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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 8
ACT: 519 TRASLADOINFORME	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
LUZ CLEMENCIA DE PÁEZ

Asunto: Radicación: 15-24867- -1
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 11955
Folios: 8

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2011/051096				
Fecha de Presentación Internacional	09/09/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	15-024867-00000-0000	06/Febrero/2015
Reivindicaciones:	15-024867-00000-0000	06/Febrero/2015
Dibujos:	15-024867-00000-0000	06/Febrero/2015
Prioridades:	US 61/403,011	09/Septiembre/2010
	US 61/456,014	29/Octubre/2010
	US 61/432,372	13/Enero/2011

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales;

por tanto, se estudiarán la totalidad de las reivindicaciones (4) que contiene la presente solicitud.

Título de la solicitud: “Uso de ácido lipasa liposomal para tratar la deficiencia de ácido lipasa liposomal en pacientes”.

El problema planteado en esta solicitud consiste en la necesidad de desarrollar tratamientos efectivos y con baja frecuencia de administración para tratar deficiencias de ácido lipasa liposomal (LAL).

Para resolver este problema técnico la solicitud en estudio proporciona métodos de tratamiento de enfermedades asociadas a deficiencias de LAL mediante la administración de una cantidad efectiva de LAL humana recombinante que tiene una estructura de glicano-N-ligado que comprende por lo menos una manosa y/o manosa-6-fosfato.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 31/135, A61K 31/366, A61K 31/397 y A61K 38/046.

4. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque no define de forma inequívoca los oligosacáridos que se unen a los residuos Asn, se sugiere a la solicitante incorporar el nombre IUPAC o la estructura condensada de los N-oligosacáridos que permitan caracterizar la glicoproteína.

La reivindicación 3 no es clara porque incluye la expresión “aproximadamente”, se sugiere a la solicitante caracterizar la proteína por las características técnicas esenciales.

La reivindicación 3 no es clara porque caracteriza la proteína por los resultados a alcanzar en ensayos de actividad enzimática. Se sugiere a la Solicitante redactar las reivindicaciones incluyendo las características técnicas esenciales de la proteína, porque de lo contrario la reivindicación incluye todas las alternativas presentes o futuras que permitan alcanzar el resultado mencionado.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: septiembre 9 de 2010, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	J Lipid Res. 2008 Aug; 49(8): 1646–1657.	Du, H., et al. Wolman disease/cholesteryl ester storage disease: efficacy of plant-produced human lysosomal acid lipase in mice	Agosto/2008
D2	Eur. J. Biochem. 219:905-914	Ameis D., Merkel M., Eckerskorn C., Greten H. "Purification, characterization and molecular cloning of human hepatic lysosomal acid lipase"	1994
D3	J. Biol. Chem. 266:22479-22484(1991)	Anderson R.A., Sando G.N. "Cloning and expression of cDNA encoding human lysosomal acid lipase/cholesteryl ester hydrolase. Similarities to gastric and lingual lipases"	25/Noviembre/1991

D4	Am. J. Hum. Genet. 77:1061–1074, 2005	Du, H., et al. “The Role of Mannosylated Enzyme and the Mannose Receptor in Enzyme Replacement Therapy”.	27/Octubre/2005
D5	US2007264249	Gene-based lipid hydrolysis therapy for atherosclerosis and related diseases	15/Noviembre/2007

El documento D1¹ divulga una enzima LAL recombinante expresada en células de *Nicotiana benthamiana* (G-hLAL) que fue clonada mediante un vector TMV. G-hLAL presenta un peso molecular (MW) aprox. 55 KDa (Fig. 1), patrones de glicosilación de Manosa-N-acetilglucosamina heterogéneos con MW entre 1000 y 1700 Da (Tabla 1) y actividad específica entre 281 y 364 U/mg proteína (Tabla 2).

El documento D2² divulga la purificación de la enzima LAL humana a partir de hígado, que presenta una estructura de 372 aminoácidos (AA) más un péptido señal de 27 AA, con un MW de 56KDa y un patrón de glicosilación con oligosacáridos complejos de manosa unidos a los residuos Asn (Resumen).

El documento D3³ enseña una enzima LAL recombinante expresada en células Cos-1 y Ltk- utilizando un vector pcD (HLAL), que presenta una estructura primaria de 398 AA con 6 sitios probables de glicosilación en los residuos Asn (Fig. 4), sustituidos por oligosacáridos complejos de manosa (Pág. 22484) y actividad específica beta-hexosaminidasa de 22 o 28 nmol/min/mg, para la enzima expresada en células Cos-1 y Ltk-, respectivamente (Pág. 22483, Tabla 1).

El documento D4⁴ revela una enzima LAL recombinante expresada en *Pichia pastoris* (phLAL) y en células CHO (chLAL) (Resumen). phLAL mostró un perfil de oligosacáridos complejos desde Man8GlcNAc2 hasta Man15GlcNAc2, mientras que chLAL presento mayoritariamente glicanos Man5GlcNAc2 (Fig. 1). phLAL presentó niveles muy bajos de ácido sialico y las dos enzimas mostraron un alto contenido de manosa-6-fosfato (Tabla 1). Estudios in vivo en ratones *lal*^{-/-} mostraron que la administración de las dos enzimas disminuye el peso del hígado y el colesterol y triglicéridos almacenados a nivel hepático (resumen).

El documento D5⁵ enseña el uso de la glicoproteína LAL humana para el tratamiento y la prevención de aterosclerosis (Resumen), la cual comprende 372 AA y 3 sitios de glicosilación en los residuos Asn15, Asn80 y Asn252 (Pág. 4, Párr. [0034]).

6. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en una lipasa aislada ácida lisosómica humana recombinante que contiene glicanos ligados a N unidos a Asn36, Asn101, Asn161, Asn273 y Asn321, de la SEQ ID NO:1, en donde los N-glicanos comprenden estructuras bi- tri- y tetraantenarias con N-acetilglucosamina, manosa y/o manosa-6-fosfato y donde los glicanos de Asn101, Asn161 y Asn273 contienen manosa-6-fosfato.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2
Una lipasa aislada ácida lisosómica humana	Una enzima lipasa ácida lisosómica humana aislada

1 Disponible en: <http://www.jlrr.org/content/49/8/1646.long>
2 Disponible en: <http://www.ncbi.nlm.nih.gov/pubmed/8112342>
3 Disponible en: <http://www.jbc.org/content/266/33/22479.full.pdf>
4 Disponible en: <http://www.ncbi.nlm.nih.gov/pubmed/16380916>
5 Disponible en: http://worldwide.espacenet.com/publicationDetails/biblio?DB=worldwide.espacenet.com&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20071115&CC=US&NR=2007264249A1&KC=A1

Características esenciales	D2
recombinante que contiene:	de hígado que comprende:
glicanos ligados a N unidos a Asn36, Asn101, Asn161, Asn273 y Asn321, de la SEQ ID NO:1, en donde los N-glicanos comprenden estructuras bi- tri- y tetraantenarias con N-acetilglucosamina, manosa y/o manosa-6-fosfato y donde los glicanos de Asn101, Asn161 y Asn273 contienen manosa-6-fosfato.	una estructura de 372 aminoácidos (AA) más un péptido señal de 27 AA, con un MW de 56KDa y un patrón de glicosilación con oligosacáridos complejos de manosa unidos a los residuos Asn (Resumen).

Las características esenciales de la enzima de la reivindicación 1 habían sido divulgadas previamente en el documento D2, el cual enseña la enzima LAL humana aislada partir de hígado, que presenta una estructura de 372 aminoácidos (AA) más un péptido señal de 27 AA (Pág. 911, Fig. 6) que presenta una identidad del 100% con la SEQ ID NO: 1 de la reivindicación 1, tal como se evidencia en el alineamiento de la figura 1. Además, esta glicoproteína presenta un MW de 56KDa y un patrón de glicosilación con oligosacáridos complejos de manosa unidos a los residuos de Asn (Resumen y Fig. 6).

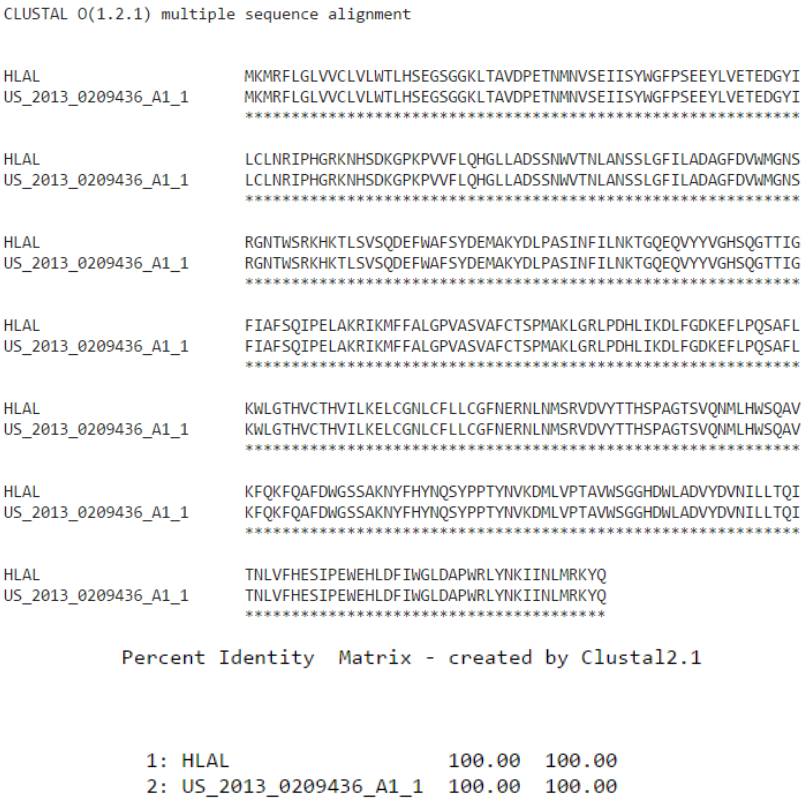


Figura. 1. Alineamiento BLAST entre las secuencias de la enzima LAL humana divulgada en el documento D2 y la secuencia SEQ ID NO: 1 de la presente solicitud.

En consecuencia, la reivindicación 1 no carece de novedad, según lo divulgado en el documento D2.

Las reivindicaciones dependientes 2 a 4 no mencionan alguna característica que haga nuevos a la enzima de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel inventivo (Art 18 D486)

Frente a D3 y D1

La reivindicación 1 consiste en una lipasa aislada ácida lisosómica humana recombinante que contiene glicanos ligados a N unidos a Asn36, Asn101, Asn161, Asn273 y Asn321, de la SEQ ID NO:1, en donde los N-glicanos comprenden estructuras bi- tri- y tetraantenarias con N-acetilglucosamina, manosa y/o manosa-6-fosfato y donde los glicanos de Asn101, Asn161 y Asn273 contienen manosa-6-fosfato.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D3	D1
Una lipasa aislada ácida lisosómica humana recombinante que contiene:	Una enzima lipasa ácida lisosómica que presenta:	Una enzima lipasa ácida lisosómica que presenta:
glicanos ligados a N unidos a Asn36, Asn101, Asn161, Asn273 y Asn321, de la SEQ ID NO:1, en donde los N-glicanos comprenden estructuras bi- tri- y tetraantenarias con N-acetilglucosamina, manosa y/o manosa-6-fosfato y donde los glicanos de Asn101, Asn161 y Asn273 contienen manosa-6-fosfato.	una estructura primaria de 398 AA con 6 sitios probables de glicosilación en los residuos Asn (Fig. 4), sustituidos por oligosacáridos complejos de manosa (Pág. 22484)	un peso molecular (MW) aprox. 55 KDa (Fig. 1), patrones de glicosilación de Manosa-N-acetilglucosamina heterogéneos con MW entre 1000 y 1700 Da (Tabla 1) y actividad específica entre 281 y 364 U/mg proteína (Tabla 2).

El documento D3 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una enzima LAL recombinante, que presenta una estructura primaria de 398 AA con 6 sitios probables de glicosilación en los residuos Asn (Fig. 4) que presenta una identidad del 100% con la SEQ ID NO: 1 de la reivindicación 1, donde los residuos Asn están sustituidos por oligosacáridos complejos de manosa (Pág. 22484).

Por otra parte, el documento D1 divulga una enzima LAL recombinante con MW aprox. 55 KDa (Fig. 1), patrones de glicosilación de Manosa-N-acetilglucosamina heterogéneos con MW entre 1000 y 1700 Da (Tabla 1) y actividad específica entre 281 y 364 U/mg proteína (Tabla 2).

La diferencia entre la invención definida en la reivindicación 1 y la enzima de D3 consiste en que en D3 no divulga las estructuras de los N-oligosacáridos.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la enzima lipasa reclamada en la solicitud porque no presenta una mayor actividad específica, según lo establecido en la descripción.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: como proveer una enzima LAL recombinante alternativa que tiene una estructura definida de un glicano-N-ligado, para obtener una glicoproteína útil en el tratamiento de enfermedades asociadas a deficiencias de LAL.

La diferencia entre la invención definida en la reivindicación 1 y la enzima de D1 consiste en que D1 no divulga la estructura de la LAL humana.

Sin embargo, el documento D3 divulga una enzima LAL recombinante que presenta una estructura primaria de 398 AA con 6 sitios probables de glicosilación en los residuos Asn (Fig. 4), donde los residuos Asn están sustituidos por oligosacáridos complejos de manosa (Pág. 22484) y actividad específica beta-hexosaminidasa de 22 o 28 nmol/min/mg (Pág. 22483, Tabla 1). Por lo tanto, una persona normalmente versada en la materia conocedora de la enzima recombinante divulgada en D3, realizaría la caracterización estructural de los N-oligosacáridos empleando los métodos de análisis de oligosacáridos por HPLC-MALDITOF descritos en el documento D1 para obtener

una enzima LAL recombinante caracterizada por una estructura de glicano-N-ligado, como la de la reivindicación 1.

En consecuencia, la reivindicación 1, carece de nivel inventivo a la luz de las enseñanzas de los documento D3 y D1.

Las reivindicaciones 2 a 4, dependientes de la reivindicación 1, no divulgan características técnicas adicionales que confieran nivel inventivo a la enzima de la reivindicación 1, porque el documento D1 divulga el MW y la actividad específica de la enzima. Por lo tanto, estas reivindicaciones, también carecen de nivel inventivo.

Frente a D4 y D5

La reivindicación 1 consiste en una lipasa aislada ácida lisosómica humana recombinante que contiene glicanos ligados a N unidos a Asn36, Asn101, Asn161, Asn273 y Asn321, de la SEQ ID NO:1, en donde los N-glicanos comprenden estructuras bi- tri- y tetraantenarias con N-acetilglucosamina, manosa y/o manosa-6-fosfato y donde los glicanos de Asn101, Asn161 y Asn273 contienen manosa-6-fosfato.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D4	D5
Una lipasa aislada ácida lisosómica humana recombinante que contiene:	Una enzima lipasa ácida lisosómica recombinante expresada en <i>Pichia pastoris</i> que presenta:	Una enzima lipasa ácida lisosómica humana que presenta una estructura conformada por:
glicanos ligados a N unidos a Asn36, Asn101, Asn161, Asn273 y Asn321, de la SEQ ID NO:1, en donde los N-glicanos comprenden estructuras bi- tri- y tetraantenarias con N-acetilglucosamina, manosa y/o manosa-6-fosfato y donde los glicanos de Asn101, Asn161 y Asn273 contienen manosa-6-fosfato.	un perfil de oligosacáridos complejos desde Man8GlcNAc2 hasta Man15GlcNAc2 (Fig. 1) bajos de ácido siálico y alto contenido de manosa-6-fosfato (Tabla 1)	372 AA con 3 sitios de glicosilación en los residuos Asn15, Asn80 y Asn252 (Pág. 4, Párr. [0034]).

El documento D4 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1, porque divulga una enzima LAL recombinante expresada en *Pichia pastoris* (pHLAL) que presenta un perfil de oligosacáridos complejos desde Man8GlcNAc2 hasta Man15GlcNAc2 (Fig. 1) con niveles muy bajos de ácido siálico y alto contenido de manosa-6-fosfato (Tabla 1).

Por otra parte, el documento D5 divulga la glicoproteína LAL para el tratamiento y la prevención de aterosclerosis (Resumen), la cual comprende 372 AA y 3 sitios de glicosilación en los residuos Asn15, Asn80 y Asn252 (Pág. 4, Párr. [0034]).

La diferencia entre la invención definida en la reivindicación 1 y la enzima de D4 consiste en que D4 no divulga la estructura primaria de la enzima LAL humana.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la enzima lipasa reclamada en la solicitud porque no presenta una mayor actividad específica, según lo establecido en la descripción.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: como proveer una enzima LAL recombinante alternativa que tiene una estructura de glicano-N-ligado que comprende por lo menos manosa y/o manosa-6-fosfato, para obtener una glicoproteína útil en el tratamiento de enfermedades asociadas a deficiencias de LAL.

La diferencia entre la invención definida en la reivindicación 1 y la enzima de D5 consiste en que en D5 no divulga las características estructurales de los N-oligosacáridos, ni tampoco el contenido de manosa-6-fosfato o ácido siálico en la glicoproteína.

Sin embargo, el documento D4 enseña una enzima LAL recombinante expresada en *Pichia pastoris* (phLAL) y células CHO (chLAL) con actividad in vivo en ratones *lal*^{-/-}, que presenta un perfil de oligosacáridos complejos de manosa y N-acetil glucosamina, así como niveles muy bajos de ácido siálico y alto contenido de manosa-6-fosfato (Tabla 1). Por lo tanto, una persona normalmente versada en la materia conocedora de la enzima recombinante divulgada en D4, realizaría la expresión de la enzima LAL humana que comprende 372 AA y 3 sitios de glicosilación en los residuos Asn15, Asn80 y Asn252 (Pág. 4, Párr. [0034]) descrita en el documento D5, para obtener una enzima LAL humana recombinante caracterizada por una estructura de glicano-N-ligado, como la de la reivindicación 1.

En consecuencia, la reivindicación 1, carece de nivel inventivo a la luz de las enseñanzas de los documento D4 y D5.

Las reivindicaciones 2 a 4, dependientes de la reivindicación 1, no divulgan características técnicas adicionales que confieran nivel inventivo a la enzima de la reivindicación 1 y por lo tanto, estas reivindicaciones, también carecen de nivel inventivo.

7. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	J Lipid Res. 2008 Aug; 49(8): 1646–1657.	1 a 4	Nivel Inventivo
D2	Eur. J. Biochem. 219:905-914	1 a 4	Novedad
D3	J. Biol. Chem. 266:22479-22484(1991)	1 a 4	Nivel Inventivo
D4	Am. J. Hum. Genet. 77:1061–1074, 2005	1 a 4	Nivel Inventivo
D5	US2007264249	1 a 4	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.


JESUS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2015-10-13

Elaboró: John Fredy Hernandez Montaño
Revisó: Sandra Carolina Crespo

Purification, characterization and molecular cloning of human hepatic lysosomal acid lipase

Detlev AMEIS¹, Martin MERKEL¹, Christoph ECKERSKORN² and Heiner GRETEN¹

¹ Department of Medicine, University Hospital Eppendorf, Hamburg, Germany

² Max-Planck-Institute for Biochemistry, Martinsried, Germany

(Received September 3, 1993) – EJB 93 1341/3

Lysosomal acid lipase (LAL) is a hydrolase essential for the intracellular degradation of cholesteryl esters and triacylglycerols. This report describes a multi-step procedure for the purification of LAL from human liver. After solubilization with non-ionic detergent, acid hydrolase activity was purified 17000-fold to apparent homogeneity by sequential chromatography on Concanavalin A Sepharose, carboxymethyl-cellulose, phenyl Superose, Mono S cation exchange and Superose 12 gel-filtration columns. This procedure yielded two silver-staining protein bands of 56 kDa and 41 kDa on SDS/PAGE. Size-exclusion chromatography of the 41-kDa protein indicated that the enzyme was catalytically competent as a monomer of approximately 38 kDa. When assayed in the presence of cholesteryl oleate or trioleoylglycerol, purified acid lipase had $V_{\max}$ values of 4390 nmol fatty acid $\cdot$ min⁻¹ $\cdot$ mg protein and 4756 nmol fatty acid $\cdot$ min⁻¹ $\cdot$ mg protein⁻¹, and apparent K_m values of 0.142 mM and 0.138 mM, respectively. The purified enzyme was most active at low pH (4.5–5.0) and required non-ionic detergent and ethylene glycol for optimal stability. Incubation of the 41-kDa acid lipase with endoglucosaminidase H reduced the molecular mass by 4–6 kDa, demonstrating Asn-linked glycosylation with high-mannose oligosaccharides. Deglycosylation did not affect enzymic activity, indicating that carbohydrates are not required for LAL activity. Based on partial peptide sequence, an oligonucleotide was synthesized and utilized to isolate LAL cDNA clones from a human liver cDNA library. A full-length LAL cDNA contained 2626 nucleotides and coded for a predicted protein of 372 amino acids, preceded by a 27 residue hydrophobic signal peptide. Hepatic LAL differed from fibroblast acid lipase at the N-terminus and revealed extensive similarities with human gastric lipase and rat lingual lipase, confirming a gene family of acid lipases. Northern hybridization using the complete LAL cDNA as a radiolabeled probe indicated striking differences in mRNA expression among human tissues. LAL mRNA was most abundant in brain, lung, kidney and mammary gland. Placenta and HeLa cells expressed intermediate amounts of LAL mRNA, while RNA extracted from liver and heart showed low levels of expression.

Lysosomal acid lipase (LAL) is a lipolytic enzyme involved in the intracellular metabolism of cholesteryl esters and triacylglycerols derived from plasma lipoproteins [1]. LAL is synthesized in a variety of cells, including fibroblasts, macrophages, lymphocytes and liver cells (reviewed in [2]). After synthesis at the endoplasmic reticulum, the enzyme is targeted to the lysosomal compartment [3]. In this subcellular location, it hydrolyzes cholesteryl esters and triacylglycerols, liberating cholesterol and free fatty acids. Cholesterol is transferred to the cytosol where it plays a central role in the cellular sterol homeostasis [4, 5]. The physiological importance of LAL in the metabolism of neutral triacylglycerols and cholesteryl esters is illustrated by two inborn errors of

metabolism. In Wolman disease [6], deficient LAL activity results in a marked intralysosomal accumulation of cholesteryl esters and triacylglycerols in most tissues of the body. Clinically, the disorder presents with hepatosplenomegaly, failure to thrive, steatorrhea and adrenal calcifications [7]. Wolman disease patients usually succumb to hepatic and adrenal failure within the first year of life. Cholesteryl ester storage disease, the other clinically recognized phenotypic expression of LAL deficiency, follows a more benign clinical course [8, 9]. However, hypercholesterolemia often occurs in cholesteryl ester storage disease patients and premature atherosclerosis may be severe [7]. In both disorders, LAL deficiency is diagnosed in homogenates of liver biopsies or circulating leukocytes.

Purification of LAL has been attempted from various human tissues, including placenta [10], and cultured fibroblasts [11]. Detailed studies of structure, function and regulation of LAL have been hampered by difficulties in purifying sufficient quantities due to its low abundance and relative instability. There have been reports on two hepatic acid lipases [12, 13], but structural information on the enzymes has not been available. Recently, cDNA clones for a human fibro-

Correspondence to D. Ameis, Department of Medicine, University Hospital Eppendorf, Martinistraße 52, D-20246 Hamburg, Germany

Fax: +49 40 4717 4330.

Abbreviations. LAL, lysosomal acid lipase; PhMeSO₂F, phenylmethylsulfonyl fluoride.

Enzyme. Lysosomal acid lipase (EC 3.1.1.13).

Note. The novel nucleotide sequence data published here have been deposited with the EMBL sequence data bank(s) and are available under accession numbers X76488.

blast acid lipase have been obtained and amino acid sequences of the protein deduced [14].

To start defining the molecular properties of human hepatic LAL and to aid in investigating the genetics of Wolman disease and cholesteryl ester storage disease, we isolated and characterized the LAL protein. In this report, we present a purification strategy for human hepatic LAL which yielded an apparently homogeneous enzyme preparation suitable for further biochemical characterization. Based on partial amino acid sequence obtained by peptide microsequencing, a full-length cDNA clone encoding human hepatic LAL was isolated and analyzed.

EXPERIMENTAL PROCEDURES

Materials

Tri-[9,10(*n*)-³H]oleoylglycerol (1.0Ci/mmol) and cholesteryl [1-¹⁴C]oleate (54 mCi/mmol) were obtained from Amersham. Trioleoylglycerol, cholesteryl oleate, oleate, lysophosphatidylcholine, α -methylmannoside, antipain and phenylmethylsulfonyl fluoride (PhMeSO₂F) were from Sigma Chemicals. Endoglucosaminidase H, chymostatin, leupeptin and pepstatin were obtained from Boehringer. Dithiothreitol was from Bethesda Research Laboratories. Concanavalin-A-Sepharose and carboxymethyl-Sepharose were purchased from Pharmacia. Sodium dodecyl sulfate, acrylamide, piperazine diacrylamide, tetramethylethylenediamine, ammonium persulfate, urea and Triton X-100 were from Biorad. Glassybond membrane was purchased from Biometra.

Tissue source

Human liver was obtained during surgical interventions for removal of hepatic metastases; liver tissue utilized was free of tumor infiltration as shown by routine pathological examination. Tissue specimens were stored frozen at -80°C prior to use.

Enzyme assays

The assay for LAL was modified from a method previously reported for lipoprotein lipase [15]. In brief, lipase activity was determined from the amount of [³H]oleate released from tri-[9, 10(*n*)-³H]oleoylglycerol. An anhydrous stock solution was prepared in glycerol by emulsifying radio-labeled trioleoylglycerol [15]. Reaction mixtures consisted of 100 μ l substrate in a final volume of 200 μ l containing 100 mM sodium acetate, pH 5.0, 0.25% Triton X-100 and 0.275 mM trioleoylglycerol [16]. Reactions were incubated for 30 min at 37°C following addition of enzyme. Reaction products were isolated by organic extraction [17]. In some experiments, assays of LAL activity were also performed using cholesteryl [1-¹⁴C]oleate as substrate. Sonicated emulsions of cholesteryl [1-¹⁴C]oleate were prepared as described [18]. Reaction mixtures consisted of 100 μ l substrate in a final volume of 300 μ l containing 100 mM sodium acetate, pH 5.0, and 0.16 mM cholesteryl oleate. Reactions were incubated for 30 min at 37°C following addition of enzyme. Reaction products were isolated by organic extraction [19]. 1 mU enzyme activity represents the release of 1 nmol fatty acid/min.

Protein determination

Protein concentrations were determined by the method of Bradford [20] with bovine serum albumin as a standard.

Enzyme purification

LAL was purified from human liver by modification and extension of a published method [13]. All steps were carried out at 4°C. To 325 g human liver, 720 ml ice-cold buffer A was added (buffer A, 10 mM sodium acetate, pH 5.0, 0.1 mM dithiothreitol, 1 μ g/ml antipain, 1 μ g/ml chymostatin, 1 μ g/ml leupeptin, 0.1 μ g/ml pepstatin, 1 mM PhMeSO₂F). The liver tissue was allowed to thaw on ice and minced. The tissue was disrupted with three strokes at maximum speed of 1 min each in an Ultra Turrax T25 blender. After centrifugation at 48000 g for 40 min, the pellet was suspended in 450 ml buffer A, containing 1% Triton X-100, and was solubilized with three strokes at maximum speed of 1 min each in an Ultra Turrax T25 blender. After centrifugation at 48000 g for 40 min, the supernatant was gently decanted and saved on ice. 450 ml buffer A containing 1% Triton X-100 was added, and the pellet was re-extracted as above. After centrifugation, the pooled supernatants were stirred with 160 ml concanavalin A Sepharose for 2 h 30 min. The resin was collected by centrifugation at 1000 $\times$ g for 2 min, and was sequentially washed with 640 ml buffer B (buffer B, 20 mM sodium acetate, pH 5.0, 1 mM dithiothreitol, 1 μ g/ml antipain, 1 μ g/ml chymostatin, 1 μ g/ml leupeptin, 0.1 μ g/ml pepstatin, 1 mM PhMeSO₂F) containing 0.5 M NaCl, 0.5% Triton X-100 and 33% ethylene glycol; 2.4 l buffer B containing 33% ethylene glycol; and 640 ml buffer B containing 33% ethylene glycol and 375 mM α -methylmannoside. LAL activity was eluted from the resin with 200 ml buffer B containing 33% ethylene glycol, 375 mM α -methylmannoside and 0.3% Triton X-100 for 5 min at 37°C. This elution was repeated eight times. Pooled supernatants (1.6 l) were loaded onto a column containing 35 ml carboxymethyl-Sepharose, equilibrated with buffer B, at a flow rate of 2 ml/min. The column was washed with 350 ml buffer B containing 3% ethylene glycol and eluted with a linear gradient of 150 ml each of buffer B containing 3% ethylene glycol and buffer B containing 0.5 M NaCl and 3% ethylene glycol. Fractions with peak LAL activity were pooled and injected onto a 1-ml phenyl Superose column (Pharmacia), equilibrated with buffer B containing 3% ethylene glycol. The column was washed with 10 ml buffer B containing 33% ethylene glycol and 0.1% Triton X-100, and eluted with a linear 25-ml gradient to buffer B containing 60% ethylene glycol and 2% Triton X-100. The flow rate was 0.1 ml/min, and 1-ml fractions were collected. Fractions with peak LAL activity were pooled and injected onto a 1-ml Mono S column (Pharmacia), equilibrated with buffer B containing 25% ethylene glycol and 0.01% Triton X-100. The column was washed with 10-ml equilibration buffer and eluted with a 30-ml linear gradient from 0 to 0.5 M NaCl in buffer B containing 25% ethylene glycol and 0.01% Triton X-100. The flow rate was 0.4 ml/min, and fractions of 1 ml were collected. Fractions 35 and 36 were pooled and concentrated to 0.2 ml on a Centricon-10 microconcentrator (Amicon). The concentrate was applied to a Superose 12 column (Pharmacia), which had been previously equilibrated in buffer B containing 25% ethylene glycol, 0.1% Triton X-100 and 0.15 M NaCl. Fractions of 0.4 ml were collected at a flow rate of 0.1 ml/min. LAL activity eluted as a single peak at 13.8 ml (fractions 35 and 36). Protein markers for standardization of the column were bovine serum albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease A (14 kDa).

SDS/PAGE and peptide microsequencing

Proteins were analyzed according to established procedures [21]. For analytical polyacrylamide gels, stacking gels of 4% acrylamide and separating gels of 12.5% acrylamide were used. Proteins were stained with silver nitrate [22]. For preparative electrophoresis, 10% separating gels were run with gel dimensions of 16 cm×16 cm×1.5 mm. After electrophoretic separation for 7 h at 55 mA and 4°C, semi-dry electroblotting was performed onto Glassybond membrane (Biometra) using 50 mM boric acid, pH 9.0, 5% methanol on the cathodic side, and 50 mM boric acid, pH 9.0, 20% methanol on the anodic side of the transfer sandwich [23]. Electrotransfer was for 2 h 30 min with 1 mA/cm². Protein was stained on the membrane with 0.1% Coomassie Blue R250 in 10% acetic acid, 40% methanol. Protein bands were excised and subjected to N-terminal amino acid sequencing by automated Edman degradation using an Applied Biosystems gas-phase microsequencer with on-line phenylthiohydantoin analyzer.

cDNA screening and analysis

A single synthetic 33-base oligonucleotide probe was designed on the basis of a 17-amino-acid peptide sequence from purified liver LAL. The oligonucleotide was designed employing codon usage tables of mammalian genes [24] and had the following sequence: 5'-CAAGCCTGTGGTGTTCCTGCAGCACGGCCTGCT-3'. A total of 1×10⁶ plaques of a human liver cDNA library in UniZAP XR (Stratagene) were screened with the ³²P-end-labeled 33-residue oligonucleotide according to standard techniques [25]. Phage plaques hybridizing to the oligonucleotide probe were subjected to two rounds of plaque purification. Plasmid was released from phage cultures by superinfection with helper phage R408 according to the supplier (Stratagene). DNA sequence determination was performed using a nested-deletion strategy (Pharmacia) and fluoro-chrome-labeled M13 primers by the dideoxynucleotide chain-termination method [26]. Sequencing products were analyzed on an Applied Biosystems 373A DNA sequencer. The cDNA clone hLAL4/1 was sequenced completely; sequences were confirmed for the complementary DNA strands. Sequence analysis and computer searches for sequence similarities were conducted using the program Tetra (Softgene) providing EMBL database release 72.

RNA analysis

Total cellular RNA of various human tissues (Clontech) was loaded onto 1% agarose gels containing 2% formaldehyde and Mops buffer. After electrophoresis for 3 h at 140 mA, gels were stained with 1 µg/ml ethidium bromide, destained, and photographed under ultraviolet illumination to document equivalent loading. RNA was then transferred to Hybond N membrane (Amersham) and hybridized to the LAL 2.6-kb cDNA labeled with ³²P (specific activity 5×10⁸ cpm/µg). After hybridization at 65°C for 18 h in 5× NaCl/Cit (NaCl/Cit, 0.15 M NaCl, 0.015 M citrate, pH 7.0), 0.12 M sodium phosphate, pH 7.2, 1% *N*-lauryl sarcosine, 0.1% SDS, 4× Denhardt's containing 100 µg/ml denatured salmon sperm DNA, blots were washed at room temperature in 2× NaCl/Cit, 0.1% SDS, and at 65°C in 2× NaCl/Cit, 0.1% SDS for 30 min. RNA standards in the range 0.36–9.5 kb (Novagen) were used to determine mRNA size. Filters were exposed to Kodak XAR film for 3 days.

RESULTS

Detergent solubilization and purification of LAL

The purification strategy for human liver employed in this study is a modification and extension of that described by Sjöberg et al. [13]. For a purification, as summarized in Table 1, 325 g liver was homogenized. The 48000 g pellet was then solubilized with Triton X-100 and subjected to protein chromatography. After batch adsorption on concanavalin A-Sepharose selecting for glycoproteins [27], chromatography on carboxymethyl-Sepharose was used for concentrating LAL activity, and hydrophobic interaction chromatography on phenyl Superose was employed for further purification. The penultimate purification step utilized a Mono S HR 5/5 column and a 0–0.5 M NaCl gradient. For the final purification step, peak fractions from the Mono S gradient elution were concentrated and chromatographed over a Superose 12 gel-filtration column. The LAL activity eluted with the major protein peak at approximately 38 kDa (Fig. 1). Fractions 34–36, which had the highest activity, contained two major peptides of 56 kDa and approximately 41 kDa, whereas fraction 37 contained the 41-kDa protein with only a trace amount of the 56-kDa polypeptide. The SDS/PAGE analyzing the sequential steps in the purification of human hepatic LAL is shown in Fig. 2. In lane 7, 0.8 µg of fraction 37 of the Superose 12 gel-filtration chromatography were analyzed, indicating that LAL is a monomeric protein of 41 kDa. The specific activity of pure LAL in a typical preparation was 3900 mU/mg (Table 1). The purification resulted in a 17000-fold increase in specific activity. Overall, the recovery of enzyme activity was in the range of 1–2% (for six purifications).

Enzymic properties

LAL is a moderately cationic protein since it required a NaCl concentration of about 0.24 M to elute from Mono S (data not shown). On chromatography over phenyl Superose the enzymic activity eluted only with 60% ethylene glycol, 2% Triton X-100, confirming previous results that LAL is a strongly hydrophobic protein [13]. To stabilize enzymic activity, a Triton X-100 concentration in excess of 0.01% was required in all steps of the isolation procedure. Ethylene glycol also stabilized LAL activity, while glycerol had no apparent effect. To further characterize purified LAL, the pH optimum for two different substrates was determined. LAL hydrolyzed both cholesteryl oleate and trioleoylglycerol over a pH range of 4.0 to 6.0, with an optimum pH of 4.5–5.0 (Fig. 3A). The pH optimum for cholesteryl oleate was slightly broader than for trioleoylglycerol. Cholesteryl-oleate-hydrolyzing and trioleoylglycerol-hydrolyzing activities of purified LAL were monitored as a function of concentration of the two substrates (Fig. 3B). Both activities yielded hyperbolic curves to which Michaelis kinetics could be applied. Maximal reaction velocities with emulsified cholesteryl oleate and trioleoylglycerol, as calculated from Lineweaver-Burke plots, were 4390 nmol fatty acid · min⁻¹ · mg protein and 4756 nmol fatty acid · mg⁻¹ · min⁻¹ with apparent *K_m* values of 0.142 mM and 0.138 mM, respectively. The effect of the amount of purified LAL on hydrolysis of cholesteryl oleate and trioleoylglycerol was determined. The hydrolytic activity was linear in the range 8–120 ng LAL (Fig. 3C). In experiments assessing the time dependence of LAL hydrolytic activity, hydrolysis for both cholesteryl oleate and trioleoylglycerol were linear for up to 60 min (Fig. 3D).

Table 1. Purification of human liver lysosomal acid lipase. 1 mU enzyme activity represents the release of 1 nmol free fatty acid/min.

Purification step	Protein	Activity	Specific activity	Yield	Purification
	mg	mU	mU/mg	%	-fold
Homogenate	18 598	4 271	0.23	100	—
Triton X-100 extract	1 987	15 932	8	373	35
Concanavalin-A-Sepharose	200	7 866	39	185	171
Carboxymethyl-Sepharose	20.1	1 185	59	28	256
Phenyl-Superose	4.6	1 150	251	27	1 095
Mono S	0.24	313	1303	7.3	5 670
Superose 12	0.016	64	3900	1.5	17 000

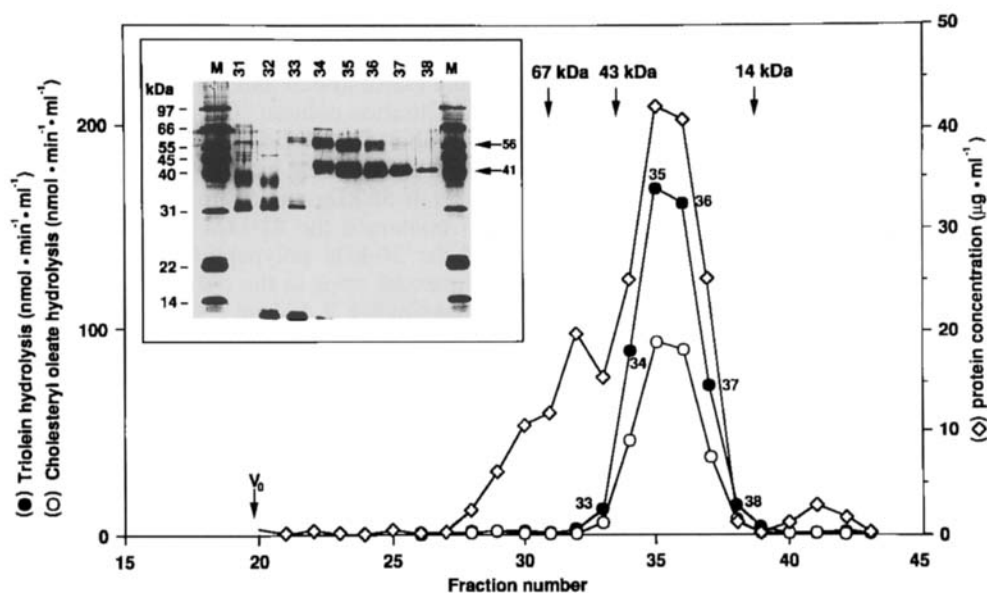


Fig. 1. Separation of human liver LAL on a gel-filtration column. Peak activity fractions from the Mono S column were concentrated to 0.2 ml on a Centricon-10 microconcentrator and chromatographed on a Superose 12 column eluted at a flow rate of 0.1 ml/min. 0.4-ml fractions were collected. The void volume (V_0) and elution volume of the molecular mass markers bovine serum albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (14 kDa) are indicated by vertical arrows. The inset shows fractions 31–38 (6- μ l aliquots) analyzed on a denaturing 12.5% SDS/polyacrylamide gel. Arrows on the right hand side of the inset identify the two major 56-kDa and 41-kDa proteins purifying with LAL activity. Triolein, trioleoylglycerol.

Structural analysis

Glycosylation of the purified 41-kDa LAL protein was investigated by incubation for 6 h at 37°C in the absence or presence of endoglucosaminidase H. Digestion with endoglucosaminidase H yielded a ladder of three bands (Fig. 4). The largest migrated with undigested protein and the smallest had a molecular mass of approximately 37 kDa. Excess of endoglucosaminidase H did not result in a complete digestion of carbohydrates. This suggested the presence of two Asn-linked oligosaccharides, accounting for approximately 5 kDa of apparent molecular mass, consistent with estimates of 4–6 kDa of Asn-linked carbohydrate in fibroblast acid lipase [11]. Acid hydrolase activity was unaffected by deglycosylation with endoglucosaminidase H (data not shown), indicating that Asn-linked carbohydrate is not essential for enzymic activity of hepatic LAL.

To determine directly the molecular identity of the two LAL protein forms observed at the Superose 12 purification step and to obtain partial amino acid sequence for oligonucleotide-based cloning of LAL, peptide microsequencing was performed. Peak fractions 34 and 35 of the Superose 12 gel-filtration chromatography (Fig. 1) were separated by prepara-

tive SDS/PAGE (Fig. 5) and blotted onto a modified glass-fiber membrane. Protein bands were excised and subjected to automated Edman degradation. The heterogeneity in relative electrophoretic mobilities was investigated by independently sequencing two protein bands from fraction 34 with molecular masses of 57 kDa and 55 kDa. Sequencing runs yielded sequences of 6 and 4 cycles, respectively. The peptide sequences indicated that the two proteins contained identical N-termini (Fig. 5). For the broad protein band in the Superose 12 fraction 35 with a molecular mass of 41–43 kDa (Fig. 5), microsequencing was performed on the upper and lower half of the protein band. The 5 amino acids GPKPV determined for the upper half of the protein were represented in the 17-residue peptide sequence GPKPVFLQHGLLADSS obtained from the bottom half. This demonstrated identical N-termini of these two LAL protein forms and suggested that the observed size heterogeneity was due to post-translational modification of a single LAL protein.

Isolation of human LAL cDNA and sequence analysis

The peptide sequence derived from the 41-kDa LAL form (Fig. 4) was used to design an LAL-specific synthetic

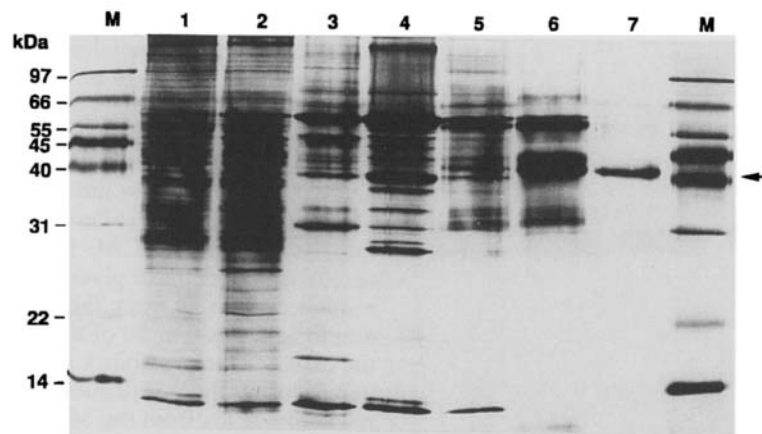


Fig. 2. Representative SDS/PAGE profile of individual chromatography steps in the LAL purification procedure. Aliquots from each step of the purification were subjected to denaturing SDS/PAGE (12.5% gel, silver stain). Lane 1, 6 μ g liver homogenate; lane 2, 6 μ g pooled fractions with major activity from Triton X-100 extraction; lane 3, 6 μ g pooled major fractions from concanavalin-A-Sepharose; lane 4, 3 μ g pooled major fractions from carboxymethyl-Sepharose; lane 5, 3 μ g pooled major fractions from phenyl Superose; lane 6, 3 μ g pooled fractions 35 and 36 from Mono S; lane 7, 0.8 μ g of the peak fraction 37 from the gel filtration on Superose 12. The 41-kDa LAL protein is indicated by an arrow on the right-hand side. Lanes M, molecular-mass standards, phosphorylase B (97 kDa), bovine serum albumin (66 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (43 kDa), aldolase (40 kDa), carboanhydrase (31 kDa), soya bean trypsin inhibitor (22 kDa) and lysosyme (14 kDa).

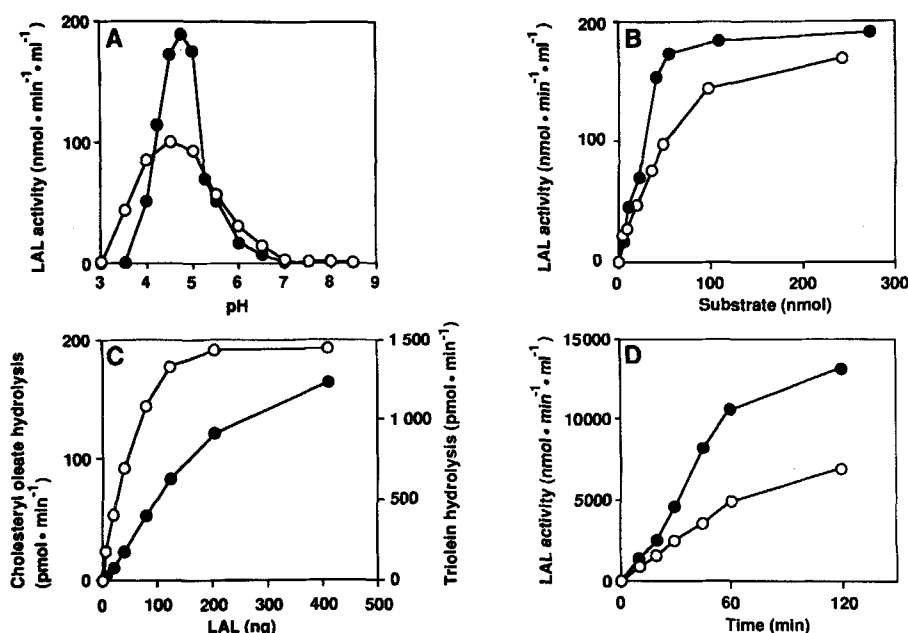


Fig. 3. Effects of pH, substrate concentration, enzyme concentration and incubation time on lipolytic activity of purified LAL. Hydrolysis of trioleoylglycerol and cholesteryl oleate (●), (○), respectively. Standard incubations except for (D) were carried out for 30 min at 37°C. The substrate concentration except for (B) was 0.275 mM trioleoylglycerol (triolein) in a reaction volume of 200 μ l or 0.16 mM cholesteryl oleate in a final reaction volume of 300 μ l. (A) Effect of pH on lipolytic activity of LAL. The incubation consisted of 41 ng LAL in 100 mM sodium acetate buffer in the pH range 3.0–5.25; 50 mM Bistris/HCl at pH 5.0–6.5; and Tris/HCl at pH 6.0–8.5. Buffers contained 0.25% Triton X-100 and tri-[9, 10- ^{3} H] oleoylglycerol or cholesteryl [1- ^{14} C] oleate. (B) Effect of substrate concentration. The incubation mixture contained 41 ng LAL, 100 mM sodium acetate, pH 5.0, 0.25% Triton X-100, and varying amounts of tri-[9, 10- ^{3} H] oleoylglycerol or cholesteryl [1- ^{14} C] oleate. (C) Effect of LAL concentration. The incubation mixture contained 100 mM sodium acetate, pH 5.0, 0.25% Triton X-100, tri-[9, 10- ^{3} H] oleoylglycerol or cholesteryl [1- ^{14} C] oleate, and varying amounts of purified enzyme. (D) Time dependence. The incubation mixture contained 41 ng LAL in 100 mM sodium acetate, pH 5.0, 0.25% Triton X-100, and tri-[9, 10- ^{3} H]oleoylglycerol or cholesteryl [1- ^{14} C] oleate. The incubation was carried out at 37°C for the various times indicated.

oligonucleotide probe in accordance with codon-frequency usage data [24]. The oligonucleotide was used to screen an oligo(dT)-primed human liver cDNA library. Two recombinant clones were isolated by hybridization to the ^{32} P-labeled oligonucleotide. The clone containing the longest cDNA in-

sert, approximately 2.6 kb, was denoted hLAL4/1 and subjected to further analysis. The nucleotide sequence was determined in both orientations using a nested-deletion strategy with exonuclease III and plasmid-specific oligonucleotide primers. Fig. 6 shows the complete cDNA sequence and the

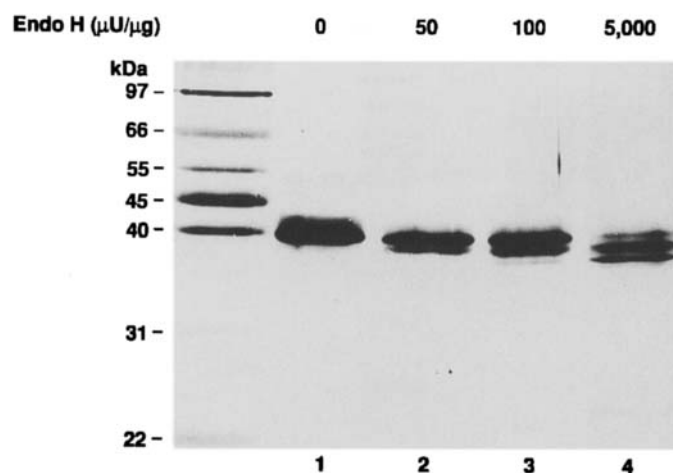


Fig. 4. Asn-linked carbohydrate in LAL. Samples (0.24 μ g) of fraction 37 of the Superose 12 gel-filtration column run (Fig. 1) were heated at 95°C for 5 min and digested at 37°C for 6 h with the indicated amounts (μ U/ μ g LAL) of endoglucosaminidase H (Boehringer Mannheim) in 20 mM sodium acetate, pH 5.0, 50 mM 2-mercaptoethanol, 0.5 mM PhMeSO₂F and 0.02% SDS. Protein was subjected to SDS/PAGE (12.5%) and silver-stained.

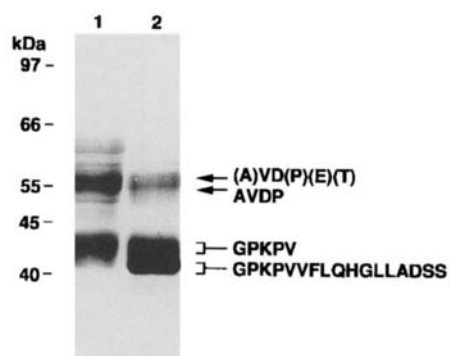


Fig. 5. N-terminal sequencing of human liver LAL. Fractions 34 and 35 of the gel-filtration chromatography on Superose 12 as shown in Fig. 1 were subjected to preparative SDS/PAGE. Following electroblotting onto a Glassybond membrane, protein bands were stained with Coomassie blue, excised, and subjected to N-terminal sequencing by automated Edman degradation using an Applied Biosystems gas-phase microsequencer with on-line phenylthiohydantoin analyzer. Lane 1, two 56-kDa and 55-kDa protein forms of fraction 34 were excised and sequenced individually, as indicated by the horizontal arrows. Peptide sequences obtained are shown on the right-hand side. Lane 2, the broad 41–43-kDa protein band was cut in half as indicated by the horizontal brackets and sequenced. The standard single-letter code for amino acids is used. Amino acids in parentheses indicate ambiguous calls on the sequencer run.

deduced amino acid sequence of LAL. The cDNA is 2626 nucleotides and contains two in-frame methionine codons at nucleotide positions 1 and 7. The first ATG triplet is likely to be used as initiator codon since the sequence immediately 5' to it (CCAGAATGA) is in good agreement with the consensus sequence (CCA/GCCATGG) for translation initiation [28], while the sequence surrounding the second ATG (TGAAAATGC) differs significantly from the consensus. The cDNA sequence displays an open reading frame of 399 amino acids and is preceded by 144 nucleotides of 5' untranslated sequence and followed by a 1255 nucleotide 3' non-coding region. A polyadenylation signal is present at nu-

cleotides 2428–2433. Sequences of the two peptides (Fig. 5) were located in the predicted amino acid sequence, verifying the identity of the clone hLAL4/1 as coding for human liver LAL. According to the position of the N-terminal peptide sequence of the 56-kDa LAL form, a 27-amino-acid hydrophobic leader peptide and a mature protein of 372 amino acids is predicted. The molecular mass of the non-glycosylated protein is 42515Da. The larger apparent molecular mass of the purified LAL 56-kDa form (Fig. 1) presumably results from extensive glycosylation. The N-terminal peptide sequence derived from the 41-kDa lipase form aligned at amino acid position 50 of the LAL protein as predicted from the cDNA. One possibility is that proteolytic degradation occurred during the purification of LAL, removing 49 N-terminal amino acids from the 56-kDa lipase. This seems unlikely, however, because the 41-kDa LAL protein was enzymically active with a high specific lipolytic activity. Moreover, the same molecular masses of 56 kDa and 41 kDa for the two LAL forms were observed on all purifications performed. The more probable interpretation seems to be that specific proteolytic processing is involved in the maturation of the N-terminus of LAL. This processing would remove 49 amino acids and release a mature lipase of 323 amino acids with a predicted non-glycosylated molecular mass of 36815Da.

Sequence comparisons of human liver LAL with human fibroblast acid lipase [14] showed that the 5' untranslated region of liver LAL extended 105 bases beyond the fibroblast sequence. Throughout the coding region, a single difference was observed in nucleotide position 46, where A is replaced by C in fibroblast lipase. This divergence resulted in threonine at position –12 in liver LAL and proline at the respective position of the fibroblast enzyme. The remaining coding region of both enzymes showed identical sequences. In the 3' non-coding region, four nucleotide differences were observed. Whether these nucleotide differences represent true polymorphisms or are due to tissue-specific isoforms is as yet undetermined. Nucleotide differences of liver and fibroblast acid lipases are summarized in Table 2.

cDNA clones for two acid hydrolases, gastric lipase and lingual lipase have been reported. Alignment of liver LAL with human gastric lipase sequences [29] showed a high degree of similarity, with 59% identical amino acids throughout the length of the protein. Human liver LAL and rat lingual lipase amino acid sequences [30] showed 58% identity. Interestingly, apart from a small region surrounding the putative active-site serine in neutral lipases (Fig. 6), no similarities were detected with neutral lipases such as hepatic lipase [31] or lipoprotein lipase [32], suggesting independent gene families for acid and neutral lipases.

LAL is synthesized by virtually all cells and tissues of the human body, including hepatocytes, fibroblasts, macrophages and lymphocytes [2]. To identify tissues which had not been investigated in terms of LAL activity and to determine relative amounts of LAL mRNA, we used the LAL cDNA as a radiolabeled probe. Northern analysis of the LAL mRNA content of various human tissues is shown in Fig. 7. A hybridization signal of 2.6 kb specific for LAL mRNA was observed in all tissues assessed (Fig. 7). The most abundant mRNA expression was detected in brain, lung, mammary gland, kidney and adrenal gland. Placenta and HeLa cells showed intermediate levels of expression, whereas heart, liver and skeletal muscle expressed low levels of LAL mRNA. Expression of skeletal muscle LAL mRNA became visible after longer exposure of the autoradiograph. To demonstrate equivalent loading of RNAs, the content of 28S

-144 TTGGCTGAACAAATAGTCCGAGGGTGGTGTCTACCGCCCTCCGACAAAGCAGACAGGCCCCCTGCAGTCCCTATCCGACCCCGCCCTGAGAGC

-44 TGGCACTCGGACTCGAGACAGCGGCCGCGAGGACAGCTCCAGATG AAA ATG CGG TTC TTG GGG TTG GTG GTC TGT ITG GTT CTC
-27 Met Lys Met Arg Phe Leu Gly Leu Val Val Cys Leu Val Leu

43 TGG ACC CTG CAT TCT GAG GGG TCT GGA GGG AAA CTG ACA GCT GTG GAT CCT GAA ACA AAC ATG AAT GTG AGT GAA
-13 Trp Thr Leu His Ser Glu Gly Ser Gly Gly Lys Leu Thr Ala Val Asp Pro Glu Thr Asn Met Asn Val Ser, Glu

118 ATT ATC TCT TAC TGG GGA TTC CCT AGT GAG GAA TAC CTA GTT GAG ACA GAA GAT GGA TAT ATT CTG TGC CTT AAC
13 Ile Ile Ser Tyr Trp Gly Phe Pro Ser Glu Glu Tyr Leu Val Glu Thr Glu Asp Gly Tyr Ile Leu Cys Leu Asn

193 CGA ATT CCT CAT GGG AGG AAG AAC CAT TCT GAC AAA GGT CCC AAA CCA GTT GTC TTC CTG CAA CAT GGC TTG CTG
38 Arg Ile Pro His Gly Arg Lys Asn His Ser, Asp Lys Gly Pro Lys Pro Val Val Phe Leu Gln His Gly Leu Leu

268 GCA GAT TCT AGT AAC TGG GTC ACA AAC CTT GGC AAC AGC AGC CTG GGC TTC ATT CTT GCT GAT GCT GGT TTT GAC
63 Ala Asp Ser Ser Asn Trp Val Thr Asn Leu Ala Asn Ser Ser, Leu Gly Phe Ile Leu Ala Asp Ala Gly Phe Asp

343 GTG TGG ATG GGC AAC AGC AGA GGA AAT ACC TGG TCT CGG AAA CAT AAG ACA CTC TCA GTT TCT CAG GAT GAA TTC
88 Val Trp Met Gly Asn Ser Arg Lys Asn Thr Trp Ser Arg Lys His Lys Thr Leu Ser Val Ser Gln Asp Lys Phe

418 TGG GCT TTC AGT TAT GAT GAG ATG GCA AAA TAT GAC CTA CCA GCT TCC ATT AAC TTC ATT CTG AAT AAA ACT GGC
113 Trp Ala Phe Ser Tyr Asp Glu Met Ala Lys Tyr Asp Leu Pro Ala Ser Ile Asn Phe Ile Leu Asn Lys Thr, Gly

493 CAA GAA CAA GTG TAT TAT GTG GGT CAT TCT CAA GGC ACC ACT ATA GGT TTT ATA GCA TTT TCA CAG ATC CCG GAT
138 Gln Glu Gln Val Tyr Tyr Val Gly His Ser Gln Gly Thr Thr Ile Gly Phe Ile Ala Phe Ser Gln Ile Pro Glu

568 CTG GCT AAA AGG ATT AAA ATG TTT TTT GCC CTG GGT CCT GTG GCT TCC GTC GCC TTC TGT ACT AGC CCT ATG GCC
163 Leu Ala Lys Arg Ile Lys Met Phe Phe Ala Leu Gly Pro Val Ala Ser Val Ala Phe Cys Thr Ser Pro Met Ala

643 AAA TTA GGA CGA TTA CCA GAT CAT CTC ATT AAG GAC TTA TTT GGA GAC AAA GAA TTT CTT CCC CAG AGT GCG TTT
188 Lys Leu Gly Arg Leu Pro Asp His Leu Ile Lys Asp Leu Phe Gly Asp Lys Glu Phe Leu Pro Gln Ser Ala Phe

718 TTG AAG TGG CTG GGT ACC CAC GTT TGC ACT CAT GTC ATA CTG AAG GAG CTC TGT GGA AAT CTC TGT TTT CTT CTG
213 Leu Lys Trp Leu Gly Thr His Val Cys Thr His Val Ile Leu Lys Glu Leu Cys Gly Asn Leu Cys Phe Ser Leu

793 TGT GGA TTT AAT GAG AGA AAT TTA AAT ATG TCT AGA GTG GAT GTA TAT ACA ACA CAT TCT CCT GCT GGA ACT TCT
238 Cys Gly Phe Asn Glu Arg Asn Leu Asn Met Ser, Arg Val Asp Val Tyr Thr Thr His Ser Pro Ala Gly Thr Ser

868 GTG CAA AAC ATG TTA CAC TGG AGC CAG GCT GTT AAA TTC CAA AAG TTT CAA GCC TTT GAC TGG GGA AGC AGT GCC
263 Val Gln Asn Met Leu His Trp Ser Gln Ala Val Lys Phe Gln Lys Phe Gln Ala Phe Asp Trp Gly Ser Ser Ala

943 AAG AAT TAT TTT CAT TAC AAC CAG AGT TAT CCT CCC ACA TAC AAT GTG AAG GAC ATG CTT GTG CCG ACT GCA GTC
288 Lys Asn Tyr Phe His Tyr Asn Gln Ser, Tyr Pro Pro Thr Tyr Asn Val Lys Asp Met Leu Val Pro Thr Ala Val

1018 TGG AGC GGG GGT CAC GAC TGG CTT GCA GAT GTC GAC GTC AAT ATC TTA CTG ACT CAG ATC ACC AAC TTG GTG
313 Trp Ser Gly Gly His Asp Trp Leu Ala Asp Val Tyr Asp Val Asn Ile Leu Leu Thr Gln Ile Thr Asn Leu Val

1093 TTC CAT GAG AGC ATT CCG GAA TGG GAG CAT CTT GAC TTC ATT TGG GGC CTG GAT GCC CCT TGG AGG CTT TAT AAT
338 Phe His Glu Ser Ile Pro Glu Trp Glu His Leu Asp Phe Ile Trp Gly Leu Asp Ala Pro Trp Arg Leu Tyr Asn

1168 AAA ATT ATT AAT CTA ATG AGG AAA TAT CAG TGA AAGCTGGACTTGAGCTGTGTACCAAGCAATGATTATGTGATGAAATGTG
363 Lys Ile Ile Asn Leu Met Arg Lys Tyr Gln End

1257 TTTCCTTATTTCTGTAAACACTTGTITTTCTTCCAGGCTTTTGTITTTTATATCAAGAAATGATAACTTTGAAGATGCCAGTTCACCTAG

1357 TTTCAATTAGAACATACAGTATTTTTCCTTTAATAGGGCTGGAATAGGAAGCCAGTGCTCAACCATAGTATTGTCTCTTTAAGTCTTTTAAATAT

1457 CACTGATGGTGAAGAGGTCATTATATCAATCTGTITTTTAAATTTAAATATATATGACTTTTTCGCTTCATAGGACAAAGTAATATATGTGTGGAA

1557 TTTTAAATTTGTGTGTCTGTAATCTGTCACTGACTTAAAGCAGGTATAAAAGTACGCACTTTTCATGTCTTTCGCTTAAAGAGCTCTGACTCTA

1657 ACGGTCTGTAGTTAGAGATCTAAATGACATTTTATCATGTITTTCTGCGAGCAGGTGCATAGTCAAAATCCAGAAATATCAAGCTGTGCCAGTAATAGG

1757 ATCTAACAATTAAATTTATCAAACTAACTGTGACAGCTGTGATTTGACACGTTTAAATGCTCAGGTAAATGAATAGTTTTCGCGCTCTTCAAAA

1857 ACAAAATGCACTGATAAAACAAAAAAGATGTITTTAAATGCTTTGAAGACTGATACACTCAACCATCTATATTCATGAGCTCTCAATTTTATGGCA

1957 GGCCATAGTCTACTTATCTGAGAAGCAAACTCCGTGGAGACTATACCACTATTTTTCGTGAGATTAAATGACTCTTGGAGCCCGCTACTGTCTGTTAT

2057 GATCACAATCTGTGGAAGCAAGCCCGCTGGTGGCCATGAGAAGTGTCTAGTTCATTTTACCCCAATGAAGTGTGAACGTGATGTTTTCGGATGCA

2157 AACTCAGCTCAGGATTCATTTTGTGTCTAGTGTATATGATCTCTTATTTTAAATACACCTGCTCAGTCCCTATGTGGGAAGTCCATATTTGTCT

2257 GCTTTTCTGAGCATCATTTCTGGACAATCTGTCCGGTGACAAATGACAATTGATGTTTTCGTGATATAATTACTTTAGCTGCATTAACAGTA

2357 CAATGCTGTGTAATGTTAATATAGGACGGGGAATACTACTTTGTAACTTTTAAAGCTTTAAACTTTTCAATAAAATTCAGTGAGACTTATAGGCC (A) 27

Fig. 6. Complete nucleotide sequence and deduced protein sequence for human LAL cDNA. Nucleotide numbering starts at +1 of the presumed initiator methionine. Nucleotides of the 5' untranslated region are numbered -1 to -144. The N-terminal amino acid of the 56-kDa LAL form is boxed. Sequences derived from peptide microsequencing are identified by underlining. Putative sites of Asn-linked glycosylation are highlighted by brackets. The putative active-site serine in position 147 and the surrounding consensus sequence of neutral lipases is denoted by dots. The polyadenylation consensus is doubly underlined.

Table 2. Nucleotide differences of liver and fibroblast LAL cDNA.

Nucleotide position	Liver LAL	Encoded amino acid	Fibroblast LAL [14]	Encoded amino acid
46	ACC	Thr12	CCC	proline
1386	C	3' non-coding	T	
2015	C	3' non-coding	T	
2109	A	3' non-coding	T	
2324	C	3' non-coding	T	

and 18S RNAs are shown in the ethidium-bromide-stained gel (Fig. 7).

DISCUSSION

The lysosomal degradation of low-density lipoproteins and other cholesterol-enriched plasma lipoproteins plays a

central role in the uptake and transfer of cholesteryl esters to intracellular sites of storage and metabolism. An acid hydrolase implicated in the cellular processing of cholesteryl esters was first studied in cultured human fibroblasts [1] and has since been observed in virtually all tissues and cells of the human body (reviewed in [2]). In this report, the purification to apparent homogeneity of LAL from human liver is

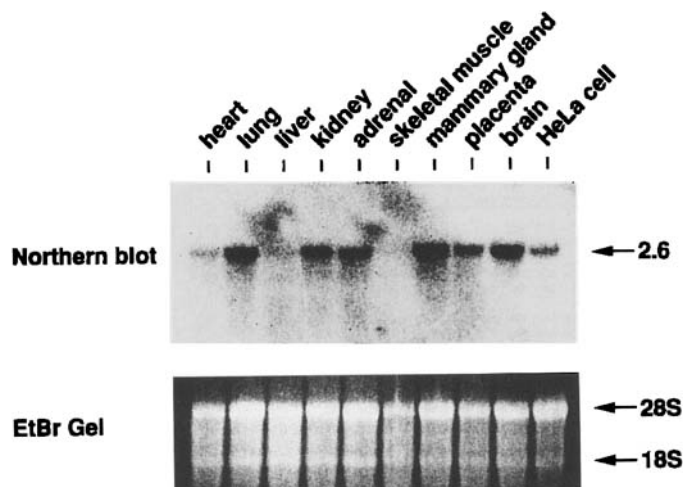


Fig. 7. Northern blot analysis of LAL mRNA in various human tissues. 15 μ g total cellular RNA was resolved on a 1% denaturing agarose gel, transferred to Hybond N membrane, and hybridized with ^{32} P-labeled LAL 2.6-kb cDNA as described in Experimental Procedures. Autoradiography was at -80°C for 3 days with two intensifying screens. The size of the LAL mRNA, in kb, is indicated on the right-hand side of the Northern blot. To document equivalent loading of RNA samples, the ethidium-bromide-stained (EtBr) gel is also shown. Ribosomal RNAs are indicated as markers.

described. The enzyme was characterized in several ways. First, using denaturing SDS/PAGE, two proteins of 56 kDa and 41 kDa were shown to purify with acid hydrolase activity. Second, gel-filtration chromatography of the native LAL protein demonstrated that the enzyme was catalytically competent as monomer of approximately 38 kDa. This result differed considerably from reports of two human hepatic acid lipases for which molecular masses of 29 kDa and 58 kDa have been described [12, 13]. Further structural information on these acid lipases has not been available, however. Third, hepatic LAL was shown to be a glycoprotein, as indicated by its binding to concanavalin-A-Sepharose and elution with α -methylmannoside. This confirmed earlier data derived from acid lipase of human placenta [10] and human [12, 13] and rat liver [33].

Based on peptide sequence from the 41-kDa LAL form, an oligonucleotide probe specific for LAL was synthesized and used to isolate clones from a human liver cDNA library. Analysis of the largest clone, hLAL4/1, established the complete nucleotide sequence of human liver LAL. The deduced amino acid sequence indicated that LAL contains 399 amino acids synthesized with a 27-amino-acid signal peptide. The predicted non-glycosylated molecular mass is 42515 Da. Six potential glycosylation sites were identified in the coding sequence of LAL. Data from endoglucosaminidase H digests indicated that at least two of the putative glycosylation sites are utilized in the mature LAL protein.

A comparison of human liver LAL with human gastric lipase [29] and rat lingual lipase [30] sequences revealed extensive similarities between the enzymes, with 59% and 58% identical amino acids, respectively. This confirmed the notion that the three lipases are members of a gene family of acid lipases [14]. Sequence comparisons of liver LAL to other lipases such as lipoprotein lipase, hepatic lipase or pancreatic lipase which have been established as members of a gene family of neutral lipases [34, 35] revealed a short stretch of similar amino acids surrounding Gly145–

His146–Ser147–Gln148–Gly149. This region was found highly conserved in all neutral lipases investigated, and site-directed mutagenesis of rat hepatic lipase [36] identified the serine residue as crucial to hydrolytic activity. The recent determination of human pancreatic lipase crystal structure [37] has described the Ser152 residue of pancreatic lipase corresponding to Ser147 of LAL as part of a catalytic triad characteristic of this group of neutral lipases. This limited sequence similarity suggests that human LAL may employ a similar or identical catalytic mechanism to hydrolyze its substrates, neutral triacylglycerol and cholesteryl esters.

Proteins with lipase activity and a pH optimum of 4.0–5.5 have been detected in tissue other than liver, including fibroblasts, macrophages and circulating lymphocytes (reviewed in [2]). To find out whether these enzymic activities represented LAL or other lipolytic enzymes and to determine the relative amounts of LAL expression, we examined RNA from various human tissues for evidence of LAL mRNA expression. A single species of mRNA with a size of 2.6 kb was detected in all tissues examined (heart, lung, liver, kidney, adrenal gland, skeletal muscle, mammary gland, placenta, brain, and HeLa cells), supporting a role of LAL as a housekeeping gene. Interestingly, the amount of expressed LAL mRNA differed widely among the tissues studied. Brain, lung, mammary gland, kidney and adrenal showed the most abundant LAL expression. Placenta and HeLa cells expressed intermediate levels of LAL mRNA, while expression in liver and heart was low. LAL mRNA in skeletal muscle was observed only after an extended autoradiography of the respective Northern analysis. With the liver LAL cDNA sequence as a molecular probe, it will now be possible determine the cell types expressing LAL mRNA and to search for tissue-specific isoforms of LAL.

N-terminal peptide microsequencing of the two 56-kDa and 41-kDa LAL protein forms purifying with acid hydrolase activity and comparison with the predicted amino acid sequence of liver LAL and fibroblast LAL [14] revealed two interesting features. First, the predicted N-terminus of the 56-kDa protein aligned with Ala7 of fibroblast acid lipase [14]. This indicated different N-termini for hepatic and fibroblast acid lipases, with resulting signal peptides of 27 and 21 amino acids for liver and fibroblast LAL, respectively. Second, the N-terminus of the 41-kDa LAL form aligned with Gly50 of the predicted LAL protein. Since the 41-kDa protein was catalytically active at high specific activity, non-specific proteolytic degradation during purification of the enzyme removing the N-terminal sequences seems unlikely, although not impossible. The more likely explanation is that the 56-kDa LAL form constitutes a precursor protein which is subject to post-translational processing. Cleavage of the 56-kDa precursor lipase carboxyl to Lys49 would then remove 49 N-terminal amino acids and yield a mature liver LAL of 323 amino acids. Studies of α -fucosidase [38], cathepsin D [39], and β -glucuronidase [40] have demonstrated an important role for a limited N-terminal proteolysis during lysosomal processing of these enzymes. Determination of whether C-terminal processing also contributes to the maturation of liver LAL, as has been demonstrated for acid phosphatase [41], will have to await C-terminal sequencing of purified LAL.

Deficient activity of LAL is associated with two inherited disorders of cholesterol metabolism, Wolman disease and cholesteryl ester storage disease [7]. Clinical and biochemical studies have delineated remarkable differences in the phenotype of the two lysosomal storage disorders with an as

yet undetermined molecular basis. In Wolman disease, LAL deficiency results in a striking overload with cholesteryl esters and neutral triacylglycerols of various tissues, most notably the liver and spleen [6]. Affected infants show massive hepatosplenomegaly and adrenal calcifications, and death usually occurs within the first 12 months of life [7]. Cholesteryl ester storage disease is characterized by a more benign clinical course; the enzyme deficiency frequently remains unrecognized until adulthood when hepatomegaly and mild hypercholesterolemia are detected [8, 9]. Most affected patients seem to be at an increased risk of developing premature atherosclerosis [7]. A critical role of LAL in the development of atherosclerosis is also supported by studies of patients with premature coronary artery disease. A significantly reduced acid lipase activity has been observed in peripheral mononuclear leukocytes of these patients [42]. These findings provide evidence that low activity of LAL may promote atherosclerotic vascular disease and may thus represent an independent risk factor in the pathogenesis of premature atherosclerosis [42, 43].

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The Role of Mannosylated Enzyme and the Mannose Receptor in Enzyme Replacement Therapy

Hong Du,¹ Mark Levine,³ Chandrashekar Ganesa,³ David P. Witte,² Edward S. Cole,³ and Gregory A. Grabowski¹

¹Division and Program in Human Genetics and ²Division of Pathology, Cincinnati Children's Hospital Research Foundation, Cincinnati; and ³Genzyme, Cambridge, MA

Lysosomal acid lipase (LAL) is the critical enzyme for the hydrolysis of triglycerides (TGs) and cholesteryl esters (CEs) in lysosomes. LAL defects cause Wolman disease (WD) and CE storage disease (CESD). An LAL null (*lal*^{-/-}) mouse model closely mimics human WD/CESD, with hepatocellular, Kupffer cell and other macrophage, and adrenal cortical storage of CEs and TGs. The effect on the cellular targeting of high-mannose and complex oligosaccharide-type oligosaccharide chains was tested with human LAL expressed in *Pichia pastoris* (phLAL) and CHO cells (chLAL), respectively. Only chLAL was internalized by cultured fibroblasts, whereas both chLAL and phLAL were taken up by macrophage mannose receptor (MMR)-positive J774E cells. After intraperitoneal injection into *lal*^{-/-} mice, phLAL and chLAL distributed to macrophages and macrophage-derived cells of various organs. chLAL was also detected in hepatocytes. Ten injections of either enzyme over 30 d into 2- and 2.5-mo-old *lal*^{-/-} mice produced normalization of hepatic color, decreased liver weight (50%–58%), and diminished hepatic cholesterol and TG storage. Lipid accumulations in macrophages were diminished with either enzyme. Only chLAL cleared lipids in hepatocytes. Mice double homozygous for the LAL and MMR deficiencies (*lal*^{-/-}; *MMR*^{-/-}) showed phLAL uptake into Kupffer cells and hepatocytes, reversal of macrophage histopathology and lipid storage in all tissues, and clearance of hepatocytes. These results implicate MMR-independent and mannose 6-phosphate receptor-independent pathways in phLAL uptake and delivery to lysosomes in vivo. In addition, these studies show specific cellular targeting and physiologic effects of differentially oligosaccharide-modified human LALs mediated by MMR and that lysosomal targeting of mannose-terminated glycoproteins occurs and storage can be eliminated effectively without MMR.

Introduction

Lysosomal acid lipase (LAL) hydrolyzes cholesteryl esters (CEs) and triglycerides (TGs) that are delivered to the lysosomes by receptor-mediated endocytosis (Goldstein et al. 1975). Mutations in the LAL gene cause two distinct phenotypes, Wolman disease (WD [MIM +278000]) and CE storage disease (CESD [MIM +278000]) (Assmann and Seedorf 2001). WD is a rare infantile-onset disorder, with an incidence of <1/300,000 live births (Meikle et al. 1999). Infants with WD display hepatosplenomegaly with massive accumulation of macrophages filled with CEs and TGs in the liver, spleen, and other organs. In addition, accumulation of CEs in the zona reticularis of the adrenal gland leads to adrenal calcification and insufficiency (Assmann and Seedorf

2001). Patients with WD develop cachexia from malabsorption related to the accumulation of engorged macrophages in the villi of the small intestine (Assmann and Seedorf 2001). The life span of patients with WD is ~6 mo. Bone marrow transplantation has had only minimal therapeutic impact (Krivit et al. 2000). CESD is a later-onset disorder and has been reported more frequently than WD. The incidence rate is unknown, but the disorder appears to be 2–3 times more common than WD. Hepatomegaly can be the only clinical manifestation. This results primarily from the accumulation of macrophages engorged with CEs (Beaudet et al. 1977). Some patients progress to develop cirrhosis, cholestasis, and atherosclerosis due to impaired cholesterol and TG homeostasis (Beaudet et al. 1977).

Deletions, insertions, or nonsense mutations in the LAL locus lead to WD with very low or absent LAL activity (Grabowski et al. 2003). In comparison, mutations found in patients with CESD have been missense changes or deletions localized to exon 10. These lead to small deletions in the C-terminus of the LAL protein (Grabowski et al. 2003). The milder CESD phenotypes correlate with the presence of detectable amounts of re-

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Address for correspondence and reprints: Dr. Gregory A. Grabowski, Cincinnati Children's Hospital Research Foundation, Division and Program in Human Genetics, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail: greg.grabowski@cchmc.org

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sidual enzyme activity (Aslanidis et al. 1996; Redonnet-Vernhet et al. 1997). Although bone marrow transplantation has been used for WD therapy, therapeutic efforts for CESD have focused on diminishing the accumulation of CEs within the liver and spleen by use of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors. Some effects on lipoprotein metabolism have been observed, but no demonstrable phenotypic or outcome effects have been reported consistently for these agents (Ginsberg et al. 1987).

An LAL null mouse model ($lal^{-/-}$) was created by gene targeting (Du et al. 1998a). The $lal^{-/-}$ mice have age-dependent progressive accumulation of CEs and TGs in the liver, spleen, small intestine, adrenal gland, and lymph nodes. The liver, small intestines, and lymph nodes are engorged with foamy macrophages filled with CEs and TGs. By age 6 mo, the $lal^{-/-}$ mice also show complete loss of white and brown adipose tissue throughout the body (Du et al. 2001a). These mice have a condition biochemically similar to WD but have survived into early adulthood (~8 mo life span).

Enzyme and gene strategies have been tested for therapeutic effects in the $lal^{-/-}$ mouse model (Du et al. 2001b, 2002). Human LAL (hLAL) produced in *Pichia pastoris* (phLAL) has shown promising results. Peritoneal administration of phLAL into $lal^{-/-}$ mice resulted in significant reductions of hepatomegaly and lipid storage in the liver, spleen, and small intestine. Interestingly, the reduction of lipid storage in the liver was primarily in Kupffer cells, whereas the lipid storage in the hepatic parenchymal cells was mostly unchanged (Du et al. 2001b). Such an effect might be expected from phLAL, because glycoproteins produced in this yeast have α -mannosyl-terminated N-linked oligosaccharides (Trimble et al. 2004). Adenoviral gene therapy in $lal^{-/-}$ mice was tested by single intravenous injections of recombinant virus containing the hLAL cDNA. The hLAL was produced primarily in the liver, with secretion into the bloodstream and uptake by visceral tissues (Du et al. 2001b). Significant corrections of lipid storage in the liver (Kupffer cells and hepatocytes), spleen, and small intestine were observed by histological and biochemical analyses (Du et al. 2002).

Here, the cellular targeting and physiologic effects of phLAL and of hLAL expressed in CHO cell lines (chLAL) are compared in $lal^{-/-}$ mice with and without intact macrophage mannose receptors (MMRs). Administration of phLAL or chLAL to $lal^{-/-}$ mice with intact MMR reduced lipid storage in macrophages. chLAL also reduced lipid storage in hepatocytes. In mice with both LAL and MMR disrupted, phLAL produced improvements in hepatocytes and visceral macrophages. These results provide insights into the effects of differential carbohydrate modifications on cellular targeting

and biological activity, as well as the redistribution of glycoproteins by mechanisms other than mannose 6-phosphate (M6P) receptors and MMRs.

Material and Methods

Animals

The mice were provided care in accordance with institutional guidelines, and all protocols were approved by the Institutional Animal Care and Use Committee at the Children's Hospital Research Foundation. The $lal^{-/-}$ mice originated from mixed genetic backgrounds of 129Sv, CF-1, and FVB (Du et al. 1998a) and were backcrossed to a FVB background for 10 generations. The $MMR^{-/-}$ mice were a gift from Dr. Michel Nussenzweig, a Howard Hughes Medical Institute Investigator at The Rockefeller University (Lee et al. 2002). The $lal^{-/-};MMR^{-/-}$ double-knockout mice were generated by crossbreeding of $lal^{-/-}$ and $MMR^{-/-}$ mice in a C57BL/6 background. Mice were housed in microisolators, under 12 h/12 h dark/light cycles. Water and food (regular chow diets) were available ad libitum. The mice were genotyped by PCR-based screening of tail DNA (Du et al. 1998a; Lee et al. 2002).

Study Design

Three cohorts of age-matched $lal^{-/-}$ mice received 79 μ g of phLAL, chLAL, or PBS injections. Two cohorts of age-matched $lal^{-/-};MMR^{-/-}$ mice received phLAL or PBS injections. Intraperitoneal (IP) injections were used in these studies, to mimic the slow infusion of enzyme replacement therapy in patients (Barton et al. 1991), rather than the intravenous boluses used in previous mouse studies. The injections of phLAL or chLAL were given to each mouse every 3 d for 30 d, for a total of 10 injections. Enzyme protein was quantified by immunoblotting assays (Du et al. 1998b). The $lal^{-/-}$ mice were harvested 2 d after the final injection, and tissues were processed for histologic, immunohistochemical, and biochemical analyses. phLAL activities were determined using 4-methylumbelliferyl-oleate (4-MUO) as substrate (Sheriff et al. 1995). All assays were conducted in duplicate. Assays were linear within the time frame used, and <10% of substrate was cleaved. One U is equal to 1 μ M of 4-MUO cleaved per min under standard assay conditions (Sheriff et al. 1995).

Enzyme Uptake Studies in J774E Macrophages and Human Fibroblast Cultures

J774E cells expressing MMR (from Dr. Philip Stahl at Washington University, St. Louis) were used previously for cellular uptake of phLAL (Du et al. 2001b). J774E cells were maintained in minimal essential medium with 6-thioguanine (60 μ M), and human fibro-

blasts were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, penicillin, and streptomycin (at 37°C; 5% CO₂). For the uptake studies, cells (2×10^5 per well) were seeded 1 d before the addition of pH₂LAL or chLAL. The cells were incubated in medium containing pH₂LAL or chLAL, with or without M6P, for 24 h and then in fresh media without LAL and were harvested at 48 h. The cells were washed twice with $1 \times$ PBS, were collected with a cell scraper, and were centrifuged (at 12,000 rpm for 1 min) at room temperature. The intracellular proteins were extracted by cell lysis (10 mM NaPO₄, 1 mM EDTA, 10 mM dithiothreitol, and 0.5% NP-40; pH 6.8) with sonication and were centrifuged (at 12,000 rpm for 10 min) at 4°C. The protein extracts were analyzed by western blot.

Immunohistochemical Staining and Western Blot Analyses

Immunohistochemical analyses of paraffin-embedded tissue sections were performed as described elsewhere (Du et al. 2001b). From the VECTASTAIN ABC kit (Vector), a second antibody to rabbit anti-hLAL antiserum (Du et al. 1996) was used and counterstained with Nuclear Fast Red. Immunoblots were conducted with anti-hLAL antiserum, as described elsewhere (Du et al. 2001b). The intracellular form of hLAL on western blots was evaluated by densitometry scanning (Pharmacia LKB Ultrascan XL).

Tissue Lipid Analyses

Total lipids were extracted from liver, spleen, and small intestine by the Folch method (Folch et al. 1957; Du et al. 1998a). TG and cholesterol amounts were measured by chemical analysis (Rudel and Morris 1973; Du et al. 2001a).

Histologic Analyses

Light-microscopic examinations of the liver, spleen, intestine, and adrenal glands were performed. The paraffin-embedded sections were stained with hematoxylin and eosin (H+E) for light-microscopic analysis. Representative sections are presented.

Monosaccharide Composition Analyses

The monosaccharide composition of the oligosaccharide structures in LAL samples was determined by acid hydrolysis. Purified pH₂LAL (80 µg) was incubated with 250 µl of 2-M trifluoroacetic acid at 120°C for 2 h. After hydrolysis, the reaction tubes were cooled on ice, were centrifuged (at 10,000 rpm for 1 min), and were evaporated to dryness in a vacuum centrifuge. The released monosaccharides were separated by high-pH

anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) on a Dionex CarboPac PA1 (4 × 250 mm) column equilibrated and run in 24 mM sodium hydroxide at a flow rate of 1 ml/min for 20 min. The results were expressed as mole of sugar per mole of LAL and were determined from linear curves of authentic, commercially available monosaccharide standards (E Y Laboratories).

M6P Content

The M6P content of the oligosaccharide structures in LAL samples were determined by acid hydrolysis. pH₂LAL (80 µg) was incubated with 6.75 M formic acid at 100°C for 1.5 h. After incubation, samples were cooled, dried in a vacuum centrifuge, and reconstituted in distilled water (200 µl). The released M6P was analyzed by HPAEC-PAD on a Dionex CarboPac PA10 (4 × 250 mm; Dionex) column by use of a 170–400-mM sodium acetate gradient in 100 mM sodium hydroxide at a flow rate of 1 ml/min for 15 min. The results were expressed as mol of M6P per mol of LAL and were determined from linear curves of authentic, commercially available M6P standards.

Sialic Acid Content

The sialic acid content of the oligosaccharide structures in LAL samples was determined by mild acid hydrolysis. LAL (50 µg) was incubated in 0.5 M formic acid (at 80°C for 60 min). The released sialic acids were separated by HPAEC-PAD on a Dionex CarboPac PA1 (4 × 250 mm) column by use of a 50–180-mM sodium acetate gradient in 100 mM sodium hydroxide at a flow rate of 1 ml/min for 20 min. The results were expressed as mol of sialic acid per mol of LAL and were determined from linear curves of authentic, commercially available sialic acid standards (Toronto Research).

Oligosaccharide Profiling

The N-linked oligosaccharides were released from chLAL and pH₂LAL samples by use of 0.2% (6 U/mg) of PNGase F and 2-mercaptoethanol (57.2 mM) in sodium phosphate, pH 7.0, at 37°C for 18–20 h. To separate the deglycosylated protein from the released oligosaccharides, the digests were centrifuged (at 14,000 rpm for 30 min) using a 10-kDa molecular weight–cutoff regenerated cellulose membrane spin-filters. The filtrates containing the oligosaccharides were dried in a vacuum centrifuge for at least 4 h. The oligosaccharides were reconstituted in 500 µl of high-performance liquid chromatography (HPLC)–grade water and were dialyzed using a 1-kDa molecular weight–cutoff cellulose acetate membrane against sodium phosphate, pH 7.0, at 25°C for 18–20 h. The dialysate was dried in a vacuum centrifuge, was reconstituted in 50 µl HPLC-grade water,

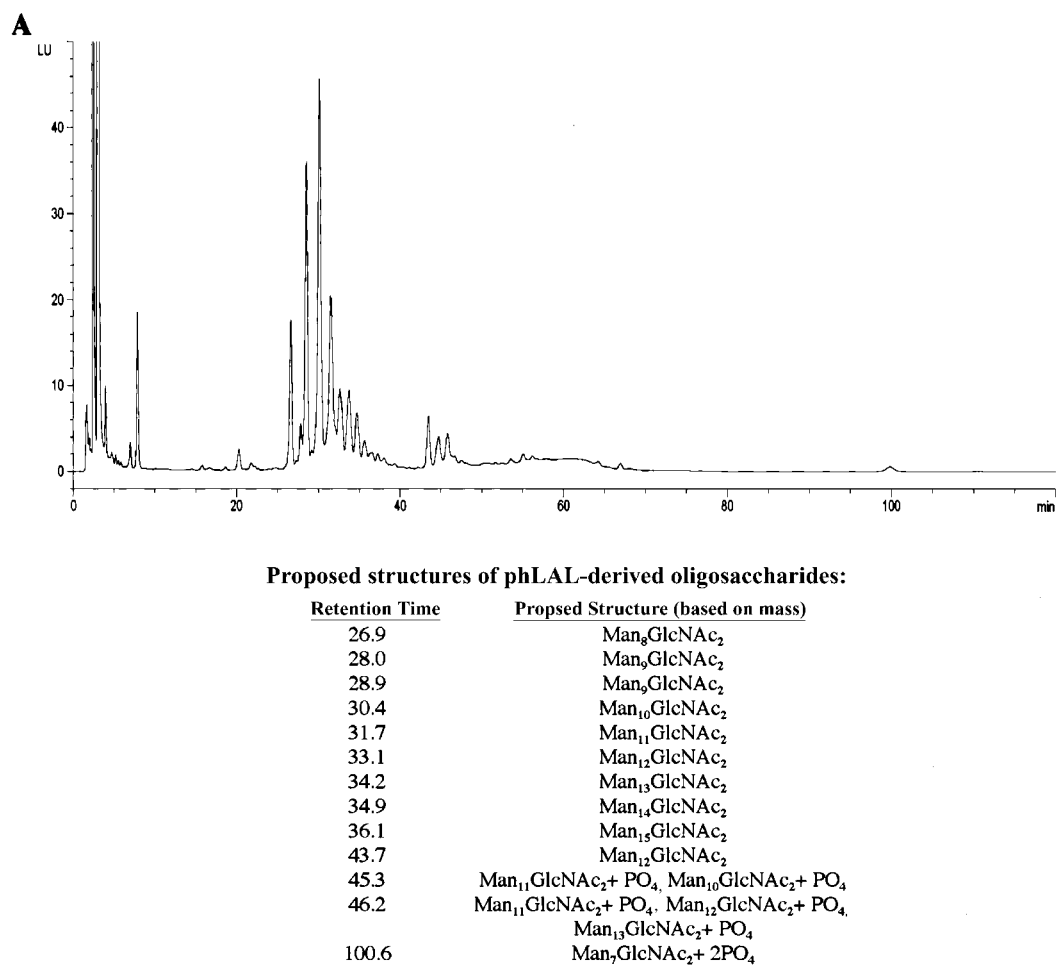


Figure 1 HPLC profiles of AA-derivatized pH₂LAL-derived (A) and chLAL-derived (B) oligosaccharides. The proposed structures of oligosaccharides are listed below each respective graph. LU = Luminescence units.

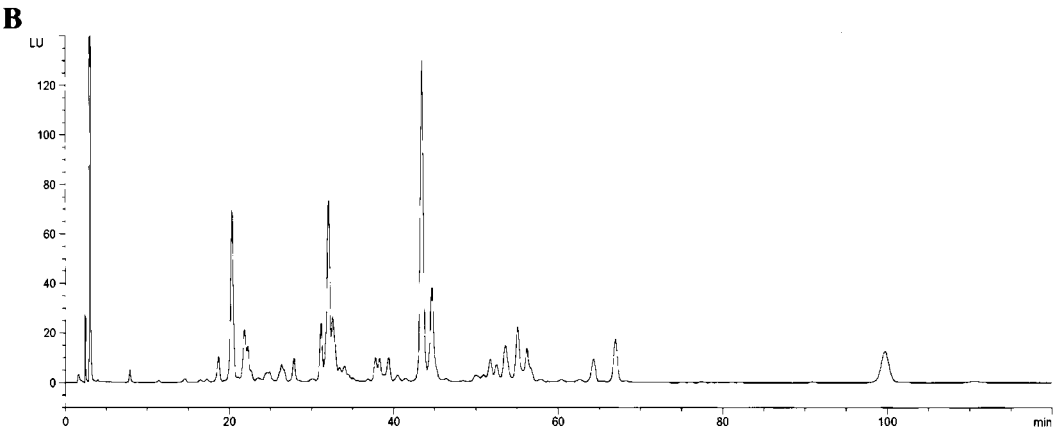
and was labeled with anthranilic acid (Van Dongen et al. 1984).

The oligosaccharides were derivatized using the protocol described by Anumula and Dhume (1998). In brief, the released oligosaccharides were resuspended in 0.1 ml HPLC-grade water and were labeled with 0.1 ml of labeling solution (30 mg anthranilic acid and 25 mg sodium cyanoborohydride in 1 ml of 4% sodium acetate and 2% boric acid in methanol) at 80°C for 75 min. After the labeling, the reactions were quenched with 1 ml of 95% acetonitrile/5% water and were loaded onto the Oasis HLB cartridges (Waters Corporation). The excess reagents were removed using 2 × 1-ml washes of 95% acetonitrile/5% water. Derivatized oligosaccharides were eluted using 0.5 ml of 20% acetonitrile/80% water, were dried, and were resuspended in 125 μl of 20% acetonitrile prior to HPLC analyses.

The AA-labeled oligosaccharides (100 μl sample/injection) were separated on a Luna NH₂ column

(4.6 × 250 mm; Phenomenex) by use of an HP1100 system equipped with a fluorescent detector (peak excitation wavelength at 230 nm; peak emission wavelength at 425 nm). The column was equilibrated in 70% solvent A (2% acetic acid and 1% inhibited tetrahydrofuran in acetonitrile), and the AA-labeled oligosaccharides were eluted at 50°C by use of a gradient consisting of 30%–95% of solvent B (5% acetic acid, 3% triethylamine, and 1% inhibited tetrahydrofuran in water) at a flow rate of 1 ml/min for 80 min. The gradient was held at 95% solvent B for an additional 38 min, to elute the bisphosphorylated species.

The HPLC chromatographic eluant containing the AA-derivatized peaks were split after column separation (~1:50) and were directly analyzed by electrospray ionization (ESI) mass spectrometry on a ZQ4000 mass spectrometer (Waters Corporation). The parameters on the mass spectrometer were capillary voltage at 3.0 kV and cone voltage at –30 V. The negative-ion ESI mass spec-



Proposed structures of chLAL-derived oligosaccharides:

Retention Time	Proposed Structure (based on mass)
18.9	Man ₃ GlcNAc ₂ + Fucose
20.5	Man ₃ GlcNAc ₂ , Man ₃ GlcNAc ₄ Gal + Fucose
22.2	Man ₃ GlcNAc ₄ Gal ₂ + Fucose
22.7	Man ₇ GlcNAc ₂
25.5	Man ₇ GlcNAc ₂
26.5	Man ₈ GlcNAc ₂
28.3	Man ₈ GlcNAc ₂
31.5	Man ₃ GlcNAc ₄ Gal + Fucose + Sialic acid (NeuAc)
32.3	Man ₃ GlcNAc ₄ Gal ₂ + Fucose + Sialic acid (NeuAc) , Man ₃ GlcNAc ₄ Gal + Sialic acid (NeuAc)
34.1	Man ₃ GlcNAc ₂ Gal + Fucose + Sialic acid (NeuAc), Man ₃ GlcNAc ₅ Gal ₃ + Fucose + Sialic acid (NeuAc)
43.7	Man ₃ GlcNAc ₄ Gal ₂ + Fucose + Sialic acid (NeuAc ₂)
44.9	Man ₃ GlcNAc ₄ Gal ₂ + NeuAc ₂
52.1	Man ₇ GlcNAc ₃ + PO ₄
52.6	Man ₈ GlcNAc ₃ + PO ₄
53.9	Man ₈ GlcNAc ₂ + PO ₄
55.4	Man ₃ GlcNAc ₅ Gal ₃ + Fucose + Sialic acid (NeuAc ₃) , Man ₆ GlcNAc ₂ + PO ₄
56.4	Man ₃ GlcNAc ₅ Gal ₃ + Fucose + Sialic acid (NeuAc ₃)
67.3	Man ₆ GlcNAc ₃ Gal + Fucose + Sialic acid (NeuAc) + PO ₄
99.4	Man ₇ GlcNAc ₂ + 2PO ₄

tra were obtained over a mass range of 500–3,750 m/z at an interval of 5 s per scan. The resulting mass spectra were obtained by summing at least six scans.

Results

Expression of phLAL and chLAL

phLAL was produced in *P. pastoris* by use of the yeast GAPDH promoter and the α factor signal peptide. chLAL was produced in a CHO cell line by use of the mouse metallothionine promoter and the hLAL endogenous leader sequence. phLAL and chLAL were secreted into culture media and were purified to 93%–96% electrophoretic purity as assessed by SDS-PAGE.

Oligosaccharide Profiling

The N-linked oligosaccharides from phLAL and chLAL samples were released using PNGase F, were fluorescently labeled with anthranilic acid, and were analyzed by liquid chromatography with online mass spectrometry. On the basis of mass:charge ratios, phLAL contained predominantly high-mannose structures: from Man₈GlcNAc₂ to Man₁₅GlcNAc₂. The most abundant oligosaccharide structures in phLAL were Man_{8–12}GlcNAc₂ (fig. 1A, retention time ~28–33 min). In CHO cells, secreted high-mannose N-glycans (Man₉GlcNAc₂ and smaller) are observed at low levels, since most of these structures typically get processed into Man₅GlcNAc₂ glycans during passage through the endoplasmic reticulum and Golgi compartments. In contrast to CHO cells, *P. pastoris* cells add mannose to this structure to produce high-mannose N-glycans, with up

to 15 mannose residues. However, in both phLAL and chLAL, some of the structures get modified by a GlcNAc-phosphotransferase, and the GlcNAc residues are subsequently removed, yielding phosphorylated structures. Some of the high-mannose structures in phLAL were either monophosphorylated (Man_{10–13}GlcNAc₂, retention times ~43–46 min) or bisphosphorylated (Man₇GlcNAc₂, retention times ~100 min).

The glycans from chLAL consisted of a mixture of complex (sialylated), oligomannose, phosphorylated oligomannose, and hybrid (sialylated and phosphorylated) structures (fig. 1B). The presence of galactose, sialic acid, and M6P in the carbohydrate composition assay confirms these glycan structures. The phosphorylated structures in chLAL were mainly Man_{6–7}GlcNAc₂, some of which still had the additional GlcNAc residue from the transferase activity. In phLAL and chLAL, compositionally identical glycans eluted at different retention times during the chromatographic separation—for example, in phLAL, Man₉GlcNAc₂ eluted at ~28–29 min, and, in chLAL, Man₇GlcNAc₂ eluted at ~23–26 min. This may be because of the different isomeric forms of the glycan, which would have the same mass:charge ratios in this assay.

Monosaccharide Composition and Sialic Acid and M6P Content of phLAL and chLAL Oligosaccharides

The oligosaccharide compositions of phLAL and chLAL were analyzed after acid hydrolysis and separation by HPAEC-PAD (table 1). Monosaccharide compositional analyses indicated that chLAL and phLAL contained ~10 mol of GlcNAc per mol of LAL, suggesting that five of six potential N-linked oligosaccharide sites are occupied. The number of experimentally determined mannose residues (~10) does not correlate with the number predicted for five occupied sites (15 mannoses would be expected if all were complex oligosaccharides). The less-than-expected mannose results may be because of the fact that the monosaccharide compositional analysis is highly variable. Also, the monosaccharide/protein (M/M) results differ from the theoretical values calculated from the actual identified oligosaccharide structures. The chLAL sample had higher amounts of fucose and galactose, corroborating the presence of complex-type structures. The phLAL sample had higher amounts of mannose that correlated with either high-mannose or pauci-mannosyl structures

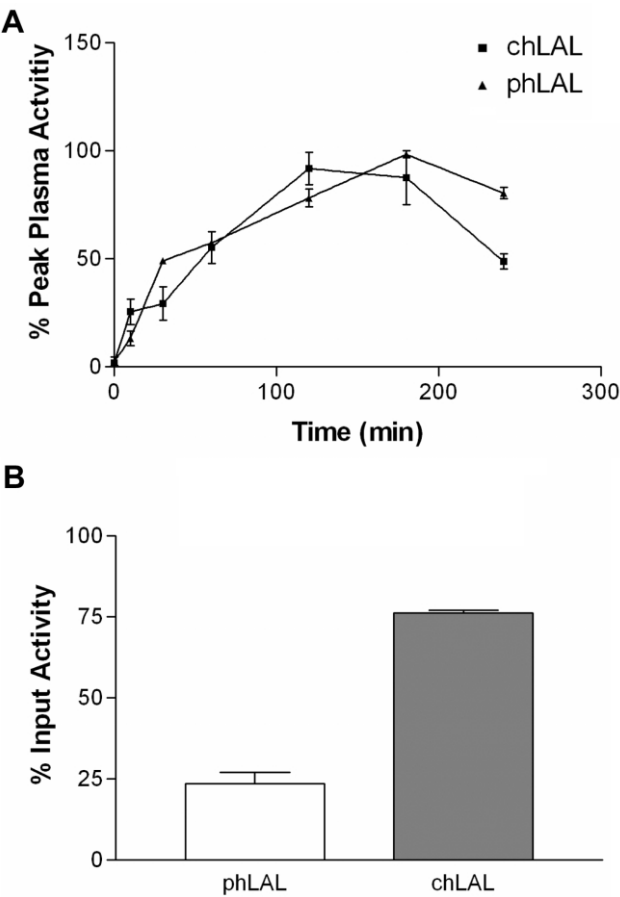


Figure 2 LAL activity in plasma (A) and LAL activity recovery in liver (B) of *lal*^{-/-} mice that received IP injections of hLAL. Plasma was collected from phLAL- or chLAL-injected mice at 0, 10, 30, 60, 120, 180, and 240 min after injection. Livers were collected at 240 min after injection. LAL activities in plasma and liver extract were determined using 4-MUO as the substrate.

in *P. pastoris*-derived samples. The phLAL samples contained no galactose. Although the monosaccharide results indicated that phLAL contained minimal sialic acid, it is well understood that *P. pastoris*, like plants, does not possess the necessary enzymes (β 1,4 galactosyltransferases and α 2,3 sialyltransferases) to remodel N-linked glycosylation of oligosaccharide chains (Lerouge et al. 1998; Montesino et al. 1998). The lack of sialylated glycan structures was confirmed in the oligosaccharide profiling assay (see the “Oligosaccharide Profiling” section). Both phLAL and chLAL contained phosphoryl-

Table 1

Monosaccharide Composition and M6P and Sialic Acid Content (per mol of hLAL Protein)

Sample	Fucose	GlcNAc	Galactose	Mannose	GlcNAc/Mannose	M6P	Sialic Acid
phLAL	.6	10.0	0	212.0	.05	1.7	.5
chLAL	1.5	11.6	4.1	10.4	1.11	2.2	5.9

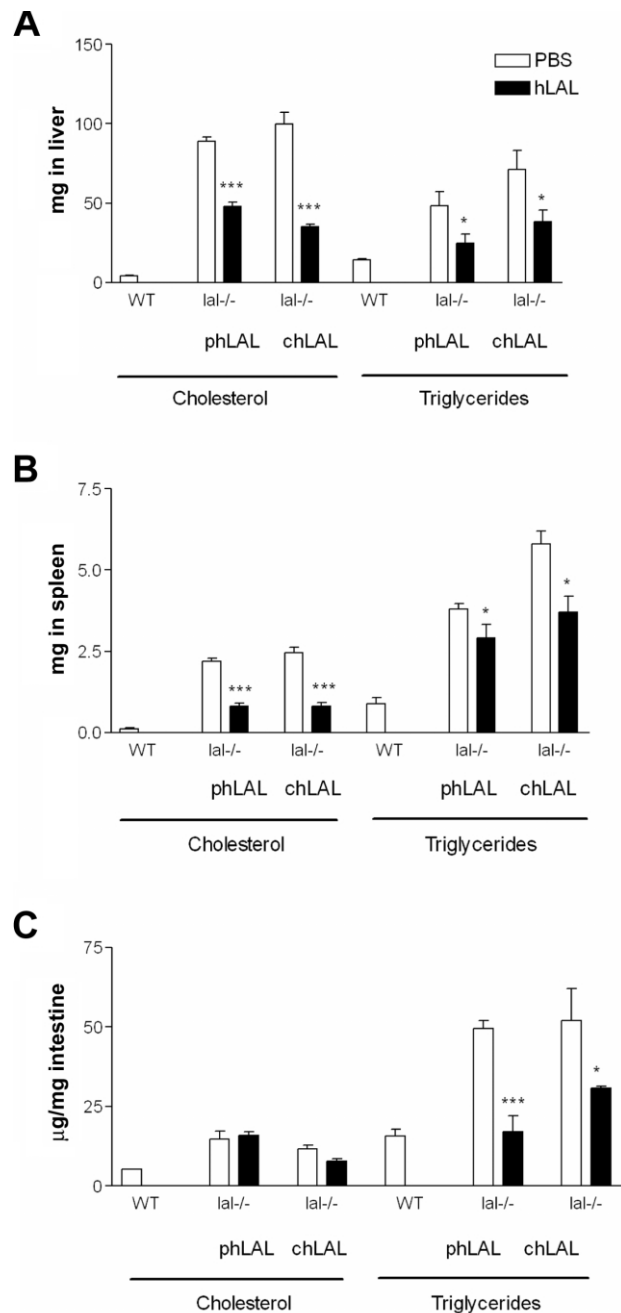


Figure 3 Tissue cholesterol and TGs in *lal*^{-/-} mice administered PBS or chLAL. Total cholesterol or total TGs per liver (A) or per spleen (B) and lipid concentration in intestine (C) was compared between PBS control mice and phLAL- or chLAL-injected mice (*n* = 6). The *P* values were from unpaired *t* tests between PBS and chLAL-treated samples. **P* < .05. ****P* < .001. WT = wild type.

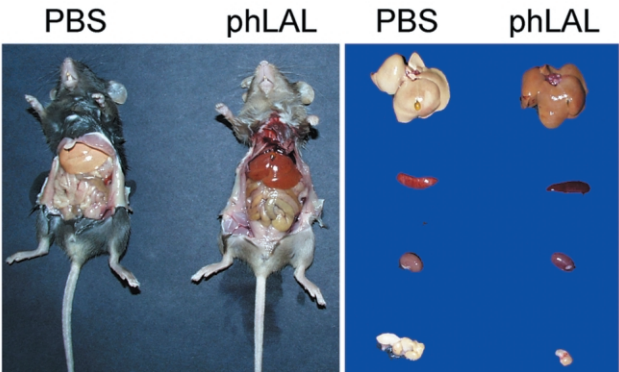


Figure 4 Gross pathology of age-matched PBS and phLAL-treated *lal*^{-/-};MMR^{-/-} mice. Ventral views (*left panel*) show the yellow fatty liver in PBS control *lal*^{-/-};MMR^{-/-} mice. In phLAL-treated *lal*^{-/-};MMR^{-/-} mice, the liver had essentially normal color. Gross view of livers, spleens, kidneys, and mesenteric lymph nodes (*right panel*, top to bottom) from PBS and phLAL-treated *lal*^{-/-};MMR^{-/-} mice. Notice that the color of these organs was nearly normal in phLAL-treated *lal*^{-/-};MMR^{-/-} mice.

ated oligomannosyl structures (1.7 and 2.2 mol M6P per mol protein, respectively). This amount of M6P is similar to values reported in the literature for other recombinant CHO-derived proteins that are taken up by the lysosomes via the M6P receptor system (Lee et al. 2003).

Plasma Profiles of Injected hLAL

The recovery of hLAL activity in plasma was determined after IP injection into *lal*^{-/-} mice with intact MMR and the sampling of blood at 0, 10, 30, 60, 120, 180, and 240 min after injection. The profiles (fig. 2A) of phLAL and chLAL in plasma were essentially identical for activities remaining in plasma from 15 to 240 min. The total LAL activity recovered in the liver at 4 h after injection was significantly greater for chLAL (76.3%) than for phLAL (23.5%) (fig. 2B).

Phenotypic and Gross Pathologic Changes

To evaluate the metabolic effects of each hLAL, *lal*^{-/-} mice were injected with PBS, phLAL, or chLAL (79 µg per dose) every third day over a 30-d period, for a total of 10 IP injections. phLAL was started at age 2 mo, and chLAL was started at age 2.5 mo. The PBS control group was staggered between the 2- and 2.5-mo-old mice. This small age difference does not produce a significantly different degree of disease involvement. Untreated *lal*^{-/-} mice developed a yellow coloration of the liver, as well as significant hepatosplenomegaly. The *lal*^{-/-} mice receiving phLAL or chLAL had livers with more normal color and weight (data not shown). The livers in age-matched wild-type mice were ~5% of body

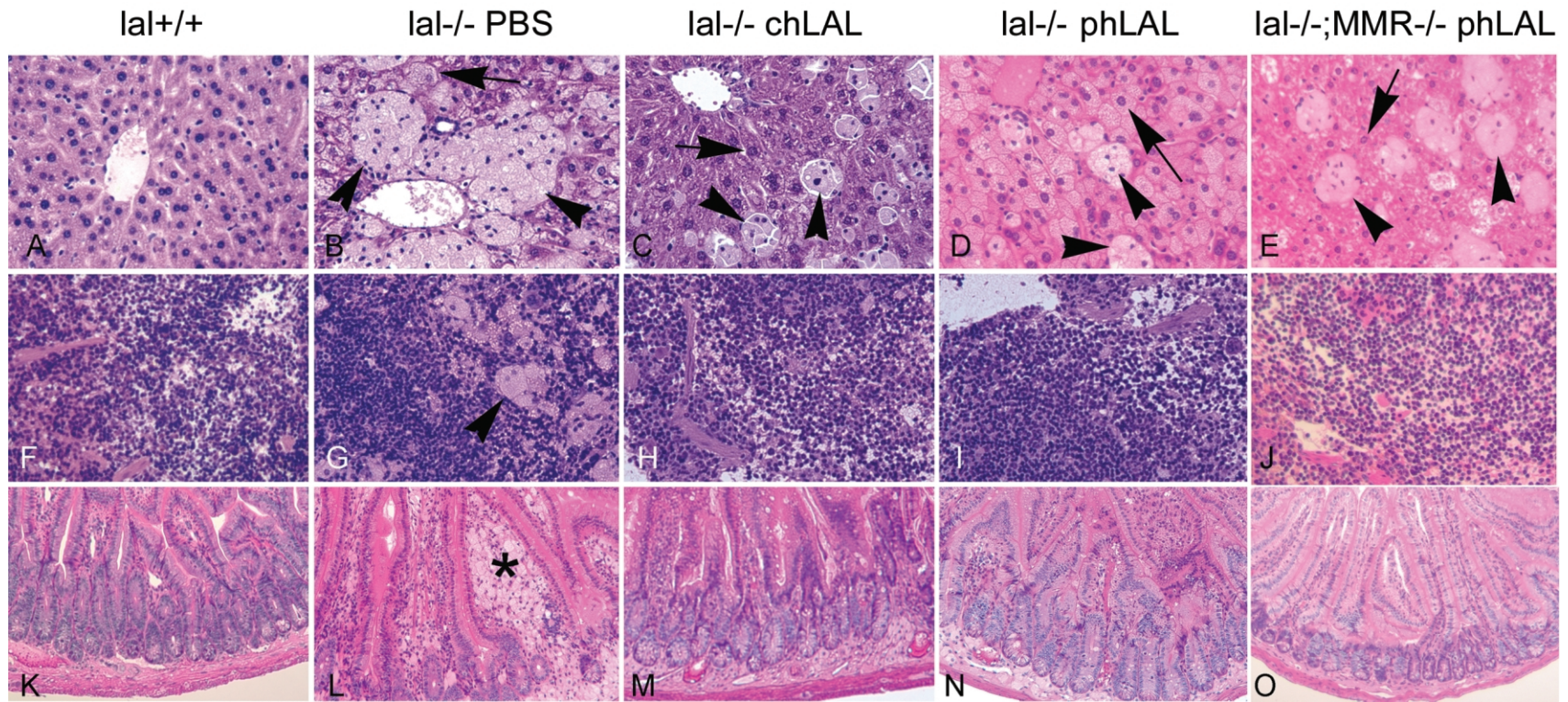


Figure 5 Histological analyses of wild-type, *lal*^{-/-};MMR^{+/+}, and *lal*^{-/-};MMR^{-/-} mice administered PBS, phLAL, or chLAL. Shown are representative H+E-stained tissue sections from age-matched wild-type (*lal*^{+/+}) mice (A, F, and K); from *lal*^{-/-};MMR^{+/+} mice injected with PBS (B, G, and L), chLAL (C, H, and M), or phLAL (D, I, and N); and from *lal*^{-/-};MMR^{-/-} mice treated with phLAL (E, J, and O). The tissues are liver (A–E), spleen (F–J), and small intestine (K–O). Arrows indicate hepatocytes, arrowheads indicate Kupffer cells, and the asterisk (*) indicates intestinal macrophages. The numbers of remaining Kupffer storage cells in the phLAL- or chLAL-treated mouse livers are similar, whereas the hepatocytes in the chLAL-treated *lal*^{-/-};MMR^{+/+} mice and the phLAL-treated *lal*^{-/-};MMR^{-/-} mice are essentially cleared of storage. Hepatocyte storage remained in the phLAL-treated *lal*^{-/-};MMR^{+/+} mice. In spleen and small intestine, the storage macrophages were nearly eliminated in all phLAL-treated mice. Original magnification, × 200.

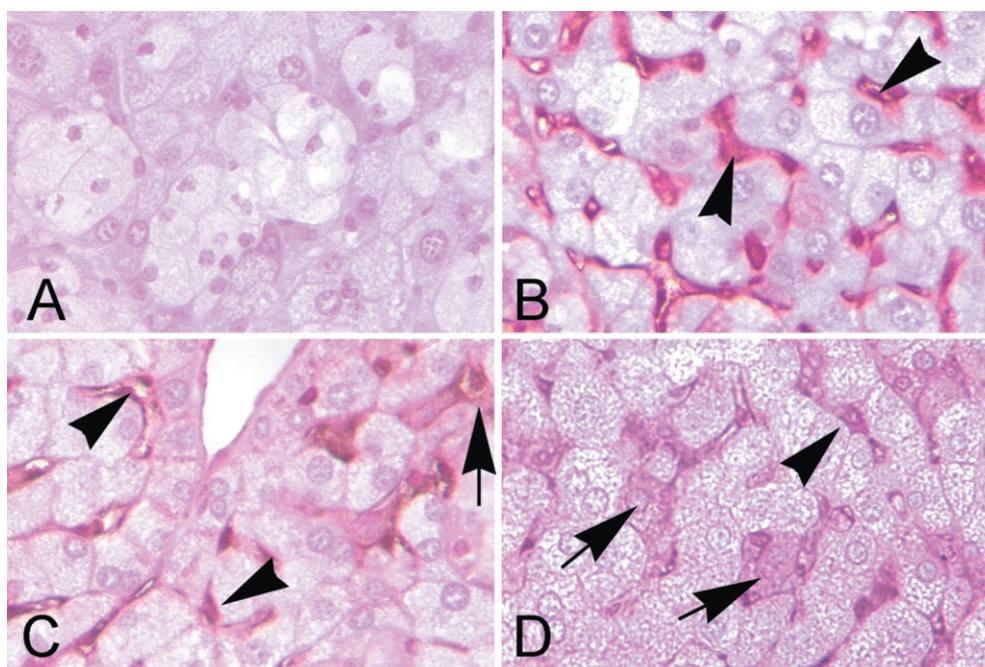


Figure 6 Anti-hLAL immunohistochemical staining of $lal^{-/-};MMR^{+/+}$ (A–C) and $lal^{-/-};MMR^{-/-}$ (D) mouse livers. $lal^{-/-};MMR^{+/+}$ mice were given IP injections of PBS (A), phLAL (B), and chLAL (C), and $lal^{-/-};MMR^{-/-}$ mice were given IP injections of phLAL (D). The amount of hLAL given to each mouse was 79 μ g. Mice were killed 4 h after injection. Liver and spleen were processed for immunohistochemical staining by use of anti-hLAL antibody. Positive signals were in Kupffer cells of the livers (B–D, arrowheads) and in hepatocytes (C and D, arrows). Original magnification, $\times 400$.

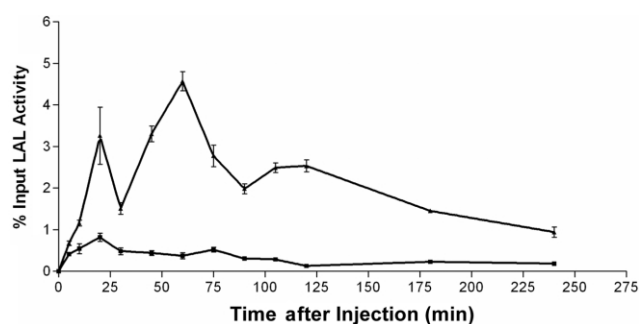


Figure 7 Comparison of plasma LAL activity between wild-type and $MMR^{-/-}$ mice after a single injected dose of hLAL. Age-matched wild-type (lower line) and $MMR^{-/-}$ (upper line) mice received a single dose (60 μ g phLAL per 25 g body weight) via IP injection. Plasma was collected from mice at 0, 5, 20, 30, 45, 60, 75, 90, 105, 120, 180, and 240 min after injection. LAL activities in plasma were determined using 4-MUO as the substrate. Plasma volume was calculated as 3.5% of body weight. The percentage activity remaining in plasma, with respect to input LAL activity, was plotted against time.

weight, whereas the livers in the untreated $lal^{-/-}$ mice were $\sim 14\%$. In phLAL- and chLAL-injected mice, the livers were 9.8% (reduced by 30%; $P = .0029$) and 7.7% (reduced by 45%; $P = .0001$) of body weight, respectively.

Biochemical Findings

Tissue cholesterol (free and esterified) and TGs from liver, spleen, and small intestine were evaluated in treated and untreated $lal^{-/-}$ mice (fig. 3). Administration of phLAL to $lal^{-/-}$ mice led to 46% reduction in total hepatic cholesterol (48.08 ± 2.67 mg [treated] vs. 89.07 ± 2.74 mg [untreated]; $P = .0003$; $n = 4$) (fig. 3A) and 63% reduction in total splenic cholesterol (0.80 ± 0.10 mg vs. 2.19 ± 0.10 mg; $P = .0008$; $n = 4$) (fig. 3B). Similar decreases in TGs were observed: 49% reduction in liver (24.84 ± 5.79 mg vs. 48.26 ± 8.97 mg; $P = .025$; $n = 4$) and 24% reduction in spleen (2.90 ± 1.43 mg vs. 3.80 ± 0.17 mg; $P = .042$; $n = 4$) (fig. 3A and 3B). Administration of chLAL to $lal^{-/-}$

mice resulted in reductions of total hepatic and splenic cholesterol by 65% (35.19 ± 1.55 mg vs. 99.94 ± 7.13 mg; $P < .0001$; $n = 6$) (fig. 3A) and 67% (0.80 ± 0.13 mg vs. 2.45 ± 0.17 mg; $P = .0012$; $n = 6$) (fig. 3B), respectively. The reduction of TGs was 47% in the liver and 36% in the spleen ($P = .040$ and $P = .017$, respectively) (fig. 3A and 3B). The cholesterol accumulation in small intestine was unchanged by phLAL or chLAL administration. However, the TG storage in small intestine was reduced by 66% and 41% with administration of phLAL and chLAL, respectively (fig. 3C). Lipids in the massively enlarged mesenteric lymph nodes were not significantly changed (3.72 ± 0.17 mg vs. 4.28 ± 0.7 mg for total cholesterol; 21.74 ± 5.59 vs. 16.53 ± 2.99 mg for TGs).

Effects of phLAL Injections into $lal^{-/-}$;MMR $^{-/-}$ Mice

To evaluate the role of MMR as a major receptor mediating the therapeutic effects of highly mannosylated phLAL, double-homozygous knockouts were generated for $lal^{-/-}$ and MMR $^{-/-}$. The phenotype of $lal^{-/-}$ mice was not altered in the background of MMR $^{-/-}$. Administration of phLAL (79 μ g per mouse, 10 IP injections given over 30 d) effectively corrected the phenotype in $lal^{-/-}$;MMR $^{-/-}$ mice. This included the correction of liver and spleen color and the reappearance of mesenteric fat

(fig. 4). The liver as a percentage of body weight was reduced in phLAL-treated $lal^{-/-}$;MMR $^{-/-}$ mice (11.1% in mice given PBS vs. 8.8% in mice given phLAL; -21%). Even more striking was the reduction in the size of mesenteric lymph nodes in the phLAL-treated mice compared with PBS control mice (0.273 g in control mice vs. 0.081 g in phLAL-treated mice; -70%)

Histologic Studies

Representative H+E-stained liver sections from $lal^{+/+}$ mice or $lal^{-/-}$ mice with or without MMR and obtained after PBS, chLAL, or phLAL administration are shown in figure 5. Regular phLAL injections led to major reductions in the size and number of lipid-filled Kupffer cells (arrowheads in fig. 5B and 5D) in $lal^{-/-}$;MMR $^{+/+}$ mice. In comparison, the lipid storage in hepatocytes appeared unaltered (arrows in fig. 5B and 5D). In the $lal^{-/-}$ mice treated with chLAL, a reduction was observed in the number and size of Kupffer storage cells, and lipid accumulation was eliminated from hepatocytes (arrow in fig. 5C). Interestingly, in $lal^{-/-}$;MMR $^{-/-}$ mice, injections of phLAL reduced the lipid storage in hepatocytes (arrows) and Kupffer cells (arrowheads) (compare fig. 5B and 5E). H+E staining of other tissues—that is, spleen and small intestine—indicated that phLAL and chLAL administration had similar effects in reducing

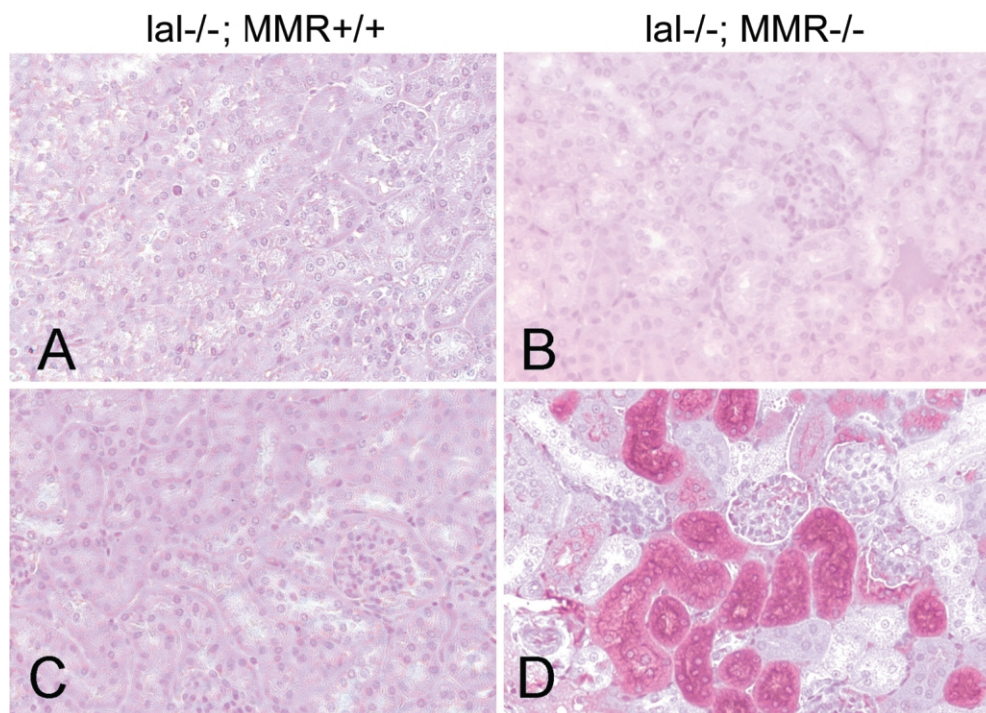


Figure 8 Anti-hLAL immunohistochemical staining of $lal^{-/-}$;MMR $^{+/+}$ (A and C) and $lal^{-/-}$;MMR $^{-/-}$ (B and D) mouse kidneys. Kidney sections from PBS-injected mice (A and B) and hLAL-injected mice (C and D) were processed for immunohistochemical staining by use of anti-hLAL antibody. Only $lal^{-/-}$;MMR $^{-/-}$ mice had positive uptake of hLAL in kidneys and only in the proximal renal tubular epithelial cells (D). Original magnification, $\times 200$.

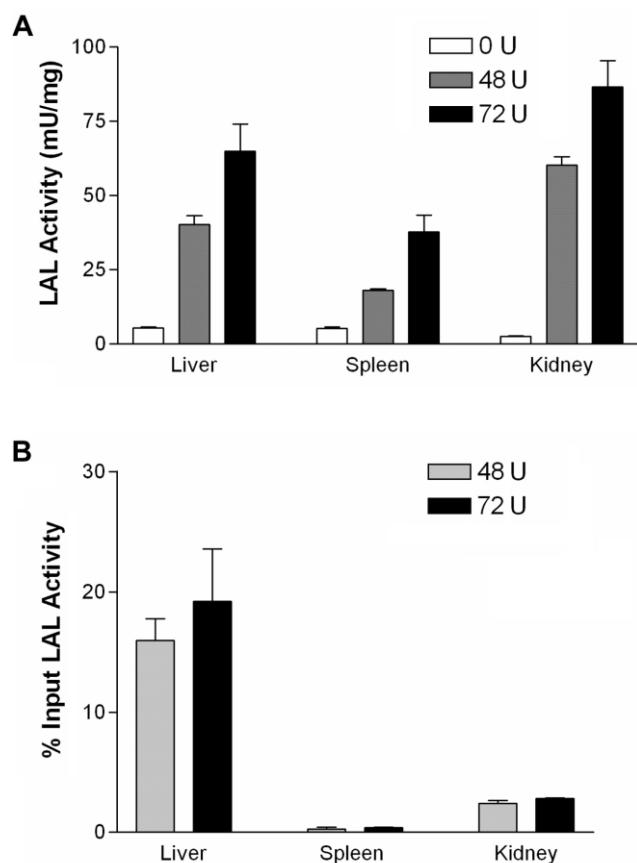


Figure 9 Tissues recovery of pHAL. A, LAL activity in tissue extracts of liver, spleen, and kidney from $lal^{-/-};MMR^{-/-}$ mice that received 48 U or 72 U of pHAL and were harvested 4 h after injection. B, LAL activity recovery in liver, spleen, and kidney as a percentage of total injected LAL activity in $lal^{-/-};MMR^{-/-}$ mice.

lipid-storage cells in $lal^{-/-};MMR^{+/+}$ and $lal^{-/-};MMR^{-/-}$ mice (fig. 5F–5J and 5K–5O). Similar to the results for pHAL, chLAL had no effect on lipid storage in lymph nodes and adrenal glands in $lal^{-/-};MMR^{+/+}$ mice (data not shown). Treatment with pHAL in $lal^{-/-};MMR^{-/-}$ mice had no histological effects on lipid storage in adrenal glands or lymph nodes, although there was a reduction in lymph node size (fig. 4).

Comparison of Cellular Uptake of pHAL and chLAL

To evaluate the cellular targeting of pHAL and chLAL in the $lal^{-/-};MMR^{+/+}$ and $lal^{-/-};MMR^{-/-}$ mice, livers were harvested and sectioned at 4 h after injection (79 μ g per mouse) and were processed for immunohistochemical staining with the use of anti-hLAL antibody. The majority of positive-staining liver cells after injection of either pHAL or chLAL was in sinusoid-lining cells (arrowheads, fig. 6B and 6C). Some hepatocyte staining was also positive in chLAL-injected mice (arrow, fig. 6C). Interestingly, positive staining for pHAL in the

liver of $lal^{-/-};MMR^{-/-}$ mice was less intense than that in livers of pHAL-injected $lal^{-/-};MMR^{+/+}$ mice. However, unlike injected $lal^{-/-};MMR^{+/+}$ mice, the $lal^{-/-};MMR^{-/-}$ mice had positive hLAL signals in both Kupffer cells and hepatocytes (arrowhead and arrows, respectively, in fig. 6D).

Plasma Pharmacokinetics of hLAL in $MMR^{+/+}$ and $MMR^{-/-}$ Mice

Since the deficiency of MMR in mice affects the uptake of multiple glycoproteins from plasma into tissues and organs (Lee et al. 2002), the pharmacokinetics of pHAL uptake was evaluated in $MMR^{+/+}$ and $MMR^{-/-}$ mice. After a single dose of pHAL, $MMR^{+/+}$ mice showed rapid uptake into tissues, with ~1% of injected LAL activity remaining in plasma after ~2 h. The $MMR^{-/-}$ mice displayed a complex plasma pharmacokinetic pattern, with two major peaks of activity at 15 and 60 min after injection (fig. 7). The clearance of LAL activity in plasma was very slow in $MMR^{-/-}$ mice, with significant enzyme activities remaining at 4 h after injection. To evaluate organ uptake of pHAL, immunohistochemical staining for hLAL was performed in $lal^{-/-};MMR^{+/+}$ and $lal^{-/-};MMR^{-/-}$ mice 4 h after injection. Interestingly, in addition to liver and spleen, the proximal renal tubular epithelial cells were strongly positive for LAL staining in the $lal^{-/-};MMR^{-/-}$ mouse kidney (fig. 8D). The kidneys were negative for hLAL staining after injection of pHAL into $lal^{-/-};MMR^{+/+}$ or of PBS into $lal^{-/-};MMR^{-/-}$ mice (fig. 8B and 8C). LAL activity in tissue extracts from $lal^{-/-};MMR^{-/-}$ mice indicated higher LAL-specific activity in the kidney than in the liver (fig. 9A), although the total recovery of pHAL was much less in the kidney than in the liver because of the size of the organs (fig. 9B). To further test whether the uptake of pHAL could be through the M6P receptor, pHAL and chLAL were incubated with human fibroblasts from an LAL-deficient patient or MMR-positive J774E cells. Human fibroblasts were negative for MMR and positive for M6P receptors. Uptake of chLAL occurred in MMR-positive J774E cells and M6P receptor-positive human fibroblasts (fig. 10A). The uptake of chLAL in fibroblasts can be competed with by M6P (fig. 10B). pHAL was internalized into J774E cells (Du et al. 2001b), but no uptake was observed in human fibroblasts (fig. 10A). The results show that the in vivo uptake of pHAL into a variety of cells can occur through receptors other than M6P receptors and MMRs.

Discussion

These data show differential glycosylation of IP-administered LALs that leads to specific physiologic and localization changes at a cellular level. These findings demonstrate that alternative oligosaccharide processing of

administered enzymes can lead to clear differential therapeutic effects at a cellular level. The presence of high mannose oligosaccharides on pHAL leads to preferential, if not exclusive, uptake by hepatic sinusoidal-lining cells, including Kupffer cells, and other visceral macrophages. This produced specific reductions of CE and TG storage in these cells of *lal*^{-/-};MMR^{+/+} mice. In comparison, the enzyme produced in CHO cells, chLAL, led to clearance of CEs and TGs in hepatocytes, Kupffer cells, other hepatic sinusoidal lining cells, and macro-

phages throughout the bodies of such mice. Importantly, equal doses of pHAL produced similar degrees of reduction in storage cells and lipids in the mice lacking both *lal* and MMR (i.e., *lal*^{-/-};MMR^{-/-} mice). However, the effects of pHAL in the background of MMR^{-/-} produced significantly different clearance effects in the liver but not in other tissues. In the *lal*^{-/-};MMR^{-/-} mice that received pHAL, the liver showed hepatocellular clearance in addition to significant reductions in lipid storage in Kupffer cells that were comparable to those in chLAL-treated *lal*^{-/-};MMR^{+/+} mice. The clearance of storage macrophages from the spleen and small intestine by pHAL was essentially complete in *lal*^{-/-};MMR^{-/-} and *lal*^{-/-};MMR^{+/+} mice. Thus, the lack of MMR altered the physiological effect of the exogenous enzyme-containing high-mannose residues to include a major effect on hepatocytes as well as macrophages. This is somewhat surprising in light of the delayed clearance of mannose-terminated glycoproteins in the MMR^{-/-} mice (Lee et al. 2002). The low-level presence of M6P receptors on macrophages may account for some uptake and effects of pHAL, but the completeness of response was greater than anticipated.

The glycoproteins produced by *P. pastoris* have either high-mannose or pauci-mannose structures that contain no galactose and terminal sialic acid residues (fig. 1). In comparison, the CHO cell-produced enzymes, chLAL and agalsidase β (Lee et al. 2003), have glycans that contain terminal sialic acid and uncapped galactose residues that would allow uptake into the lysosomes via the asialoglycoprotein receptor present on hepatocytes (Hudgin et al. 1974; Halberg et al. 1987). Indeed, the CHO cell-produced enzyme agalsidase β has an oligosaccharide content similar to that of chLAL (Lee et al. 2003). Significantly, both agalsidase β and chLAL were deficient in Man₇₋₁₀GlcNAc₂ structures and did not contain Man₁₀₋₁₅GlcNAc₂ structures. Also, pHAL had little fucosylation, compared with that of chLAL. The majority of pHAL oligosaccharides contained Man₈₋₁₅GlcNAc₂ structures and were similar to those observed in bile salt-stimulated lipase and other proteins produced in *P. pastoris* (Yamada et al. 1994; Kalidas et al. 2001; Hirose et al. 2002; Henriksson et al. 2003). The redistribution of pHAL in the MMR^{-/-} mice could be mediated through the M6P receptor but could also be mediated through other receptors or mechanisms in hepatocytes, such as pinocytosis (Lee et al. 2002). The presence of other mannose receptors with lower affinity for the structures in pHAL cannot be excluded.

The poor distribution of mannosylated BSA to liver and spleen in the MMR^{-/-} mice, along with retention in plasma, supported the MMR as the major controller of plasma levels of mannosylated glycoproteins (Lee et al. 2002). MMR was suggested to play a minor role in

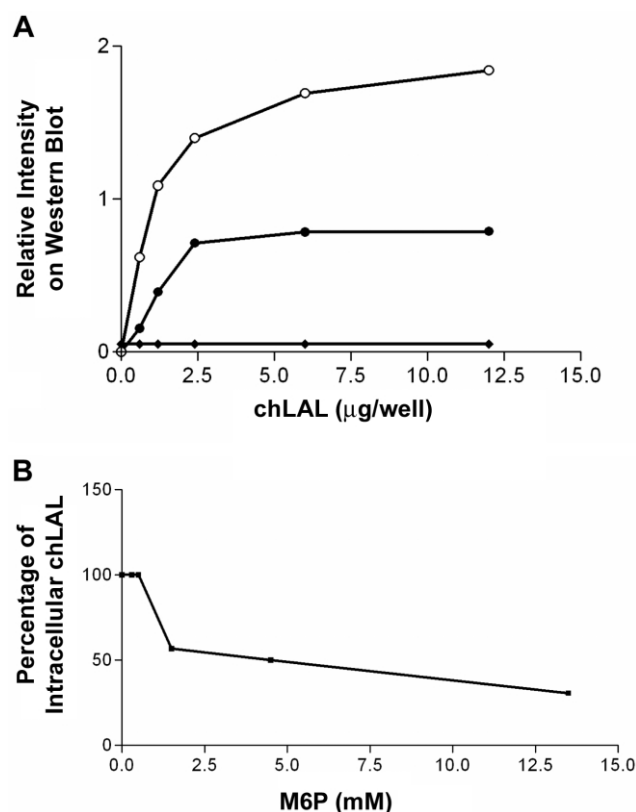


Figure 10 Cellular uptake of chLAL and pHAL in murine macrophages and human fibroblasts. **A**, Western blot analysis of cellular uptake of chLAL and pHAL. Varying amounts of chLAL (unblackened and blackened circles) or pHAL (diamonds) were placed in media surrounding murine macrophage J774E cells (unblackened circles) or human fibroblasts (blackened circles and diamonds) for 24 h. After the media was replaced with fresh media without supplemented LAL, the cells were harvested (at 48 h) and were processed for western blot analysis. The intracellular form of hLAL was evaluated by densitometry scanning. Uptake of chLAL occurred in mannose receptor-positive J774E macrophages (unblackened circles) and M6P receptor-positive human fibroblasts from an LAL-deficient patient (blackened circles). No uptake was observed in human fibroblasts incubated with pHAL (diamonds). **B**, Competition of cellular uptake of chLAL in human fibroblasts by M6P. Various amounts of M6P and 12 μg of chLAL were coincubated with human fibroblasts for 24 h. Cells were processed in the same way as described for panel A. The densitometry signal of intracellular hLAL at 0 mM of M6P was defined as 100%. Notice that ~1.5 mM of M6P inhibited ~50% of chLAL uptake in fibroblasts.

the control of intracellular glycoproteins, particularly lysosomal enzymes; however, measurements were made against a high background of various enzymatic activities, and physiologic roles and specific cellular targeting were not assessed. In contrast, in this case, the physiologic effects of pH-LAL in the *lal*^{-/-}; *MMR*^{-/-} background and the delivery of this enzyme to macrophages show that more complex mannosyl structures mediate a different distribution than monomannose-containing glycoproteins. The detection of pH-LAL in kidney-proximal tubule epithelial cells may well represent reabsorption of filtered hLAL as a result of the prolonged retention of the enzyme in plasma of these mice.

The physiologic effects of pH-LAL in the *lal*^{-/-}; *MMR*^{-/-} mice suggest the presence of other uptake mechanisms that can recognize mannosylated oligosaccharides for internalization to the hepatic parenchymal cell lysosome. This was evident by clearance of neutral lipid storage in hepatocytes by pH-LAL in these mice. Importantly, pH-LAL was taken up selectively by J774E cells that expressed MMR, whereas no uptake was noted in J774A cells that did not express MMR (Du et al. 2001b). Thus, additional receptors in natural macrophages in the liver, spleen, and small intestine could be present for uptake of the highly mannosylated pH-LAL. M6P receptors are present in liver parenchymal cells and might account for some of the uptake in the absence of MMR (Geuze et al. 1984). However, the lack of uptake of pH-LAL into human WD fibroblasts, which are positive for M6P receptors and negative for MMR, makes this unlikely (fig. 10C). Additionally, since the pH-LAL contains longer high-mannose oligosaccharides, the protein could interact with mannose-binding protein in the plasma, leading to subsequent binding of complement factors that stimulate uptake into macrophages as well as hepatocytes and hepatic endothelial cells (Matsuo et al. 1994).

These data demonstrate the physiologic importance of targeting glycoproteins via specific uptake mechanisms for a therapeutic effect on lysosomal storage. These effects are mediated preferentially by MMR, but additional uptake mediated through the M6P receptor and/or endocytosis or pinocytosis may be evident when the MMR is missing or blocked. Such observations may have direct relevance to the treatment of macrophage-storage diseases if the observation of downregulation of MMR in Gaucher storage cells (Boven et al. 2004) is consistent and is shown for other macrophage-storage diseases. Such observations would indicate a redirection to a different population of receptors and/or cells, leading to alternative uptake or differential uptake into various cell types, depending on the expression of MMR at particular stages of the disease process. Clearly, such pathophysiology requires elucidation for the improvement of therapy for patients.

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Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.gov/Omim/> (for WD and CESD)

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Cloning and Expression of cDNA Encoding Human Lysosomal Acid Lipase/Cholesteryl Ester Hydrolase

SIMILARITIES TO GASTRIC AND LINGUAL LIPASES*

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Richard A. Anderson^{‡§} and Gloria N. Sando[¶]

From the [‡]Molecular Genetics Program, Departments of Medicine and of Comparative Medicine, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27157 and the [¶]Department of Internal Medicine and Arteriosclerosis Specialized Center of Research, University of Iowa, Iowa City, Iowa 52242

Molecular cloning of a full-length cDNA for human lysosomal acid lipase/cholesteryl ester hydrolase (EC 3.1.1.13) reveals that it is structurally related to previously described enteric acid lipases, but lacks significant homology with any characterized neutral lipases. The lysosomal enzyme catalyzes the deacylation of triacylglycerol and cholesteryl ester core lipids of endocytosed low density lipoproteins; this activity is deficient in patients with Wolman disease and cholesteryl ester storage disease. Its amino acid sequence, as deduced from the 2.6-kilobase cDNA nucleotide sequence, is 58 and 57% identical to those of human gastric lipase and rat lingual lipase, respectively, both of which are involved in the preduodenal breakdown of ingested triglycerides. Notable differences in the primary structure of the lysosomal lipase that may account for discrete catalytic and transport properties include the presence of 3 new cysteine residues, in addition to the 3 that are conserved in this lipase gene family, and of two additional potential *N*-linked glycosylation sites. Transfection of the cDNA into Cos-1 cells resulted in the expression of acid lipase activity with the substrate range of the native enzyme at a level that was greater than 40 times the endogenous activity.

Lysosomal acid lipase/cholesteryl ester hydrolase is required for the breakdown of cholesteryl esters and triglycerides that cells acquire from receptor-mediated uptake of low density lipoprotein. The enzyme plays a key intracellular role in supplying cholesterol for cell growth and membrane function and in the regulation of processes that are mediated by cellular cholesterol flux, including internalization of low density lipoprotein and cholesterol biosynthesis and esterification (1). The low level of human lysosomal acid lipase (HLAL)¹ in

patients afflicted with the hereditary allelic lysosomal storage disorders Wolman disease (WD) and cholesteryl ester storage disease (CESD) is associated with massive intracellular storage of cholesteryl esters and derangements in the control of cholesterol production (2). The predisposition of CESD patients to the development of premature atherosclerotic disease (2) represents a link to more general clinical concerns involving the control of lipid metabolism and adds to evidence that supports a role for altered HLAL function as a determinant of atherosclerotic disease risk in the population at large (3–6).

Although the importance of lysosomal acid lipase activity in lipoprotein metabolism and in the determination of cell cholesterol levels has been recognized, very little is known about the enzyme's catalytic mechanism or regulation. The elucidation of the details of acid lipase cell biology and enzymology has been hindered by the difficulty of isolating and purifying sufficient amounts of this low abundance protein for biochemical analysis. Similar low levels of immunologically cross-reactive acid lipase activity are detectable, however, in most cell types including human fibroblasts, smooth muscle cells, and endothelial cells, as well as in established human cell lines such as the hepatocarcinoma cell line HepG2, the colon adenocarcinoma line CaCo-2, and the monocyte-macrophage lines U-937 and THP-1. We succeeded in purifying small amounts of the secreted form of HLAL, which had not been exposed to lysosomal processing, from fibroblast microcarrier spinner cultures (7). These nearly homogeneous preparations enabled the production of specific monoclonal antibodies and permitted a limited characterization of structural and functional properties of the enzyme. When the purified lipase was applied to HLAL-deficient WD or CESD fibroblasts in culture, it was taken up via the mannose 6-phosphate recognition system and packaged into lysosomes where it functioned to correct the abnormal cholesteryl ester accumulation in the diseased cells (7–9).

In order to produce a sufficient quantity of the enzyme for detailed analysis of its biochemical and physiological properties and to elucidate the molecular defect(s) that cause WD and CESD, the molecular cloning of HLAL was undertaken. There has been a preliminary report of the isolation of a partial length cDNA clone for HLAL by O'Brien *et al.* (10), but a further characterization has not appeared.

MATERIALS AND METHODS

HLAL Peptides—The enzyme was isolated from fibroblast secretions and purified through the hydroxylapatite step as previously described (7), then further purified using an immunoaffinity chro-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) M74775.

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¹ The abbreviations used are: HLAL, human lysosomal acid lipase/cholesteryl ester hydrolase; WD, Wolman disease; CESD, cholesteryl ester storage disease; PCR, polymerase chain reaction.

matography procedure with a monoclonal antibody²-directed against HLAL. Tryptic peptide sequences were determined at the Yale Protein and Nucleic Acid Chemistry Facility with the application of microsequencing techniques.

Polymerase Chain Reaction (PCR)—Avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories) with an oligo(dT) primer was used to synthesize first strand cDNA from 5 µg of total cellular RNA which had been isolated from U-937-cultured monocyte-macrophage cells using a guanidine isothiocyanate/CsCl protocol (11). A PCR reaction was performed using 8 ng of the first strand cDNA in a total volume of 40 µl with the buffer, salt, nucleotide, primer, and *Taq* polymerase concentrations suggested by the supplier of the enzyme (United States Biochemicals). The sequences of the primers used in the PCR amplification, GGAATTC(A/G)TG(G/C)AGCAT(A/G)TT(T/C)TG and GGAATTCAT(C/T)AA-(G/A)ATGTT(T/C)TT(T/C)GC, were derived by reverse translation of regions of the HLAL peptide sequences with the lowest number of codon degeneracies. These were synthesized with the inclusion of limited degeneracy after consulting species-specific, preferred codon usage tabulations. *Eco*RI restriction endonuclease recognition sequences were included to facilitate the insertion of amplified fragments into cloning vectors. After a denaturing step, 40 thermal cycles were programmed for 1 min at 94 °C, 2 min at 34 °C, and 2 min at 72 °C. Reactions were terminated and electrophoresed into a 1.5% low melting temperature agarose gel. An approximately 300-base pair band was detected by staining with ethidium bromide and excised. The fragment was further amplified by mixing 20 µl of the melted gel slice in a 100-µl PCR reaction as above, but with 2.5 mM MgCl₂ and 1.5 µM oligonucleotide primers. After another round of gel purification, the fragment was purified by phenol extraction, mixed with vector pIB130, digested with *Eco*RI, and ligated with T4 DNA ligase. Transformed colonies that represented recombinants were screened with nucleotide sequencing using [α -³²S]dATP in a double-stranded template version of the Sequenase dideoxy sequencing protocol (United States Biochemicals).

RNA Analysis—Messenger RNA was purified from HepG2 cells with a batch oligo(dT) cellulose procedure (Invitrogen), electrophoresed into a 1% agarose gel, and blotted onto a nylon membrane using standard techniques (12). The membrane was hybridized with the PCR-derived partial cDNA and then stripped and reprobed with an α -actin fragment from pHM α -1 (13). Both probes were labeled to a similar specific activity (2×10^8 dpm/µg) by incorporating [α -³²P]dCTP with a random priming protocol.

Library Screening—A stock of a human fibroblast cDNA library was provided by the laboratory of P. Berg, Stanford University (14). Colonies were screened at a density of 20,000/15-cm plate and transferred to duplicate nitrocellulose filters as described in standard protocols (12) for hybridization with the ³²P-labeled PCR-derived cDNA fragment. Nucleotide sequences of the inserts in the positive clones were determined from both directions with a series of oligonucleotide primers corresponding to previously sequenced regions of the insert.

Expression of HLAL cDNA—The full-length HLAL cDNA insert in the pcD vector was transfected into Cos-1 and Ltk⁻ cells using a standard calcium phosphate coprecipitation protocol (15) except that CsCl gradient-purified plasmid DNA was left on the cells for 14 h. The Cos-1 cell line (ATCC), derived from the established African green monkey kidney cell line CV-1, constitutively expresses an integrated copy of the SV40 T-antigen enabling replication of transfected pcD plasmid, which contains an SV40 viral replication origin, as well as an SV40 early promoter to drive over-expression of insert sequences (14). The Ltk⁻ cell line is a subclone of the established mouse L-cell fibroblast line. Ten µg of plasmid were added to cells which were initially 70% confluent in a 60-mm dish. The cells were allowed to express the transfected material for 24–72 h before analysis of intracellular and secreted acid lipase activity. The medium was aspirated and centrifuged to remove cell debris. The cells were harvested by scraping from the plates, rinsed in phosphate-buffered saline, and pelleted by a brief centrifugation. Extracts were prepared by sonication of cells in saline. Assays of acid lipase and β -hexosaminidase activity and of cell protein content were conducted as was previously described (7, 9). Acid lipase activity was determined with use of a fluorogenic substrate, 4-methylumbelliferyl oleate, and with the radiolabeled substrates, cholesteryl oleate and triolein, dispersed in bile salt and phospholipid.

RESULTS

As the initial step in our cDNA cloning strategy, we obtained amino acid sequences of three tryptic peptides from immunoaffinity purified lysosomal acid lipase. A significant finding was evident immediately when the sequences of the tryptic peptides (Fig. 1) were found to be 47 and 53%, 74 and 68%, and 73% each identical to the amino acid sequence of segments of the previously analyzed acid triacylglycerol hydrolases, human gastric lipase and rat lingual lipase, respectively (16, 17). Human gastric lipase (379 amino acids) and rat lingual lipase (377 amino acids) are glycosylated proteins similar in size to the ~43-kDa value determined for the secreted form of HLAL by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the protein following removal of high mannose oligosaccharides with endoglycosidase H (7).

The high level of sequence identity with the published human gastric lipase and rat lingual lipase sequences facilitated the construction of oligonucleotide primers for PCR by indicating the probable amino- to carboxyl-terminal orientation of the HLAL peptides. The amino acid sequence on which the forward primer was based, Ile-Lys-Met-Phe-Phe-Ala, came from the first tryptic peptide in Fig. 1, with the addition of an Ile-Lys element at the amino terminus based on their conservation in human gastric lipase and rat lingual lipase. That used for the reverse primer was Gln-Asn-Met-Leu-His from the second tryptic peptide. PCR reactions on first strand cDNA prepared from total cell RNA isolated from cultured U-937 human monocytes were performed using the degenerate sequence primers and low stringency annealing conditions. Although the particular primer molecules that were incorporated into the two separate recombinant plasmid clones that contained HLAL-coding material showed sequence variation, the sequences of the 274 nucleotides of included, PCR-amplified material were identical to each other. The deduced amino acid sequence encoded by the PCR fragment showed that the 13 amino acids adjacent to the primers at either end agreed with the amino acid sequence determined by Edman degradation of the HLAL tryptic peptides. When the nucleotide and deduced amino acid sequences of the amplified cDNA fragment were aligned with the published human gastric lipase and rat lingual lipase sequences using the HLAL tryptic peptide sequences as anchors, the fragment size was exactly that predicted based on the assumption that alignment was conserved. Ten additional recombinant plasmids resulting from the ligation reaction be-

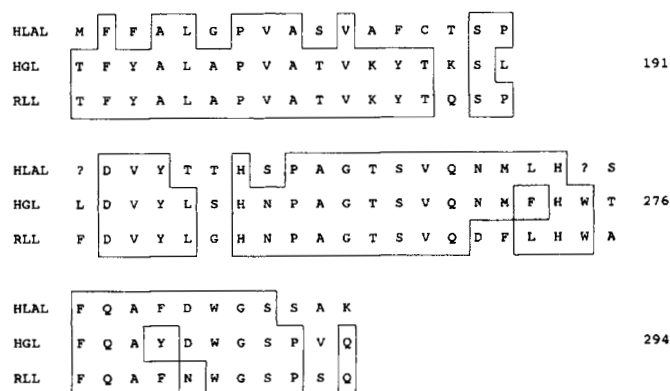


FIG. 1. Amino acid sequences of human lysosomal acid lipase/cholesteryl ester hydrolase tryptic peptides compared to regions in previously described enteric acid lipases. Numbers on the right refer to positions in the amino acid sequence of the mature human gastric lipase (HGL) protein. Sequence identities are boxes. RLL, rat lingual lipase.

² C. Dahle and G. N. Sando, manuscript in preparation.

tween the PCR products and vector DNA were screened; none showed any resemblance to acid lipase sequences or any meaningful length of any open reading frame beyond the primers.

An autoradiograph of a Northern blot of poly(A)⁺ mRNA isolated from cultured HepG2 cells showed that the labeled HLAL partial cDNA hybridized to a single band which migrated through the gel at a rate consistent with a 2600–2700 nucleotide long RNA (Fig. 2). A faint band of the same apparent size was present in total cell RNA from cultured, THP-1 cells (not shown). The HLAL message is larger than the coding sequence requirements for a polypeptide of the molecular weight of the secreted human fibroblast lysosomal acid lipase and it is larger than the 1500 nucleotide sizes reported for the human gastric lipase and rat lingual lipase mRNAs (16, 17).

When the labeled PCR fragment was used to probe a Southern blot of genomic DNA from hamster/human somatic cell hybrids (not shown) containing portions of chromosome 10 as the only human constituent, a pattern indicative of hybridization to a single copy human sequence was found. This is consistent with biochemical analyses employing somatic cell hybrids that have previously localized LIPA, the gene locus for HLAL, to human chromosome 10 (18).

The HLAL cDNA fragment was subsequently used to screen 2.5×10^5 colonies from a human fibroblast cDNA library in the Okayama-Berg pcD vector. The three positive colonies were purified to homogeneity and their plasmids characterized; the restriction site map and sequencing strategy for the insert in the largest recombinant plasmid,

pHLAL11, is diagrammed in Fig. 3 and its nucleotide sequence is shown in Fig. 4. The segment shown flanked by the underlined PCR primer sites (nucleotides 637–910) was identical to the sequence of the PCR-derived cDNA segment. Information to code for all of the original tryptic peptide sequences was present in the pHLAL11 insert. The coding sequences examined from the inserts in the two other clones that screened positive were identical to the pHLAL11 results, but ended at positions equivalent to nucleotides 390 and 458 in Fig. 4. Their 3'-untranslated regions were also the same length and had the same sequence as that of pHLAL11. This long 3'-noncoding sequence accounts for the larger size of the HLAL message in comparison to the mRNAs encoding the two other acid lipases and indicates that the HLAL message does not extend significantly further in the 5' direction.

A search of the GenBank and EMBL databases (19) indicated that the HLAL amino acid sequence has no significant similarities to those published for any other proteins, including those of other lipases, except human gastric lipase and rat lingual lipase. The amino acid sequence deduced from the HLAL cDNA insert showed 58 and 57% identity with the deduced human gastric lipase and rat lingual lipase peptide sequences, respectively. Across the overlapping coding regions, the HLAL message had a nucleotide sequence that was 64 and 63% identical to the two other acid lipases. These values are smaller than the 76% amino acid sequence identity and 80% nucleotide sequence identity that human gastric lipase and rat lingual lipase show to each other over this region.

Transfection of Cos-1 cells with the HLAL-specific cDNA increased their intracellular acid lipase activity to approximately twice that of controls after 24 h and to as much as 20-fold after 72 h (Table I). The results obtained with each of three different substrates known to be hydrolyzed by the native enzyme were similar; variations in the relative increase of activity in the different assay systems may be explained by basal levels of other fatty acyl hydrolases that differentially hydrolyzed these substrates. Cos-1 cells that were maintained for 72 h after transfection secreted more than 100 times the acid 4-methylumbelliferyl oleate hydrolase activity secreted by controls (not shown). This extracellular activity was over twice the intracellular level in the HLAL-transfected cells, indicating that a large fraction of the recombinant lipase escaped lysosomal packaging. Summing the intra- and extracellular values indicated a greater than 40-fold increase in the production of acid lipase by the transfected Cos cells. Lesser, but significant increases in acid lipase activity were induced in transfected Ltk⁻ murine fibroblasts. pHLAL11-transfected Ltk⁻ cells secreted 10 times more acid lipase activity than did controls; this corresponded to roughly half the level of the induced intracellular activity. The endogenous activity of

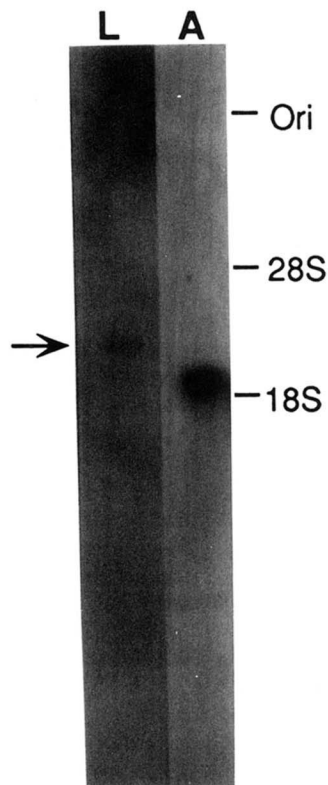


FIG. 2. Autoradiograph of a Northern blot of poly(A)⁺ RNA from HepG2 cells. Only a single lane loaded with 5 μ g of mRNA is shown; initially it was probed with the labeled HLAL insert from pHLAL11 (shown as L), then stripped and reprobed with an α -actin fragment (shown as A) labeled to the same specific activity. The autoradiographic signal in lane L resulted from a 36-h exposure and that in lane A from a 12-h exposure. The position of rRNA size markers are shown on the right.

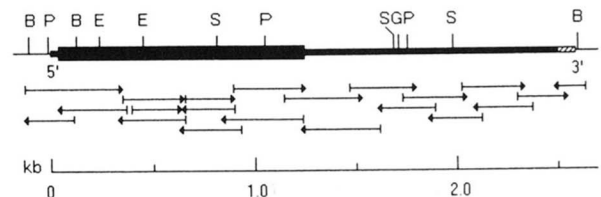


FIG. 3. Restriction map and sequencing strategy for pHLAL11. The thick bar represents the cloned insert with the broadest portion delineating the coding sequence and the hatched region indicating poly(A). Restriction sites are noted as B, BamHI; E, EcoRI; G, BglII; P, PstI; S, SacI. Arrows indicate length and direction of sequenced areas. Only 70 nucleotides near the 3' end of the insert were not sequenced in both directions; these were confirmed to be identical from the 5' direction in all three clones.

HLAL, along with the other two acid lipases, does, however,

TABLE I

Intracellular expression of acid lipase/cholesteryl ester hydrolase activity in transfected Cos-1 and Ltk⁻ cells

Lipase activity was determined with 4-methylumbelliferyl oleate (4-MeUmbO), cholesteryl oleate (CO) and triolein (TO); values represent the mean $\pm$ S.E. of the determinations on three individual cultures when error limits are shown and otherwise are the average of duplicate determinations on single cultures.

Cells	DNA	Time ^a <i>h</i>	Specific activity (nmol)(min) ⁻¹ (mg) ⁻¹			
			4-MeUmbO	CO	TO	β -Hex ^b
Cos-1	pHLAL11	24	10.5 $\pm$ 0.9	0.26 $\pm$ 0.03	0.37 $\pm$ 0.04	7.8 $\pm$ 0.5
	CMV β gal	24	5.4 $\pm$ 0.3	0.12 $\pm$ 0.01	0.27 $\pm$ 0.03	6.1 $\pm$ 1.0
	None	24	6.2 $\pm$ 0.1	0.13 $\pm$ 0.01	0.33 $\pm$ 0.01	8.6 $\pm$ 0.4
Cos-1	pHLAL11	72	186 $\pm$ 40	0.86 $\pm$ 0.15	1.11 $\pm$ 0.27	14.0 $\pm$ 1.2
	CMV β gal	72	12 ^c	0.12	0.26	14.4
	None	72	12 ^c	0.14	0.39	21.8
Ltk ⁻	pHLAL11	72	94 $\pm$ 26	0.98 $\pm$ 0.50	0.58 $\pm$ 0.12	26.3 $\pm$ 4.8
	CMV β gal	72	45	0.09	0.30	24.3
	None	72	41	0.07	0.24	28.0

^a Cells were cultured for the indicated period of time after removal of plasmid DNA.

^b Endogenous β -hexosaminidase activity.

^c Activity of 4-methylumbelliferyl oleate acid hydrolase in untransfected and CMV β gal transfected COS-1 cells incubated under identical conditions ranged from 8.0–12.5 (nmol)(min)⁻¹ (mg)⁻¹ in several experiments.

share the presence of the esterase-associated amino acid sequence motifs, -Gly-Xaa-Ser-Xaa-Gly-, with most other lipases and with lecithin-cholesterol acyltransferase (28). There are two of these pentapeptides in each of the acid lipases; they appear at HLAL residues 97–101 and 151–155 (Fig. 4). Until recently, it was thought the primary role of these pentapeptide elements was to mediate interfacial binding; experiments employing chemical modification, site-specific mutagenesis, and x-ray crystallography have now, however, implicated the central serines in at least some of these lipase structures as active site catalytic residues (30–32). Chemical modification studies in our laboratory showing that boronic acids (7) and diethyl-*p*-nitrophenyl phosphate³ inhibit HLAL activity suggest that serine residues may have a role in the catalytic mechanism of HLAL also.

As would be expected for a protein that has a broader catalytic specificity and a different cellular compartmentalization, the deduced amino acid sequence of HLAL has regions that are particularly distinct from human gastric lipase and rat lingual lipase. In the stretch extending from residue 186 to residue 250, there is a rearrangement in the pattern of charged residues and the addition of 2 of the extra cysteines seen in HLAL. The predicted sequence for HLAL has 6 cysteine residues with another in the signal peptide region, while rat lingual lipase has 4 and human gastric lipase has 3 (16, 17). The presence of the cysteine at residue position 240 (Fig. 4), along with the others that are conserved in this region of the gastric and lingual enzymes, produces an array of 4 cysteines over an 18-residue span. The involvement of 1 or more of these cysteines in the catalytic mechanism of HLAL would be consistent with the observations that sulfhydryl reagents inactivate the enzyme's catalytic activity and that thiols are necessary for the stability of purified HLAL (7, 33, 34), as well as with the studies that have documented the involvement of a cysteine in catalysis by human gastric lipase (35). The presence of the extra cysteines in the lysosomal enzyme may relate specifically to its cholesteryl ester hydrolase activity, a function which has not been documented for the gastric and lingual enzymes (36). A pair of free cysteines has been shown to mediate the cholesterol esterification reaction catalyzed by lecithin-cholesterol acyltransferase (37); the participation of both serine and cysteine residues in the

internal transacylation reaction of that enzyme may serve as a model for the catalytic mechanism of cholesteryl ester hydrolysis by HLAL.

An understanding of the catalytic mechanisms of lysosomal acid lipase may be facilitated by exploiting the HLAL cDNA to explicate the "experiment of nature" manifested in WD and CESD. While both disease states are characterized by deficiencies in lysosomal acid lipase activity, they exhibit markedly different clinical and biochemical phenotypes (2). Those affected with the WD variant die in the first year of life with massive, widespread, intracellular storage of both triglycerides and cholesteryl esters. Some patients with CESD live normally into middle-age manifesting only an enlarged liver (38). Histologic examination of the liver shows cells with marked intralysosomal and cytoplasmic accumulations of cholesteryl esters. There is also widespread, but less dramatic, intracellular accumulations of cholesteryl esters in many other organs and in vascular tissue. While it may be that the phenotypic differences between WD and CESD arise from variations in low residual levels of intracellular acid lipase activity, the possibility that the triacylglycerol lipase and the cholesterol esterase functions of HLAL *in vivo* are differentially affected by WD and CESD mutations has not been excluded. *In vitro* studies have consistently shown that both activities are severely depressed in material from WD and CESD patients, but the possibility that the cholesteryl ester and triglyceride hydrolase activities of the enzyme may utilize distinct mechanistic elements is supported by the observation that pretreatment of purified HLAL with thiols over a range of concentrations resulted in differential inactivation of the two functions.⁴ An analysis of the range of natural mutations in the presumably different mixes of alleles at the lysosomal acid lipase loci in WD and CESD cells, in conjunction with the information from investigations of human gastric lipase catalytic mechanisms could efficiently and explicitly delineate the nature of the active site residues that determine the activities of HLAL.

The existence of the relatively long, A-T rich, 3'-untranslated tail on the HLAL mRNA represents a striking divergence from the pattern of mRNAs for the other acid lipases. This structure may affect the stability or translatability of the HLAL message and may be involved in the modulation

³ G. N. Sando and H. L. Brockman, unpublished data.

⁴ G. N. Sando, unpublished data.

of levels of the intracellular lipase.

HLAL is an example of a lysosomal enzyme that shows a high level of sequence identity to enzymes with similar functions that are targeted to non-lysosomal destinations. Some other such pairs include α -glucosidase and intestinal sucrase-isomaltase (39), arylsulfatase A and steroid sulfatase (40), and cathepsin D and pepsinogen (41). The degree of sequence identity between HLAL and the enteric acid lipases, however, is notably higher than that observed between most other such pairs. The sequence conservation implies that there are stringent functional limitations on the structure of the enzymes in the acid lipase gene family and indicates that the alterations that produce the broader substrate specificity and the different subcellular localization of the lysosomal acid lipase do not require major alterations in protein primary structure.

In regard to signals that determine localization of the enzymes, all three of the characterized acid lipases contain asparagine-linked high mannose oligosaccharides as assessed by sensitivity to endoglycosidase H (7, 16, 17). There are six potential N-linked glycosylation signals in the HLAL sequence; four of them are near analogous points in the enteric acid lipase sequences while two are at least 20 residues away from potential sites in the two other enzymes. A different glycosylation pattern for HLAL and a different arrangement of charged residues may relate to the action of the phosphotransferase that mediates assembly of the mannose 6-phosphate recognition marker necessary for lysosomal targeting of the acid lipase (41).

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INFORME DE BÚSQUEDA DE PATENTES PARA EL No de Radicación: 15-24867						
CRITERIOS PARA LA BÚSQUEDA (anexar pantallazo o reporte en PDF, junto con los boleanos utilizados)						
Palabras clave utilizadas		Lysosomal acid lipase, cholesteryl ester hydrolase, Acid cholesteryl ester hydrolase, LAL, Cholesteryl esterase, wolman disease, cholesteryl ester storage disease				
CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA		A61K38 y C12N9				
BASES DE DATOS CONSULTADAS.						
USPTO		LENS				
ESPACENET						
NCBI						
UniPROT						
INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS (documentos patentes, literatura no patente etc...)						
Oficina	Fecha del examen o de reporte de búsqueda de documentos	Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos)	Reiv. afectadas	RELEVANCIA (requisito que afecta)	FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA	
					SI	NO
EUROPEA	26/06/2014	US2007264249	1 a 4	Nivel Inventivo	X	
		WO2011133960				X
		H. DU ET AL: "Wolman disease/cholesteryl ester storage disease: efficacy of plant-produced human lysosomal acid lipase in mice", THE JOURNAL OF LIPID RESEARCH, vol. 49, no. 8, 1 January 2008 (2008-01-01), pages 1646-1657, XP55016778, ISSN: 0022-2275, DOI: 10.1194/jlr.M700482-JLR200	1 a 4	Nivel Inventivo	X	
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BÚSQUEDA DE SECUENCIAS						
Número de depósito / patente y N° de secuencia						
% de identidad						
SEQ ID NO (de la solicitud)	NCBI	The Lens	EMBL-EBI	DDJB	Genome Quest	
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	99%	100				
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	99%	100				
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1	AKI69946.1				
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Alignments  Download  GenPept  Graphics  Distance tree of results  Multiple alignment 							
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☒	lysosomal acid lipase/cholesterol ester hydrolase isoform 1 precursor [Homo sapiens]	1369	1369	100%	0.0	100%	NP_000226.2
☐	lipase A precursor variant [Homo sapiens]	1366	1366	100%	0.0	99%	BAD96480.1
☐	lysosomal acid lipase/cholesterol esterase [Homo sapiens]	1364	1364	100%	0.0	99%	AAA59519.1
☐	lysosomal acid lipase [Homo sapiens]	1362	1362	100%	0.0	99%	AAB60328.1
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☐	LIPA [synthetic construct]	1354	1354	100%	0.0	99%	AKI69946.1

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