Biogenesis of Nonspecific Lipid Transfer Protein and Sterol Carrier Protein x

STUDIES USING PEROXISOME ASSEMBLY-DEFECTIVE pex CELL MUTANTS*

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Nonspecific lipid transfer protein (nsLTP; also called sterol carrier protein 2) with a molecular mass of 13 kDa is synthesized as a larger 15-kDa precursor (pre-nsLTP) with an N-terminal 20-amino acid extension presequence, as well as with the peroxisome targeting signal type 1 (PTS1), Ala-Lys-Leu, at the C terminus. The precursor pre-nsLTP is processed to mature nsLTP by proteolytic removal of the presequence, most likely after being imported into peroxisomes. Sterol carrier protein x (SCPx), a 59-kDa branched-chain fatty acid thiolase of peroxisomes, contains the entire pre-nsLTP moiety at the C-terminal part and is converted to the 46-kDa form and nsLTP after the transport to peroxisomes. We investigated which of these two potential topogenic sequence functions in biogenesis of nsLTP and SCPx. Morphological and biochemical analyses, making use of Chinese hamster ovary cell pex mutants such as the PTS1 receptor-impaired pex5 and PTS2 import-defective pex7, as well as green fluorescent protein chimeras, revealed that both pre-nsLTP and SCPx are imported into peroxisomes by the Pex5p-mediated PTS1 pathway. Nearly half of the pre-nsLTP remains in the cytosol, as assessed by subcellular fractionation of the wild-type Chinese hamster ovary cells. In an in vitro binding assay, only mature nsLTP, but not pre-nsLTP, from the cell lysates interacted with the Pex5p. It is likely, therefore, that modulation of the C-terminal PTS1 by the presequence gives rise to cytoplasmic localization of pre-nsLTP.

The mammalian nonspecific lipid transfer protein (nsLTP) functions in vitro as a carrier protein in the transfer of a variety of lipids, such as phospholipids and cholesterol (for a review, see Ref. 1). It is identical to sterol carrier protein 2, stimulates the enzymatic conversion of lanosterol to cholesterol during the biosynthesis of cholesterol, and enhances the synthesis of bile acids (1). But, the physiological role of nsLTP is not yet well defined. nsLTP is localized in peroxisomes, as well as the cytoplasm, in several organs such as rat liver (2, 3) and adrenal gland (1). nsLTP is synthesized as a larger precursor, termed pre-nsLTP, with a mass of 15 kDa on free polyribosomes (4) and then processed to its mature form of 13 kDa (4, 5), apparently after transport into peroxisomes (2, 4). Two types of cDNA for nsLTP of rat liver were cloned (6–8), one encoding an 15-kDa pre-nsLTP consisting of 143 amino acids, a precursor form of 123-amino acid mature nsLTP (9, 10), and the other coding for a 547-amino acid protein with a mass of 59 kDa, termed sterol carrier protein x (SCPx) (see Fig. 1A).

pre-nsLTP and SCPx are expressed from one gene by alternative transcription initiation by two distinct promoters (11). The N-terminal, 20-amino acid extra sequence of pre-nsLTP is cleaved off to form mature nsLTP in peroxisomes (4, 12). Interestingly, nsLTP was recently shown to interact with acyl-CoA oxidase (AOx) (13), the first-step enzyme of peroxisomal β-oxidation system, suggesting that nsLTP may function in transfer of the substrates such as fatty acyl-CoA derivatives to AOX. The sequence for SCPx contains the full sequence of pre-nsLTP at the C-terminal part and is suggested to be partly converted to the N-terminal part protein of 46 kDa and nsLTP (1, 14). It has been demonstrated that SCPx functions as a peroxisomal branched-chain β-ketothiolase (15–17).

The import of most peroxisomal matrix proteins is mediated by well characterized cis-acting peroxisomal targeting signals (PTSs), C-terminal Ser-Lys-Leu (SKL) motif PTS1 (18, 19) and N-terminal cleavable presequence PTS2 (20–22). Other types of PTS have been postulated to exist but have not been identified yet. It is evident that pre-nsLTP contains a cleavable N-terminal 20-amino acid presequence resembling PTS2, as well as PTS1 tripeptide Ala-Lys-Leu (AKL), whereas SCPx possesses internally this 20-amino acid presequence of nsLTP and the C-terminal AKL. However, despite numerous biochemical findings such as those related to the lipid transfer activity, biogenesis of nsLTP and SCPx has not been well defined at the molecular and cellular levels.

To address the underlying mechanisms by which nsLTP and SCPx are transported into peroxisomes, we investigated biogenesis of these proteins at morphological, as well as biochemical, levels using several types of peroxisome biogenesis-defective cell mutants and the PTS1 receptor Pex5p. We herein report that import of nsLTP and SCPx was affected in pex5 mutant of Chinese hamster ovary (CHO) cells deficient in import of PTS1 and PTS2, whereas in PTS2 import-defective pex7 mutant both proteins were imported into peroxisomes as effi-
Isopycnic ultracentrifugation on a sucrose density gradient using PNS tant, termed cytosolic, fraction was prepared by centrifuging a post-antibody conjugated to rhodamine (Chemicon). Labeled sheep anti-rabbit IgG antibody (Cappel), or donkey anti-goat IgG hydrogenase antibody (29). Antigen antibody complexes were detected used goat anti-rat catalase antibody (28) and rabbit anti-malate dehydrogenase antibody was purchased from rabbit anti-green fluorescent protein (GFP) antibody was constructed as a template cDNA encoding pre-nsLTP (6) in pUcD2 and a set of primers, a forward primer nsLTP-I, CCTCTAGAAGAAT-I site of pUcD2Hyg I- SalI site of pUcD2Hyg I site of pUEX3 (Amersham Pharmacia Biotech). The polymerase chain reaction product under- Sal, 3’-underline, Sal site) and a reverse 5’-GTCGACATACCTGGGACCCCGAG- GCC-3’ (underline, SalI site). The polymerase chain reaction product was cloned into pGEM-T Easy vector (Promega) and cleaved with SalI and SalI. The SalI-SalI fragment encompassing an N-terminal site consisting of amino acid residues at 7–300 of 59-kDa SCPx was inserted into the SalI-SalI site of pUEX3 (Amersham Pharmacia Biotech). The resultant plasmid for lacZ fusion protein was expressed in Escherichia coli. Antiserum specific for SCPx was raised in rabbit by conventional subcutaneous injection of β-galactosidase-SCPx fusion protein. Rabbit anti-green fluorescent protein (GFP) antibody was purchased from CLONTECH.

**Morphological Analysis**—nsLTP and SCPx in CHO cells were visualized by indirect immunofluorescence light microscopy using rabbit antibodies to nsLTP and SCPx, respectively, as described (27). We also used goat anti-rat catalase antibody (28) and rabbit anti-malate dehydrogenase antibody (29). Antigen-antibody complexes were detected under a Carl Zeiss Axioskop FL microscope by fluorescein isothiocyanate-labeled sheep anti-rabbit IgG antibody (Cappel), Texas Red-labeled sheep anti-rabbit IgG antibody (Cappel), or donkey anti-goat IgG antibody conjugated to rhodamine (Chemicon).

**Subcellular Fractionation**—Subcellular fractions of rat liver and CHO cells were prepared as described (19, 30). A high speed supernatant, termed cytosolic, fraction was prepared by centrifuging a post-nuclear supernatant fraction (PNS) for 40 min at 100,000 × g (30). Isopionic ultracentrifugation on a sucrose density gradient using PNS from CHO cells was done as described (30).

**In Vitro Binding Assay**—Cell lysates were prepared from CHO-K1 cells using 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 25 μg/ml each of leupeptin and antipain, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol as described (30). In vitro binding assays were performed by incubation of cell lysates with glutathione S-transferase (GST) fused to the longer isoform of Pex5p (Pex5pL) and GST-Pex5pL-G335E with a mutation at G335E derived from a pex2 mutant, ZP105, as described (30). Bound proteins were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) and immunoblot.

**Expression of GFP Fusion Protein**—DNA fragment for the N-terminal presequence of nsLTP was obtained by polymerase chain reaction using as a template cDNA encoding pre-nsLTP (6) in pUCD2 and a set of primers, a forward primer nsLTP-I site of pUcD2Hyg I- SalI site of pUEX3 (Amersham Pharmacia Biotech). The antibody specifically recognizes 59- and 46-kDa proteins in PNS, as well as in purified peroxisomes (Fig. 1B, lanes 1 and 2). The 46-kDa protein is most likely to be derived from 59-kDa SCPx by proteolytic conversion (1, 14). Using anti-nsLTP antibody, 13-kDa, proteins were detected in PNS and peroxisomes (Fig. 1B, lanes 3 and 4), consistent with our earlier observation (2, 4). In cytosolic fraction ~15 kDa of protein reacted with this antibody, in addition to 13- and 59-kDa polypeptides (lane 5), both presumably from broken peroxisomes during homogenization of liver (2). We interpreted these results to mean that nsLTP and 59-kDa SCPx cross-reactive to anti-nsLTP antibody are localized in peroxisomes, and pre-nsLTP is present in the cytosol. It is more likely that SCPx is partly processed to produce nsLTP and an N-terminal 46-kDa thiolase protein of SCPx (1, 14). These data demonstrate that both antibodies are specific.

### EXPERIMENTAL PROCEDURES

**Cell Lines**—Wild-type CHO-K1 and pex mutants, including pex2 (23), pex5 (24), and pex7 (25) (Table I), were cultured as described (26).

**Antibodies**—Affinity-purified rabbit anti-rat nsLTP antibody was as described (2, 4). Rat cDNA (TM-33/24) encoding the 59-kDa SCPx protein (6) was amplified by polymerase chain reaction using SCPx primers, a forward 5’-CCCGGAAACTCTCGCCCGCTGGCC-3’ (underline, Sal site) and a reverse 5’-GTCGACATACCTGGGACCCCGAG-GCC-3’ (underline, Sal site). The polymerase chain reaction product was cloned into pGEM-T Easy vector (Promega) and cleaved with SalI and SalI. The SalI-SalI fragment encompassing an N-terminal site consisting of amino acid residues at 7–300 of 59-kDa SCPx was inserted into the SalI-SalI site of pUEX3 (Amersham Pharmacia Biotech). The resultant plasmid for lacZ fusion protein was expressed in Escherichia coli. Antiserum specific for SCPx was raised in rabbit by conventional subcutaneous injection of β-galactosidase-SCPx fusion protein. Rabbit anti-green fluorescent protein (GFP) antibody was purchased from CLONTECH.

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### RESULTS

**Anti-SCPx Antibody**—We raised antiserum against SCPx by immunizing a rabbit with SCPx (residues at 7–300; see Fig. 1A) fused to E. coli β-galactosidase. The antibody specifically recognizes 59- and 46-kDa proteins in PNS, as well as in purified peroxisomes (Fig. 1B, lanes 1 and 2). The 46-kDa protein is most likely to be derived from 59-kDa SCPx by proteolytic conversion (1, 14). Using anti-nsLTP antibody, 13-kDa, as well as 59-kDa, proteins were detected in PNS and peroxisomes (Fig. 1B, lanes 3 and 4), consistent with our earlier observation (2, 4). In cytosolic fraction ~15 kDa of protein reacted with this antibody, in addition to 13- and 59-kDa polypeptides (lane 5), both presumably from broken peroxisomes during homogenization of liver (2). We interpreted these results to mean that nsLTP and 59-kDa SCPx cross-reactive to anti-nsLTP antibody are localized in peroxisomes, and pre-nsLTP is present in the cytosol. It is more likely that SCPx is partly processed to produce nsLTP and an N-terminal 46-kDa thiolase protein of SCPx (1, 14). These data demonstrate that both antibodies are specific.

### Table I

| pex Mutant | CHO mutant | Complementing gene | Phenotype (import) | Reference |
|------------|------------|--------------------|-------------------|-----------|
| pex2       | Z65        | PEX2               | −                  | (23)      |
| pex5       | Z105       | PEX5               | −                  | (24,30)   |
| pex7       | ZTG207     | PEX7               | +                  | (25)      |
**Modulation of Peroxisomal Import of nsLTP**

**Morphological Analysis of nsLTP and SCPx—Wild-type and pex mutant CHO cells were stained using anti-nsLTP antibody.** In CHO-K1 and pex7 mutant ZPG207 defective in PTS2 import (25), nsLTP was detected in a punctate staining pattern (Fig. 2A, a and d), in a superimposable manner with that obtained using anti-catalase antibody (e and h), thereby suggesting that nsLTP was localized to peroxisomes. On the other hand, in pex6 Z65 and pex5 Z105 mutants defective in PTS1 and PTS2 (Fig. 2B, b and e), nsLTP appeared to be stained partly in a diffused pattern in the cytoplasm (Fig. 2A, b and c), as was the case for catalase (f and g) (24, 26). A membrane-associated pattern, presumably representing SCPx (see Fig. 3), is also visible by staining with anti-SCPx antibody in Z65 and Z105 (Fig. 2A, a and d). These results collectively suggest that nsLTP and SCPx cross-reacting with anti-nLTP are imported by the PTS1, rather than PTS2, pathway. Moreover, in a recently isolated pex5 CHO cell mutant ZPG201 with a phenotype showing a defect solely in PTS2 import (31), punctate staining pattern was likewise discernible upon cell staining using anti-nsLTP antibody, in a superimposable manner with catalase-positive structures, hence confirming the import of nsLTP via the PTS1 pathway (data not shown).

These four types of CHO cells were stained using an antibody specific for SCPx. In CHO-K1, SCPx was detected in punctate structures that were superimposable upon those stained for catalase, hence indicating peroxisomal localization (Fig. 2B, a and e). Similarly, SCPx was localized in particles in ZPG207, as was the case for catalase, strongly suggesting that SCPx is transported into peroxisomes (Fig. 2B, d and h). Similar punctate structures were detected when pex5 ZPG201 was stained with anti-SCPx antibody (data not shown). In contrast, in pex2 Z65 and pex5 Z105 where catalase (Fig. 2B, f and g) and PTS1 proteins (data not shown) were in the cytoplasm, SCPx was partly, if not entirely, stained in a punctate pattern (b and c). In the same sets of cells that were permeabilized with 25 μg/ml of digitonin, under which plasma membranes were selectively destabilized and intraperoxisomal proteins were inaccessible to exogenous antibodies (28, 32), SCPx was not detectable as a particle-associated form in any of four types of CHO cells including wild-type, pex7, pex2, and pex5 cells (Fig. 2B, i–l). Catalase was not accessible in digitonin-permeabilized CHO-K1 and pex7 ZPG207 (m and p), whereas catalase in pex2 Z65 and pex5 Z105 cells was apparently visible in the cyto-

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**Fig. 2. Intracellular localization of nsLTP and SCPx.** A, wild-type and peroxisome biogenesis-defective mutant CHO cells were dual-stained using antibodies to nsLTP (a–d) and catalase (e–h). Cells were as follows: a and e, CHO-K1; b and f, a pex2 mutant Z65; c and g, a pex5 mutant Z105; d and h, a pex7 mutant ZPG207. Magnification, ×630; scale, 20 μm. B, CHO cells were fixed, treated with 1% Triton X-100 (a–h) or 25 μg/ml of digitonin (i–p), under which only plasma membranes are permeabilized, and were double-stained with antibodies to SCPx (a–d and i–l) and catalase (e–h and m–p). Cells were as follows: a, e, i, and m, CHO-K1; b, f, j, and n, a pex2 Z65; c, g, k, and o, a pex5 Z105; d, h, l, and p, a pex7 ZPG207. Scale, 20 μm. Note that SCPx (i–l) and a matrix enzyme catalase (m and p) were not discernible.

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**Fig. 3. Subcellular localization of nsLTP and SCPx in pex mutant cells.** A, cytosolic (S) and organellar (P) fractions from PNS of wild-type and pex mutant CHO cells (8 × 10⁶ cells each) were analyzed by SDS-PAGE and immunoblot. Immunoblot was performed using antibodies to nsLTP (top two panels), SCPx (middle panel), and AoX (bottom panel). Immunoblot pattern using anti-SCPx antibody was essentially as in Fig. 1B (right panel); only parts showing SCPx and nsLTP were shown as a composite. Solid and open arrowheads indicate SCPx and 46-kDa protein (nsSCPx) derived from SCPx; P and M, a larger precursor (pre-nsLTP) and mature protein of nsLTP, respectively. A, B, and C show 75-, 53-, and 22-kDa components of AoX; dots, nonspecific bands (36). B, protease protection assay. PNS from pex5 Z105 was treated with proteinase K for 30 min on ice. The digestion was terminated with 1 mM phenylmethylsulfonyl fluoride. The reaction mixture was centrifuged to separate cytosolic (S) and organellar (P) fractions, which were analyzed by SDS-PAGE and immunoblot using antibodies to SCPx and Pex14p. PNS fractions analyzed were from 5 × 10⁶ cells each. PNS was mock-treated (lanes 1 and 2) or treated with 5, 10, and 20 μg/ml of proteinase K in the absence (lanes 3–5) or presence (lane 6) of 1% Triton X-100. Only P fractions, each from proteinase K-treated PNS, were loaded. In lane 6, total reaction mixture was analyzed. C, isopycnic subcellular fractionation. PNS fractions from CHO-K1 (upper panel) and pex2 Z65 (lower panel) (2 × 10⁶ cells each) were fractionated by isopycnic ultracentrifugation on a sucrose density gradient. The gradient was collected into 19 tubes (0.5 ml each). An equal volume (15 μl) of each fraction was analyzed by SDS-PAGE, followed by immunoblot with antiserum against SCPx, Pex14p, and Pex5p. Results are presented in the direction of lower to higher density of sucrose, from left to right.
plasm (n and o), slightly more vividly than the other cell types. We interpreted these data to mean that SCPx is associated with and possibly imported into endomembranes in pex2 and pex5 cell mutants.

Subcellular Fractionation Analysis—Intracellular localization of nsLTP and SCPx was also investigated by subcellular fractionation of CHO cells. When PNS of CHO-K1 cells was fractionated, nsLTP protein was found in both the cytosolic fraction and organelle pellets by immunoblotting with anti-nsLTP antibody (Fig. 3A, top panel, lanes 1 and 2). Subcellular localization of nsLTP and pre-nsLTP was distinct; pre-nsLTP was exclusively in the organelle fraction, apparently in peroxisomes (see Fig. 2). SCPx cross-reacting with anti-nsLTP antibody was exclusively in the organelle pellets (lane 2), presumably peroxisomes (see Fig. 2), as reported previously (2, 33). A similar distribution pattern of pre-nsLTP, nsLTP, and SCPx was observed in pex7 ZPG207 (Fig. 3A, middle panel, lanes 1, 2, 7, and 8). In contrast, only pre-nsLTP, but not mature nsLTP, was present and exclusively in the cytosolic fraction from pex2 Z65 and pex5 ZP105 (top lower panel, lanes 3–6), whereas SCPx was detected by anti-nsLTP antibodies in the organelle fraction in both mutants (top upper panel, lanes 4 and 6) as in CHO-K1 and ZPG207. Next, using SCPx-specific antibody, SCPx and 46-kDa protein, presumably the processed form of 59-kDa SCPx, were discernible in the organelle fraction of both CHO-K1 and ZPG207 (Fig. 3B, middle panel, lanes 1, 2, 7, and 8), thereby implying that SCPx is imported into peroxisomes and partly converted to 46-kDa protein plus (presumably) nsLTP. In contrast, there was only the 59-kDa form of SCPx detectable in membrane pellet fractions of Z65 and ZP105 cells (middle panel, lanes 3–6), implying that there was no processing activity in these mutants.

AOx, a PTS1 protein, consists of 75-kDa A, 53-kDa B, and 22-kDa C polypeptide components and exists as a hetero-oligomer comprising A8, B6, ABC, and A2B2 (26, 34). In normal cells, proteolytic conversion of AOx-A component to B and C polypeptides occurs in peroxisomes (26, 35). A, B, and C components of AOx were evident in CHO-K1 and ZPG207, indicative of normal biogenesis of AOx (Fig. 3A, bottom panel, lanes 1, 2, 7, and 8), consistent with our earlier observation (25). In Z65 and ZP105, AOx-A, but not the B and C components, was detectable in cytosolic fraction at a lower level (bottom panel, lanes 3–6), indicating failure on the conversion of AOx, as in our earlier observation (27, 28, 36).

To determine the subcellular, as well as intraorganellar, localization of SCPx in pex mutants such as pex5 ZP105, we took another approach, a so-called protease protection assay. SCPx in PNS of ZP105 cells was resistant to the treatment with exogenously added proteinase K and was sedimentable (Fig. 3B, upper panel, lanes 1–5), whereas a peroxisomal membrane peroxin Pex14p (37) was sensitive to the digestion (lower panel), thereby strongly suggesting that SCPx was inside the organelles. Treatment with Triton X-100 prior to the protease digestion abolished the resistance, thereby confirming that SCPx was localized in membranous vesicles. Essentially the same results were obtained using pex2 Z65 cells (data not shown).

To confirm the findings described above with respect to the intracellular location of SCPx, PNS from CHO-K1 and a pex2 Z65 was fractionated by isopycnic ultracentrifugation on a sucrose density gradient. From CHO-K1, both 59- and 46-kDa forms of SCPx cosedimented with Pex14p, indicating that SCPx was localized and processed in peroxisomes (Fig. 3C, upper panel). Pex5p mostly remained nearly at the top of gradient, but a small portion of Pex5p cosedimented with peroxisomes, indicating a largely cytoplasmic PTS1 receptor (30, 38).

In Pex2p-defective Z65 (23), 59-kDa SCPx was sedimented and distributed from the middle to the bottom of the gradient (Fig. 3C, lower panel). Pex14p representing peroxisomal remnants (37) sedimented to a lighter density part of the gradient, with which a larger part of Pex5p cosedimented, consistent with our earlier observation (23). We interpreted these findings to mean that SCPx was localized to endomembranes, partly associated with peroxisomal remnants, in Z65 cells. SCPx appeared to be associated with mitochondria, as well (see Fig. 2B).

Binding to the PTS1 Receptor Pex5p—To investigate whether nsLTP and SCPx interact with the PTS1 receptor Pex5p, cell lysates from CHO-K1 were incubated with GST-Pex5pL fusion protein of wild-type Pex5pL, as well as ZP105-type mutant GST-Pex5p-L-G335E (GST-G335E), were incubated with cell lysates of CHO-K1 (3 × 106 cells each). After thoroughly washing, proteins bound to glutathione-Sepharose were analyzed by SDS-PAGE. SCPx and nsLTP were detected by immunoblot using anti-nsLTP antibody; AOx was assessed as a control PTS1 protein. Arrowhead indicates SCPx; P and M show pre-nsLTP and mature protein of nsLTP, respectively. A, B, and C designate polypeptide components of AOx. Lanes 1 and 2, cytosolic (S) and organelle (P) fractions from PNS of CHO-K1 (one-tenth aliquot each used for the pull-down assay); lanes 3–5, bound proteins to GST and GST-Pex5p fusion proteins as indicated. Note that SCPx and nsLTP, but not pre-nsLTP, specifically bound to Pex5pL.

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Intracellular Localization of GFP Fusion Protein—To elucidate whether the presences of pre-nsLTP and/or C-terminal AKL function as a sufficient PTS, we constructed two types of GFP-fusion proteins, one fused N-terminally to the 25-amino acid residues including the 20-amino acid presequence plus 5 amino acid residues at the C-terminal end. These chimeras were transiently expressed in CHO-K1, pex2 Z65,
A GFP fusion protein was assessed by fluorescence (and chondria was done as in transfected with cDNA for pre-GFP cDNA. Detection of GFP and mitochondria were Western blots using anti-GFP antibody (a). Peroxisomes were as-detected in particulates and colocalized with catalase, hence indicating that pre-GFP-AKL was targeted to peroxi-somes (Fig. 5h). Peroxisomes were ass-essed by immunostaining of catalase (e-h). Scale, 20 μm. C, pex2 Z65 and pex5 ZP105 cells were transfected with cDNA for pre-GFP-AKL. GFP fusion protein was assessed by fluorescence (a and b). Mitochon-dria were detected by staining with anti-malate dehydrogenase (a-MDH) antibody (c and d). Scale, 20 μm. D, CHO-K1 and a pex2 Z65 were transfected with cDNA for pre-GFP cDNA. Detection of GFP and mitochondria was done as in C. Scale, 20 μm.

pex5 ZP105, and pex7 ZPG207 cells. In CHO-K1, pre-GFP-AKL was detected in particulates and colocalized with catalase, hence indicating that pre-GFP-AKL was targeted to peroxi-somes (Fig. 5b, a and c). pre-GFP-AKL was likewise in parti-cles, in catalase-positive but PTS2-negative “peroxisomes” in ZPG207 (25) (Fig. 5b, d and h). In contrast, pre-GFP-AKL appeared to remain partly in the cytoplasm, like catalase, in pex2 and pex5 mutants (Fig. 5b, b, c, f, and g). In these mu-tants, pre-GFP-AKL was also undetectable in a membrane-assoc-iated form seen for nsLTP and SCPx (see Fig. 2, A and B), which was superimposable on that assessed by cell staining using antibody to malate dehydrogenase, a mitochondrial marker enzyme (Fig. 5c). Accordingly, the data strongly sug-gested that pre-GFP-AKL was mostly localized to mitochondria in pex2 and pex5 mutants. Taken together, pre-GFP-AKL was transported to peroxisomes in a PTS1-dependent and Pex5p-mediated pathway in normal and PTS2-import-defective pex7 cells. Moreover, we likewise investigated whether the prese-quence of pre-GFP-AKL functions as a topogenic signal. pre-GFP-AKL was detected in a superimposable manner with malate dehydrogenase-positive particles, presumably mitochondria, in both wild-type and pex2 CHO cells (Fig. 5d), suggesting that the 20-amino acid presequence functions per se as a mitochondrial targeting sequence.

Interaction of pre-GFP-AKL with Pex5p—The expression level of pre-GFP-AKL and pre-GFP-AKL was also verified by immuno-blot of the cell lysates. Both proteins were detected with an expected size and at a similar level in wild-type CHO-K1 and a pex2 Z65 (Fig. 6a, lanes 2, 3, 5, and 6). As a control GFP-AKL (28) was also expressed, which was detectable in both types of cells (lanes 1 and 4), and was targeted to peroxisomes in CHO-K1 but remained in the cytoplasm in Z65 (data not shown), in good agreement with our earlier observation (28). The distinct mobility in SDS-PAGE of these three GFP fusion proteins suggested that the presequence of pre-GFP-AKL and pre-GFP-AKL was not cleaved off, even after imported to peroxi-somes and mitochondria, respectively. The processing site of these chimera may not be recognized by a potential processing protease. Next, we investigated whether pre-GFP-AKL inter-acts with Pex5p. Control GFP-AKL bound to GST-Pex5pL, but not to GST (Fig. 6b, lanes 1–3), consistent with peroxisomal localization (data not shown) (28), pre-GFP-AKL, but not pre-GFP, from CHO-K1 cell-lysatess was likewise pulled down by GST-Pex5pL, not by GST (Fig. 6b, lanes 4–9), thereby indicat-ing that, in contrast to pre-nsLTP, the PTS1 of pre-GFP-AKL was readily recognized by Pex5p, in agreement with morpho-logical findings (see Fig. 5b).

**DISCUSSION**

pre-nsLTP can be classified as a unique protein among a number of peroxisomal proteins with respect to its topogenic sequence. The cleavable presequence locates at the N terminus, similar to PTS2 of several peroxisomal enzymes such as 3-ketoacyl-CoA thiolase (20, 21), and the C terminus carries a typical PTS1 tripeptide motif, AKL. Moreover, the amino acid sequence, -AAPT|SS- (-, continuing to adjacent residues), near the processing site of pre-nsLTP to form mature nsLTP resembles the sequence of PTS2-thiolase, -AAPC|SS- (-, continuing to adjacent residues), implying that pre-nsLTP and PTS2 proteins may share a potential, yet unidentified processing protease present in peroxisomes. The nsLTP presequence is
relatively positive-charged like PTS2, but it does not contain the consensus PTS2 sequence -R(K)/L(V/I)X(H/Q)/L(Ala). This implies that the presequence may be a novel-type PTS. We investigated in the present work which of two potential PTS sequences destines pre-nsLTP to its final intracellular location in vivo. With the use of several CHO pex mutants, import of pre-nsLTP was verified, pre-nsLTP was affected in import in pex2 and pex5 mutants with a phenotype showing import defect of both PTS1 and PTS2 proteins, where pre-nsLTP remained in the cytosol. Other groups of investigators reported that nsLTP is likely to be degraded or barely detectable in ZR82, a pex2 CHO mutant (33), as well as in fibroblasts from a patient with Zellweger syndrome (12), although pre-nsLTP was not fully described. In pex7 cell mutant impaired in PTS2 import, pre-nsLTP was translocated as efficiently as in the wild-type CHO-K1. Transport of pre-GFP-AKL was likewise re-established in wild-type and pex7 mutant CHO cells, consistent with the morphological phenotype as assessed for pre-nsLTP. Taken together, we conclude that pre-nsLTP is imported by the Pex5p-mediated PTS1 pathway.

To our surprise, pre-GFP-AKL was targeted to mitochondria when expressed in pex2 and pex5 mutants. These results imply that pre-GFP-AKL behaves differently from pre-nsLTP; PTS1 of pre-nsLTP could not be recognized by Pex5p in vitro, whereas AKL of pre-GFP-AKL readily interacted with Pex5p. Despite such biochemical properties, pre-nsLTP is transported to peroxisomes by a Pex5p-dependent pathway in vivo. Moreover, in mutant cells absent from Pex5p, pre-nsLTP remains in the cytoplasm, whereas pre-GFP-AKL is readily transported to mitochondria at least in an over-expression system. The mitochondrial topogenic activity appears to be interfered with in the presence of pre-nsLTP. Accordingly, the PTS1 and presequence of pre-nsLTP are most likely to be mutually regulated by unknown mechanisms. One possibility includes modulation of PTS1 of pre-nsLTP by direct steric effect of the presequence or that mediated by a cytosolic factor. Alternatively, PTS1 of pre-nsLTP may be partly modified, whereby a portion of total pre-nsLTP no longer interacts with Pex5p and remains in the cytoplasm. In the case of pre-GFP-AKL, the tripeptide AKL is more likely to be exposed to the surface of the molecule, thereby being readily recognized by Pex5p. The presequence may be unable to modulate the PTS1, possibly because of the configuration of this GFP chimera. Furthermore, we identified the presequence as a mitochondrial targeting signal, based on the observation that pre-GFP was targeted to mitochondria in normal and mutant CHO cells.

It is noteworthy that the N-terminal sequence encompassing the 20-amino acid presequence plus Ser at position 21 was predicted as a mitochondrial targeting sequence (44% probability) using a recognition program, PSORT II, minimally requiring 21 amino acid residues to be analyzed, where the prediction also included cytoplasmic (26%) and peroxisomal (4%) localization. Secondary structure analysis of the 20-amino acid presequence using a Chou-Fasman program predicted an a-helix between residues at 3–10 and b-sheet structure between the residues 10–17. These characteristics may contribute as a mitochondrial import signal. Therefore, it is tempting to infer that the presequence may interact with the C-terminal PTS1 and regulate peroxisomal transport of pre-nsLTP, possibly in a concerted manner with other cytosolic factors such as chaperones. How the PTS1 and mitochondrial targeting signal-like presequence are regulated in biogenesis of nsLTP in vivo remains to be defined.

pre-nsLTP is rather stably present in the cytosol as assessed by subcellular fractionation study and morphological analysis. The modulated presequence may function in vivo as a signal for pre-nsLTP to be a cytosolic protein. If synthesized pre-nsLTP remains in the cytoplasm, nsLTP in peroxisomes may be all derived from SCPx instead of pre-nsLTP by proteolytic conversion. Although we can not exclude this possibility, this may be less likely based on the findings in pulse-chase experiments, where processing of pre-nsLTP to nsLTP was noted (2, 12). The physiological consequences of pre-nsLTP in the cytosol, nearly at an equal level as nsLTP in peroxisomes in normal CHO cells, also remain to be investigated. The level of cytosolic pre-nsLTP may vary, depending on the cell types from various tissues and organs that are involved in lipid metabolism and steroid biosynthesis (1, 14). It is also notable that a role of nsLTP and SCPx in peroxisomal a-oxidation of phytanic acid was implicated from the study using nsLTP/SCPx gene null mice (39).

SCPx is exclusively localized in peroxisomes where it is converted to the 46-kDa fragment and nsLTP. SCPx is also transported with use of C-terminal AKL via a Pex5p-mediated PTS1 pathway. In PEX5-deficient mutant cells, SCPx is associated with endmembranes such as peroxisomal remnants and mitochondria. It is noteworthy that not only the 59-kDa form but also the 46-kDa fragment are enzymatically active as a branched-chain fatty acid thiolase (15, 17, 40), as revealed using specimens from patients with peroxisome-defective disorders such as Zellweger syndrome. Localization of SCPx on endomembranes may be required for such enzymatic activity of the unprocessed form. However, its underlying mechanisms are presently unclear. Exact cleavage sites initially at position 404–405 or 424–425 or both (see Fig. 1A), as well as physiological significance of this intraperoxisomal processing of SCPx, are not well understood at present. Interestingly, P-44 with thiolase activity and ZK892.2 apparently corresponding to the 46-kDa form of SCPx and nsLTP in mammals, respectively, have both been identified in Caenorhabditis elegans (41), whereas an orthologue of 59-kDa SCPx has not been detected. Whether accumulation of SCPx in protease-resistant form in the membrane fraction in pex5 and pex2 mutants, as noted in this work, is physiologically relevant or related to clinical phenotypes of peroxisome biogenesis disorders also remains to be defined.

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Biogenesis of Nonspecific Lipid Transfer Protein and Sterol Carrier Protein x: STUDIES USING PEROXISOME ASSEMBLY-DEFECTIVE pex CELL MUTANTS

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