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# Novel Acyl-CoA Synthetase in Adrenoleukodystrophy Target Tissues

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**X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disorder characterized by demyelination of white matter. The X-ALD gene product adrenoleukodystrophy protein (ALDP) is expressed broadly among various tissues. However, deficiency of functional ALDP exclusively impairs brain, adrenal gland, and testis. Thus, loss of ALDP function is assumed to involve inactivation of a putative mediating factor that functions in a tissue-specific manner. Here we cloned a mouse cDNA encoding a novel protein, Lipidosin, that possesses long-chain acyl-CoA synthetase (LCAS) activity. Lipidosin is expressed exclusively in mouse brain, adrenal gland, and testis, which are affected by X-ALD. LCAS activity of Lipidosin was diminished by mutation of conserved amino acids within the AMP-binding domain. Mutation of the Drosophila homologue of Lipidosin has been reported to cause neuronal degeneration. Thus, Lipidosin may mediate the link between ALDP dysfunction and the impairment of fatty acid metabolism in X-ALD.** 

***Key Words:*** **long-chain acyl-CoA synthetase; adrenoleukodystrophy; very-long-chain fatty acid; long-chain fatty acid; lipidosis; adrenomyeloneuropathy; Addison disease; long-chain acyl-CoA; very-long-chain acyl-CoA; *Bubblegum*.**

X-linked adrenoleukodystrophy (X-ALD) is an inherited disorder characterized biochemically by the accumulation of very-long-chain fatty acids (VLCFA) in tissues and plasma (1–3). Though X-ALD affects mainly the nervous system, adrenal cortex, and testis, clinical manifestations of X-ALD are highly variable (1, 2). The major forms of X-ALD are childhood cerebral X-ALD, adrenomyeloneuropathy (AMN), and Addison disease. Childhood X-ALD is characterized by progressive demyelination within the white matter. AMN involves mainly the long tracts of the spinal cord and peripheral nerves. In Addison disease, adrenocortical insufficiency is the only clinical symptom. All forms of X-ALD are associated with the accumulation of VLCFA, which is due to impaired $\beta$-oxidation of VLCFA. The activation of VLCFA by very-long-chain acyl-CoA synthetase (VLCAS) is essential for its $\beta$-oxidation. Thus, VLCAS has been presumed to be responsible for X-ALD. Surprisingly, however, the X-ALD gene encodes a member of the family of ABC transporters, adrenoleukodystrophy protein (ALDP) (4). Although it is established that the loss of ALDP function is responsible for the abnormality in VLCFA metabolism, the relationship between ALDP function and X-ALD phenotypes remains to be elucidated. Although expression of ALDP is broad among tissues (4, 5) and the loss of its function results in accumulation of VLCFA in several tissues, X-ALD mainly affects brain, adrenal gland and testis. It remains unknown why brain, adrenal gland, and testis only should be sensitive in X-ALD, but their exclusive impairment in X-ALD implies a putative secondary factor that mediates ALDP function and fatty acid metabolism in a tissue-specific manner.

In the present study, we identified a novel protein, Lipidosin, that is capable of acyl-CoA synthetase activity and is exclusively expressed in tissues affected by X-ALD. Lipidosin catalyzes long-chain fatty acids (LCFA, 12–22 carbon chain) to long-chain acyl-CoA. Further, dysfunction of the Drosophila homologue of Lipidosin, *Bubblegum*, causes neuronal degeneration accompanied by accumulation of VLCFA (6). Lipidosin may be a mediator linking ALDP dysfunction and the impaired fatty acid metabolism in X-ALD.

## MATERIALS AND METHODS

*cDNA cloning, sequencing, and plasmid constructions.* The #54 (Lipidosin) fragment was cloned through differential screening using a PCR-subtraction kit (CLONTECH Lab., USA) according to manu-

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facturer's instructions. 5′ RACE and CapSite cloning were performed using mouse brain cDNA (Marathon-Ready cDNA kit, CLONTECH Lab) and CapSite mouse brain cDNA (Nippon Gene, Japan) as templates. In order to clone a complete open reading frame by RT-PCR, mouse brain mRNA prepared with a Quick mRNA preparation kit (Amersham Pharmacia Biotech, USA) was reverse transcribed at 60°C using a BcaBEST RNA PCR kit (TAKARA, Japan). The amplified fragment was subcloned into a pT7Blue-T vector (Invitrogen, USA), and its nucleotide sequences were determined with a DNA sequencer ABI377 (Applied Biosystems, USA) according to manufacturer's instructions.

For the enforced expression in mammalian cells, Lipidosin cDNA containing complete open reading frame was cloned into an expression vector, pcDNA3.1/hygro (Invitrogen). Plasmids used for transfection were prepared with a QIAGEN column (QIAGEN, Germany).

*Northern blot analysis.* A mouse Lipidosin cDNA fragment including an open reading frame was amplified with specific primers and then purified with a Gene Clean Kit III (BIO101, USA). Mouse MTN Blot (7763-1, CLONTECH Lab) was probed with the mouse Lipidosin cDNA fragment labeled with [$^{32}$P]dCTP using a Random Prime Labeling Kit (Amersham Pharmacia Biotech). Hybridization was performed essentially according to the method described previously (7, 8). In brief, the membrane was hybridized with $^{32}$P-labeled Lipidosin cDNA for 12 h at 65°C in a hybridization buffer containing 0.25 M $Na_2HPO_4$, 1 mM EDTA, and 7% SDS. Then, it was washed for 15 min at 65°C with a low-stringency wash buffer containing 25 mM $Na_2HPO_4$, 1 mM EDTA, and 5% SDS and a high-stringency wash buffer containing 25 mM $Na_2HPO_4$, 1 mM EDTA, and 1% SDS, respectively. Signals were detected by a Fuji image analyzer (BAS 1000, Fuji Film, Japan).

*Cell culture and transfection.* Culture conditions of INC2 and COM3 cells were described previously (8, 9). COS7 cells were cultured in 10% fetal calf serum/Dulbecco's modified Eagle medium supplemented with 4.5 g/L glucose under 10% $CO_2$ at 37°C (10). For transfection, $1 \times 10^5$ COS7 cells were plated in a single well of a 6-well plate according to the manufacturer's instructions. On the next day, 2 $\mu$g plasmids was mixed with 3 $\mu$l transfection reagent (FuGENE6, Roche Diagnostic, Germany), and then added to a single well of a 6-well plate. Cells were harvested 2 days later.

*Quantification of acyl-CoA synthetase activity.* The standard reaction buffer included 50 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM MgCl, 10 mM ATP, 0.1% Triton X-100, and 1× protease inhibitor (Complete, Roche Diagnostic). Transfected COS7 cells were washed with PBS and trypsinized with 0.05% trypsin–0.53 mM EDTA (Life Technologies, USA). Then the cells collected from 6 wells of a 6-well plate were pooled in 150 $\mu$l of ice-cold reaction buffer. Cells were homogenized using a sonicator (Bioruptor, Olympus, Japan) at maximum power for 100 s. Fatty acids were dissolved in 1% Triton X-100 and 1% isopropanol. The reaction mixture, which included cell lysates, 0.2 mM palmitic acid or lignoceric acid, and 0.5 mM CoA in the reaction buffer was incubated for 5 min at 35°C. Protein concentrations of cell lysates were determined with a protein assay kit (Bio-Rad, USA) using BSA as a standard.

Quantification of acyl-CoA by HPLC was performed essentially according to the method described by Kono *et al.* (11). Briefly, samples were applied to reverse-phase HPLC on a LUNA 5 $\mu$ C18(2) column (150 × 4.6 mm, Phenomenex, USA). The C16-CoA was eluted by acetonitrile/50 mM potassium phosphate (pH 8.0) isocratically (45:55, v/v) at a flow rate of 1.0 ml/min, and the C24-CoA was eluted by a gradient system (45:55 to 80:20) at 2.0 ml/min. The effluent was monitored with a Shimadzu LC-10AD at 260 nm. *n*-Heptadecanoyl-CoA (C17-CoA) was used as a standard for quantification. All assays were performed within the range where the reaction proceeded linearly with time and the initial rate of reaction was proportional to the amount of cell lysate added.

*Site-directed mutagenesis.* Site-directed mutagenesis was performed essentially according to the method of Imai (12). Mutation primers used were as follows: reverse primer, 54-mt 1: CGT GTT GGC TGA TGT GTA GAC; forward primer, 54-mt 3: GGA AAC CCC ATG GGT GTG ATG.

*Antibody preparation and immunoblot analysis.* Anti-Lipidosin monoclonal antibody was prepared from a mouse immunized with GST-Lipidosin fusion protein. Characterization of the antibody will be published elsewhere (Hashimoto *et al.*, submitted). Immunoblotting analysis was done as described previously (9).

## RESULTS AND DISCUSSION

### *Cloning of a Gene Encoding a Putative Novel Acyl-CoA Synthetase*

A fragment of a novel gene called #54 expressed exclusively in nondifferentiating myoblasts was identified through differential screening between nondifferentiating and differentiating mouse myoblasts named INC2 and COM3, respectively (9). Nucleotide sequences of the #54 fragment implied that it was the 3′ flanking region of a novel gene. Preliminary Northern blot analysis revealed that the #54 gene was highly expressed in mouse brain. Thus, the upstream sequences of #54 were cloned from mouse brain cDNAs by 5′ RACE and CapSite cloning techniques (13). Nucleotide sequences containing the whole open reading frame were determined from cloned upstream fragments. Finally the almost full-length cDNA fragment of #54 containing the complete open reading frame with partial 5′ and 3′ flanking regions was cloned from mouse brain mRNA by RT-PCR technique (Fig. 1A).

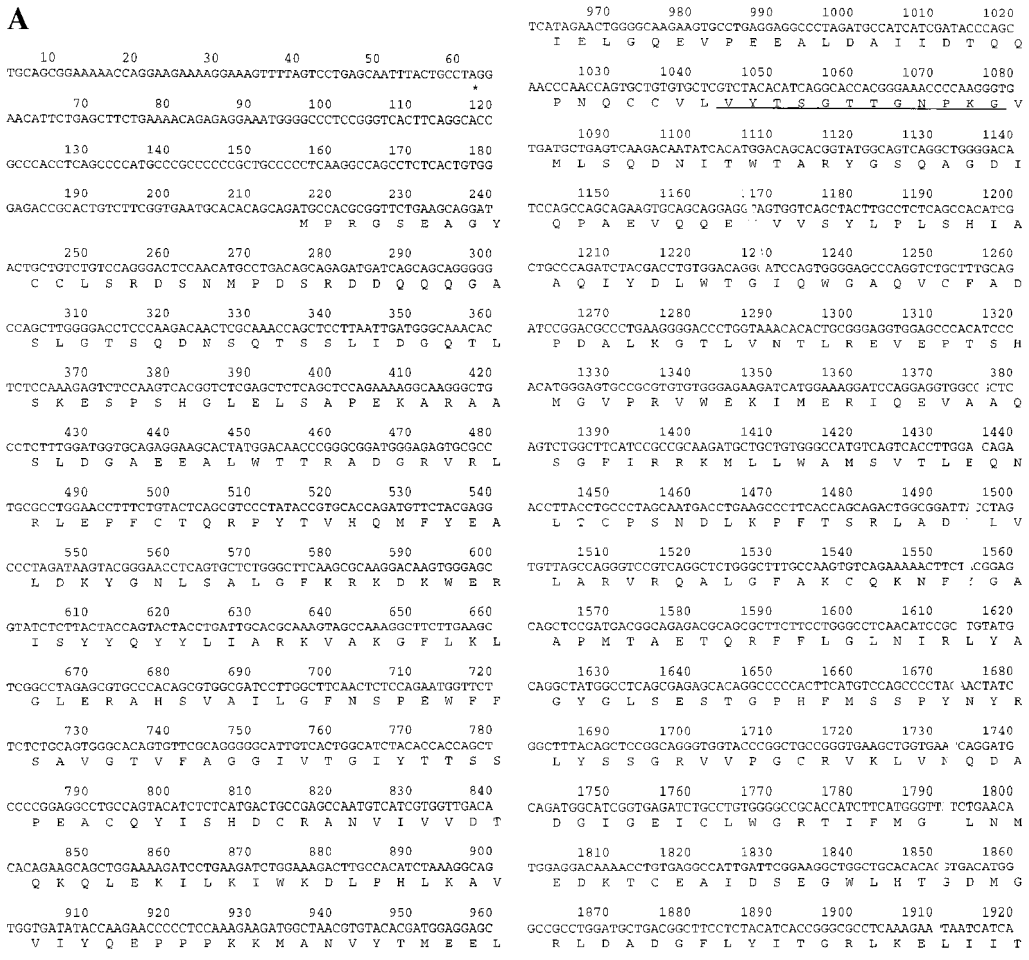
The #54 gene showed low (less than 30%) but significant partial homology with LCAS previously reported in both mammals and non-mammals. Furthermore, the AMP-binding motif (14) that is strongly conserved among thioesterification enzymes including LCAS was present in the #54 gene product (the amino acids underlined in Fig. 1A). Thus, the #54 was assumed to encode a novel acyl-CoA synthetase.

### *AMP-Binding Domain-Dependent Long-Chain Acyl-CoA Synthetase Activity of #54 Gene Product*

The acyl-CoA synthetase activity of #54 was determined because it showed significant but low homology with previously reported LCAS. An expression plasmid containing mouse #54 cDNA was transfected into COS7 cells in order to express #54 transiently. The acyl-CoA synthetase activity in COS7 cell lysates was determined by HPLC quantification of the acyl-CoA produced. When palmitic acid (C16) was used as the substrate, #54-expressing COS7 cells showed very high LCAS activity (Fig. 2A). In contrast, VLCAS activity was not detected in #54-expressing cells using lignoceric acid (C24) as the substrate (Fig. 2B).

The AMP-binding domain present in #54 is conserved among various thioesterification enzymes of nonmammals as well as mammals. However, it has been unclear whether this domain is essential to

**A**



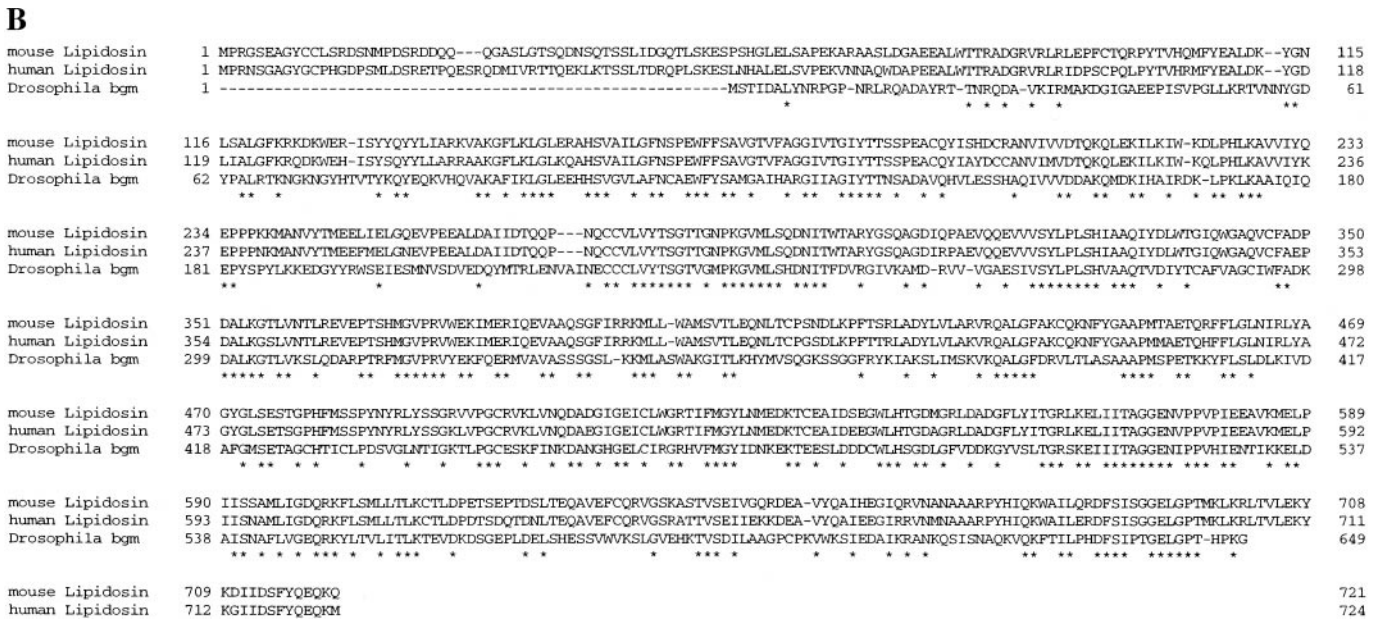



**FIG. 1**—*Continued*

enzymatic activity of the thioesterification enzymes. To clarify the role of the domain in LCAS activity of #54, the highly conserved 281st glycine and 287th lysine in the AMP-binding domain were converted to alanine and methionine, respectively. The mutated #54 (represented as Lipidosin AM in Fig. 2A) was expressed in COS7 cells, and LCAS activity of the cell lysate was determined. The protein levels of the mutant #54 in COS7 cell lysate were similar to those of wild-type #54 (represented as Lipidosin in Fig. 2A). However, LCAS activity was not detected in the lysate prepared from the mutant #54-expressing COS7 cells. Therefore, the AMP-binding domain is essential for the LCAS activity of #54. This is the first evidence showing the essential role of the AMP-binding domain in enzymatic activity of thioesterification enzymes containing this motif.

### *Gene Product of #54 as a Putative Lipidosis-Related Protein*

During the cDNA cloning of #54, we found a partial sequence of its human homologue (KIAA0631) in databases. Then, an almost complete human #54 homologue cDNA containing the complete open reading frame was cloned in our laboratory (Moriya-Sato *et al.*, submitted).

We also found the Drosophila homologue of #54 in genome databases. Then the corresponding fruit fly mutant was searched in FlyBase (http://flybase.bio.indiana.edu/) on the basis of the chromosomal position of fly #54 homologue. Finally we found the Drosophila homologue of #54, *Bubblegum* (6). A fruit fly with a mutation in the *Bubblegum* gene shows the neurodegeneration and the accumulation of VLCFA that is also reported in X-ALD (1, 2). Both the LCAS activity of #54 gene product and the lipidosis in the fly mutant, *Bubblegum*, strongly suggested that #54 gene was involved in mammalian lipidosis such as X-ALD. Thus, we named #54 Lipidosin (putative lipidosis-related protein).

Homology of the amino acid sequences between mouse or human and fruit fly Lipidosin/*Bubblegum* was approximately 50% (Fig. 1B). There was neither a membrane-bound domain nor any subcellular localization signals bound to the nucleus, peroxisome, and mitochondria in Lipidosin protein. Mouse and human Lipidosin proteins contained 721 and 724 amino acids, respectively. The predicted molecular sizes of mouse and human Lipidosin proteins were 80.5 and 81 kDa, respectively. An approximately 80-kDa band corresponding to mouse Lipidosin was recognized by anti-Lipidosin monoclonal antibody in mouse Lipidosin-expressing COS7 cells (Fig. 2A) as well as in mouse brain, adrenal gland, and testis (Fig. 3B). Human Lipidosin was also detected as an appropriate band (Moriya-Sato *et al.*, submitted).

**FIG. 1.** (A) Nucleotide sequences and deduced amino acid sequence of mouse Lipidosin (#54). Amino acids in the AMP-binding domain are underlined and stop codons are indicated by asterisks. (B) Alignment of amino acid sequence of mouse Lipidosin with sequence of human Lipidosin and Drosophila Lipidosin/*Bubblegum*. Identical amino acids are indicated by asterisks.

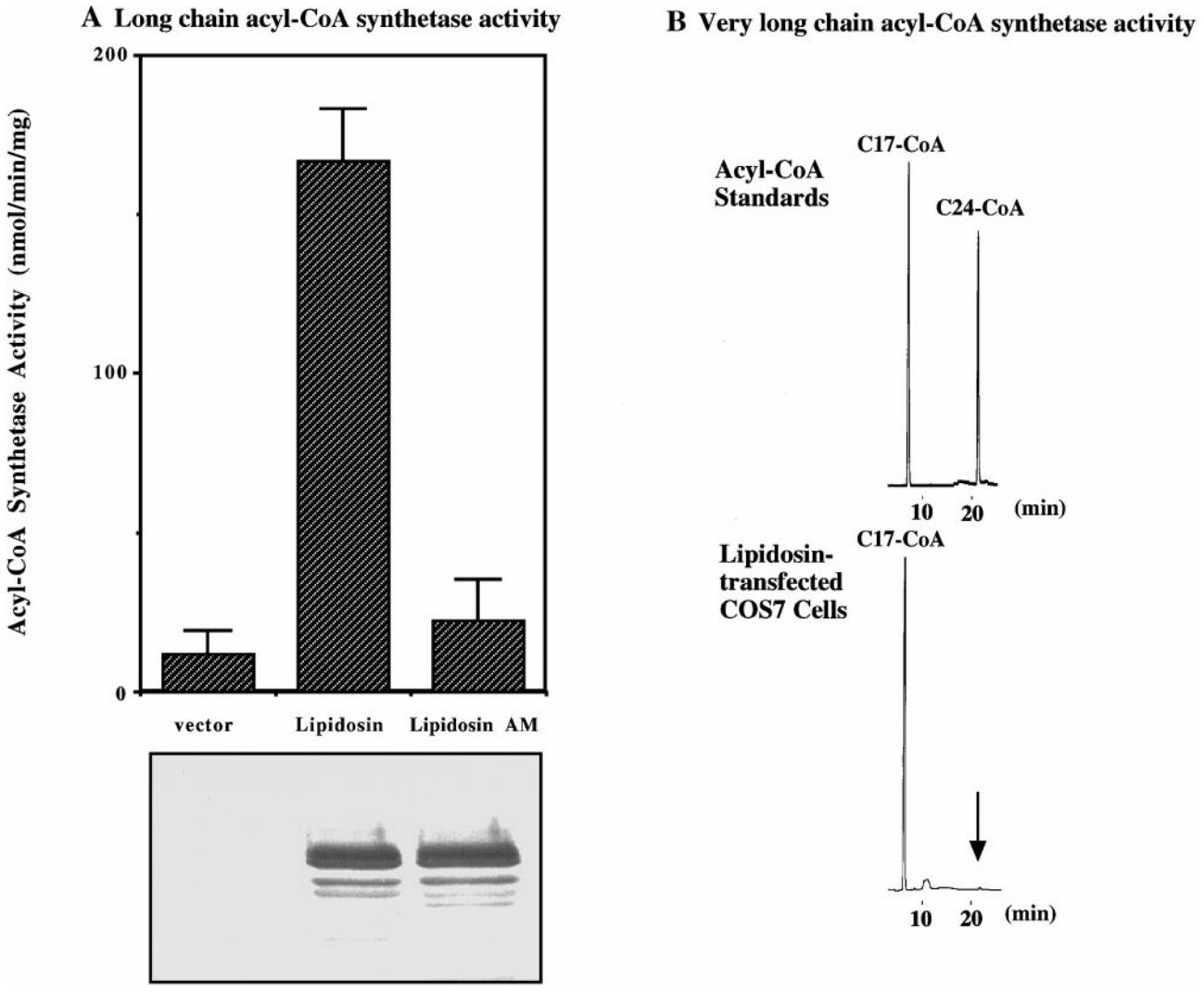



**FIG. 2.** Acyl-CoA synthetase activity of Lipidosin. (A) AMP-binding domain-dependent long chain acyl-CoA synthetase activity of Lipidosin. COS7 cells were transiently transfected with pcDNA3.1/hygro alone, or vector subcloned with mouse wild-type Lipidosin (#54) or mutated Lipidosin (Lipidosin AM). Two days later, cells were harvested and stored at −80°C for later assay of acyl-CoA synthetase activity. The average and SD estimated from three independent experiments are shown in the upper panel. To determine the Lipidosin protein levels in each samples, 2 $\mu$g protein of each sample was analyzed by immunoblot analysis. An almost equivalent amount of Lipidosin was detected in both wild-type and mutated Lipidosin expressing COS7 cells. Rapidly migrating faint bands that seemed to be attributable to degradation of Lipidosin were observed only in Lipidosin-expressing COS7 cells. (B) Very long chain acyl-CoA synthetase activity of Lipidosin. Upper panel, authentic acyl-CoAs including C17-CoA and C24-CoA were chromatographed on a reverse-phase HPLC column as described under Materials and Methods. Lower panel, HPLC analysis of reaction products formed when COS-7 cells transiently expressing Lipidosin were incubated with lignoceric acid. For quantification, authentic C17-CoA was added to the reaction mixture after incubation. Numbers under the base line represents retention time. An arrow shows the expected retention time of C24-CoA.

*Tissue-Specific Expression of Lipidosin Gene in Mice*

The ALDP gene on the X-chromosome is mutated in X-ALD patients (4). White matter, adrenal cortex and testis exclusively are impaired in X-ALD (1, 2), although ALDP is broadly expressed among various tissues (5). Therefore, it has been assumed that one or more putative secondary/mediating factor(s) are involved in the tissue-specific impairment caused by ALDP dysfunction. To assess the possibility that Lipidosin is the secondary factor involved in X-ALD disease, we determined expression of the Lipidosin gene in various mouse tissues by Northern blot analysis. Lipidosin was strongly expressed in brain and testis, but it was not detected in heart, spleen, lung, liver, skeletal muscle, or kidney (Fig. 3A). The size of the Lipidosin transcript was approximately 3.0 kb and no splicing variant was detected. Though expression of the Lipidosin gene in adrenal gland has not

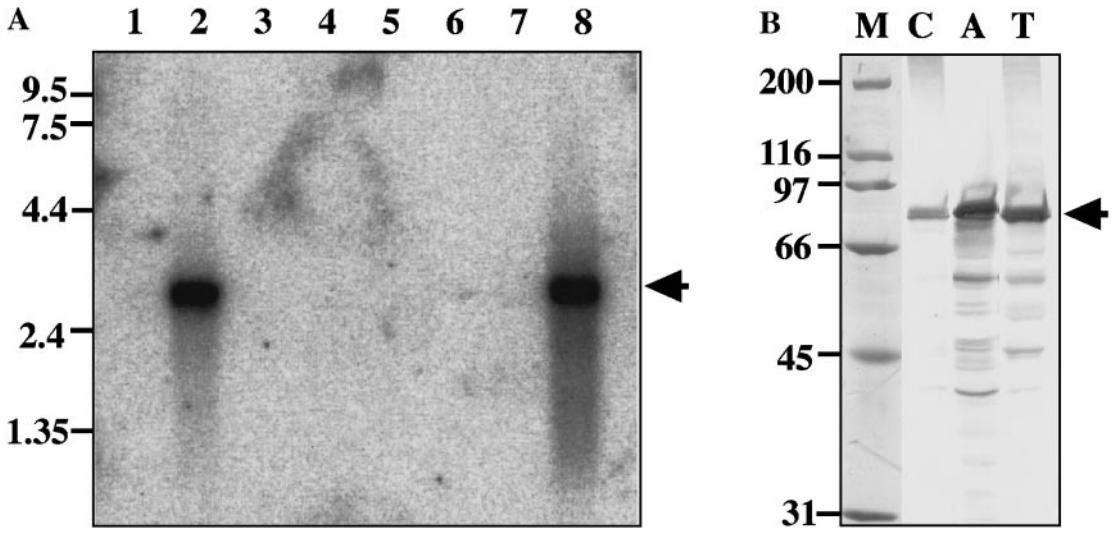



**FIG. 3.** Exclusive expression of Lipidosin in tissues affected by X-ALD. (A) Mouse multiple tissue Northern blot (CLONTECH Lab.) was probed with $^{32}$P-labeled mouse Lipidosin cDNA. An approximately 3.0-kb transcript (arrow) was detected exclusively in brain and testis. The positions of size markers are shown on the left (values in kb). Lanes 1–8, heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis, respectively. (B) Total proteins were extracted from 3-month-old Balb/c mouse brain (cerebrum), adrenal gland, and testis. Twenty micrograms of cellular protein was analyzed by immunoblotting. Approximately 80-kDa doublet bands of Lipidosin were detected with anti-Lipidosin monoclonal antibody (arrow). The positions of size markers are shown on the left (values in Da). M, size markers; C, cerebrum; A, adrenal gland; T, testis.

been determined yet, an 80-kDa Lipidosin protein was identified by a specific antibody against Lipidosin in adrenal gland as well as brain and testis (Fig. 3B). Taken together with the results, Lipidosin is expressed exclusively in tissues that are selectively affected by X-ALD.

*Putative Role of Lipidosin in Human X-ALD*

In the present study, we found LCAS activity of mouse Lipidosin but did not detect VLCAS activity. During the preparation of this manuscript, Steinberg *et al.* (15) reported human Lipidosin/*Bubblegum* cDNA sequences. In contrast to our results, they showed the VLCAS activity of human Lipidosin as well as the LCAS activity by radioisotope assay although the VLCAS activity was approximately 2% of the LCAS activity. The detection level of HPLC is assumed to be 1 nmol/min/mg protein whereas the reported VLCAS activity of human Lipidosin was 0.92 nmol/min/mg protein (15). Thus, the present results and those reported by Steinberg *et al.* are not contradictory. Furthermore, it is not conceivable that VLCAS activity of that very low magnitude represents the biological significance of Lipidosin as a VLCAS, although the possibility cannot be excluded that the VLCAS activity of Lipidosin is much higher *in situ* than in *in vitro* enzyme assay. In any event, Lipidosin can be assumed to play a role as a LCAS in brain, adrenal gland, and testis in a tissue-specific manner.

Lipidosin is a novel protein showing LCAS activity, but its amino acid sequences are not very similar to those of previously reported LCAS. Thus, Lipidosin might belong to a new subfamily of LCAS (15). Lipidosin is expressed exclusively in brain, adrenal gland, and testis, which are known to be impaired in human X-ALD patients. The mechanisms of the tissue-specific damage in X-ALD have been puzzling. The mutant phenotype of Drosophila Lipidosin homologue, *Bubblegum*, implies that Lipidosin is responsible for the neuronal degeneration and VLCFA accumulation that appear in human X-ALD. It is critical to clarify the mechanisms by which the deficiency of a fruit fly Lipidosin, *Bubblegum*, causes accumulation of VLCFA and neuronal degeneration. The ALD target tissue-specific distribution of the novel LCAS revealed here, Lipidosin, strongly suggests that Lipidosin is a possible candidate involved in tissue-specific impairment in human X-ALD.

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