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Nonspecific Lipid Transfer Protein (Sterol Carrier Protein-2) Defective in Patients with Deficient Peroxisomes

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ABSTRACT. **The biosynthesis and intracellular localization of nonspecific lipid transfer protein (nsLTP) in control human subjects and in patients with peroxisome-deficient disorders were investigated. The molecular mass of human nsLTP was indistinguishable from that of rat nsLTP (13 kDa) by an immunoblot analysis. Intracellular localization was identical with that of catalase, a marker enzyme of peroxisomal matrix, by a double immunofluorescence study. The nsLTP was deficient in liver tissues or fibroblasts from patients with peroxisome-deficient disorders such as Zellweger syndrome and neonatal adrenoleukodystrophy (ALD). Pulse-chase experiments showed that nsLTP was synthesized as a large precursor in both the control and Zellweger fibroblasts. However, the processing to the 13 kDa mature protein was disturbed and the degradation was rapid in Zellweger fibroblasts. After somatic cell fusion using Zellweger fibroblasts from different genetic groups, the processing was normalized. These results suggest that the biosynthesis and localization of human nsLTP are similar to those of rat nsLTP and that the defect of nsLTP in peroxisome-deficient disorders is a phenomenon secondary to an abnormal transport mechanism of peroxisomal proteins. The defect of nsLTP may play an important role in metabolic disturbances in bile acid synthesis and steroidogenesis in peroxisome-deficient disorders.**

Nonspecific lipid transfer protein (nsLTP), a compound identical with sterol carrier protein 2, is localized in the matrix of rat liver peroxisomes (18). Although the physiological role of nsLTP is unclear, this protein appears to function at several steps in the metabolism of cholesterol (13). The nsLTP transfers phospholipids, cholesterol and glycosphingolipids *in vitro* (2). Since peroxisomes oxidize 3α,7α,12α,trihydroxy-5β-cholestanoic acid to cholic acid (8), and part of the 3-hydroxy-3-methylglutaryl coenzyme A reductase, a rate-limiting enzyme of cholesterol formation, is located in peroxisomes (9), nsLTP is thought to play an important role in these metabolic events.

The intracellular localization of nsLTP was analyzed by several investigators. Van der Krift *et al.* (20) first demonstrated the presence of a 58 kDa protein in peroxisomes. However, the relationship between 58 kDa protein and nsLTP remains to be determined. Tsuneoka *et al.* (18) clarified that mature rat nsLTP was predominantly localized in peroxisomes. Rat nsLTP is synthesized as a larger precursor polypeptide of 14.5 kDa on free polysomes and is then processed to its mature form of 13 kDa (3).

These basic investigations prompted us to determine whether or not nsLTP plays a significant role in the pathogenesis of peroxisome-deficient disorders such as Zellweger's cerebro-hepato-renal syndrome and neonatal adrenoleukodystrophy (ALD). Zellweger syndrome and neonatal ALD are fatal autosomal recessive diseases characterized by the absence of peroxisomes (4) and multiple metabolic disturbances, including defects of peroxisomal β-oxidation enzymes (15), the first step of plasmalogen biosynthesis (7), phytanic acid oxidation (12) and others (see reference 22). Although accumulation of bile acid intermediates in patients with Zellweger syndrome and neonatal ALD (6) was considered to be mainly caused by defects in peroxisomal β-oxidation enzymes, the possible role of defective nsLTP in these metabolic disturbances must be given attention. Decreased formation of plasmalogen (7), and adrenal dysfunction in these patients (5) may relate to the metabolic roles of nsLTP. Although the deficiency of nsLTP in patients with Zellweger syndrome was reported (19), precise molecular mechanisms related to the defect of

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Abbreviations: nsLTP, nonspecific lipid transfer protein; ALD, adrenoleukodystrophy; MEM, minimal essential medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; P12, 12-(1′-pyrene) dodecanoic acid

nsLTP are not well understood. We investigated the biosynthesis and intracellular localization of nsLTP in tissues from control subjects and in those from patients without peroxisomes. We obtained evidence of a defective processing and transport of nsLTP in the patients.

MATERIALS AND METHODS

Materials. Purification of rat nsLTP and preparation of affinity-purified antibodies were as described (18). Antibodies against human erythrocyte catalase and rat enoyl-CoA hydratase were the kind gifts of Prof. T. Hashimoto (Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Japan). Alkaline phosphatase conjugated goat anti-rabbit IgG and the immunoblotting kit were obtained from Promega Biotec (Madison, WI, USA) Fluorescein isothiocyanate (FITC)-conjugated goat F (ab')2 anti-rabbit IgG was from TAGO (Burlingame, CA, USA). Rhodamin-conjugated goat anti-guinea pig IgG was from Cappel (West Chester, PA, USA). The ^{14}C-labelled methylated protein and [^{35}S]methionine (1000 Ci/mmol) were purchased from Amersham (Buckinghamshire, England). Protease inhibitors were from Peptide Institute (Osaka, Japan). Inactivated *Staphylococcus aureus* cells were from Sanraku (Tokyo, Japan). EN^{3}HANCE^{TM} was obtained from NEN (Boston, MA, USA). The 12-(1′-pyrene) dodecanoic acid (P12) was from Molecular Probes (Eugene, OR, USA).

Immunoblot analysis. Liver specimens from a control human subject (a biopsied sample taken at the time of hepatectomy), two patients with Zellweger syndrome (one each of a biopsied and autopsied liver), an infant with neonatal ALD (autopsied liver), and from a patient with a probable defect of peroxisomal β-oxidation enzyme and detectable peroxisomes (autopsied liver) were homogenized separately with 50 mM potassium phosphate buffer, pH 8.0/0.1% Triton X-100/protease inhibitors (10 μg/ml of leupeptin, pepstatin, chymostatin, bestatin and E-64). After centrifugation at 10,000 × g for 20 min, the supernatant was mixed with an equal volume of 125 mM Tris-Cl, pH 6.8/5% SDS/0.32 M 2-mercaptoethanol/20% glycerol/0.005% bromophenol blue

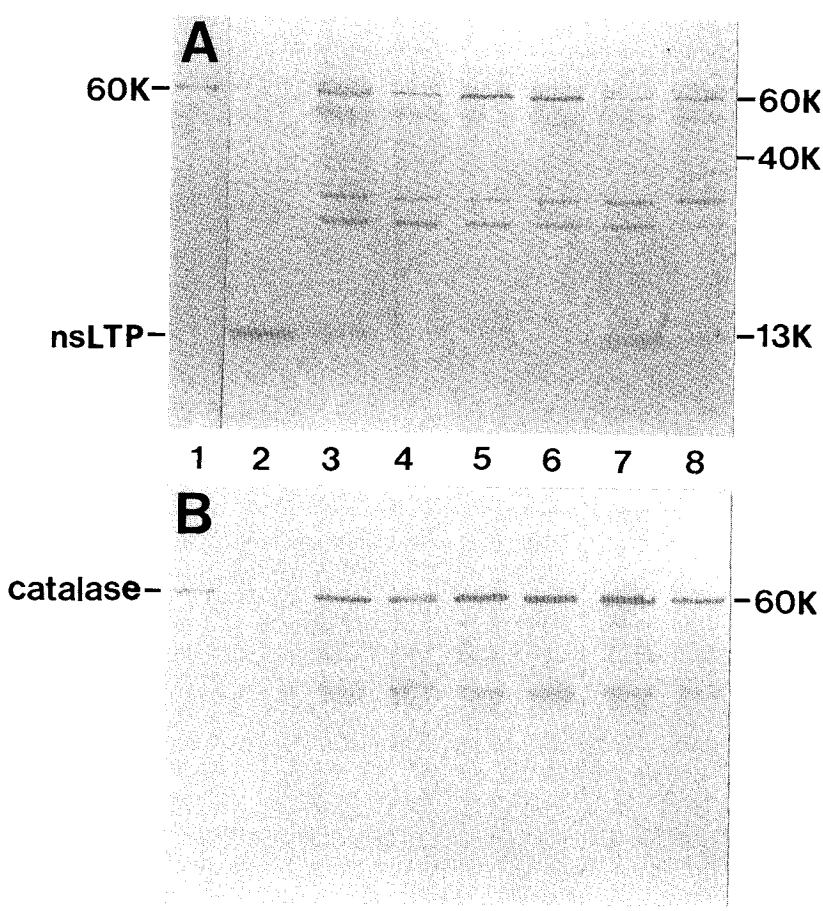

Fig. 1. Immunoblot analysis of human liver extract. Immunoblotting was carried out with affinity-purified anti-rat nsLTP IgG (A) and anti-human catalase IgG (B). Lanes: 1, purified human catalase (10 ng) treated with anti-catalase IgG in both A and B; 2, purified rat nsLTP (10 ng); 3 and 8, control liver extract (20 μg); 4 and 5, Zellweger liver extract (20 μg); 6, neonatal ALD liver extract (20 μg), 7; liver extract of a patient with a probable deficiency of peroxisomal β-oxidation enzyme (20 μg).

(sample buffer), boiled for 3 min and subjected to SDS/PAGE (12%) (11). After western blotting (16), blocking was done with 5% bovine serum albumin, and the preparation was incubated with anti-rat nsLTP IgG or anti-human catalase IgG, then reincubated with alkaline phosphatase conjugated goat anti-rabbit IgG. Color development was performed with the use of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Immunofluorescence staining. Skin fibroblasts from the patients or from the control were cultivated on cover slips with Eagle's MEM supplemented with 10% FBS. After washing with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde/0.1 M potassium phosphate buffer, pH 7.4 for 1 h, washed with PBS and permeabilized with 0.1% Triton X-100/PBS for 15 min. After blocking with 2% FBS/0.1% Triton X-100/PBS for 1 h, the cells were incubated with rabbit anti-rat nsLTP IgG or anti-human catalase IgG for 2 h, washed 4 times with PBS then reincubated with FITC-conjugated goat F(ab')2 anti-rabbit IgG in a dark box for 1 h. Cover slips were washed 4 times with PBS, mounted on slide glass with 0.1% paraphenylenediamine/0.1 M Tris-Cl, pH 8.5/90% nonfluorescent glycerin and observed under a fluorescence microscope (Axiophot, Zeiss, West Germany) (10). For a double immunofluorescence staining, guinea pig anti-rat nsLTP, rhodamine-conjugated goat anti-guinea pig IgG and a laser fluorescence microscope MRC-500 (Nihon Bio-Rad, Tokyo, Japan) were used.

Cell fusion and P12/UV selection. Two cell lines from the patients with Zellweger syndrome and who belonged to the different genetic groups (unpublished data) were mixed and cultivated. The cells were then fused by treatment with 45% polyethylene glycol/MEM for 1 min, washed 4 times with MEM, and cultivated for 3 days. Immunofluorescence staining of the fused cells was performed as described above. Complemented fused cells were selected by P12/UV killing of cells without peroxisomes as described (23), and used for pulse-chase experiments.

Pulse-chase experiments. Fibroblasts from a patient with

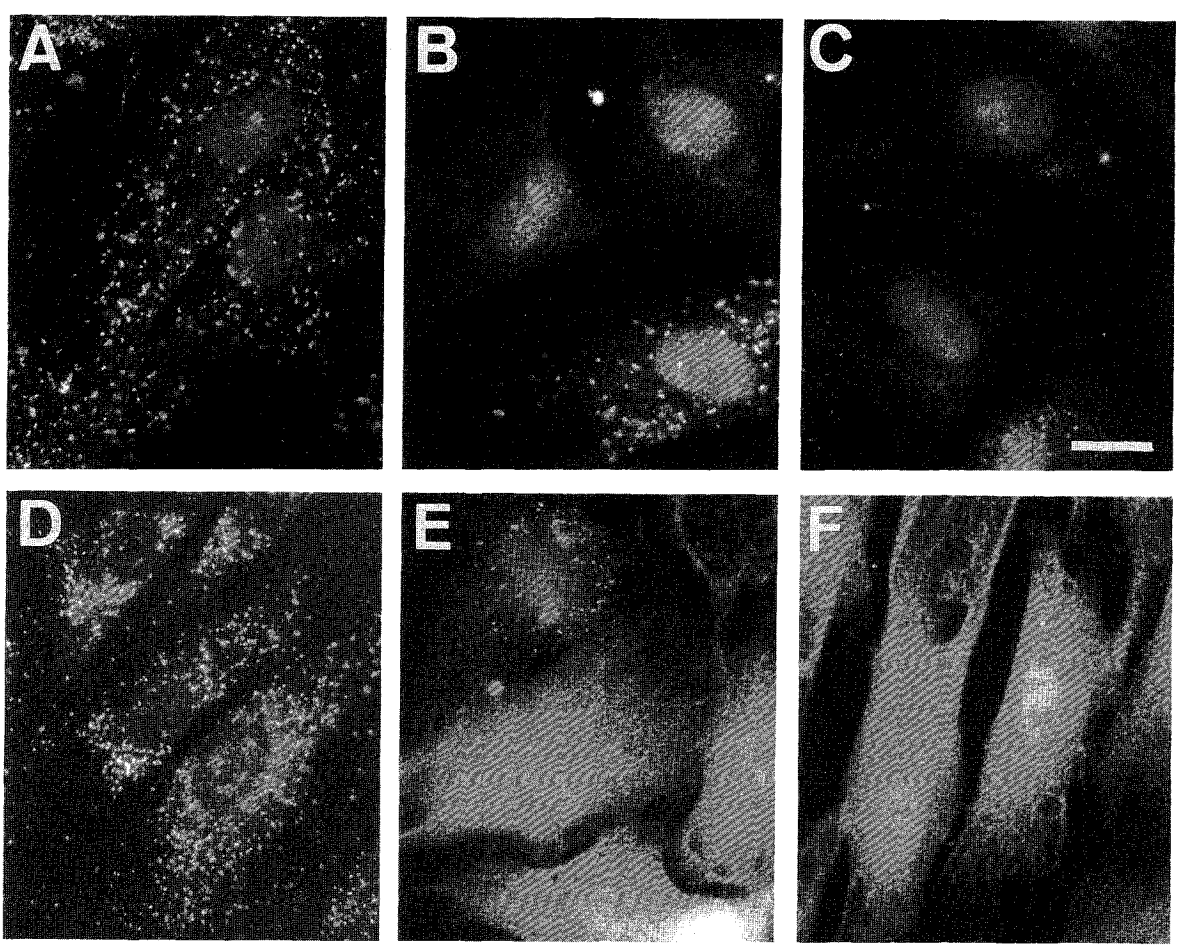

Fig. 2. Immunofluorescence staining of human skin fibroblasts. Cells were treated first with anti-rat nsLTP IgG (A, B, C,) or anti-human catalase IgG (D, E, F), then incubated with FITC-conjugated goat anti-rabbit IgG. A and D: control fibroblasts; B and E; neonatal ALD; C and F: Zellweger syndrome. Bar = 10 μm.

Zellweger syndrom, a control subject and P12/UV selected cells were cultivated in 8 cm^2 dishes. Preincubation with methionine-free MEM supplemented with 5% dialyzed FBS was performed for 1 h. Subsequently the medium was replaced with freshly prepared 0.6 ml of the same medium supplemented with 5% dialyzed FBS and 0.3 mCi of [^{35}S]methionine, and pulse-labelled for 1 h. Chase experiments were performed with MEM supplemented with 10% FBS and L-methionine for 6 h, 24 h and 72 h. After pulse-labelling and chase, the cell monolayer was washed with PBS, lysed with 10 mM Tris-Cl, pH 7.4/2 mM EDTA/0.1% SDS/0.1% Triton X-100, freeze-thawed 3 times and centrifuged. Immunoprecipitation with anti-nsLTP and mitochondrial enoyl-CoA hydratase, SDS/PAGE and fluorography were performed as described (14).

RESULTS

Immunoblot analysis. Human nsLTP was detected in the liver extracts from the control (Fig. 1A, lanes 3 and 8) and the patient with a probable deficiency of peroxisomal β-oxidation enzyme (lane 7) using anti-rat nsLTP IgG. The molecular mass (about 13 kDa) was indistinguishable from that of purified rat nsLTP (lane 2). In the liver from the patients with Zellweger syndrome and neonatal ALD, nsLTP was hardly detectable (lanes 4, 5 and 6). A 60 kDa protein with a molecular mass similar to that of catalase (lane 1, stained with anti-human catalase IgG) and two smaller bands (about 30 kDa) were detected in both the control and the patients. Antibody against nsLTP did not cross-react with the purified catalase (data not shown). Catalase was present in tissues from both the control and the patients (Fig. 1B). Anti-catalase IgG did not cross-react with the purified nsLTP (Fig. 1B, lane 2).

Immunofluorescence staining. In the control fibroblasts, nsLTP was stained in a granular pattern (Fig. 2A), similar to that seen with catalase, a marker enzyme of the peroxisomal matrix (Fig. 2D). This staining pattern differed from those of other organelles such as mitochondria and lysosomes (data not shown). In fibroblasts from the patient with neonatal ALD, nsLTP was not detected in the majority of cells. However, it was detected in a very small number of cells (less than 10%)

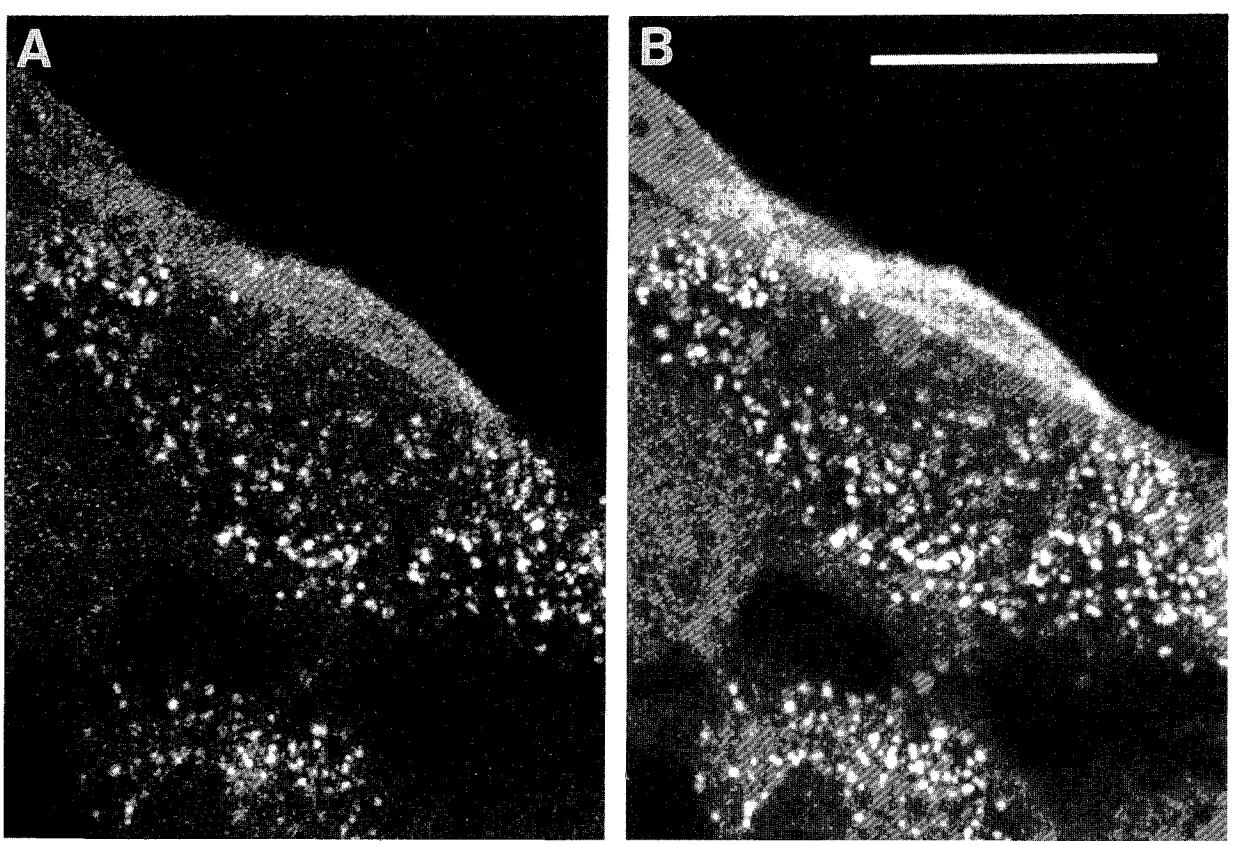

Fig. 3. Double immunofluorescence staining of fused fibroblasts. Zellweger fibroblasts from two genetically different groups were fused with polyethylene glycol and cultured on a cover slip for 3 days, and a double immunofluorescence staining was carried out. A: stained with guinea pig anti-rat nsLTP IgG and rhodamine-conjugated goat anti-guinea pig IgG; B: stained with rabbit anti-human catalase IgG and FITC-conjugated goat anti-rabbit IgG. Bar = 10 μm.

(Fig. 2B), a finding which agreed well with the result of catalase staining (Fig. 2E). In fibroblasts from the patient with Zellweger syndrome, nsLTP was hardly detected either in peroxisomal granules or in the cytosol (Fig. 2C). Catalase was present in the cytosol of Zellweger fibroblasts (Fig. 2F).

After cell fusion using fibroblasts from two genetically different Zellweger patients, peroxisomes were formed and visualized by a double immunofluorescence staining using guinea pig anti-rat nsLTP and rabbit anti-human catalase. Localization of nsLTP (Fig. 3A) and catalase (Fig. 3B) was very similar. The results using control fibroblasts or neonatal ALD cells also support the localization of nsLTP in peroxisomes (data not shown).

Pulse-chase experiments. In the control fibroblasts, a 60 kDa protein (open arrowhead), a precursor protein (arrow) and a thick mature nsLTP were detected in the pulse experiment (Fig. 4, lane 3). These bands disappeared when 10 μg of rat nsLTP was added during the immunoprecipitation. (lane 1, same sample as lane 3). The molecular masses of the precursor (about 14.5 kDa) and the mature protein (about 13 kDa) were considered to be idetical with those of rat proteins. The precursor is thought to be rapidly processed to the mature protein. In the chase experiments, the precursor disappeared and the mature protein was stable even after a 72 h chase. A 60 kDa protein was faintly detected in a 6 h chase (lane 4), then it disappeared with a 24 h chase. The competition experiment (lane 2, same sample as lane 4) indicated that this is not catalase since the bands of 60 kDa protein and mature nsLTP did not disappear with the addition of catalase.

The precursor of nsLTP was synthesized in fibroblasts from the patients with Zellweger syndrome (Fig. 5A). However, the newly synthesized nsLTP precursor could not be processed to the mature protein. After a 6 h chase, the precursor rapidly degraded and the mature protein was not detected. On the other hand, the precursor was completely processed to the mature protein in

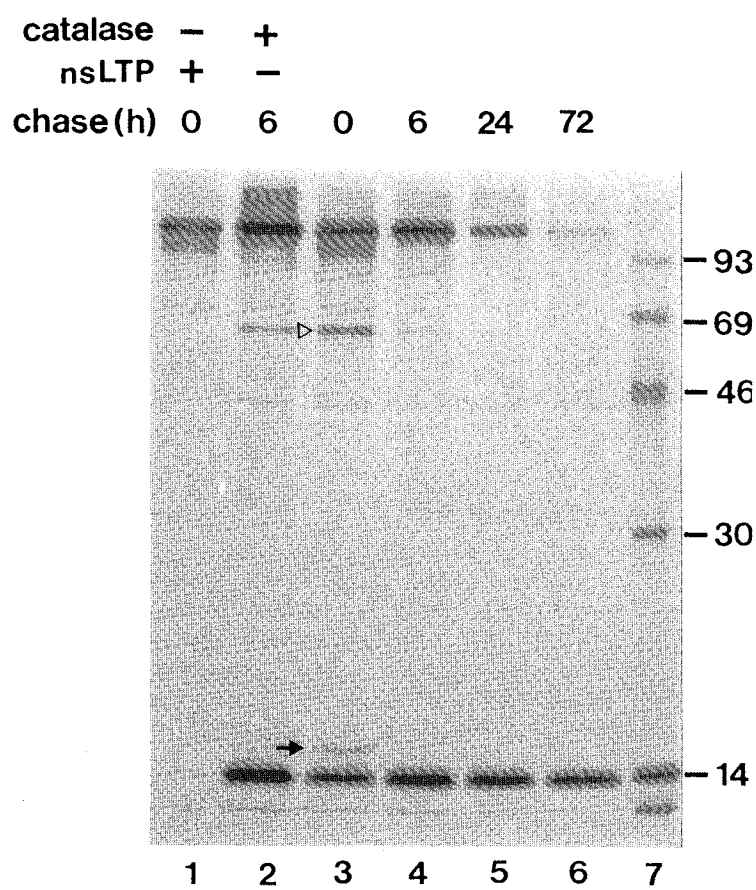

Fig. 4. Pulse-chase experiments of control fibroblasts. Fibroblasts were pulse-labelled with [^{35}S]methionine and chased for 6, 24 and 72 h as described under MATERIALS AND METHODS. Lanes: 1, competition experiment with 10 μg of purified nsLTP (same sample as lane 3); 2, competition with 10 μg of purified catalase (same sample as lane 4); 3, pulse-labelling for 1 h; 4, chase for 6 h; 5, chase for 24 h; 6, chase for 72 h; 7, molecular mass standards. The arrow indicates the precursor protein of nsLTP. Open arrowhead shows the 60 kDa protein.

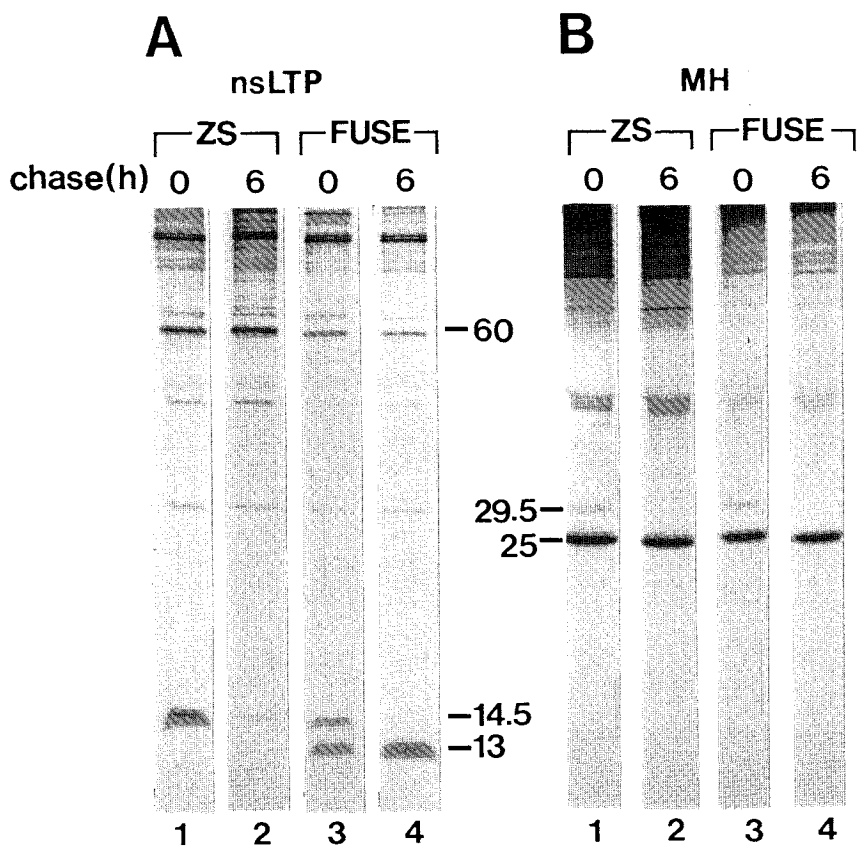

Fig. 5. Pulse-chase experiments of Zellweger fibroblasts and complemented fused cells selected by P12/UV killing. A: nsLTP; B: mitochondrial enoyl-CoA hydratase (MH); ZS: Zellweger fibroblasts; FUSE: P12/UV selected fused fibroblasts. Lanes: 1 and 3, pulse-labelling for 1 h; 2 and 4, chase for 6 h.

the complemented fused cells which were selected by P12/UV killing, albeit the amount of the mature protein in the pulse being less than that of the control fibroblasts. The 60 kDa protein was detected after a 6 h chase in both the patient and the complemented fused cells. Synthesis and processing of mitochondrial enoyl-CoA hydratase were normal throughout (Fig. 5B).

DISCUSSION

In case of biosynthesis and intracellular localization of nsLTP in the rat liver, it was elucidated that nsLTP was synthesized as a large precursor (14.5 kDa) on free polysomes, transported into peroxisomes and presumably proteolytically processed to a 13 kDa mature protein (3). The nsLTP was determined to be localized mainly in the peroxisomal matrix by immunoelectron microscopic examinations and immunoblot analysis (18).

In the present work, we obtained evidence for the biosynthesis and intracellular localization of human nsLTP. Human nsLTP is also synthesized as a precursor which is about 1.5 kDa larger than the mature nsLTP, and it seems to be localized in peroxisomes from the data of immunofluorescence studies. We also have evidence for the localization of human nsLTP in peroxisomes by immunoelectronmicroscopic examinations (data not shown). As in the case of rat (3), the precursor is completely processed to its mature form within 1 h. The mature nsLTP remains stable for at least 72 h.

The relationship between nsLTP and 60 kDa protein remains to be elucidated. Limited proteolysis of 60 kDa protein showed that this is not a oligomer of nsLTP (3). However, the result of competition experiment suggests that 60 kDa protein may be a nsLTP-related protein. The 60 kDa protein is not likely to be a catalase, a marker enzyme of peroxisomal matrix with a 60 kDa mass, since purified catalase was not detected with anti-nsLTP IgG and the 60 kDa protein did not disappear when purified catalase was added during immunoprecipitation. Although van Amerongen *et al.* found a 40 kDa protein which cross-reacted with anti-nsLTP (19), it was not detected in our study. On the other hand, two smaller bands (about 30 kDa) were found in our immu-

noblot analysis using liver extract. This may be based on the difference of antibodies.

A deficiency in nsLTP in patients with Zellweger syndrome was first reported by van Amerongen *et al.* (19), as based on immunoblot analyses of liver extracts. Chinese hamster ovary cells deficient in peroxisomes also lacked nsLTP (21). However, the localization and biosynthesis of nsLTP were apparently not examined in these reports. The present study clearly shows the lack of nsLTP in patients with Zellweger syndrome and neonatal ALD. The precursor form of nsLTP was synthesized in patients with Zellweger syndrome, but it was not transported into peroxisomes and was not processed to the mature protein, as in the case of β-oxidation enzymes (14). The precursor is considered to be degraded rapidly in Zellweger fibroblasts. From the data on the immunofluorescence staining, nsLTP is partially localized into peroxisomes in a very small number of neonatal ALD cells as in the case of catalase. Heterogeneity may be present among neonatal ALD cells. The results of somatic cell fusion indicate that the biosynthesis of nsLTP is normalized after functional peroxisomes are generated. The defect of nsLTP is probably not to a primary lesion in Zellweger syndrome or neonatal ALD but rather a phenomenon secondary to a deficiency in the peroxisomes.

The results of the 60 (58) kDa protein were rather complicated. It was detected in our patients and in mutant Chinese hamster ovary cells (21), whereas Amerongen *et al.* (19) reported the deficiency of the 60 kDa protein in Zellweger syndrome. Further investigation on this unknown protein is needed.

The defect of nsLTP is probably linked to the pathophysiology of peroxisome-deficient disorders. As shown in rat experiments, nsLTP may function in cholesterol metabolism. Although the defect of biosynthesis of bile acids in patients without peroxisomes is considered to be mainly caused by defects in peroxisomal β-oxidation enzymes, the defect of nsLTP may decrease transport of bile acid precursors into the peroxisomes. Administration of adrenocorticotropin increases the number of peroxisomes in the guinea pig adrenal gland (1) and induces nsLTP in rat adrenocortical cells (17). Adrenal dysfunction or low reaction of steroid hormones against adrenocorticotropin in peroxisome-deficient disorders, as possibly related to the defective biosynthesis of nsLTP, deserves further attention.

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