursor and/or mature forms of thiolase (precursor form at 37°C and both forms at 30°C) were detected in the LM fraction of mutant cells. Component A of Aox was also detected in the LM fraction of the mutant cells at both temperatures.

Thiolase and Aox Are in Inside Peroxisomes—To determine the distribution of thiolase and Aox in peroxisomes, the LM fraction was digested with proteinase K (Fig. 6). In wild type cells, thiolase (mature form) and Aox (components A and B) were protected from the digestion in the absence of Triton X-100 under the conditions where PMP70 facing the cytoplasmic side in the peroxisomal membrane was degraded. In the presence of Triton X-100, thiolase was completely degraded and Aox was digested into component B with a slightly smaller molecular mass. Since component B of rat liver is known to resistant to trypsin digestion (*35*), it is plausible that component B is resistant to proteinase K-treatment in the presence of Triton X-100. When the LM fraction of mutant cells cultured at 37°C or 30°C was treated with proteinase K in the absence of Triton X-100, the precursor and/or mature forms of thiolase and component A of Aox were protected

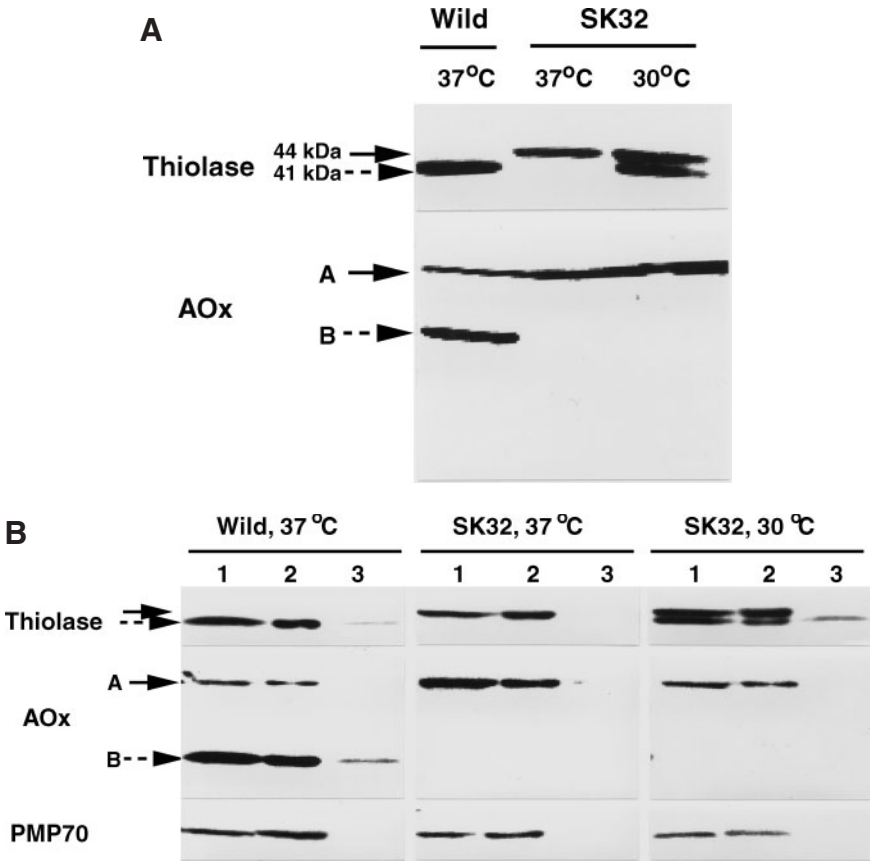

Fig. 5. **The retarded maturation and subcellular distribution of thiolase and Aox in SK32 cells.** (A) The PN fraction (75 μg protein) of cells cultured at the indicated temperature was subjected to SDS-PAGE and then analyzed by immunoblotting using anti-thiolase or anti-Aox antibodies, followed by binding of ^{125}I-protein A. Arrows indicate the precursor form (44 kDa) and mature form (41 kDa) of thiolase, or components A and B of Aox. (B) Cells were cultured at the indicated temperature, harvested and then homogenized in STE buffer. PN (1), LM (2) and post-LM (3) fractions were separated by centrifugation. The distributions of thiolase, Aox and PMP70 were analyzed by SDS-PAGE and immunoblotting using ^{125}I-protein A. The PN fraction (75 μg protein) and equivalent amounts of the LM and post-LM fractions were examined.

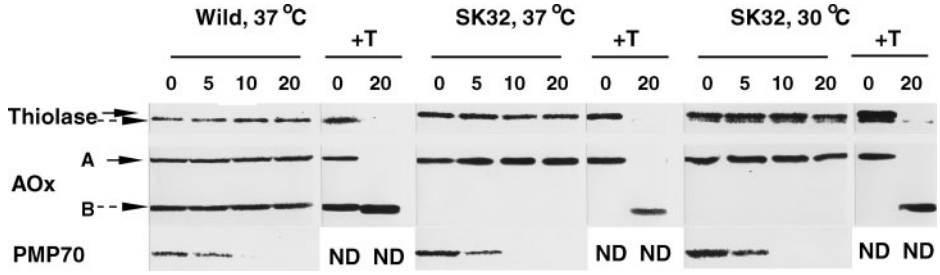

Fig. 6. **Thiolase and Aox localized in peroxisomes of SK32 cells were protected against proteinase K digestion.** The LM fraction (20 μg) of cells cultured at 37°C or 30°C was incubated in protease inhibitor–free STE buffer containing the indicated concentrations (μg/ml) of proteinase K on ice for 30 min in the absence of Triton X-100. The reactions were stopped by the addition of PMSF, followed by immediate boiling after the addition of ×2 Laemmli buffer. The treated samples were subjected to SDS-PAGE and immunoblot analysis using the antibodies and ^{125}I-proteinA. PMP70 is susceptible to the digestion in the absence of TritonX-100 and not detectable (ND) in the presence of Triton X-100.

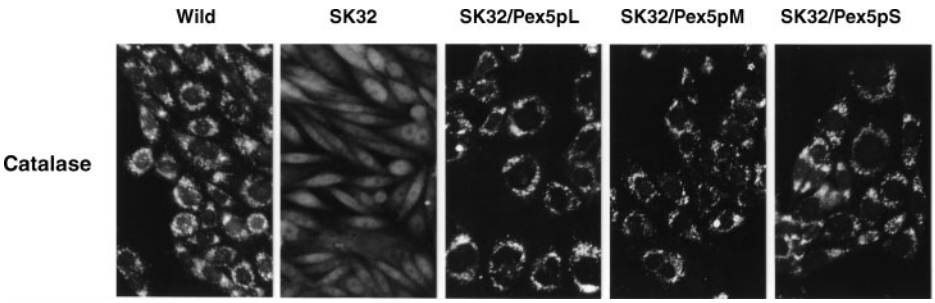

Fig. 7. **Peroxisomal restoration of catalase in transformed SK32 cells stably expressing the wild type Pex5p isoform.** Wild type CHO cells, parental mutant SK32 cells and the three transformed SK32 cell lines stably producing each of the wild type Pex5p isoforms were cultured at 37°C and then observed as to immunofluorescence after staining with anti-catalase antibodies.

from the digestion similarly to as in wild type cells. The presence of Triton X-100, the digestion resulted in the disappearance of thiolase and the appearance of component B. This protection indicated that these proteins in the mutant cells were located inside peroxisomes. In addition, this peroxisomal translocation suggested that the processing reactions for maturation (thiolase) and conversion (Aox) could occur in peroxisomes after they had been translocated into peroxisomes as unprocessed forms. The peroxisomal translocation of thiolase and Aox in SK32 cells suggests that reduced amounts of Pex5p isoforms are still sufficient for the translocation of both proteins (Figs. 3 and 5).

Establishment of SK32 Cells Stably Producing Pex5p Isoforms—A decrease in any endogenous isoform will allow assessment of the functions of Pex5p isoforms by overexpression in SK32 cells. We next isolated a transformed cell line stably expressing each wild type isoform in order to elucidate the functions of isoforms, especially of the novel Pex5pM isoform. The transformant cell lines, which are referred to as SK32/Pex5pL, SK32/Pex5pM and SK32/PexpS, respectively, produced several fold more L, M or S isoform than wild type cells did (Fig. 3A, lanes 2 and 5–7). In accordance with the stable expression, catalase became to localize in peroxisomes in each transformed cell line (Fig. 7). The subcellular distributions of Pex5p isoforms were studied in wild type and transformed cells (Fig. 8A). Almost all the Pex5p isoforms were distributed in the post-LM fraction of wild type cells. In the transformed cells, each isoform was preferentially recovered in the post-LM fraction but a small part was found in the LM fraction. The proteinase K-treatment of the LM fraction demonstrated that Pex5p isoforms were susceptible to digestion under the conditions where component A of Aox was protected from the digestion (Fig. 8B). The antibody used was raised against the C-terminal 19 amino acids of Pex5p. The susceptibility therefore indicated that proteinase K could gain access to the C-terminal portion of the isoforms located on peroxisomes. Gouveia, A.M. *et al*. recently showed that the N-terminus of Pex5p located in peroxisomes was exposed to the cytosol (*37*). Therefore, it seems likely that the most of the isoforms is disposed on the outside of peroxisomes.

Since the peroxisomal translocation of catalase was restored in each transformed cell line, every wild type isoform was able to function as a receptor for translocation of the enzyme into peroxisomes (Fig. 7). However, the isoforms exhibited different involvement in the maturation processes for thiolase and Aox. The production of the M or S isoform restored the maturation of both enzymes but that of the L isoform did not (Fig. 9A). Interestingly, the maturation of thiolase was completely prevented at 30°C in SK32/Pex5pL cells even though partial maturation was observed at this temperature in parental SK32 mutant cells (Fig. 9B), indicating that the L isoform antagonizes the maturation of thiolase.

Thiolase and Aox, whether processed or not, were protected from proteinase K-digestion in the LM fractions of the three transformed cell lines under the conditions where PMP70 was degraded (Fig. 9C), indicating that they were located inside peroxisomes. The mechanism by which Pex5p isoforms are involved in the maturation steps of two enzymes is presently unknown. Nevertheless, the data presented here demonstrate that the novel isoform, Pex5pM, actually functions in the peroxisomal

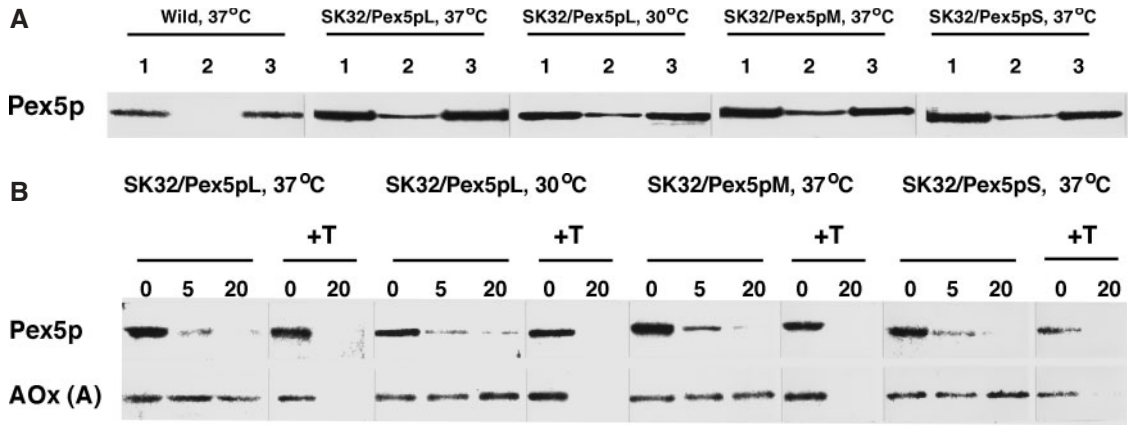

Fig. 8. **Subcellular localization and proteinase K digestion of Pex5p isoforms.** (A) Cells were cultured at 37°C, harvested and then homogenized in STE buffer. PN (1), LM (2), and post-LM (3) fractions were separated by centrifugations. The PN fraction (75 μg protein) and equivalent amounts of the LM and post-LM fractions were subjected to SDS-PAGE and immunoblot analysis using anti-Pex5p antibodies and ^{125}I-protein A. (B) Cells were cultured at the indicated temperature, harvested, homogenized and then fractionated by centrifugation. The LM fraction (20 μg) was digested with the indicated concentration (μg/ml) of proteinase K in the absence or presence (+T) of 2% (w/v) Triton X-100. The conditions for analysis were the same as in Fig. 6 except that anti-Pex5p antibodies were used for immunoblotting. The protection of component A of Aox is presented to show the integrity of the peroxisomes prepared. In +T, the conversion of component A of Aox to B is omitted from the figure.

translocation of catalase, and the processing of thiolase and Aox.

DISCUSSION

Mammalian Pex5pL and S isoforms are involved in the peroxisomal translocation of PTS1 and/or PTS2 proteins (*24*, *25*). PTS1 proteins are translocated through the binding of the SKL motif with the tetratricopeptide repeats (TPRs) of Pex5p isoforms (*38*). PTS2 proteins are translocated through the binding of the Pex5pL isoform and Pex7p recognizing the cargo-proteins (*26*). Both types of peroxisomal proteins are docked through the binding of Pex5p isoforms with Pex14p on the membrane, and then imported into the matrix through the cooperative functions of peroxins constructing the translocation apparatus. In fact, Pex5p and these several peroxins have been isolated as subunits of the same protein complex from peroxisomes in both rat liver (*39*, *40*) and yeast (*41*).

In this study, we revealed a novel isoform Pex5pM created from the PEX5 gene in addition to two isoforms of L and S. Pex5pM is shorter by 7–amino acids than Pex5pL and longer by 30–amino acids than Pex5pS (Fig. 1). The frequencies of the cDNAs derived from some sources (cDNA library and RT-PCR) suggest that the expression for the Pex5pM isoform will be rather low. However, the function of the Pex5pM isoform is significant. The wild type Pex5pM in SK32/Pex5pM cells is able to translocate catalase (Fig. 7), and restores the retarded maturation of thiolase and Aox into the processed forms (Fig. 9A). In the present study, we have shown that the Pex5pM isoform could be expressed in human fibroblasts. It is interesting to speculate that in humans the Pex5pM isoform also plays a role in these processes.

The present study also shows the following unique features of the intracellular fate of thiolase (PTS2 protein) and Aox (PTS1 protein) in SK32 cells. Although the protein levels of Pex5p isoforms were markedly reduced (Fig. 3), thiolase and Aox were still translocated inside peroxisomes (Figs. 5 and 6). In the case of fibroblasts derived from knockout mice, in which Pex5p was completely abolished, the cytosolic mislocalization of both PTS1 proteins and thiolase was demonstrated (*42*). Therefore, the residual amounts of Pex5p isoforms produced in the mutant cells are sufficient to deliver thiolase and Aox into peroxisomes. How are the two enzymes translocated efficiently into peroxisomes of mutant cells, although catalase and GFP-SKL are not? In the previous study we demonstrated that blue fluorescent protein (BFP) appending either PTS1 or PTS2 exhibited cytosolic localization in SK32 cells while BFP appending both PTSs exhibited peroxisomal localization (*28*). This finding indicates that a cargo-protein bound by both Pex5p (receptor for PTS1) and Pex7p (receptor for PTS2) is delivered efficiently into peroxisomes of the mutant cells. Thiolase and Aox might exist as a complex, which makes the association of Pex5p and Pex7p easy, and increases the efficiency of the translocation. In fact, there have been several reports that peroxisomal proteins are transported as oligomeric forms into the organelles (*43*, *44*). Another possibility is that thiolase and Aox might hold an additional PTS besides the canonical PTS. The consensus sequences of PTS1 and PTS2 are (S/A/C)(K/R/H)(L/M) at the carboxyl-terminus and (R/K)(L/V/I)X_5(H/Q)(L/A) at the amino-terminus, respectively (*2–5*). We therefore searched for these sequences, and found SRL (amino acid positions 176–178) and AKL (positions 268–270) in rat thiolase, and KIQDKAVQA (amino acid positions 545–553) in rat Aox. These divergent sequences are conserved at the corresponding positions in mouse and man, and might be functional as supplementary PTSs that translocate the respective enzymes, but this remains to be proved.

Another unique feature is that the unprocessed forms of thiolase and Aox are accumulated in peroxisomes in SK32 cells (Fig. 5). Furthermore, processing of thiolase was temperature-sensitive and a considerable part was converted to the mature size at 30°C, but Aox was not (Fig. 5). This observation suggests that thiolase and Aox

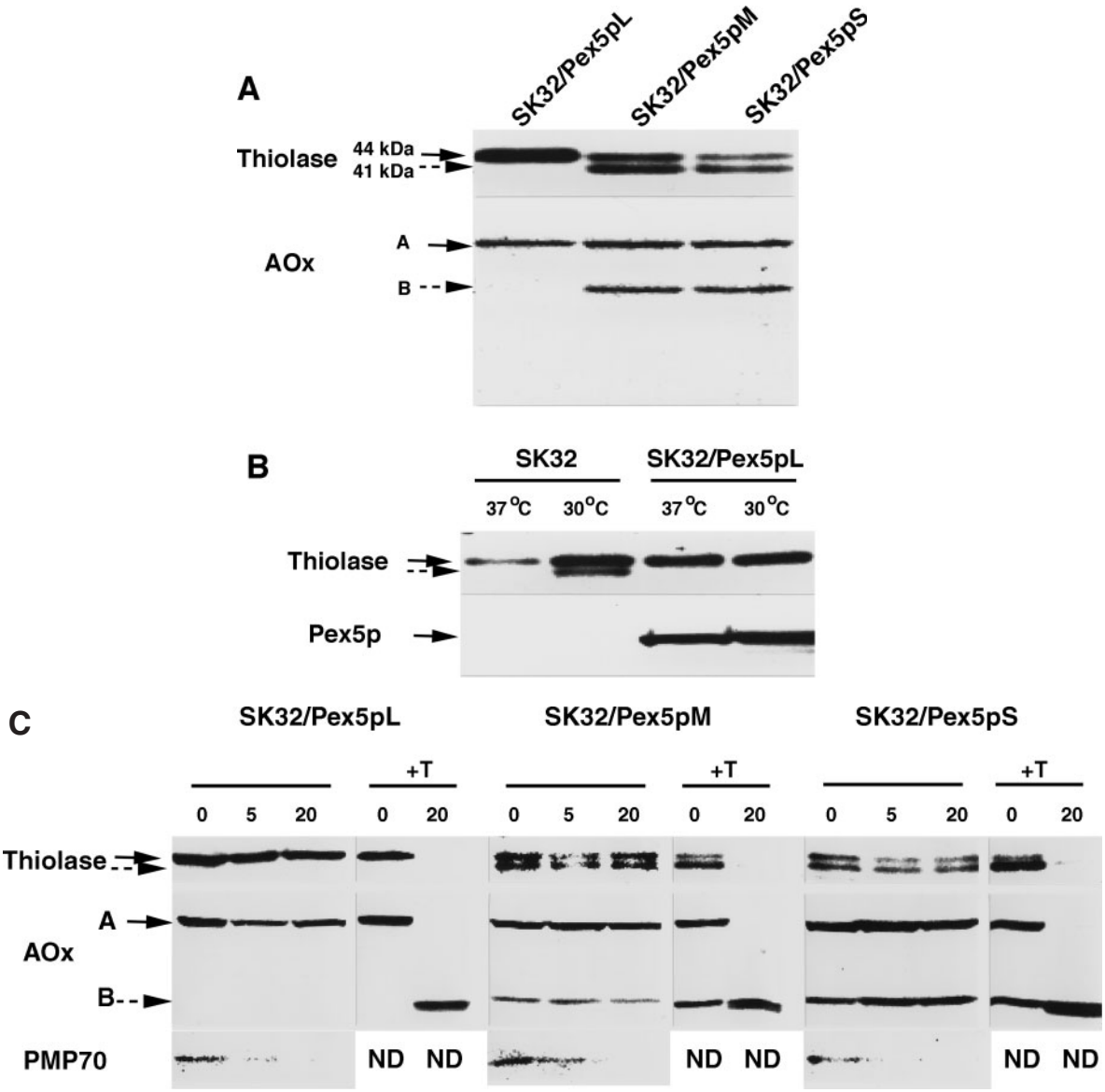

Fig. 9. **Restoration of processing events for thiolase and Aox in transformed Sk32 cells.** (A) The three transformed cell lines were cultured at 37°C, harvested, homogenized and then fractionated. The LM fraction (20 μg protein) was subjected to SDS-PAGE and immunoblot analysis using antibodies and ^{125}I-protein A to detect thiolase and Aox. Arrows indicate the precursor form (44 kDa) and mature form (41 kDa) of thiolase, or components A and B of Aox. (B) Cells were cultured at 37°C or 30°C. The PN fraction (75 μg protein) was subjected to SDS-PAGE and immunoblot analysis using anti-thiolase or anti-Pex5p antibodies, followed by binding of ^{125}I-protein A. The precursor form (dotted line with an arrowhead) and mature form (solid line with an arrowhead) of thiolase and Pex5p are indicated. (C) Cells were cultured at 37°C, harvested, homogenized and then fractionated. The LM fraction (20 μg protein) was digested with the indicated concentration (μg/ml) of proteinase K in the absence or presence (+T) of 2% (w/v) Triton X-100, and then subjected to SDS-PAGE and immunoblot analysis. PMP70 is susceptible to the digestion in the absence of Triton X-100 and not detectable (ND) in the presence of Triton X-100.

are converted into the processed forms in peroxisomes and that different proteases could be responsible for the reactions, although the responsible protease(s) has not been identified. Recently, we demonstrated that two proteases, insulin-degrading enzyme and lon protease, exist in rat liver peroxisomes (*45*, *46*). It is interesting as to whether or not these proteases are involved in the processing. In addition, the retarded maturation in SK32 cells indicates that the normal functions of Pex5p isoforms are involved in the processes.

As SK32 cells show unique features in the targeting of thiolase and Aox to peroxisomes, we tried to examine the functions of individual Pex5p isoforms by the overexpression of each isoform in the mutant cells. As expected, the wild type Pex5pL, M or S isoform restored the targeting of catalase to the peroxisomes. On the other hand, the wild type Pex5pM and S restored the retarded maturation of thiolase and Aox in SK32/Pex5pM and SK32/Pex5pS cells, but the wild type L isoform did not in SK32/Pex5pL cells (Fig. 9A). Furthermore, surprisingly, Pex5pL inhibited

restoration of the processing event for thiolase, which was observed at 30°C in the parental SK32 cells (Figs. 5A *vs.* 9B). It is known that Pex5p is mostly localized in the cytosol, and Pex5p associated with a cargo protein is translocated into the peroxisomal matrix and recycled to the cytosol during the import cycles (*17*, *37*). This inhibition might be explained by an abnormal distribution of overproduced Pex5pL in the mutant cells. Actually, almost all Pex5p was recovered in the post-LM fraction of wild type cells and a part of Pex5pL in the LM fraction of the transformed cells (Fig. 8A). However, the amount of Pex5pL in the LM fraction was almost equal to those of Pex5pM and S, and Pex5pL was digested by proteinase K like Pex5pM and S were under the conditions under which Aox was protected from proteinase K treatment (Fig. 8B). Therefore, it is unlikely that specific insertion of Pex5pL into the peroxisomal membranes affects the processing of thiolase and Aox. The seven amino acid residues, positions 216–222, in the Pex5pL isoform that are lacking in the M and S form might play a significant role in the maturation of thiolase and Aox. Very recently, Gouveia *et al*. suggested that the cycle of Pex5p during the transport of cargo proteins between the cytosol and peroxisomes is regulated directly or indirectly by Pex5p itself but not by a peroxisomal docking/translocation machinery (*37*). The existence of these seven amino acid residues of the L isoform might affect the efficiency of the release of cargo proteins from Pex5p and Pex7p or translocation of peptidase(s) across peroxisomal membranes. We found that the precursor form of thiolase was resistant to alkaline Na_2CO_3 extraction in peroxisomes of SK32/Pex5pL cells, but considerably susceptible in those of SK32/Pex5pM and SK32/Pex5pS cells (data not shown). The abundant production of the L isoform might give rise to the appearance of the insoluble form of thiolase in peroxisomal membranes through the depression of the maturation in SK32/Pex5pL cells. However, these studies were limited to CHO cells, and the inhibitory effect of Pex5pL on the maturation of thiolase and Aox from other mammalian cells remains to be determined.

In this paper, we report that a novel isoform of Pex5p exists in CHO cells and functions as a receptor of cargo-proteins. It will be interesting to determine whether or not it binds with Pex7p, a cytosolic receptor for PTS2 proteins, because the binding site for Pex7p is located between positions 190 and 233 of the L isoform (*14*). Furthermore, we demonstrate that Pex5pL, but not Pex5p M or S, inhibited the maturation of thiolase and Aox in peroxisomes, although it occurs under conditions under which Pex5p is overexpressed. At present, there is no information as to how Pex5p is translocated to peroxisomes together with cargo-proteins as well as Pex7p, or how the cargo-proteins are released. There is also no evidence as to what stage of translocation or after a translocation peptidase(s) processes the thiolase and Aox. Therefore, mutant cell lines such as SK32/Pex5pL, SK32/Pex5pM and SK32/Pex5pS cells will be good tools for characterization of the translocation mechanism for peroxisomal proteins depending on Pex5p.

This paper is a posthumous work of Dr. Masaki Ito who died untimely last year, and is dedicated to his family.

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