Tissue and cellular specific expression of murine lysosomal acid lipase mRNA and protein

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Abstract Lysosomal acid lipase (LAL) is essential to the intracellular control of cholesterol and triglyceride catabolism via the low density lipoprotein (LDL) delivery of these neutral lipids to the lysosome. Deficiency of LAL in humans leads to Wolman disease and cholesteryl ester storage disease that result, respectively, in the intralysosomal storage of both neutral lipids or only cholesteryl esters. The mouse and human LAL cDNAs were cloned. The deduced amino acid sequences from the mouse and human LAL had high similarity (95%) and identity (75%) including conservation of the active center motifs (G-X-S-X-G) and five potential N-glycosylation consensus sequences. Tissue specific expression of LAL mRNA and protein in mouse tissues was evaluated by in situ hybridization and immunofluorescence staining, respectively. The LAL mRNA was expressed at low levels in most tissues. High level expression was found in hepatocytes and splenic and thymic cells. Very high level expression was observed in cells of the small intestinal villi, the zona fasciculata and reticularis of the adrenal cortex, pancreatic acini, and renal tubular epithelium. Significant levels of expression were detected in epithelial cells of choroid plexus in developing mouse embryo by day 12, in liver and lung by day 14, and in small intestine and kidney by day 16. Similar distribution of LAL protein was observed by immunofluorescence stain. Our results show that the expression of LAL is regulated in a tissue- and cell-specific manner that corresponds to the pathologic involvement in Wolman disease.—Du, H., D. P. Witte, and G. A. Grabowski. Tissue and cellular specific expression of murine lysosomal acid lipase mRNA and protein. *J. Lipid Res.* 1996. **37:** 937–949.

Supplementary key words gene expression • in situ hybridization • immunofluorescence • cholesteryl esterase • triglyceridase

Lysosomal acid lipase (LAL) is required for the hydrolysis of cholesteryl esters and triglycerides that are delivered to lysosomes by low density lipoprotein (LDL) receptor-mediated endocytosis (1, 2). The human LAL (hLAL) from fibroblasts (3), liver (4) or heterologously expressed in insect cell (5) or COS cells (6) has specificity for cholesteryl ester and tri-, di-, and monoglycerides. By liberating free cholesterol for inhibition of HMG-CoA reductase, LAL contributes to the homeostatic control of plasma lipoprotein levels and to the prevention of cellular lipid overloading in liver, spleen, macrophages, and smooth muscle cells of the arterial walls. The genetic deficiency of LAL leads to two human diseases, Wolman disease (WD) and cholesteryl ester storage disease (CESD) (3). In WD, both triglycerides and cholesteryl esters are massively accumulated in patients' liver, spleen, and adrenal glands. Steatorrhea and abdominal distention also occur (7). This disease is fatal before the age of 1 year. CESD presents with hepatomegaly and has a less aggressive course. Triglyceride and cholesteryl ester accumulation in a variety of tissues leads to death or disability during early life or adolescence (7, 8). These diseases are associated with arterial lipid plaques and development of premature atherosclerosis.

The structural gene for hLAL maps to chromosome 10 q 23.2, contains 10 exons, and is about 36.5 kb in size (9–11). The full-length cDNA encoding hLAL is about 2.5 kb in length with 1200 nt. of coding sequence (6). This corresponds well to the single 2.6 kb mRNA found in most human tissues (4). The full-length LAL cDNA predicts a protein of 399 amino acids (4, 6), but the co-translational cleavage sites for the signal peptide may differ when hLAL is expressed in fibroblasts (6), liver (4), or insect cells (5). This results in the synthesis of a 372 or 378 amino acid mature protein (4, 6). Glycosylation of LAL is required for mannose-6-phosphate-mediated lysosomal targeting and for the development of a catalytically active conformer (5). LAL activity and mRNA are expressed in all tissues with the highest levels

Abbreviations: LAL, lysosomal acid lipase; hLAL, human LAL; mLAL, mouse LAL; CESD, cholesteryl ester storage disease; WD, Wolman disease; hGAPD, human glyceraldehyde-3-phosphate dehydrogenase; TBS, Tris-buffered saline; IPTG, isopropyl-β-D-thiogalactosidase.

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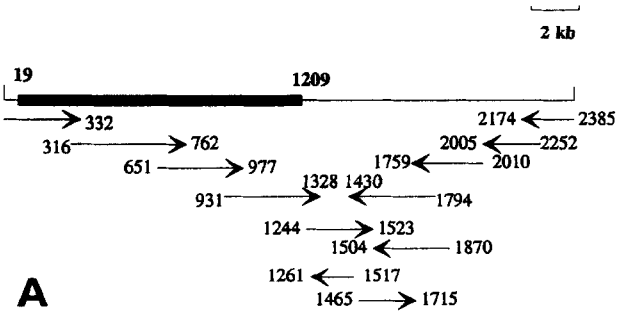

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mLAL   1 [CGTGCCCACTACCGGAAGATGCAACTCCAGGGCCTGGTGTTCGTTTTCACCATTGGGATC   60
hLAL  29 GACAGCTCCAGAATGAAAATGCGGTTCTTGGGGTTGGTGGTCTGTTTGGTTCTCTGGCCC   88

mLAL     CTGCTTTCTCGGGTGCCCACGGGAACTGTATCAGCTGTGGATCCTGAAGTCAACATGAAT   120
hLAL     CTGCATTCTGAGGGGTCTGGAGGGAAACTGACAGCTGTGGATCCTGAAACAAACATGAAT   148

mLAL     GTGACCGAGATAATCATGCGCTGGGGATATCCTGGTGAGGAACACTCGGTCCTGACAGGA   180
hLAL     GTGAGTGAAATTATCTCTTACTGGGGATTCCCTAGTGAGGAATACCTAGTTGAGACAGAA   208

mLAL     GATGGCTATATTCTGAGTATTCACCGAATCCCTCGTGGGTGGAAGAACCATTTTGGCAAA   240
hLAL     GATGGATATATTCTGTGCCTTAACCGAATTCCTCATGGGAGGAAGAACCATTCTGACAAA   268

mLAL     GGTCCCAGACCAGTTGTGTATCTTCAGCACGGCTTGCTGGCAGATTCTAGTAACTGGGTC   300
hLAL     GGTCCCAAACCAGTTGTCTTCCTGCAACATGGCTTGCTGGCAGATTCTAGTAACTGGGTC   328

mLAL     ACAAACATTGACAACAGCAGCTTGGGCTTCCTCCTGGCGGATCGTGGCTTTGATGTGTGG   360
hLAL     ACAAACCTTGCCAACAGCAGCCTGGGCTTCATTCTTGCTGATGCTGGTTTTGACGTGTGG   388

mLAL     ATGGGAAACAGCAGAGGAAACACCTGGTCTCTGAAGCACAAGACTCTTTCAGTTTCTCAG   420
hLAL     ATGGGCAACAGCAGAGGAAATACCTGGTCTCGGAAACATAAGACACTCTCAGTTTCTCAG   448

mLAL     GATGAGTTCTGGGCCTTCAGTTTTGATGAGATGGCAAAATATGACCTACCTGCTTCAATT   480
hLAL     GATGAATTCTGGGCTTTCAGTTATGATGAGATGGCAAAATATGACCTACCAGCTTCCATT   508

mLAL     AACTACATTTTGAATAAAACTGGCCAAGAACAAATCTATTATGTGGGTCATTCTCAAGGC   540
hLAL     AACTTCATTCTGAATAAAACTGGCCAAGAACAAGTGTATTATGTGGGTCATTCTCAAGGC   568

mLAL     TGCACCATAGGTTTCATAGCTTTTTCACAGATGCCTGAGTTGGCAAAGAAGATTAAAATG   600
hLAL     ACCACTATAGGTTTTATAGCATTTTCACAGATCCCTGAGCTGGCTAAAAGGATTAAAATG   628

mLAL     TTTTTAGTCTTGGCTCCCGTGTTGTCTCTCAATTTTGCTTCAGGCCCGCTACTTCAATTG   660
hLAL     TTTTTTGCCCTGGGTCCTGTGGCTTCCGTCGCCTTCTGTACTAGCCCTATGGCCAAATTA   688

mLAL     GGACGCTTGCCAGACCCTCTTCTCAAGGACATGTTTGGACAGAAGCAATTTCTCCCTCAG   720
hLAL     GGACGATTACCAGATCATCTCATTAAGGACTTATTTGGAGACAAAGAATTTCTTCCCCAG   748

mLAL     AGTGCGATGCTGAAGTGGCTGAGCATTCACGTTTGCACCCACGTCATCATGAAGGAGCTT   780
hLAL     AGTGCGTTTTTGAAGTGGCTGGGTACCCACGTTTGCACTCATGTCATACTGAAGGAGCTC   808

mLAL     TGCGCAAACGTCTTCTTCCTGCTATGTGGCTTCAATGAGAAGAATTTAAATATGT]CTAGA   840
hLAL     TGTGGAAATCTCTGTTTTCTTCTGTGTGGATTTAATGAGAGAAATTTAAATATGT CTAGA   868

mLAL     GTGGATGTGTATACAACACACTGCCCTGC-GGAACTTCTCGTGCAAAACATGTTACACTG   899
hLAL     GTGGATGTATATACAACACATTCTCCTGCTGGAACTTCT-GTGCAAAACATGTTACACTG   927

mLAL     GGGTCAGGTTTTTAAATACCGTAAGCTTCAAGCTTTTGACTGGGGAAGCAGTGAAAAGAA   959
hLAL     GAGCCAGGCTGTTAAATTCCAAAAGTTTCAAGCCTTTGACTGGGGAAGCAGTGCCAAGAA   987
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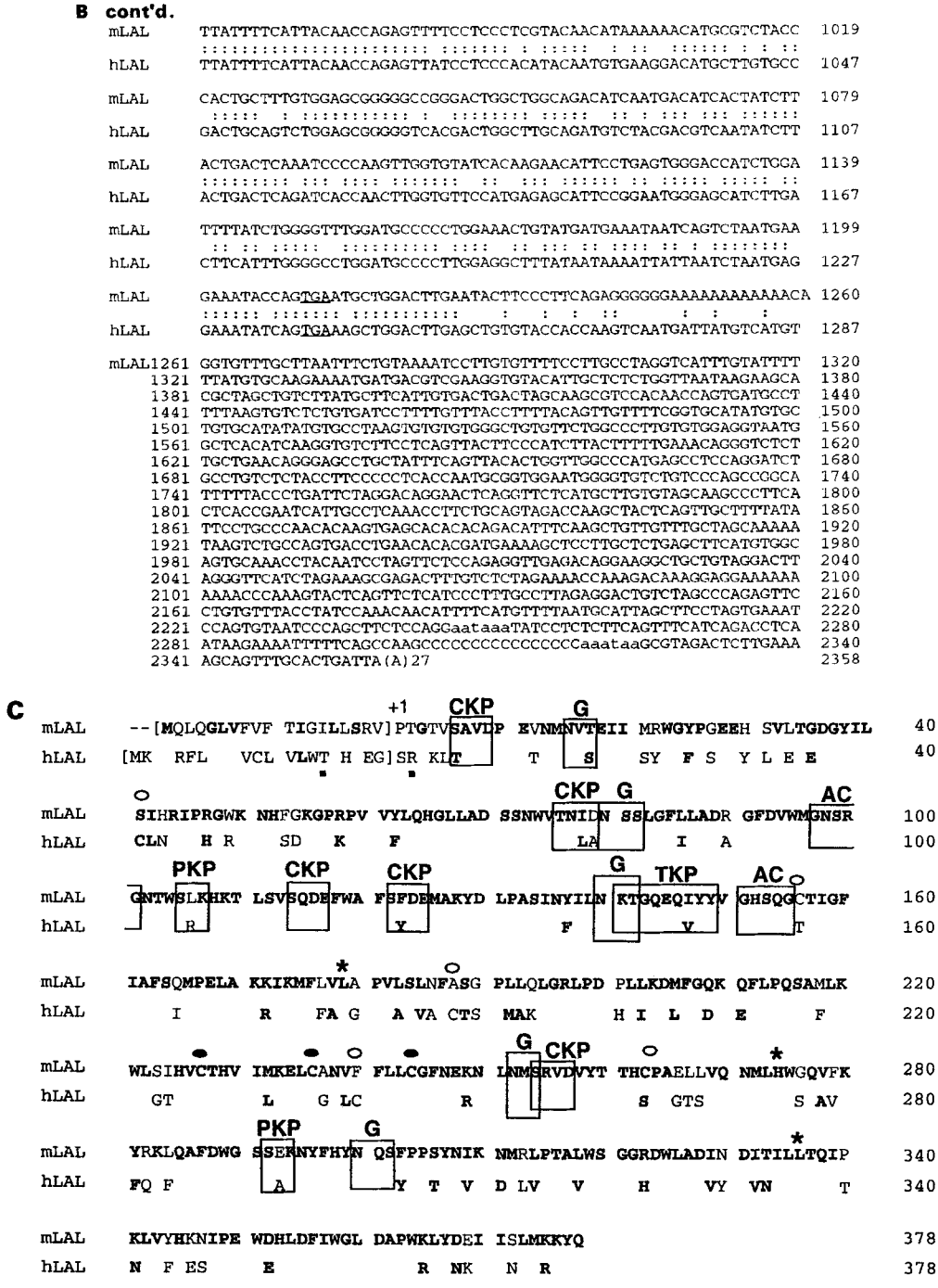


Fig. 1. Sequencing strategy and comparison of mLAL and hLAL. A) Sequencing strategy for the mLAL cDNA. Arrows indicate the direction of sequencing reactions. Numbers indicate the resulting base sequences. The bold line indicates the coding region of the mLAL. B) cDNA sequence comparing of mLAL (top) and hLAL (bottom). For hLAL only the coding region is shown. The initiation codon (ATG) and stop codon (TGA) are underlined. The polyadenylation signals are indicated by lower case letters and the exact boundaries of riboprobes are indicated by []. C) Amino acid sequence homology of mLAL (top) and hLAL (bottom). For hLAL, only the amino acid differences from mLAL are shown. Two amino acid polymorphisms identified here in hLAL are shown at position -6 and +2 and marked (■). The putative signal sequence is shown in []. An alternative signal sequence and processed form of hLAL begins at amino acid +6 and +35 (4). Identical or conserved amino acids are in bold according to the grouping (K, R, H), (D, E, N, Q), (A, I, L, V, M), (F, W, Y) (S, T) (C), (G) and (P). Amino acid residues are numbered on the right with the first residue after the putative signal peptide cleavage point as +1. Putative structural motifs are boxed (G, glycosylation consensus sequence; AC, active center; PKP, protein kinase C phosphorylation site; CKP, Casein kinase II phosphorylation site; TYP, tyrosine kinase phosphorylation site). Three point mutations in CESD are marked (*); conserved and nonconserved cysteines are marked as (●) and (○) respectively.

in whole organ extracts from lung, kidney, adrenal gland, and mammary gland (4). The temporal and spatial expression of LAL mRNA has not been evaluated.

To gain insight into the tissue specific pathology of WD and CESD and into LAL's role in pathogenesis, we studied the tissue and cellular expression of LAL in the mouse. The mouse LAL (mLAL) cDNA was cloned and shown to have high homology with human LAL (hLAL) in the coding region. By in situ hybridization and immunofluorescence, LAL mRNA and protein were shown to have tissue and cellular specific expression.

METHODS AND MATERIALS

Cloning of hLAL and mLAL cDNAs

A mouse liver cDNA library in λ-ZAP was from Stratagene. Screening was followed by standard protocols (12) using PCR generated probes from the coding region of our hLAL cDNA. The hLAL cDNA was cloned by screening of the human liver cDNA library using PCR-generated probe according to the published cDNA sequence (6). Double-stranded DNA sequencing was with Sequenase (United States Biochemicals Corporation, Cleveland, OH). For riboprobes an 835 nt. fragment containing 75% of the coding mLAL region (cDNA 1-835) (Fig. 1B), was subcloned by digestion of Xba I and religation in pBluescript (Stratagene, La Jolla, CA). Sense and antisense riboprobes were synthesized from the T_7 and T_3 promoters, respectively, using [^{35}S]-UTP (800 Ci/mmol, Amersham, Arlington Heights, IL) and linearized template DNA (1 μg; Xba I for sense and Hind III for antisense). Labeling was with a RNA transcription kit (Stratagene, La Jolla, CA). Unincorporated nucleotides were removed with a Quick Spin column (Boehringer Mannheim Corporation, Indianapolis, IN). The specific activity of the probes was 10^8 cpm/μg.

In situ mRNA hybridization

Tissues for in situ hybridization were from C57BL6 or C57BL10 adult mice (Harlan Sprague-Dawley Inc.) and prepared as described (13). Briefly, tissues are fixed with 4% paraformaldehyde in 10 mM phosphate-buffered saline overnight, cryoprotected in 30% sucrose for overnight, embedded in ornithine carbamoyltransferase compound (Miles, Inc., Charlotte, NC), and snap frozen in liquid nitrogen. Embedded tissue blocks were stored at -80°C until use. The frozen sections (7 μm) on silane-coated slides were air-dried and postfixed for 1 h. The tissue sections then were treated with 20 μg/ml proteinase K and acetylated. The treated tissues were prehybridized at 42°C for 15 min in 50% formamide hybridization mixture (2 × NaCl/Cit [NaCl/sodium citrate (SSC); 0.15 M/0.015 M], 1 × Denhardt's, 10% dextran sulfate, and 0.75 mg/ml of carrier DNA and tRNA). They were then hybridized in the hybridization mixture at 42°C with sense or antisense probes and sealed under coverslips overnight. After hybridization, the sections were washed three times (10 min each) in 1 × SSC and 1 mM of DTT at 50°C, then treated with RNase A (50 μg/ml) and RNase T1 (500 U/ml) at 37°C for 30 min. These treatments were followed by a series of high stringency washes: at 50°C for 30 min in 50% formamide and 2 × SSC twice, in 1 × SSC once, in 0.5 × SSC once, and then at 55°C in 0.1 × SSC. All wash solutions contain 1 mM DTT. After dehydration by graded ethanol rinses, the slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY, 1 g/ml in 0.5 M ammonium acetate), exposed for 1 to 2 weeks at 4°C, and developed in Kodak D19 developer for 5 min, rinsed in

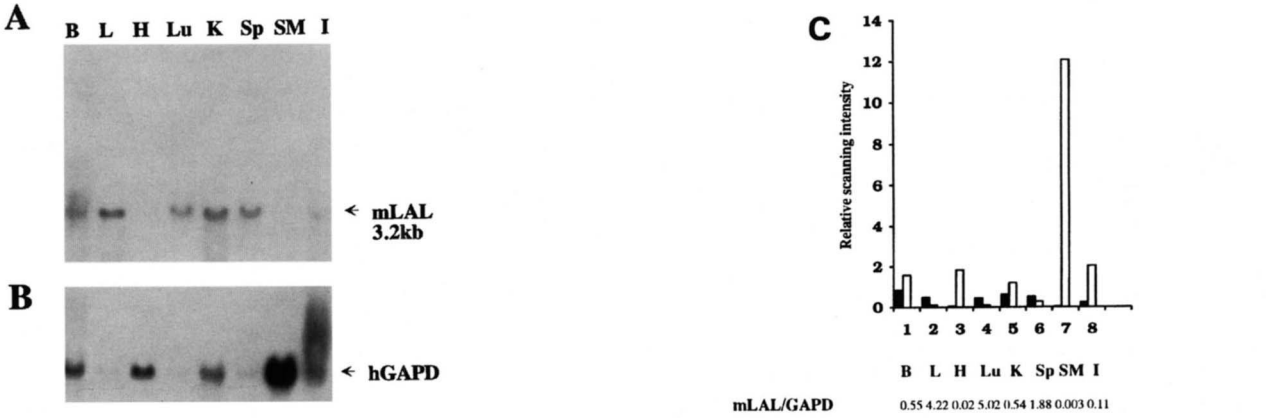


Fig. 2. Northern blot analysis of mLAL mRNA in various mouse tissues. A) A nylon membrane contain 20 μg total RNA per lane was hybridized with ^{32}P-labeled mLAL 2.3-kb cDNA as described in Methods and Materials. Autoradiograph was at -80°C for 4 days with an intensifying screen. Size was determined from an RNA ladder. B) As a relative control, the membrane was stripped off and rehybridized with ^{32}P-labeled Hind III/Xba I (548 bp) fragment of human GAPD cDNA (20). C) Transmission densitometry of signals from mLAL and GAPD hybridization. The black bars represent the intensity of mLAL signal and clear bars represent the intensity of GAPD signals. The ratios of mLAL and GAPD signals are indicated at the bottom. B: brain; L: liver; H: heart; Lu: lung; K: kidney; Sp: spleen; SM: smooth muscle and I: intestine.

water for 1 min, and fixed in Kodak fixer for 5 min. All slides were counterstained with hematoxylin and eosin for light microscopic studies.

Northern analysis

Nylon membranes containing 20 μg of total RNA from each of the various mouse tissues (BIOS Laboratories) were hybridized to the ^{32}P-labeled 2.3 kb mLAL cDNA. A high specific activity probe (4.5×10^9 cpm/μg) was prepared with random primers (*redi*primer kit, Amersham Corp., Arlington Heights, IL). After hybridization overnight at 42°C in 5 × SSC, 5 × Denhardt's, 2% SDS, and 100 μg/ml denatured salmon sperm DNA, blots were washed at room temperature in 0.25 × SSC, 0.2% SDS for 20 min and at 50°C for 20 min. Filters were exposed to Fuji X-ray film for 4 days with an intensifying screen. The probe was strip-washed in 0.1 × SSC/0.2% SDS at 95°C for 3 min and rehybridized to human glyceraldehyde-3-phosphate dehydrogenase (hGAPD) cDNA probe under the same conditions for a reference standard.

Expression of hLAL in *E. coli* and antibody production

The bacterial His • Tag fusion protein expression vector pET21-a (+) (22) (Novagen, Madison, WI) was used for overexpression of the hLAL cDNA in *E. coli*. The entire coding region of hLAL was cloned into the expression vector in-frame with the His • Tag. The upstream primer contains a Sal I site (5′-TATCTCGAGGATCTGGAGGGGAAACTGACAGCT-3′) and the downstream primer contains a Hind III site (5′-CGGAAGCTTCTGATATTTCCTCATTAGATT-3′) to generate PCR products of the LAL coding region without the putative leader peptide. The PCR product was digested with Sal I and Hind III followed by gel purification of DNA fragment, ligation, and transformation. The entire coding region for fusion protein hLAL was sequenced to ensure no mutations were introduced by PCR. The recombinant hLAL was accumulated as inclusion bodies after isopropyl-β-D-thiogalactoside (IPTG) (1 mM) induction for 4 h in BL21 (DE3) cells (Novagen). The inclusion bodies were solubilized in 7 M urea and purified on a nickel-chelating resin column (Novagen). The eluate from this affinity column is >95% pure with M_r ~39,000 by silver staining SDS-PAGE (data not shown). This bacterial-generated hLAL was inactive but the purified antigen was used to raise polyclonal antibodies in New Zealand white rabbits (2.3 kg) as follows. Primary immunization was with intradermal (10 areas) injections of 300 μg (total) of hLAL emulsified in adjuvant. Boosts were done every 2 to 3 weeks with 60 μg of antigen injected intramuscularly. Serum was collected 10 to 15 days after each boost. The antibody titers from each bleeding were done by enzyme-linked immunosorbent assay (ELISA) using affinity column purified recombinant hLAL protein as an antigen and horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum (Chemicon, Temecula, CA) as a secondary antibody. The anti-serum used in the studies had a titer of $1{:}10^6$ using 2 ng of purified protein. The specificity of the antibody was evaluated by Western blot using recombinant LAL or lysates from normal human fibroblasts. Specific hLAL bands at M_r ~41,000, and ~46,000 were detected. The antibody also detected specific bands of mLAL in Western analyses of NIH 3T3 and mouse embryo fibroblast cells.

Immunofluorescence analyses

Immunofluorescence staining was carried out as described (14). Briefly, the tissue sections (5–7 μm) were treated with 0.2% Triton X-100 in Tris-buffered saline (TBS) blocked with 3% goat serum in TBS at 37°C for 30 min, incubated with rabbit preimmune serum or anti-serum against hLAL (1:250 dilution) at 37°C for 1.5 h and fluorescein-conjugated goat antibody to rabbit IgG F(AB′)^2 (1:20 dilution) (Organon Teknika, Durham, NC) at 37°C for 1 h. The slides were washed in TBS after each antibody incubation and protected from fluorescence quenching by Antifade (Oncor, Gaithersburg, MD).

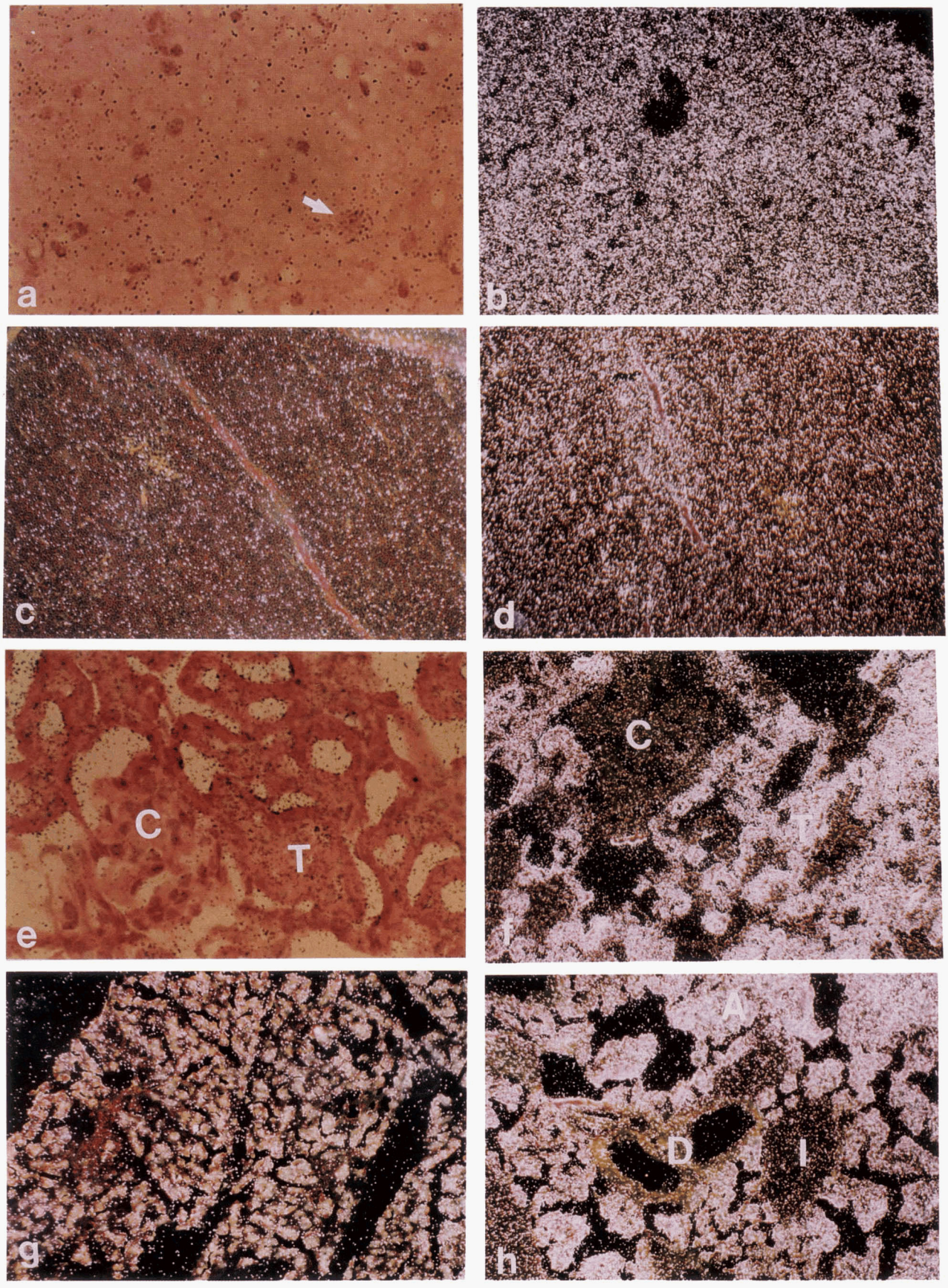
RESULTS

Molecular cloning and characterization of hLAL and mLAL cDNA

The hLAL cDNA was isolated by screening of human liver cDNA library with a PCR-generated probe according to the published hLAL cDNA sequence. The cDNA encoded a protein that was identical to that reported by Anderson and Sando (6) except for two polymorphic variants at amino acid -6 and +2 (15). For mLAL, 20 positive initial plaques were identified and 10 were purified for analyses. Three full-length coding region and three partial cDNA inserts were sequenced using the strategy in **Fig. 1A.** The full length mLAL insertions (2358 bp) had an open reading frame from 19 to 1212 that encodes for 397 amino acids (Figs. 1B and 1C). The nucleotide homology of mLAL and hLAL cDNA sequences is 79.4% in the coding region and divergent in the 3′ untranslated region (48.7%) (Fig. 1B).

The initiating methionine (Met) of mLAL corresponds to the third in-frame Met in the hLAL sequence

[2]The novel nucleotide sequence data of mLAL have been deposited with the EMBL sequence data bank under accession number Z31689.



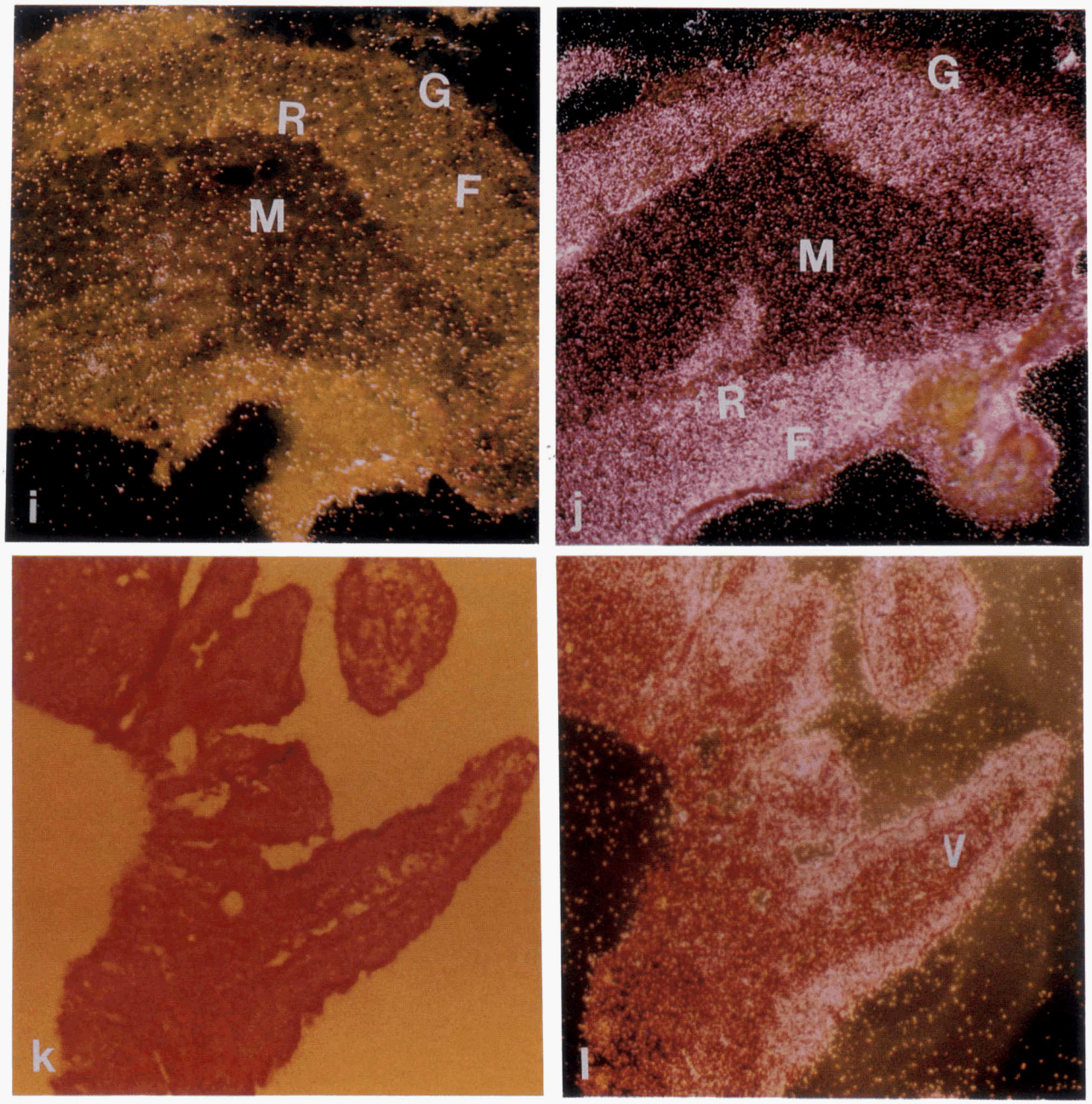


Fig. 3. In situ hybridization analysis of mLAL expression in mouse tissues. Dark field (b, d, f, h, j, l) and bright field (a, e, k) in situ hybridization with the anti-sense mLAL cRNA probe. The darkfield (c, g, i) are sense transcript hybridization. Liver (a, b), LAL mRNA expression signal in hepatocytes (indicated by arrow) and sinusoidal lining cells. Thymus (c, d), intensity signals of LAL mRNA were present in the cell clusters that represent activated macrophages or component of the reticuloendothelial cells and medium level of signal present uniformly in cortex and medulla. Kidney (e, f), strong signals were present in proximal renal tubules (T), but not in renal corpuscles (C). Pancreas (g, h), strong signals were present only in acinar cells (A) and background level signal in islets (I) and duct cells (D). Adrenal gland (i, j), high level signal was present in zona fascilculata (F) and zona reticularis (R), and basal signal in zona glomerulosa (G) and medulla (M). Small intestine villi (k, l), high level mLAL mRNA signal present only in epithelial cells lining the villi (V) (haematoxylin/eosin counterstain; original magnifications 100X).

(Fig. 1B). Therefore, two 5′ amino acid residues in hLAL are missing in mLAL. Except for these two 5′ residues, the amino acid sequences of mLAL and hLAL could be aligned without deletions or insertions. The amino acid sequences of mLAL and our hLAL (16) showed 75% identity and 95% similarity (Fig. 1C). All the positions and/or sequences of predicted motifs were conserved between hLAL and mLAL. These included two putative active centers (G-X-S-X-G) (AC), five potential N-glycosylation consensus sequences (G), two protein kinase C

phosphorylation sites (PKP), one tyrosine kinase phosphorylation site (TKP), and four casein kinase phosphorylation sites (CKP). Three of the five cysteines are conserved suggesting the presence of a single disulfide bond in LAL. Three point mutations, L179P, L336P and H274Y, on hLAL have been reported from patients with CESD (9, 16–19). These were at conserved positions in mLAL and hLAL.

Expression of LAL mRNA in mouse tissues

By Northern analysis hLAL mRNA is expressed in most tissues with high levels in human brain, lung, mammary gland, kidney, and adrenal gland, intermediate levels in placenta and HeLa cells and low levels in heart, liver, and skeletal muscle (4). We confirmed these findings (data not shown). The LAL expression levels in mouse and human tissues were compared by Northern analysis of the mLAL mRNA in various whole mouse tissues (**Fig. 2A**). A specific hybridization signal of 3.2 kb of mLAL mRNA was detected in all tissues. However, expression of mLAL in the heart and smooth muscle could only be detected after long exposures. The steady state level of mRNA is relatively high in brain, lung, kidney, spleen, and liver, intermediate in duodenum, and low in heart and skeletal muscle. The relative quantity (Fig. 2C) and intact quality (Fig. 2B) of total RNAs were shown by hybridization to the hGAPD probe (20).

Whole tissue analyses of mRNA may obscure differential expression within its heterogeneous cell populations. To evaluate potential cell specific expression, in situ hybridization was done on adult murine tissues. In liver (**Figs. 3a, b**), thymus (Figs. 3c, d), and spleen (not shown), LAL mRNA was strongly detected in all cell types. In liver, hepatocytes and sinusoidal lining cells showed intense positivity (Fig. 3b). Lymphocytes and macrophages of spleen also were intensely positive. In thymus, the expression was generally uniform throughout the cortex and medulla although occasional isolated cells or cell clusters show increased signal intensity. These cells may represent activated macrophages or other components of the reticuloendothelial system (Fig. 3d).

In the kidney, high levels of mLAL mRNA expression were detected predominantly in the proximal renal tubules, but not in renal corpuscles, medullary rays of cortex or medulla (Figs. 3e and 3f). In the pancreas, the mLAL mRNA was highly expressed only in acinar cells (Fig. 3h) compared to the background levels in the islets and duct cells. The low level nonspecific sense RNA hybridization could not be completely eliminated in this tissue (Fig. 3g). In the adrenal gland mLAL mRNA expression was high in the zona fasciculata and reticularis (Fig. 3j). Only background signal levels were detected in the subcapsular zona glomerulosa of the adrenal cortex as well as in the adrenal medulla (Figs. 3i and 3j).

In small intestine, the LAL mRNA signal was specifically in villous columnar enterocytes (absorptive cells) and goblet cells (Figs. 3k and 3l). This high expression was detected only in the epithelial cells lining the villi and not in the glands at the base of the villi or in the lamina propria, muscularis mucosae, submucosa and muscularis externa. The signal of LAL mRNA was more evident in the tissue section from jejunum than that from duodenum (data not shown). In the uterus the mLAL mRNA signal was high only in macrophages

TABLE 1. Summary of in situ mRNA hybridization of mLAL

Tissues	mLAL mRNA Specific Expression	Expression Level
Liver	hepatocytes and sinusoidal linin cells	+++
Spleen		+
Heart		+
Kidney	cortical tubules	++++
Adrenal gland	zona fasciculata and reticularis of cortex	++++
Pancreas	acinar cells	++++
Ovary		+
Thymus	lymphocytes and reticular cells	+
Small intestine	epithlial cells in villi	+++
Salivary gland		+
Tongue		+
Seminal vesicles		+
Uterus	macrophage	+++
Testis		+

+, background level signal; ++, low level signal but higher than background; +++, high level signal; ++++, very strong signal.

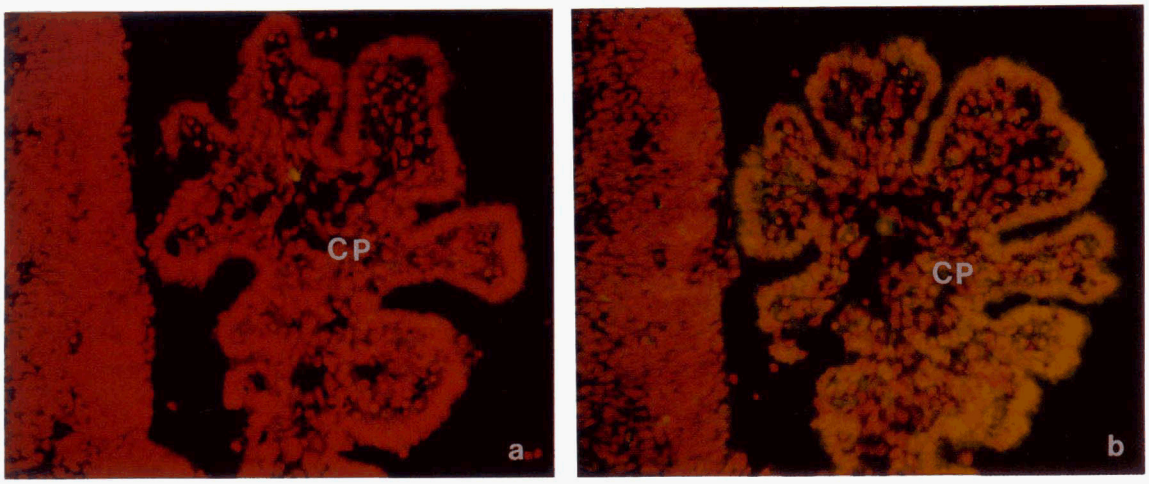


Fig. 4. Cellular specific expression of mLAL in choroid plexus in developing mouse embryo by day 12. Immunofluorescence staining of mLAL in day 12 embryonic choroid plexus with preimmune serum (a) and with immune serum (b). The expression is specifically detected in the epithelial cells.

particularly when associated with hemosiderin deposits. Other cells had background level of the signal.

A survey of a variety of other tissues showed only basal level of expression. This included a variety of cell types within the lung, heart, vascular endothelial cells, salivary gland, tongue, seminal vesicles, testis, and ovary (**Table 1**).

The expression of mLAL mRNA and protein was also evaluated in the developing mouse embryo from day 11 to day 16. The expression signal was detected in the embryonic liver and lung by day 14 (data not shown), and in the kidney and small intestine by day 16 (data not shown). Interestingly, in the day 12 embryo, expression was detected only in choroid plexus epithelium of the embryonic brain (**Fig. 4**). No other tissue has detectable signal beyond the background level at this time of development.

Expression of LAL protein in mouse tissues

The antibody raised to the bacterial expressed hLAL cross-reacted with mLAL protein in Western blots (**Fig. 5**). In human fibroblasts, two molecular species of LAL were detected with M_r ~41,000 and 46,000. N-glycanase treatment produced a single M_r ~41,000 form from this source (data not shown). In 3T3 cells only one form of hLAL was detected with M_r ~41,000. To evaluate the cellular distribution of mLAL expression, immunofluorescence studies were conducted in adult mouse tissue. Expression of mLAL protein was very similar to that of mRNA detected by in situ hybridization, i.e., liver, thymus, and spleen had high level expression in parenchymal and nonparanchymal cells. In the kidney cortex, adrenal cortex, and villi of small intestine, the protein levels were highest in the same cellular distribution as with mRNA expression (**Fig. 6**), e.g., the zona fasciculata and zona reticularis of the adrenal cortex and renal tubular epithelial cells of the cortex.

DISCUSSION

To explore the enzymatic basis for the pathologic manifestations in WD and CESD, the cDNAs for mLAL and hLAL were cloned and shown to encode highly homologous proteins. Using the mLAL cDNA, tissue and cellular specific transcription levels were detected by in situ hybridization for mRNA. The highest levels were found in the general distribution of the most severe organ involvement in WD. High level expression was found in hepatic sinusoidal lining cells including endothelial cells, but the vascular endothelium in other tissues had relatively low mRNA level. In addition, several reports (9, 16–19, 21) have identified mutations in the hLAL locus in patients with WD or CESD. These results implicate the deficiency of LAL as a major predisposing factor for these two diseases. However, the differential tissue expression of mLAL indicates that its level of expression and/or other factors are important in determining the degree and distribution of pathologic involvement in LAL deficiency diseases.

LAL cDNAs encode highly homologous proteins in mouse and human. The predicted mature LAL proteins from the two species had 75% amino acid identity and about 95% similarity. LAL cDNA from rat has been

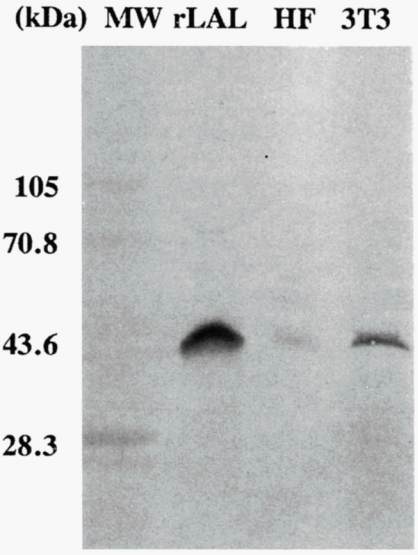


Fig. 5. Immunoblot of LAL in human and mouse cell lines. The specific interaction of hLAL antibody with recombinant hLAL and natural human and mouse LAL. Recombinant hLAL expressed in *E. coli* (rLAL), whole cell extracts from human fibroblast (HF), and NIH/3T3 cell (3T3) were loaded on Phast gel (12.5% SDS PAGE) for the Western blot. The membrane was incubated with immune serum (1:4000 dilution) and goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad). MW, standards lane.

reported recently and has 81.7% and 73.9% predicted amino acid identity to mLAL and hLAL, respectively. All three LALs share 95% similarity (22). This degree of conservation is similar to that observed for several other lysosomal proteins including acid β-glucosidase (23, 24), α-galactosidase A (25), N-acetyl-galactosaminidase (26), sphingomyelinase (27), hexosaminidase A and B (28, 29), and α-glucosidase (30). The rat LAL cDNA encodes the same initiation Met as mLAL. It is possible that the translation initiation of hLAL is at the third in-frame Met, as the Kozak sequences preceeding the first and third Met in hLAL cDNA are weak in both cases in terms of consensus (Fig. 1B).

With LAL, several putative functional domains also are conserved in either placement and/or sequence. There are six glycosylation consensus sequences in hLAL. Four out of six (third to sixth) were conserved in placement and sequence between mouse and human LAL. The first site beginning at amino acid 15 was the exception in which a conservative substitution of a serine for threonine was present in hLAL compared to mLAL. The second glycosylation site at amino acid 51 of hLAL is not conserved in mLAL, indicating that the glycosylation of this site may not be occupied or important for catalytic function. Human LAL has six cystine residues, three of which are conserved in mLAL. Cysteine 41 (C41) in the hLAL and C263 in mLAL were replaced by serine in the LAL of the other species. As serine is an isosteric substitution for cysteine, these cysteines in mLAL and hLAL probably do not participate in catalysis or disulfide formation. These results also indicate that two of the other three conserved cysteines in mLAL could participate in a single intrachain disulfide bond. In this regard, C156 in mLAL has a nonconservative substitution, threonine, in the human sequence, indicating that it is unlikely that cysteine in the mouse will participate in disulfide bond formation. As with other lipases (31, 32), putative active center sequence GXSXG sequences are conserved in mLAL and hLAL. These consensus sequences cover residues 97 to 101 and 151 to 155. The presence of these two active centers have led to the suggestion that they may participate in the specificity for either cholesteryl

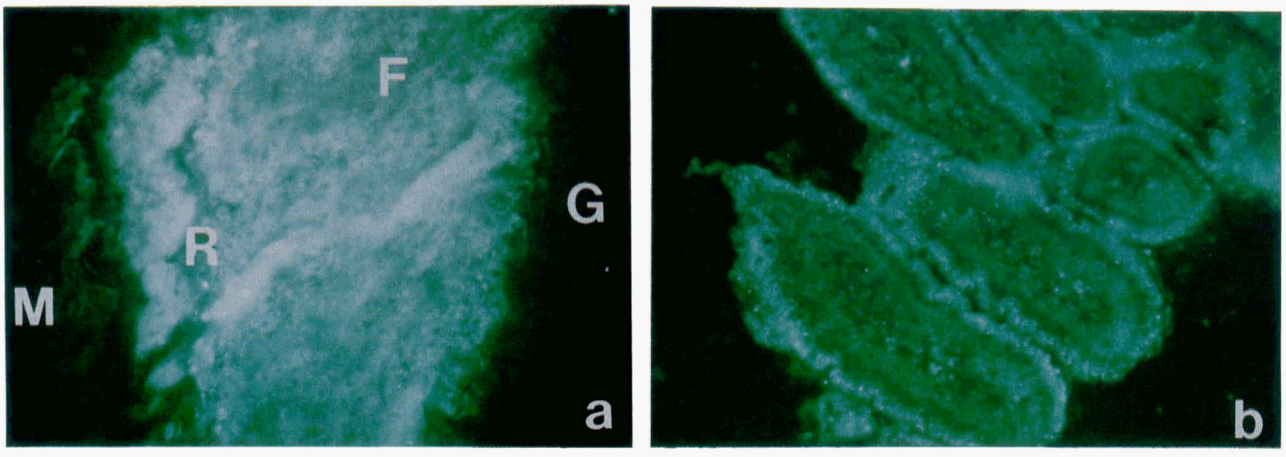


Fig. 6. Tissue specific distribution of mLAL protein. Immunofluorescence staining of mLAL in adult mouse tissue; (a) adrenal gland, very intensive fluorescence signal present in zona fasciculata (F) and zona reticularis (R), but not in zona glomerulosa (G) and medulla (M); (b) intestine, a cross-section of small intestine villi, the signals present in the epithelial cells lining the villi. The sections are analyzed on a Microphot-FXA Nikon fluorescence microscope (original magnification 200×).

esters or triglycerides (33–35). Our recent heterologous expression of mutagenized hLAL has indicated that S153 is the most likely nucleophile for hLAL and, by inference, for mLAL (5). Several phosphorylation domains are conserved in placement, as well as sequence with conservative substitutions indicating that these could be present and functional in both enzymes. Finally, some mutations that have been identified in WD and CESD at amino acids 179 and 336 that are at a conserved leucine in the mouse sequence and heterologous expression of the mutagenized proteins containing Pro179 and Pro336 substitutions at the respective residues result in highly malfunctional hLALs (5).

As the majority of lysosomal hydrolases are thought to participate in housekeeping functions within various cells, the specific high level cellular expression of mLAL in various tissues including adrenal, liver, and gut, was unexpected. Only the prosaposin locus that produces a multi-functional protein including a precursor, which is neurotrophic (36), and four homologous saposin A, B, C, and D, amino acid activator proteins (37, 38) has been shown to be highly spatially and temporally regulated (39). mLAL shows higher levels of expression in those tissues that are involved in WD. In particular, the high level expression throughout the liver, in intestinal villi, choroid plexus, particularly of the jejunum, and of the zona fasciculata of the adrenal gland are primarily involved in WD. The liver pathology includes the accumulation of massive amounts of triglyceride and cholesteryl esters within hepatocytes and Kupffer cells. This is consistent with our finding of generalized high level expression in most cells and regions of the liver including parenchymal cells and sinusoidal lining cells. Interestingly, the villi of the small intestine have massive accumulation of triglycerides in WD; mLAL was expressed at high levels in these regions. In WD patients infiltration of foam cells into the lamina propria leads to distorted architecture of the villi of the small intestine, and is associated with malabsorption (40, 41). Also, the high level of expression of mLAL mRNA was found in the epithelial cells and goblet cells, but not in the glandular cells at the base of the villi in the lamina propria or in the muscularis components of the small intestine. The lipid storage cells in WD are found mainly in lamina propria of the mucosa (40). This finding suggests that in WD and CESD patients the lipid absorbed by epithelium is transported to the inner cell layer of the lamina propria and the region of lacteals. These lipids then accumulate as foam cells beneath the epithelial layer. This pattern is consistent with maturation dependent expression as the villus cells differentiate along the crypt to villus tip axis. Similarly, the high level mLAL mRNA expression in kidney was detected in the proximal renal tubules, not in renal corpuscles (also called glomeruli), although the lipid droplets storage cells were found in mesangial cells of the glomeruli and tubules appear normal (42). These results strongly support that LAL is a secreted enzyme that can be transported to other cells via a mannose-6-phosphate receptor and targeted to the lysosome (43, 44), and can hydrolyze the cholesteryl esters and triglycerides in adjacent cells that only express background level of enzyme.

The high level expression of mLAL in the zona fasciculata of the adrenal cortex correlates well with the massive lipid deposits of the adrenal glands and calcification in WD. Neutral fats, fatty acids, cholesterol, and phospholipids in the adrenal gland are metabolized in the zona fasciculata (45). The high level of mLAL mRNA and protein suggests a potential role of mLAL in the regulation of cholesterol metabolism in this organ and, potentially, in glycocorticoid biosynthesis. Distinctly lower expression in the zona glomerulosa, which is responsible for mineralocorticoid production, suggests a lesser role for this enzyme in the supply of cholesterol for the formation of these latter compounds. Curiously, patients with CESD, who preferentially accumulate cholesteryl ester, show no major pathology of the adrenal glands (7). Consequently, other factors must be influencing the pathophysiology of adrenal gland involvement rather than a straightforward accumulation of substrates. This is also true in acinar cells of the pancreas which show high level of expression of mLAL mRNA and yet pathologic involvement of this organ has not been reported in either CESD or WD. These findings implicate factors in addition to mRNA and residual enzyme activity in the pathogenesis of CESD and WD. An additional example is the lack of high level expression of mLAL mRNA or protein in vascular endothelial cells, but the presence of atheromas in CESD patients. This is also evident from the mutations that have been reported in such patients; in particular, there are two patients who have deletion mutations of both hLAL alleles and yet these patients have CESD (46). In comparison, the reported mutation in a Wolman disease patient is a point mutation, L179P, with a heteroallele of a single base insertion in exon 6 that produces a frame shift and premature stop codon at 189 (9).

Previously, the enzymatic bases of the phenotypes of CESD and WD were interpreted by the threshold hypothesis which postulates that small differences in the level of residual activity lead to the different clinical manifestations. Thus, a low level of esterase activity of LAL in CESD would lead to the manifestations of that disease in the tissues with high level exposure and flux of cholesteryl esters. The complete block of cholesteryl esterase and triglyceridase would produce WD. Although the threshold hypothesis may provide the broad

outline for the different phenotypes, an expansion of the hypothesis is needed incorporate tissue specific substrate(s) flux and enzyme capacity to account for tissue and cellular specific disease effects.

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