Page d'identification du jury

Université de Montréal
Faculté des études supérieures

Cette these intitulé:

Human Lysosomal Sialidase: Biogenesis, Structure and Molecular Pathology in Childhood Inherited Disorders, Galactosialidosis and Sialidosis

présenté par:

Kiven Erique Lukong

a été évalué par un jury composé des personnes suivantes:

President
Dr. Stephen Mishnick

Membres du jury
Dr. Alexey Pshezetsky (Directeur)
Dr. Michel Potier (Co-directeur)
Dr. Grant Mitchel
Dr. John Mort (examinateur externe)

Représentant du Doyen
Dr. P. Manjunath

Université de Montréal

Human Lysosomal Sialidase: Biogenesis, Structure and Molecular Pathology in Childhood Inherited Disorders, Galactosialidosis and Sialidosis.

par

Kiven Erique Lukong

Département de biochimie
Faculté de médecine

Thèse présenté à la faculté des études supérieures
en vue de l'obtention du grade de
Philosophiae Doctor (Ph.D)
en biochimie

Décembre, 2000

RESUMÉ

Le lysosome, décrite pour la première fois par de Duve en 1955, est l'organite cellulaire du catabolisme indispensable au recyclage des déchets cellulaires. Il est rempli d'enzymes hydrolytiques capable de dégrader presque toutes les macromolécules (lipides, polysaccharides, protéines, acides nucléiques et substances mixtes). Le lysosome est entouré d'une membrane imperméable contenant une ATPase translocatrice de protons qui conserve l'environnement acide facilitant les réactions hydrolytiques. Pour empêcher l'action hydrolytique des hydrolases a l'égard de la cellule, la majorité des hydrolases lysosomiales sont synthétisées sous forme de précurseurs inactifs. Dans le lysosome, ces précurseurs sont ensuite activés par une ou plusieurs étapes de maturations par protéolyse partielle. Toujours à l'intérieur des lysosomes certaines enzymes sont protégés contre la protéolyse en s'associant en structures supramoléculaire formant une matrice lysosomiale (Koenig, 1962).

La biosynthèse des protéines lysosomiales c'est un processus complexe. Les enzymes hydrolytiques et la membrane du lysosome sont produits et la plupart N-glycosyléés par le réticulum endoplasmique rugueux, puis transférés séparément dans l'appareil de Golgi où leur traitement se poursuit. Les oligosaccharides des enzymes hydrolytiques sont modifiées par l'addition des mannose-6-phosphates qui sont ensuit reconnues par les récepteurs mannose-6-phosphates (MPR). Cette interaction permet la ségrégation et ciblages des enzymes vers le lysosome. Le ciblage des protéines des membranes

lysosomiales nécessite un autre mécanisme. Selon plusieurs auteurs ces protéines telles que LAMP ("lysosomal associated membrane protéine"), LIMP ("Lysosomal integral membrane protéine") et LAP ("lysosomal acid phosphatase") portent dans leurs C-terminus un signal (Tyr-X-X-hydrphobe) qui interagit avec un complexe de protéines nommées adapteurs. Cette interaction permet l'internalisation des protéines des membranes lysosomiaux et leurs ciblage ver les lysosomes. Étant donne la complexité de la biosynthèse des protéines lysosomiales, une obstruction du processus pourrait provoquer une ou plusieurs maladie de surcharge lysosomiale.

Le concept de maladies de surcharge lysosmiale a été introduit par Hers (1965). Il a appelé maladies lysosomiales, les diverses pathologies qui sont causées par l'absence héréditaire d'une hydrolase acide présente dans le lysosome. Ces surcharges se rencontrent lorsqu'une ou plusieurs hydrolases lysosomiales ont perdu leur activité enzymatique à cause d'une mutation génétique. Lorsque le matériel séquestré ne peut pas être dégradé, il s'accumule dans le système vacuolaire ce qui, à la longue, peut causer la mort cellulaire (Hers 1966, 1972; Dingle, 1968; Van Hoof, 1976).

Les maladies lysosomales sont des maladies génétiques rares affectant moins de 10 000 personnes le monde entier. Les symptômes de ces maladies se traduisent en général par une atteinte progressive et irréversible des facultés physiques et mentales. Il existe une quarantaine de maladies lysosomales, regroupées selon les caractéristiques cliniques et biochimiques de la maladie

(Table 1). Chacune de ces maladies correspond au déficit spécifique d'une ou plus enzymes lysosomiales.

Dans le cadre de ce projet, notre intérêt repose sur la sialidase lysosomiale humaine ou α-neuraminidase (SIAL; E C 25 65 50) impliquée dans deux maladies de surcharge lysosomiales, la galactosialidose et la sialidose.

Dans le lysosome, SIAL existe en complexe multienzymatique de très haute masse moléculaire (1.27 MDa) comprenant la cathepsine A/protéine de protection lysosomiale (CathA; EC 3.4.16.5), la ß-galactosidase (GAL; EC 3.2.1.23) et la N-acetylgalactosamine-6-sulfate (GALNS) (d'Azzo et al., 1982; Hoogeveen et al., 1983; Van der Horst et al., 1989; Pshezhetsky and Potier, 1994, 1996).

Le gène codant la **CathA** couvre 7.5 kb, comprenant 15 exons. Elle est localisée dans le chromosome 20 (20q13.1) (Shimmoto et al., 1996). CathA est issu d'un précurseur de 54 kDa donc la séquence contient deux sites de glycosylation. Dans le lysosome, la protéine est maturée par l'excision d'un polypeptide d'environ 2 kDa formant une protéine de deux chaînes de 32 et 20 kDa (D'Azzo, 1982).

La CathA joue un rôle majeur dans le complexe mult-enzymatique. Elle assure l'activité et stabilité de GAL et SIAL dans les lysosomes. Elle est appelée donc protéine protectrice à cause de sa capacité de protéger les autres composants du complexe contre la protéolyse intralysosomiale, (d'Azzo et al., 1982; Hoogeveen et al., 1983; Van der Horst et al., 1989; Pshezhetsky and Potier, 1996).

Une déficience primaire en CathA cause la **galactosialidose**, une maladie héréditaire de surcharge lysosomiale transmise de facon autosomique récessive (Gollberg et al., 1971; Suzuki et al., 1982, 1991; pour une revue voir d'Azzo et al., 1994). Cette déficience déclenche une déficience secondaire combinée en GAL et SIAL. Il existe trois phénotypes cliniques : la forme congénitale ou infantile précoce avec syndrome oedémateux-ascitique et hépatosplénomégalie, atteinte neurologique, insuffisance rénale, dysmorphie faciale, anomalies squelettiques et oculaires (tache rouge cerise et cécité précoce); la forme infantile tardive caractérisée par une détérioration mentale absente ou mineure. et la forme juvénile/adulte (Japon surtout), avec atteinte neurologique lentement progressive, dysmorphie, atteintes osseuses et oculaires (tache rouge cerise et opacités cornéennes) et angiokératomes. Le diagnostic biologique repose sur la mise en évidence d'un profil caractéristique sur une chromatographie des oligosaccharides urinaires, confirmée par la mesure d'activité de la SIAL et de la GAL ou de la CathA dans les fibroblastes, le trophoblaste ou les amniocytes.

La **sialidose** est une maladie de surcharge lysosomiale rare, du groupe de oligosaccharidoses ou glycoprotéinoses. Elle est due au déficit en SIAL responsable d'une surcharge tissulaire sialyloligosaccharides. La transmission est récessive autosomique. On distingue: la sialidose de type 1, normomorphique ou 'cherry-red-spot, myoclonus' syndrome, débutant entre 8 et 25 ans, avec rétinopathie mais pas de dégradation psychique, et comitialité pouvant s'associer aux myoclonies, et la sialidose de type 2 ou sialidose dysmorphique infantile (mucolipidose de type 1 décrite par Spranger). La

dysmorphie faciale et la dysostose multiple rappellent celles de la maladie de Hürler. Le retard mental s'installe, ainsi que parfois une atteinte rénale (néphrosialidose). La tache rouge cerise est constante après 3 ans. Il existe une variante congénitale anténatale (pouvant se révéler sous forme d'anasarque foeto-placentaire) ou néonatale (avec syndrome oedémateux-ascitique et hépatosplénomégalie), et, à l'opposé, une forme juvénile plus progressive, avec myoclonies et angiokératomes. Comme pour la galactosialidose, le diagnostic biologique de la sialidose repose sur la mise en évidence d'un profil caractéristique sur une chromatograhie des oligosaccharides urinaires, confirmée par la mesure d'activité de la SIAL dans les fibroblastes, le trophoblaste ou les amniocytes.

La SIAL fait partir d'une large famille des sialidases retrouvées chez les bactéries, les virus, les protozoaires et les vertébré (Roggentin et al., 1993; Miyagi et al., 1993; Colman 1994; Chou et al., 1996). Ces sialidases sont essentiellement responsables de l'hydrolyse des acides sialiques dans les sialoglycoconjugates. Les analyses des structures primaires ont indiqué que les sialidases bactériennes ont une homologie d'environ 35% et contiennent tous le motif FY/RIP et une série de séquences consensus (Ser/Thr-X-Asp-(X)-Gly-X-Thr-Trp/Phe) nommée "Asp boxes" (Roggentin et al., 1993; Warner et al., 1993). Les structures crystalographiques démontrent que ces sialidases sont formées de six groupes de quatre feuillets β liés antiparallélement. Les six groupes sont disposés en forme des arbres d'hélice (Caskel et al., 1995).

Il existe trois types de sialidase chez les mammifères: la sialidase cytosolique, la sialidase du membrane plasmatique et la sialidase lysosomiale (Miyagi et al., 1990a, 1993; Sato and Miyagi, 1995; Kopitz et al., 1997; Miyagi et al., 1999; Frisch and Neufeld, 1979; Cantz 1982, 1991). Toutes ces sialidases sont actives dans des conditions acidiques, mais se diffèrent par leur localisation subcellulaire ainsi que par leur spécificité de substrat. La sialidase du membrane plasmatique par exemple a une stricte spécificité pour les gangliosides, tandis que la sialidase cytosolique est active contre les glycoprotéinees, les substrats artificiels ainsi que les gangliosides. La sialidase lysosomiale quant a elle est active contre la majorité de glycoconjugates et les substrats artificiels.

La purification et la caractérisation de la sialidase lysosomiale humaine (SIAL) a été un défi pendant des années surtout à cause de la labilité de l'enzyme jusqu'à c'est que son association avec le complexe multienzymatique a été découvert. (Verheijen et al., 1982, 1985). Mais c'est relativement récent que le gène de SIAL à été cloné (Bonten et al., 1996; Pshezhetsky et. al., 1997; Milner et al., 1997) Le gène a été localisé en chromosome 6p21.13.

Le ADNc code pour une protéine de 415 acides aminés avec une masse moléculaire estimée de 45.4 kDa. Les premiers 47 acide aminés représentent le peptide signal. La protéine contient le motif FY/RIP et quatre "Asp boxes", conservés dans les sialidases bactériennes. L'homologie de séquence entre la SIAL et les sialidase bactériennes est environ 35%. La SIAL à trios sites potentiels de glycosylation et les résidus du site active sont aussi conservés dans les sialidases de la bactérie et la souris (Crennell et al., 1993; Pshezhetsky et al.,

1997; Igdoura et al., 1998). L'identification du gène de la SIAL a permis l'étude du déficit moléculaire de la SIAL.

Dans le cadre de ce projet, nous avons étudier la biogenèse, la structure et la pathologie moléculaire de la SIAL dans la galactosialidsose et la sialidose. Nous avons réalisé les objectives suivants:

1) **Etudier le mécanisme moléculaire de la SIAL dans la galactosialidose.** Nous avons exprimé la SIAL dans les cellules fibroblasts et utilisé l'immunofluorescence et la microscopie immunoelectronique pour la localisation intracellulaire l'enzyme dans les cellules, le "Pulse-chase" et le "western blot" pour évaluer la glycosylation, la maturation et la stabilité de la SIAL. Nous avons démontré le précurseur de la SIAL a une masse moléculaire de 45.5 kDa et après la glycosylation l'enzyme mature obtient une masse de 48.3 kDa. La protéine est exprimée surtout dans le lysosome et la membrane plasmatique dans les fibroblasts galactosialidoses, mais la demi-vie de l'enzyme chute rapidement de 2.7 h dans les cellules normales à 30 min. Nous avons également démontre que la protéine est dégradée par conséquent en deux formes inactives de 38.7 et 24 kDa. Ensemble, les résultants indiquent que la déficience de la SIAL dans la galactosialidose est issu du clivage anormal et la degradation rapide de l'enzyme.
2) **Identifier et caracteriser des mutations dans le gene de la sialidase.** Nous avons utilisé "single-strand conformation

polymorphism" (SSCP), les analyses heteroduplexes et "allele-specific oligonucleotide hybridization" (ASO) et le séquençage direct pour identifier et confirmer onze nouvelles mutations chez des patients d'origine ethnique différente. Les mutations comprennent une mutation, 623delG, qui cause un changement du cadre de lecture par l'introduction d'un codon de terminaision, et huit missenses (679G→A (Gly227Arg), 893C→T (Ala298Val), 203G→T (Gly68Val), 544A→G (Ser182Gly) 808C→T (Leu270Phe), 982G→A (Gly328Ser), 649G→A (Val217Met) et 727G→A (Gly243Arg)). Nous avons exprimé les sialidase mutantes dans les cellules COS-7 et fibroblasts afin d'évaluer les effets des mutations sur la biosynthese, l'activité catalytique, la maturation, la stabilité, la localisation intracéllulaire et l'integrité du complexe multienzymatique. Nous avons également crée un modele de la structure tertiaire de la sialidase lysosomiale et l'utilisé pour prédire les changements possible de conformation de l'enzyme du a des mutations missenses identifiées. Bref, nos modèles de la SIAL, les mutations causant les phenotypes moins sevère sont distribué aléatoirement, tandis que les mutations identifiées dans les patients atteint de la sialidose sevère de type II sont concentrées dans une région de la structure. Dans cette région on rétrouve les mutations Gly227Arg, Leu270Phe, Ala298Val et Gly328Ser ainsi que les mutations Arg294Ser, Leu231His, Gly218Ala, Trp240Arg et Pro316Ser identifiées par

d'autres groupes (Bonten et al., 1996, 2000; Sakuraba, communication privee). Nous avons ensuite vérifié l'hypothèse que cette région soit l'interface de l'interaction de la SIAL avec la CathA dans le complexe lysosomiale. et nous avons démontré que Gly328Ser, Leu270Phe, Ala298Val et Phe260Tyr ne sont pas protégées par la CathA et sont dégrades rapidement.

3) **Identifier le mécanisme de ciblage de la SIAL vers le lysosome.**

La SIAL a trios sites de N-glycosylation et devrait en principe atteint le lysosome par voie du récepteur mannose-6-phosphate. Néanmoins, van der Spoel et al. (1998) a démontré que la SIAL est faiblement phosphorylée et est ciblée vers le lysosome comme complexe avec la CathA. Mais, selon Vinogradova et al. (1998), la SIAL est partiellement ciblée vers la membrane plasmatique. Plusieurs rapports ont indiqué que le motif Tyr-X-X-Φ retrouve dans le C-terminal des protéines membranaires lysosomiales telles que la "LAMP" et l'acide phosphatase lysosomiale est impliqué dans le ciblage vers le lysosome (Lehmann et al., 1992; Hunziker and Geuze, 1996; Honings et al., 1996). La SIAL contient le motif Tyr-Gly-Thr-Leu dans sont C-terminal (Pshezhetsky et al., 1997). Nous avons muté les résidus Tyr et Leu pour l'Ala et exprimé les SIALs mutés dans les cellules Cos-7 pour vérifier si le motif est impliqué dans le ciblage lysosomal de la SIAL. Les résultants d'immunohistochimie et fractionnation cellulaire confirment que la

SIAL est ciblée vers le lysosome et la membrane plasmatique par voie du signal C-terminal.

Ces études devraient accroître de façon significative nos connaissances sur la biologie et la biochimie du lysosome. Elles vont contribuer à définir le mécanisme pathogène des maladies héréditaires sévères, la galactosialidose et la sialidose, dans le but ultime de trouver des méthodes de traitements. L'analyse mutationelle nous permet de développer des méthodes diagnostiques basées sur l'ADN et les corrélations phenotype-genotype. De plus, ces études éclaircissent nos connaissances de la biogenèse du lysosome et la biosynthèse des enzymes lysosomiaux, surtout le concept de l'organisation et stabilité du complexe multienzymatique impliquant la SIAL, GAL, CathA et GALNS.

TABLE OF CONTENTS

List of Figures

Chapter 4: Lysosomal sorting of sialidase

Article 6

List of Tables

Chapter 5: Discussion and general conclusion

List of Abbreviations

4MU-NeuAc	4 methylumbelliferyl-alpha-D-N-acetylneuramic acid
DNA	Dinucleic acid
CathA	Cathepsin A
CD-MPR, MPR46	Cation-dependent Man-6-P receptor
CI-MPR, MPR300	Cation-independent Man-6-P receptor
GAL	β-galactosidase
GALNS	N-acetylgalactosamine-6-sulfatase
GlcNAc-1-P	N-acetylglucosamine 1-phosphate
GS	Galactosialidosis
K Da	KiloDalton
LAMP	Lysosomal associated membrane protein
LAP	Lysosomal acid phosphatase
LDL	Low density lipoprotein
LGP-85	Lysosomal membrane glycoprotein
LIMP	Lysosomal integral membrane protein
LSD	Lysosomal storage disease
Man-6-P	Mannose-6-phosphate
MPS	Mucopolysaccharidosis
NEU3	Human plasma-membrane-associated sialidase
RER	Rough endoplasmic reticulum
SAP	Sphingolipid activator protein
SIAL	Human lysosomal sialidase
TIP47	Tail-interacting 47-kDa protein
Tr	Transferrin
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine

AMINO ACID CODES

Name	Three-letter Code	One-letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Acknowledgement

I am very grateful to my director Dr. Alexey Pshezhetsky for the opportunity to work in his laboratory and for supporting me relentlessly through this project. I am also indebted to Dr. Michel Potier who gave me the confidence and inspiration to resume research after a long interval.

I thank Dr. Marc-Andre Elsliger for his indispensable role in provide the structural analyses presented in this work. I also acknowledge Drs Tina Buchholz, George Thomas, William Carey, Anna Tylki-Szymanska, Barbara Czartoryska, German Rodríguez Criado and Silvia Palmeri for providing samples from sialidosis patients.

I am also grateful to all my laboratory colleagues especially Catherine Richard, Karine Landry and Yuan Chang for their close collaboration, and Dr Carlos Morales and Stephane Lefrançois for their participation in immunofluorescence and immunoelectron microscopy.

Finally, I would like to thank all the members of my entire family especially my wife and my children for standing by me through thick and thin in making my dream come true.

This work was supported by the operating grants from the Medical Recherche Council of Canada, Fond de la Recherche en Sante de Quebec and Vaincre les Maladies Lysosomales Foundation (France) to Dr. Alexey Pshezhetsky.

CHAPTER 1

INTRODUCTION

General Review

1.1 Biogenesis of Lysosomes

Lysosomes were discovered in 1949 by Belgian researcher Christian de Duve. They are cytoplasmic organelles harboring over 100 hydrolytic enzymes such as nucleases, proteases, lipases, glycosidases, phosphatases, sulfatases and phospholipases. These enzymes have an acidic pH optimum and are involved in the degradation of essentially all types of biological macromolecules including lipids, polysaccharides, proteins, nucleic acids, and even whole bacteria and worn out and nonfunctioning organelles like the mitochondria.

The main pathways involved in the biogenesis of lysosomes are shown in Figure 1. The first pathway is followed by macromolecules taken up by receptor-mediated endocytosis. The endocytosed material, which includes the receptor, ligand and associated membrane, is initially delivered into small irregularly shaped intracellular vesicles called early endosomes. Some of the ingested molecules are selectively retrieved and recycled to the plasma membrane and rest of the vesicle becomes the late endosome. The late endosome then fuses with vesicles containing lysosomal hydrolases from the Golgi apparatus to form mature lysosome. The lower pH in the lysosome favors the release and the hydrolytic digestion of the endocytosed molecules. A second pathway leads to the degradation of worn out organelles such as mitochondria in a process called autophagy. In this process, an organelle is enclosed by membranes derived from the ER, creating an autophagosome which then fuses with vesicles carrying lysosomal enzymes and becomes a mature lysosome. The third pathway involves the uptake and degradation of large particles and microorganisms like bacteria by specialized cells (phagocytes) the process of phagocytosis. These phagocytes

(macrophages and neutrophils in vertebrates) engulf objects to form phagosomes, which, like autophagosomes, are then converted to lysosomes.

The biogenesis of lysosomes is therefore a complex process which requires that specific sets of soluble hydrolases and membrane proteins, synthesized in the rough endoplasmic reticulum (RER), be segregated from proteins with other subcellular destinations and be transferred to developing or mature lysosomes. Any failure in the biogenesis of lysosomes due to genetic mutation can result in a severe inherited disorder, characterized by the absence of one or more lysosomal enzymes. These disorders are called lysosomal storage disorders because of the accumulation of undigested macromolecules in the lysosomes of the affected tissues.

In 1967, Leroy and DeMars identified large, phase-dense inclusions in the fibroblasts of a patient with a disorder that clinically resembled Hurler Syndrome. Because these cells were called inclusion cells (I-cells), the disorder became known as I-cell disease. Hickman and Neufeld (1972) discovered that multiple deficiency of lysosomal enzymes in I-cell disease (also called mucolipidosis II) results from a deficiency in a recognition marker that is common to all soluble lysosomal enzymes and required for their targeting to the lysosomes. This observation provided the basis that eventually led to the identification of the lysosomal marker, mannose-6-phosphate (Kaplan et al., 1977) and its two distinct receptors of 275-300 kDa and 46 kDa respectively. To date, two principally different mechanisms for the targeting of lysosomal hydrolases have been described (Neufeld, 1991). They involve the difference in the sorting of membrane-bound lysosomal enzymes and soluble lysosomal enzymes (Natowicz et al, 1979).

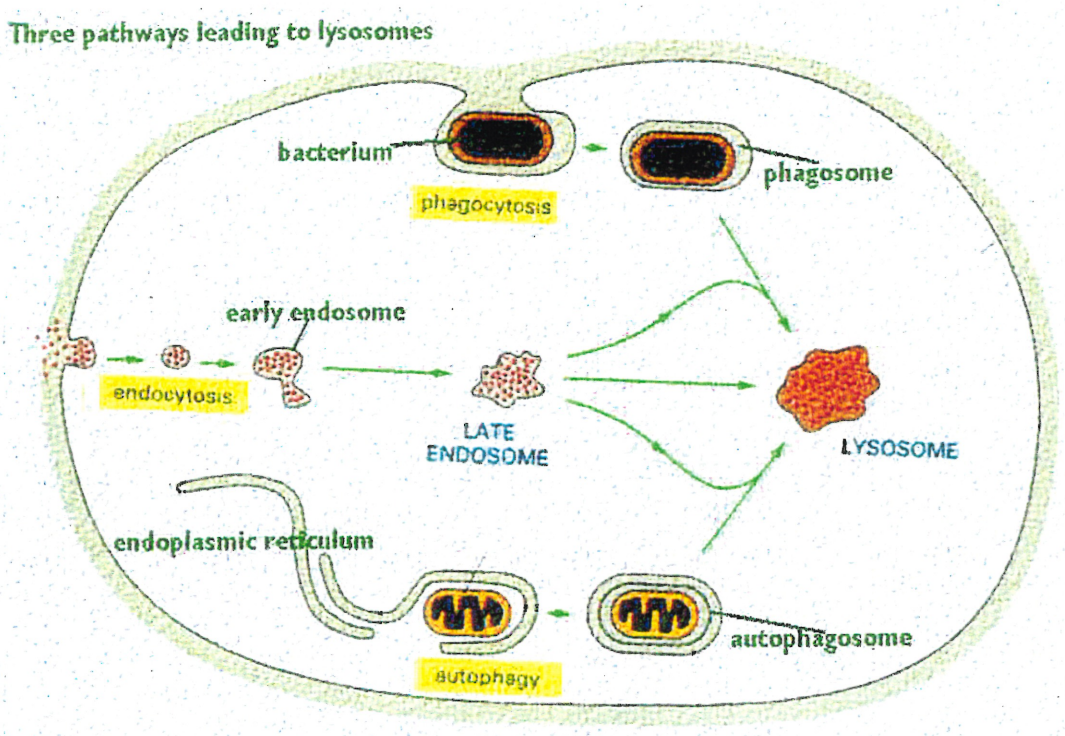

Figure 1. Biogenesis of lysosomes. (Molecular Biology of the Cell by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts, and James D. Watson, Garland Publishing, NY 1994).

1.1.1 Lysosomal membrane

The lysosomal membrane serves as a selective permeability barrier between the lysosomal lumen and the cytoplasm and is equipped with carriers, transport systems and a proton pump that maintains the lumen acidic. Macromolecules that enter the lysosomes by endocytosis or autophagy are prevented from egress by the lysosomal membrane. Certain small molecules and degradation products easily cross the lysosomal membrane. Monosaccharides seem to cross the membrane by carrier-mediated systems. Sialic acid transfer for example is mediated by a specific carrier, sialic acid transporter protein. A deficiency in this carrier causes Salla disease, characterized by sialic acid accumulation within the lysosomes. Sanfilippo C syndrome is also another disease caused by a defect in a membrane-associated enzyme, acetyl CoA: alpha glycosaminide N-acetyltransferase, which is normally involved in the acetylation of heparan sulfate. Acetylation is necessary for heparan sulfate degradation and in Sanfilippo C syndrome, heparan sulfate accumulates in the lysosomes (Bame and Rome, 1985).

The lysosomal surface of the lysosomal membrane is rich in complex oligosaccharides bearing sialic acid residues, which are thought to protect the surface from the attack of lysosomal hydrolases. The sialic acid moieties probably establish a Donnan potential for protons that contribute to the low pH of the lysosomes (Chretin, 1982). Characterization of the cytoplasmic surface of the lysosomal membrane is still in the early stages. Polyclonal and monoclonal antibodies prepared against the lysosomal membrane have led to the identification of several integral or associated membrane

proteins. They include lysosomal-associated membrane proteins (LAMPs) and lysosomal integral membrane proteins (LIMPs) (reviewed in Hunziker and Geuze, 1996).

1.1.2 Biosynthesis of lysosomes enzymes

1.1.2.1 Sorting of lysosomal membrane proteins

Membrane-bound lysosomal proteins are glycoproteins rich in N-linked oligosaccharides, most of which are of the complex type. These proteins are therefore synthesized and glycosylated in the RER and their oligosaccharides modified in the Golgi apparatus.

The sorting of membrane-bound lysosomal proteins like glucocerebrosidase (Aerts *et al.*, 1988), LIMPs and LAMPs (Barriocanal *et al.*, 1986), and lysosomal acid phosphatase (LAP) is independent of mannose-6-phosphate receptors which mediate transport of soluble lysosomal enzymes. It involves the internalization of the proteins from the plasma membrane via clathrin-coated pits and transportation to the lysosomes by a mechanism requiring the association of Gly-Tyr-X-X-hydrophobic amino acid motif at the carboxyl terminus of their cytoplasmic domain with μ2-subunit of HA-2 adaptor complex (Guarnieri *et al.*, 1993; Pearse et al., 2000). This same pathway is also used by the LAP, which is synthesized as a transmembrane protein (Braun *et al.*, 1989) transported to lysosomes via the cell surface. In lysosomes LAP is released from the membrane by proteolytic processing, which involves at least two cleavages at the C terminus of LAP. The first cleavage is catalysed by a thiol proteinase at the outside of the lysosomal membrane and removes the bulk of the cytoplasmic tail of LAP. The second cleavage is catalysed by an aspartyl proteinase inside the lysosomes and

releases the luminal part of LAP from the membrane-spanning domain (Gottschalk *et al.*, 1989).

Further evidence of a distinct sorting mechanism for the membrane-associated proteins is supported by the presence of normal levels of β-glucocerebrosidase and acid phosphatase in I-cell fibroblasts. These enzymes are not phosphorylated in normal cells (Erickson *et al.*, 1985). Moreover, pulse-labeling and cell fractionation experiments with rat kidney cultured cells treated with tunicamycin to inhibit glycosylation have indicated that newly synthesized plasma membrane proteins lacking N-linked oligosaccharides were nonetheless rapidly transported to the lysosome (Barriocanal *et al.*, 1986). These studies with tunicamycin confirmed that the sorting of lysosomal membrane proteins is not coupled obligatorily to the transport of soluble lysosomal enzymes, which require the carbohydrate recognition signal: mannose-6-phosphate (Man-6-P).

1.1.2.2 Sorting of soluble lysosomal proteins

Whereas little is known of the sorting process of membrane-bound lysosomal proteins, much progress has been made towards an understanding of the mechanisms by which newly synthesized soluble lysosomal enzymes are sorted from secretory proteins. Lysosomal proteins as well as most secretory proteins are glycoproteins synthesized in polyribosomes bound to the RER membranes. Each of these proteins contains a hydrophobic signal peptide at the N-terminus which interacts with a signal recognition particle, an 11 S ribonucleoprotein, and thereby initiates the vectoral transfer of the nascent protein across the RER membrane into the lumen of that organelle (Erickson *et al.*, 1981).

The initial stages of glycoprotein synthesis involve a large precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ (three glucose, nine mannose and two N-acetylglucosamine residues), assembled with a pyrophosphate link to a ligand carrier, dolichol (Dol), and then transferred *en bloc* to the target asparagine residue of the precursor glycoprotein. Still in the RER, the signal peptide is cleaved, and the processing of the asparagine-linked (or N-linked) oligosaccharide begins. Glucosidases I and II excise three glucoses, and mannosidase cleaves one of the mannose residues from the sugar chain.

The proteins then move, by vesicular transport, to the Golgi stack where they undergo a number of post-translational modifications and are sorted for targeting to the proper destination - lysosome, secretory granule or plasma membrane. The Golgi complex consists of a stack of flat cisternae functionally divided into three regions: cis, medial and trans Golgi. The cis part normally receives products of biosynthesis from the RER. During the passage through the Golgi apparatus, the oligosaccharide chain residues on secretory and membrane glycoproteins are processed stepwise to complex-type units containing sialic acid. This processing involves the removal of five mannose residues and the addition of two molecules each of N-acetylglucosamine, galactose and sialic acid.

Most of the oligosaccharides on lysosomal enzymes undergo a different series of modifications that introduce the Man-6-P marker. The Man-6-P distinguishes lysosomal from secretory glycoproteins and is responsible for addressing them via one of two Man-6-P receptors to their lysosomal destinations. The mechanism by which the Man-6-P marker is incorporated into lysosomal enzymes is a two-step process requiring the sequential action of two enzymes (reviewed by Kornfeld 1987). The first step involves

the enzyme UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine 1-phosphotransferase (phosphotransferase), a multisubunit protein (Boa et al., 1996; Raas-Rothschild et al., 2000) which transfers N-acetylglucosamine 1-phosphate (GlcNAc-1-P) from the nucleotide sugar uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) to the C-6 hydroxyl position of specific mannose residues on the high mannose oligosaccharides of newly synthesized lysosomal enzymes, to give rise to a phosphodiester intermediate. This modification of mannose residues in the cis-Golgi protects them from cleavage by mannosidases in the medial Golgi. In the medial Golgi, the second enzyme, a specific phosphodiesterase called N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (phosphodiesterase) (Kornfeld et al., 1999) removes the terminal N-acetylglucosamine (GlcNAc) residue uncovering the phosphate, thus exposing the phosphomannosyl signal. Subsequently, the lysosomal hydrolases bind to the mannose-6-phosphate receptors in the trans-Golgi network, thus remaining intracellularly.

Two described Man-6-P receptors differ in their binding properties and divalent cation requirements (Hoflack *et al.*, 1985). The first identified was a 275-300 kDa cation-independent Man-6-P receptor (CI-MPR, MPR300). This receptor is a type I transmembrane glycoprotein with a segment of 17 kDa exposed on the cytoplasmic side of the membrane. CI-MPR has an insulin-like growth factor type II receptor function and participates in the endocytosis of extracellular Man-6-P-containing proteins. It has been detected in the Golgi complex, coated vesicles, endosomes, and the plasma membrane and the importance of CI-MPR for lysosomal enzyme trafficking was demonstrated by

the massive mistargeting of lysosomal enzyme precursors in homozygote embryos in chimeric mice lacking CI-MPR (Sahagian *et al.*, 1984).

The second receptor, a cation-dependent Man-6-P receptor (CD-MPR, MPR46), is a glycoprotein composed of three subunits of 46 kDa each (Hoflack *et al.*, 1985). CD-MPR was first discovered when it was noted that endothelial cells, deficient in the CI-MPR, contained high levels of enzymes localized in lysosomes (Robbins *et al.*, 1981). The CI-MPR seems to have a dominant role in lysosomal targeting while the function of the CD-MPR is less clear. CD-MPR like CI-MPR is also a transmembrane protein with a cytoplasmic domain of 69 residues. Both CI-MPR and CD-MPR, like the integral membrane proteins and endocytosed receptors, have sorting determinants in their cytoplasmic domains. The trafficking of CI-MPR is mediated by the tyrosine- and di-leucine-based motifs, while the CD-MPR requires two distinct motifs: a dominant FPHLAF sequence and a weak YRGV sequence (Collawn et al., 1991; reviewed in Le Borgne and Hoflack, 1998; Schweizer et al., 2000). The affinity of these receptors to Man-6-P moieties of lysosomal hydrolases is pH dependent. At neutral or slightly acidic pH, the receptors bind strongly to their ligand, whereas the strongly acidic lysosomal medium favours dissociation of the ligand (Dahms et al., 1989). Both receptors are recycled back to the Golgi complex through interaction with the tail-interacting 47-kDa protein (TIP47), which binds the cytoplasmic domains of the CD-MPR and CI-MPR and mediates their transport from endosomes to the Golgi complex (Orsel et al. 2000).

Studies with the cells of CD-MPR-deficient mice suggested the existence of an alternative targeting mechanism (Koster *et al.*, 1994). This carbohydrate-independent traffic of proteins is probably controlled by protein kinase as suggested on the basis of specific inhibition of lysozyme and cathepsin D targeting to lysosomes by protein kinase

C inhibitors (Radons *et al.*, 1994). Several authors have also suggested that Man-6-P receptor-independent lysosomal targeting of several soluble enzymes including cathepsins L and D (McIntyre and Erickson, 1991) and sphingolipid activator protein (SAP) (Rijnboutt *et al.*, 1991) may involve the association of their propeptides with Golgi membranes. However, subsequent studies (Conner *et al.*, 1992) have failed to demonstrate the role of cathepsin D propeptide in sorting to lysosomes. Moreover, according to Glickman and Kornfeld (1993), Man-6-P independent sorting of cathepsin D involves a specific recognition of an exposed polypeptide determinant in its carboxyl-terminal lobe.

1.2 Lysosomal Storage Diseases

In 1965, Hers introduced the concept of lysosomal storage diseases (LSD) to describe how the deficiency of a lysosomal enzyme, α-glucosidase, could be lethal in Pompe disease. The undegraded substrate would gradually accumulate within the lysosomes, increasing the size and number of organelles and leading eventually to cellular death and the malfunctioning of the affected organ (Hers, 1972; Van Hoof, 1976). Some LSD like the I-cell disease are caused by the defect of a common protein, resulting in the loss of a host of lysosomal enzymes. LSD may manifest several clinical forms with different onset (infantile, juvenile or adult) or severity. Clinical features generally vary between different disorders but the most common characteristics include hepatosplenomegaly, neuronal deterioration, skeletal complications, coarse faces, blindness and growth retardation. A molecular defect of genes coding for lysosomal

enzymes may affect the enzyme's synthesis, processing, routing, folding, maturation, activation, stability and oligomerization or complex formation.

LSD are grouped into various classes based on the clinical and biochemical manifestations of the disease. They include sphingolipidosis, mucopolysaccharidosis, mucolipidosis, glycoprotein storage diseases, lysosomal membrane transport disorders and others types (**Table 1**). At least 41 genetically distinct, biochemically related LSD caused by the absence of one or more lysosomal hydrolases have been described (reviewed in Neufeld, 1991). Individually, these disorders are considered rare and each one typically affects fewer than 10,000 people worldwide, although high prevalence values have been reported in some populations (Meikle et al., 1999) Most LSD are inherited in an autosomal recessive manner, with the exception of Fabry disease and mucopolysaccharidosis (MPS) type II, which show X-linked recessive inheritance.

The biggest group of lysosomal storage diseases is associated with deficiencies of glycosidases involved in the catabolism of sugar chains of glycolipids, oligosaccharides and glycoproteins. This group includes single enzyme deficiencies as in Gaucher, Tay-Sachs and Sandhoff diseases, and the functional deficiencies of multiple enzymes caused by genetic mutations in sphingolipid activator proteins (saposins, G_{M2}-activator protein) which stimulate the activity of glycosidases against gangliosides and glycosphingolipids.

Both Tay-Sachs and Sandhoff diseases are examples of sphingolipidoses. They are caused by a failure in the degradation of G_{M2}-ganglioside. In the lysosomes, three genes are required in this process. They are HEX A and HEX B genes encoding the alpha and beta subunits of hexosaminidase A, and the gene encoding the G_{M2}-activator, a lipid-binding protein that presents the G_{M2} substrate to the enzyme. A defect in the

HEX A gene causes Tay-Sachs disease, an autosomal recessive, progressive neurodegenerative disorder, characterized by the developmental retardation, followed by paralysis, dementia and blindness and is usually fatal by age 2 or 3 years. Although less than 0.3 % of the general population are carriers of the disease, the frequency is ten times higher (3 %) in Ashkenazi Jews (Petersen *et al.,* 1983). A defect in the HEX B gene causes the clinically related Sandhoff disease.

Gaucher disease, another sphingolipidosis, is the most common lysosomal storage disease with an estimated carrier frequency of 4.6% among Ashkenazi Jews in Israel (Matoth *et al.,* 1987). It is autosomal recessive and caused by deficiency of glucocerebrosidase, resulting in the lysosomal accumulation of glucosylceramide.

Disorders of lysosomal membrane transporters include Nieman-Pick type C involving a cholesterol esterification defect, sialic acid storage disease (sialic acid transport protein deficiency) and cystinosis (cystine transport protein deficiency).

Galactosialidosis and sialidosis are of particular interest because they involve cathepsin A, β-galactosidase and sialidase, which are components of the 1.27 MDa lysosomal multienzyme complex.

Table 1

Lysosomal storage diseases

Disease	Defect	Stored Substance	Chromosomal Location
1. Sphingolipidoses			
A. Gangliosidoses			
G_{M1} –gangliosidosis	β-galactosidase	G_{M1} gangliosides	3
Tay-Sachs	β-hexosaminidase A	G_{M2} gangliosides	15
Sandhoff total	β-hexosaminidase A&B	G_{M2} gangliosides	5
Farber disease	ceramidase	ceramide	8
B. Leucodystrophies			
Krabbe	galactocerebrosidase	galactocerabroside	14
Metachromatic	arylsulphatase	sulphatides	22
C. Visceral storage disease			
Gaucher	β-glucocerebrosidase	glucosylceramide	1
Niemann-Pick types A&B	sphingomyelinase	sphingomyeline	18
Fabry	α-galactosidase	globosides	**X**
2. Glycoprotein Storage Diseases			

Galactosialidosis	cathepsin A	sialyloligosaccharides	20
Fucosidosis	α-fucosidase	fucosyloligosaccharides	1
α-Mannosidosis	α-mannosidase	mannose/oligosaccharides	19
β-Mannosidosis	α-mannosidase	mannose/oligosaccharides	4
Schindler Disease	α-N-acetylgalactosaminidase	trisaccharides with terminal α-N-acetylgalactosamine	22
Aspartylglucosaminuria	N-aspartyl β-glucosaminidase	aspartylglucosamine asparagines	4

3. Mucopolysaccharidoses

I- Hurler	α-iduronidase	heparan/dermatan sulfates	4
II - Hunter	iduronate-sulphatase	heparan/dermatan sulfates	**X**
IIIA - Sanfilippo A	heparin sulphamidase	heparan sulfate	17
IIIB - Sanfilippo B	N-acetyl α-glucosaminidase	heparan sulfate	17
IIIC - Sanfilippo C	Acetyl-CoA: α-glucosamide N-acetyl transferase	heparan sulfate	14
IIID - Sanfilippo D	N -acetyl glucosamine-6-sulfatase	heparan sulfate	12
IVA - Morquio A	N-acetyl galactosamine-6-sulfatase	keratan sulfate	16
IVB - Morquio B	α -galactosidase	keratan sulfate	3
VI - Maroteaux–Lamy	arylsulphatase B	dermatan sulfate	5

VII - Sly	β-glucuronidase	keratan and dermatan sulfate	7
4. Mucolipidoses(ML)			
Sialidosis(MLI)	α-neuraminidase	sialyloligosaccharides	6
I-cell disease(MLII)	N-acetyl glucosamine phosphostransferase	heparan sulfate	4
Pseudo-Hurler polydystrophy(MLIII)	N-acetyl glucosamine phosphoryl transferase	heparan sulfate	4
MLIV	mucolipin 1	gangliosides/hyaluronic	19
5. Other types			
Wolman and Cholesteryl Ester Storage Disease	acid lipase	cholesteryl esters	10
Mucosulphatidosis	sulphatase deficiencies		
Pompe	α-1:4-glucosidase	glycogen α-1-4 linked oligosaccharides	17
Infantile Batten	palmitoyl protein thioesterase	lipofuscins	1
Late infantile Batten	pepstatin-insensitive protease	lipofuscins	13
Juvenile Batten	cathepsin H; phospholipase A	lipofuscins	16

Pycnodysostosis	cathepsin K	(IGF1 secretion)	1

6. Lysosomal membrane transport defects

Sialic acid storage disease	sialic acid transport protein	free sialic acid /glucuronic acid	6
Cystinosis	cystine transport protein	free cystine	17
Niemann-Pick disease type C	cholesterol esterification defect	sphingomyelin	18

1.2.1 Galactosialidosis

Galactosialidosis (GS), an autosomal recessive disease caused by a primary defect of protective protein/cathepsin A (CathA), is unique among LSD because it also results in a combined secondary deficiency of two lysosomal glycosidases, β-galactosidase (GAL) and neuraminidase/sialidase (SIAL) (Goldberg et al., 1971; Suzuki et al., 1984, Zhang and Callahan, 1994; d'Azzo et al.,1995). GS is characterized by typical lysosomal storage disease manifestations including coarse facies, macular cherry-red spots, mental retardation, corneal opacities, vertebral changes, bone marrow foam cells and vacuolated lymphocytes.

There are three phenotypically distinct forms of GS according to the severity and age of onset: an early infantile, late infantile and juvenile/adult types. The early infantile onset manifest prenatally as non-immunologic hydrops fetalis, the excessive accumulation of serous fluid in the subcutaneous tissues and serous cavities of the fetus (Stone and Sidransky, 1999), or postnatally as ascites, massive edema, proteinuria, viceromegaly, skeletal dysplasia and early death from cardiac failure, kidney failure or airway obstruction (Wenger et al., 1978; Lowden and O'Brien, 1979). Some early-infantile GS have demonstrated cytopenias (abnormal decrease in number of cells in the blood) (Olcay et al., 1998) and punctate calcification in the epiphyses of the femora, calcanei, and sacrum (Patel et al., 1999). The late-infantile onset is associated with hepatosplenomegaly, growth retardation, cardiac involvement and absence of neurologic signs. The juvenile/adult onset is relatively mild and patients with this subtype

of GS may survive to adulthood, often without mental retardation or viceromegaly, but with ataxia, myoclonus and angiokeratoma. About 70 % of GS cases can be classified in this group and the majority of reported patients are of Japanese origin (Suzuki *et al.*, 1988; Takano *et al.*, 1991). Parental consanguinity has been reported in half of the families.

The correct biochemical characterization of GS came about after a series of studies on G_{M1}-gangliosidosis by various groups. G_{M1}-gangliosidosis (GAL deficiency) was one the first LSD with the identified biochemical mechanism (Okada, S. and O'Brien, 1968). This discovery facilitated rapid diagnosis of many G_{M1}-gangliosidosis patients. Among those two reported by Pinsky et al. (1974) and Loonen et al. (1974) happened to have GAL deficiency but showed an abnormal clinical phenotype with normal intelligence and late development of psychomotor deterioration. Somatic cell hybridization studies performed by Galjaard et al. (1975) showed that these variants are caused by the defects in a gene different from that of GAL. Wenger et al. (1978) investigating similar case found a combined deficiency of two lysosomal enzymes, GAL and SIAL. All reported patients belonged to the same complementation group, which allowed designating the condition as a distinct disorder further named galactosialidosis (GS) (Andria et al., 1981). For several years, the primary molecular defect in GS was thought to be identical to that in single SIAL deficiency, sialidosis (Cantz et al., 1977), until Hoogeveen et al. (1980, 1981) showed that hybridization and even co-culturing of fibroblasts from GS and sialidosis patients resulted in partial correction of SIAL and GAL activity. These studies suggested an existence of a protein "corrective factor" secreted by normal, sialidosis or G_{M1}-gangliosidosis cells, but absent in those of GS patients. Further studies showed a 10-fold enhanced cellular degradation of GAL in

galactosialidosis fibroblasts (van Diggelen et al., 1981) that could be prevented by the addition to the cell medium of either a fraction containing the "corrective factor" or by the inhibition of lysosomal proteases (Suzuki, et al. 1981, d'Azzo et al, 1982).

CathA precursor is a 54 kDa protein which is processed in the lysosomes to the mature form containing two protein chains of 32 and 20 kDa. D'Azzo et al. (1982) showed that both the 32 kDa protein and its 54 kDa precursor are genetically absent in the cells of all GS patients and that administration of the 54 kDa precursor to these cells restores the normal level of GAL protein. The 32 kDa subunit was identified as the "corrective factor" missing in GS cells (d'Azzo *et al.*, 1982).

An understanding of the molecular defects in GS became possible after the isolation and characterization of the cDNA encoding human lysosomal CathA. The gene spans 7.5 kb on human chromosome 20 (20q13.1) and comprises 15 exons (Galjart et al., 1988; Shimmoto et al., 1996). The protein is synthesized as a 542 amino acid precursor, glycosylated at Asn 117 in the ER, transported via the mannose-6-phosphate receptor (Morreau et al., 1992) to the lysosomes where it is activated by processing into its two chains of 32 and 20 kDa (Bonten et al., 1995).

The cloning and characterization of the CathA gene, and the resolution of the crystal structure (Rudenko et al., 1995) of the protein enabled the study of the biochemical and structural implications of CathA deficiency in galactosialidosis. Several molecular lesions of the CathA gene in all subtypes of galactosialidosis have been identified. For example, Zhou et al. (1996) identified three novel mutations, V104M, L208P and G411S, in patients with early infantile GS. These mutations prevent the phosphorylation of the CathA precursor and thereby its transport to the lysosome. Examples of late infantile GS mutations include M378T, which generates a new Asn-

linked glycosylation site and Y221N, which decreases the stability of CathA in the lysosome (Zhou et al., 1996). A 2-nucleotide deletion, C517delTT, and an intronic mutation, IVS8+9C-G, resulting in splice defect, generated frameshifts and a protein truncation (Richard et al., 1998). Among Japanese patients with juvenile/adult onset the most frequent mutations are a Y249N change (Fukuhara et al., 1992), and an IVS7, A-G, +3, EX7DEL mutation, resulting in skipping of exon 7 in the mRNA (Shimmoto et al., 1990).

The amino acid sequence of the Cath A has about 30% identity with the other serine carboxypeptidases: yeast carboxypeptidase Y and wheat carboxypeptidase-II (Galjart *et al.*, 1988, Elsliger and Potier, 1994), so the X-ray atomic coordinates of the wheat enzyme were used to model the CathA structure (Elsliger and Potier, 1994). Recently, the X-ray structure of the CathA precursor, expressed in a baculovirus system was determined with a 2.2-2.4 Å resolution (Rudenko *et al.*, 1995). The structure is similar to those of plant and yeast carboxypeptidases and show that CathA belongs to a so-called α/ß-hydrolase family (Remington, 1993). The protein contains a core domain and a cap domain. The core domain consists of a central ten-stranded ß-sheet which is flanked by ten α-helixes and two small ß-strands on both sides. The cap domain consists of three α-helixes and three-stranded mixed ß-sheet. The catalytic triad in the active site is formed by the Ser^{150}, His^{429} and Asp^{372} residues. At acidic pH the enzyme forms 95-98 kDa homodimers, of which the X-ray structure is also known (Rudenko *et al.*, 1995). The pI for the human enzyme is 5.4.

The resolution of the X-ray structure of the CathA precursor (Rudenko et al., 1995) enabled a comprehensive analysis of structural changes induced by the mutations

in CathA molecules (Rudenko et al., 1998). The analysis revealed a correlation between the effects of mutation on protein structure and the clinical phenotype of the affected patients. None of the mutations occurred in the active site or at the protein surface. Of 11 amino acid substitutions modeled, 9 found in patients affected with severe early or late infantile type of GS (Q21R, S23Y, W37R, S62L, V104M, L208P, Y367C, M378T, and G411S) were located in the central core domain of CathA. These substitutions introduced unsatisfied charged groups, hydrogen bonds or bulkier side chains to the protein core; or would create cavities in protein interiors and interfaces. All these changes would dramatically change the folding of mutant CathA, resulting in the impaired sorting and rapid degradation. In contrast, the other 2 mutations (F412V and Y221N associated with a more moderate clinical outcome were located in the α-helical cap domain of the enzyme and predicted to have a milder effect on protein structure. For several mutations (Q21R, W37R, S62L, Y221N, Y367C and F412V) homologous modeling of CathA structure (Elsliger and Potier, 1994) has also made similar predictions.

1.2.2 SIALIDOSIS

Sialidosis belongs to a subgroup of LSD known as mucolipidoses. It is a rare autosomal recessive disorder caused by the primary deficiency SIAL. SIAL is a glycohydrolytic enzyme that catalyzes the removal of terminal sialic acid residues from sialoglycoconjugates. SIAL deficiency results in lysosomal accumulation and excessive urinary excretion of sialyloligosaccharides (reviewed in Cantz and Ulrich-Bott, 1990).

Primary deficiencies of sialidase were first described in the late 1970s and variously referred to as mucolipidosis I (Cantz et al., 1977), the cherry-red spot myclonus syndrome (O'Brien et al., 1977), the Goldberg syndrome (Thomas et al., 1978) and nephrosialidosis (Maroteaux et al., 1978). However, Lowden and O'Brien (1979) provided the nosology, sialidosis, and its classification into two phenotypically distinct subtypes based on dysmorphic features, severity and age of onset: sialidosis type I and sialidosis type II. **Sialidosis type I** (Goldberg syndrome or non-dysmorphic type) is a mild and late-onset form characterized by bilateral macular cherry-red spots, debilitating myoclonus (sudden involuntary muscle contractions) and progressive visual impairment (Durand et al., 1977; Soggs et al., 1979; Rapin et al., 1979; O'Brien, 1979; Federico et al., 1980). These symptoms usually do not appear until the second decade of life. O'Brien and Warner (1980) noted that sialidosis type I was more frequent in Italians and that sialidase in these patients had a normal Km. **Sialidosis type II** (mucolipidosis I, lipomucopolysaccharidosis or dysmorphic type) is a severe and infantile onset form associated with skeletal dysphasia, Hurleroid features (mildly coarse facial features), dysostosis multiplex (bone malformations), mental retardation, hepatosplenomegaly and death in the first decade of life (Kelly et al, 1977; Winter et al., 1980; Oohira et al., 1985). Earlier reports indicated that most type II patients were of Japanese origin and sialidase in these patients had a low Km (O'Brien and Warner, 1980). In a recent study, Nishiyama et al. (1997) reported a cerebral blood flow and glucose metabolism decrease in the occipital lobe region of Japanese patients with the adult onset sialidosis. Other symptoms may include abdominal swelling; lack of muscle tone (hypotonia); loss of muscle mass (atrophy); irregular, involuntary spastic movements with choreoathetosis and spasticity; the protrusion of a portion of the intestines through an abnormal opening

in the muscular wall of the abdomen (inguinal hernia) (Provenzale et al., 1995) **Nephrosialidosis** (congenital sialidosis), a phenotypic variant of sialidosis type II, presents type II symptoms as well as severe hydrops fetalis, ascites and proteinuria. Death usually results from edema, dysproteinuria and bleeding (Maroteaux et al., 1978; Tylki-Szymanska et al. 1996). In addition to the above clinical features, (**Article 4,** Buchholz et al., 2000) report severe dilated coronary arteries, excessive retinal tortuosity and an erythematous, macular rash in an infant with congenital sialidosis.

Sialidosis is a very rare lysosomal storage disorder with no significant prevalence in a select population. There have been no published reports on the global incidence of sialidosis since Tipton et al. noted at least 11 cases in 1978. However, according to the Mucopolysaccharadosis (MPS) society, sialidosis occurrence is between 1 in 250000 and 1 in 4.2 million (www.mpssociety.ca/diseases). Various studies based on geographic and/or ethnic distribution of lysosomal storage diseases have been conducted. In the Netherlands, only 49 cases of mucolipidoses and oligosaccharidoses were diagnosed between 1970 and 1996 with a combined birth prevalence is 1.0 per 100,000 live births (Poorthuis et al., 1999). In Australia 27 different lysosomal storage diseases were diagnosed in 545 patients and a prevelance ranging from 1 in 57 000 live births for Gaucher disease to 1 in 4.2 million live births for sialidosis was reported (Meikle et al., 1999). The overall incidence for all types of LSD in this study was approximately 1 in 7700 live births. Since the Australian population has mainly a British ancestry, with a minor contribution from other European countries and Asia, Meikle et al. believe their results could be extrapolated to white non-Hispanic populations in the United States, Canada and the United Kingdom. At least fifteen cases of sialidosis type

II have been reported in Japan (Nishiyama et al., 1997; **Article 3**, Naganawa et al., 2000).

1.3 Sialidases (neuraminidases)

Sialidases alternatively named exo-alpha sialidases, N-acylneuraminate glycohydrolases, N-acetyl neuramyl hydrolases or neuraminidases (EC 3.2.1.18) are glycosydases that hydrolyze the alpha-2,3-, alpha-2,6- and alpha-2,8 linkages of terminal sialic acid residues in various sialoglycoconjugates including oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates.

Sialidases are widely distributed in vertebrates and also in microorganisms such as viruses, bacteria, fungi and protozoa, most of which are unable to produce sialic acid themselves (Miyagi et al., 1993; Warner et al., 1993, Colman 1994; Schenkman et al., 1994; Chou et al., 1996). In microorganisms, sialidases are thought to be important in nutrition, using free sialic acid as a good source of energy (Corfield, 1992). In certain cases the bacterial sialidases are involved in pathogenesis. For example, Vibrio cholerae sialidase removes sialic acid from higher order gangliosides to produce G_{M1}, the binding site for cholera toxin (Galen, 1992; Corfield, 1992; Tang et al., 1996). In vertebrates, sialidases modulate cellular invents such as activation, differentiation, maturation and growth, all of which involve the changes of sialic acid level (Schauer T, 1985).

1.3.1 Mammalian sialidases

In mammals, sialidases were initially grouped into four groups based on their subcellular localization, substrate preference and pH optimum (Verheijen, 1983; Miyagi et al., 1990, 1993; Bonten et al., 1996 and Pshezhetsky et al., 1997). They included cytosolic, plasma membrane, lysosomal membrane and intralysosomal sialidases. However, as described below, the latter two turn out to be the same protein, here called lysosomal sialidase.

1.3.1.2 *Cytosolic sialidase*

Cytosolic sialidase was first cloned from rat skeletal muscle and Chinese hamster ovary cells and found to be optimally active at pH 6.0 (Miyagi et al., 1990a, 1993; Sato and Miyagi, 1995). Using a sequence homology-based approach, Monti et al. (1999) identified the human gene, named NEU2, mapping it to chromosome 2q37. NEU2 encoded protein is a 380-amino-acid polypeptide with two Asp-containing motifs with conserved amino acid consensus sequences (G-X-D-X-G-X-X-W/F) and a YRIP sequence in the amino terminal part of the primary structure. The protein is a functional sialidase with a molecular mass of 42 kDa and a pH optimum of 5.6. Its cytosolic localization was demonstrated by cell fractionation. These cytosolic sialidases have a broad specificity and are active against α,2 3-sialylated oligosaccharides, glycopeptides and gangliosides (such as G_{M3}, G_{D1a} and G_{D1b}-gangliosides, but not G_{M1} and G_{M2}-gangliosides) as well as the artificial fluorometric substrate, 4 methylumbelliferyl-alpha-D-N-acetylneuramic acid (4MU-NeuAc) (Miyagi and Tsuiki, 1985). The exact biological role of this enzyme, which is expressed mostly in muscle

cells especially during their diffentiation is not known (Sato and Miyagi, 1996; Akita et al., 1997). However, it was suggested that this enzyme may cleave G_{M3}-ganglioside, associated with the cytoskeleton leading the alteration of cytoskeletal functions. The cytosolic sialidase activity of melanoma cells inversely correlates with their invasive and metastatic potential (Tokuyama et al., 1997).

1.3.1.2 *Plasma membrane sialidase*

Plasma membrane sialidase, also referred to as G_{M1}-ganglioside sialidase or G_{M1} sialidase as well as ganglioside sialidase is an integral membrane protein with a strict specificity for gangliosides such as G_{M1}, G_{D1a} and other polysialogangliosides (Schneider-Jakob and Cantz, 1991) but not glycoproteins or oligosaccharides (Kopitz et al., 1997; Miyagi et al., 1999). Recently, Miyagi et al. (1999) cloned and expressed a ganglioside sialidase cDNA isolated from bovine brain cDNA library. The enzyme encodes a 428-amino acid protein with a deduced molecular mass of 47.9 kDa. The enzyme contains a transmembrane domain and three Asp boxes. The enzyme is not affected in GS or sialidosis (Zeigler et al., 1989). Plasma membrane sialidase has a pH optimum of 4.6 and is activated by non-ionic detergents such as Triton-100. It has been recognized as an important modulator of cellular functions including cell-cell and cell-matrix interactions, cell proliferation, differentiation and oncogenic transformation (Hakomori and Igarashi., 1993; Kopitz et al., 1996, 1998). In ganglions, the enzyme is probably involved in neuritogenesis, synaptogenesis and neuronal survival (Tettamanti and Robin, 1993; Kopitz et al., 1994, 1997). Monti et al. (2000) cloned cDNA of human plasma-membrane-associated sialidase (NEU3), which has a 78% sequence homology

to the bovine protein (Miyagi et al., 1999). Human NEU3 is also a 428-residue protein highly active against ganglioside substrates, but has a pH optimum of 3.8.

1.3.1.3 *Lysosomal sialidase*

Lysosomal sialidase or lysosomal N-acetyl-alpha-neuraminidase is a hydrolytic glycoprotein with pH optimum of 4.2. It catalyzes the removal of terminal sialic acid residues from oligosaccharides, gangliosides, glycolipids and glycoproteins, but inactive against glycoproteins such as fetuin or submixiallary mucin (Miyagi and Tsuiki, 1984, Schneider-Jakob and Cantz, 1991, Hiraiwa et al. 1987, 1988). The released sialic acid triggers further degradation of the sugar moiety. The human lysosomal sialidase (SIAL) preferentially cleaves α,2-3 and α,2-6 sialyl bonds in sialoconjugates (Frisch and Neufeld, 1979). Some authors reported that in addition to soluble (luminal) sialidase, lysosomes also contain another enzyme, associated with their membranes. **Lysosomal membrane sialidase** has not been characterized, however, Miyagi et al. (1990) who immunoprecitated the enzyme from rat liver, reported that lysosomal membrane sialidase has an acidic pH optimum like its plasma membrane counterpart. The enzyme has a broad specificity in hydrolyzing gangliosides as well as fetuin and sialyllactose 4MU-NeuAc (Kopitz et al., 1996). Since lysosomal sialidase is often completely deficient in the cells of patients affected with the inherited disease, sialidosis (Bonten et al., 1996; Pshezhetsky et al., 1997) both lysosomal membrane and intralysosomal sialidases are probably products of the same gene. Furthermore, T lymphocyte activation is associated with an increase of sialidase activity on cell surface (Chen et al., 1997, 2000). However, in SM/J or SM/B10 mouse strains with lysosomal sialidase gene defect, no T-cell

activation occurs, proving also that a single gene codes for both lysosomal sialidases (Carrillo et al., 1997; Rottier et al., 1998).

1.3.2 Human lysosomal sialidase

Earlier attempts to purify and characterize SIAL were complicated by the extreme lability and the membrane-bound character of the enzyme. Studies by Verheijen et al. (1982, 1985) indicated that in bovine testis, SIAL copurifies with CathA and GAL and that SIAL is activated by CathA. These findings suggested that in the lysosomes of mammalian tissues the three glycoproteins: CathA, GAL and SIAL form a large molecular complex. Morreau et al. (1992) suggested that GAL and CathA associate soon after synthesis in the ER and co-migrate in the lysosomes, where they acquire their active and stable conformations. The components of the complex can be copurified using GAL or CathA affinity matrices (Potier et al., 1990; Pshezhetsky and Potier, 1994). Pshezhetsky and Potier,(1994) purified human placental CathA on agarose-Phe-Leu affinity and concluded that CathA forms 1270-kDa complex with CathA, GAL and N-acetylgalactosamine-6-sulfatase (GALNS). Only a small percentage of total CathA and GAL activities occur in the complex, which however contains all SIAL activity. This explains the difficulty in isolating SIAL activity separately from the complex (Hoogeveen et al., 1983; Warner el., 1990; Hubbes et al., 1992).

1.3.2.1 *Activity and Specificity*

Characterization of the storage products in urine and cultured fibroblasts from patients affected with sialidosis revealed that sialylated oligosaccharides are a major

natural substrate for SIAL (Strecker et al., 1977; Dorland et al., 1978, van Pelt et. al., 1988). The involvement of SIAL in the hydrolysis of sialylated gangliosides has been a matter of debate for a long time. The results on the analysis of the storage products in the autopsy materials from sialidosis and GS patients were controversial. For some patients several fold increase of G_{M3} and G_{D3} gangliosides was reported in systemic organs (Ulrich-Bott et al., 1987) and brain (Yoshino et al., 1990), although in the other similar cases (Sakuraba et al., 1983) or in the knock-out mouse model of GS (Zhou et al., 1995) storage of gangliosides was not observed. The cultured fibroblasts of sialidosis and galactosialidosis patients treated with radioactively labeled GM1-ganglioside accumulated G_{M3}-ganglioside (Mancini et al., 1986) that strongly suggested that SIAL is involved in vivo in degradation of this glycolipid.

Further studies (Schneider-Jakob and Cantz, 1991, Hiraiwa et al. 1987, 1988) showed that the hydrolysis of gangliosides by SIAL depends on presence of detergent, sodium cholate or taurodeoxycholate (Triton X-100 that activated plasma membrane sialidase does not have effect on SIAL), suggesting that in vivo this reaction requires activator proteins. That was confirmed by Fingerhut et al. (1992), who showed that SIAL cleaved G_{M3}, G_{D1a}, and G_{T1b}-gangliosides in the presence of Saposin B also called sulfatide activator protein. The complete hydrolysis of G_{D1b}-ganglioside to lactosylceramide by glycoprotein fraction from human placenta containing essentially all soluble lysosomal enzymes required the presence of two activators, Saposin B that activated reactions catalysed by GAL and SIAL and G_{M2}-activator that activated reaction of G_{M2} to G_{M3} conversion by HEX A. Gangliosides G_{D1b}, G_{M1} and G_{M2} were extremely poor substrates for sialidase (Fingerhut et al., 1992).

However the last conclusion was reconsidered when asialylated G_{M1} and G_{M2} gangliosides, G_{A1} and G_{A2}, respectively were found among major storage products in knock-out mouse models of G_{M1}-gangliosidosis (Matsuda et al., 1997) and Sandhoff disease (combined deficiency of HEX A and B) (Huang et al., 1997).

Sandhoff mice succumbed to a profound neurodegenerative disease by 4-6 months of age, resembling human phenotype while Tay-Sachs mice depleted of hexosaminidase A only remained asymptomatic to at least 1 year of age because G_{A2}-ganglioside was further efficiently cleaved by hexosaminidase B (Huang et al., 1997). Therefore mouse models of Tay-Sachs disease have revealed a metabolic bypass of the genetic defect based on the more potent activity of SIAL towards G_{M2}. To determine whether increasing the level of SIAL would produce a similar effect in human Tay-Sachs cells, Igdoura et al (1999) introduced a human sialidase cDNA into neuroglia cells derived from a Tay-Sachs fetus and demonstrated a dramatic reduction in the accumulated G_{M2}. These studies proved involvement of SIAL in the hydrolysis of G_{M2} and suggested a new method for the treatment of human Tay-Sachs disease.

1.3.2.2 *Biological role of SIAL*

As discussed above, the role of SIAL in the intralysosomal catabolism of sialylated glycolipids and glycoproteins is well-established. Multiple data, however, suggest that SIAL plays a pivotal role in cellular signalling. In particular, SIAL of T lymphocytes converts, so called, vitamin D3-binding protein (also known as group specific component or Gc protein) into a macrophage activating factor (MAF), that is necessary for the inflammation-primed activation of macrophages (Yamamoto and Homma, 1991; Yamamoto et al., 1993, 1996; Naraparaju and Yamamoto, 1994). In

addition, SIAL of T cells is required for production of the cytokine IL-4, the potent regulator of many hemopoetic and nonhemopoetically derived cells and tissues. Sialidase is involved in both early production of IL-4 and the IL-4 priming processing of conventional T cells to become active IL-4 producers (Chen et al., 1997, 2000). The T-cells derived from SM/j and B10/SM strains of mice, deficient in lysosomal SIAL due to the point missense mutation in *Neu-1* gene (Carrillo et al., 1997; Rottier et al., 1998) failed both to convert Gc to MAF and synthezise IL-4, whereas B-cells of these mice were not able to produce IgG1 and IgE after immunization with pertussis toxin (Yamamoto et al., 1993; Landolfi et al., 1985; Chen et al., 1997). Most probably the function of SIAL is connected with desialylation of surface antigen-presenting molecules such as MHC class I, required to render T cells responsive to antigen presenting cells (Landolfi and Cook, 1986).

1.3.2.3 *Cloning and characterization of human lysosomal sialidase*

Bacterial sialidases share an overall sequence identity of about 35% and all contain the so-called F/YRIP domain situated in the amino terminal and several conserved "Asp boxes" (consensus sequence Ser/Thr-X-Asp-X-Gly-X-X-Trp/Phe), repeated three to five times at topologically identical positions (Roggentin et al., 1989; Rothe el.,1991). Structural analyses indicate that the active site of these enzymes is located within identical monomers of about 40 kDa, each made up of six antiparallel beta sheets arranged as the blades of a propeller (Gaskell et al., 1995).

Using the bacterial conserved sequences and taking advantage of the rapid progress of the Human Genome Project, several groups simultaneously cloned and sequenced human (Bonten et al., 1996; Pshezhetsky et. al., 1997; Milner et al., 1997)

and mouse (Carillo et al., 1997; Igdoura et al., 1998, Rottier et al., 1998). Pshezhetsky et al. (1997), for example, searched the expressed sequences tags database (dbEST) for human analogues and found overlapping clones of human fetal brain, spleen and placenta. Using primer complementary to the sequences of two overlapping clones, they obtained a complete cDNA by RT-PCR amplification. In the dbEST databank, Bonten et al. (1996) also identified a full-length SIAL cDNA clone that recognized an mRNA of 1894 nucleotides long. The mRNA was highly expressed in the pancreas, kidney and skeletal muscle, but low in the brain. Both Bonten et al. (1996) and Pshezhetsky et al. (1997) localized the gene by in situ hybridization on chromosome 6p21.3 within the human major histocompatibility complex as previously suggested by Oohira et al. (1985).

The SIAL cDNA showed an open reading frame (ORF) of 1245 nucleotides, encoding a protein of 415 amino acids. The first 47 amino acids represent the signal peptide which comprise a positively charge amino region, a central hydrophobic core and a polar carboxy-terminal domain. The protein contains a F/YRIP domain and four Asp boxes characteristic of bacterial and rodent cytosolic sialidases (Roggentin et al., 1993; Carillo et al., 1997). This region is highly conserved between the human and bacterial sialidases with the extent of homology ranging from 32% to 38%. Ironically, the human sialidase is more homologous to the most bacterial sialidases than to the cytosolic sialidase from Chinese hamster of rat (Roggentin et al., 1993).

The human sialidase has three potential N-glycosylation sites at positions 185, 343 and 352. The active site residues are also conserved in bacterial, rodent and human sialidases. The conserved residues are at Arg 37, 246 and 309 in *Salmonella typhymurium*, Arg 78, 280 and 347 in humans, and Arg 72, 274 and 341 in mouse

(Crennell et al., 1993; Pshezhetsky et al., 1997; Igdoura et al., 1998). The predicted molecular mass of the SIAL protein is 45.4 kD (Bonten et al., 1996) and recent studies demonstrate that after the cleavage of the signal peptide and N-glycosylation, the mature and active form of the protein has a mass of 48.3 kDa (Vinogradova et al., 1998).

1.3.2.4 Mutations in the sialidase gene

Since the identification of the SIAL cDNA (Bonten et al., 1996; Pshezhetsky et al. 1997; Milner et al., 1997) considerable progress has been made in the understanding of the molecular defects and biochemical mechanism of sialidosis. In two siblings with sialidosis type I, Bonten et al. (1996) identified a heterozygous **1258G T** transversion, which introduced a premature TAG termination codon at amino acid 377 and caused a C-terminal truncation of 38 amino acids. In another patient with sialidosis type II, they found a compound heterozygosity for a **401T G** transversion (Leu91Arg) in one allele and the other allele contained a **1337delG** deletion that caused a frameshift at amino acid 403 and a 69-amino acid extension of the protein. The extension produced a 53-kDa protein immunoprecipitated from the patient's fibroblasts. Pshezhetsky et al., (1997) identified two missense mutations in sialidosis type II patients: a **779 T A** (Phe260Tyr) transversion and a **1088 T C** transition (Leu363Pro) in a cell line, GM01718A, obtained from a 2-month old infant with sialidosis type II. Another mutation was a frameshift caused by an ACTG duplication after nucleotide 7 (**7insACTG**) in the GM11604 cell line from a sialidosis type II patient. In the SM/J mice with a deficiency of the Neu-1 neuraminidase, Rottier et al. (1998) identified a 625C A (Leu209Ile) transversion, which accounts for the partial deficiency of lysosomal sialidase.

1.3.2.5 *Processing and lysosomal targeting of human sialidase*

As reviewed above, SIAL is translated from a single splice mRNA of 1245 bp, encoding a 415 amino acid precursor protein. After the cleavage of 47-amino acid N-terminal signal peptide and glycosylation it becomes a 48.3 kDa mature active enzyme present in the multienzyme lysosomal complex. However, the exact mechanism of sorting of the sialidase precursor until now remained unclear. Comparing the intracellular distribution of human sialidase expressed in COS-1 cells transfected with sialidase cDNA alone or co-transfected with sialidase and human CathA cDNA, Van der Spoel et al. (1998) suggested that sialidase associates with CathA precursor shortly after synthesis and that this complex is targeted to the lysosome using a mannose-6-phosphate receptor-dependent pathway. In the absence of CathA, SIAL is partially secreted and partially segregates to endosomal compartment (van der Spoel et al., 1998). In contrast, numerous data demonstrated the existence of two pools of SIAL in the lysosome, soluble and membrane associated. Both forms are absent in cultured cells of sialidosis patients and are, therefore encoded by the same gene (Miyagi et al., 1990, 1992, 1993; Verheijen et al, 1983). In addition the activation of T lymphocytes is associated with several-fold increase of sialidase activity on the cell surface. The activation does not happen in T-cells obtained form SM/J or SM/B10 mouse strains containing a mutation in SIAL gene (Landolfi et al., 1985; Naraparaju and Yamamoto, 1994), which proves that sialidase expressed on the cell surface is the product of the same gene. Analysis of the deduced amino acid sequence of SIAL (Pshezhetsky et al., 1997) revealed that the C-terminal tetrapeptide, ^{412}YGTL^{415} has similarity to the

internalization signals of several endocyted surface receptors and lysosomal membrane proteins. Tyr-X-X-Φ (hydrophobic residue) internalization signals have previously been reported in cytoplasmic domains of several internalized membrane proteins including glucocerebrosidase LAMP-I, LAMP-II, lysosomal membrane glycoprotein (LGP-85), low density lipoprotein (LDL), transferrin, asyaloglycoprotein, polymeric immunoglobulin and cation-independent mannose-6-phosphate receptor, (reviewed above and in Peters and Figura, 1994, Pearse et al., 2000; Hirst and Robinson, 1998).

1.4 Research Objectives

To sumarize, a cell may contain several hundred lysosomes which harbour a wide variety of soluble hydrolytic enzymes that function at an acidic pH and are capable of digesting essentially all types of biologic macromolecules. Once synthesized in the ER, proteins are generally glycosylated and translocated to the Golgi where soluble proteins are either secreted or targeted to the lysosomes. In the Golgi, lysosomal enzymes are modified by N-acetyl glucosamine phosphotransferase which participates in the synthesis of the mannose-6-phosphate marker. This marker then addresses the enzymes to their lysosomal destination. Other lysosomal enzymes like acid phosphatase and glucocerebrosidase lack the mannose-6-phosphate marker but are routed to the lysosome by an alternative pathway involving a C-terminal targeting signal.

It is becoming increasing clear that lysosomal enzymes associate to form supramolecular structures as a measure of protection against hydrolyses by other enzymes within the lysosome and/or to facilitate the sequential catabolism of heterogeneous macromolecules. It is therefore conceivable that a defect in one or more

of the components of the complex may disrupt the integrity of the complex, exposing the individual components to degradation. A classic example is the 1.27 MDa lysosomal complex comprising CathA, GAL, SIAL and GALNS. CathA is involved in the protection and activation of both GAL and SIAL. A deficiency of CathA results in the secondary deficiency of both GAL and SIAL in the lysosomal storage disorder galactosialidosis. Primary deficiency of SIAL causes another lysosomal storage disorder, sialidosis.

In this project, our objective was to study the biogenesis and structure of human lysosomal sialidase as well as its molecular pathology in two rare inherited disorders, galactosialidosis and sialidosis. Our main goals were as follows:

1) **Study of the biogenesis and intracellular localization of SIAL in the fibroblasts of GS patients**.

To understand the molecular mechanism of SIAL deficiency in GS cells that lack CathA, we expressed the enzyme in the fibroblasts of GS patient. We have studied the intracellular localization of SIAL using both immunofluorescent and immunoelectron microscopy. Metabolic labeling was used to address the maturation and stability of SIAL.

2) **Study of the mechanism of sialidase targeting to the lysosome**.

Numerous reports have demonstrated that lysosomal integral membrane proteins, are targeted to the lysosomes via a mechanism involving the interaction of the internalization Tyr-X-X-Φ C-terminal motif (Hunziker and Geuze, 1996; Honings et al., 1996). Since this signal (V^{411}**YGTL**^{415}) also occurs in sialidase, we hypothesized that the enzyme may be routed to the lysosome by a similar pathway. To determine its involvement in targeting, we mutated both Tyr and Leu, key residues in the signal,

to Ala and studied the expression of the mutants by cellular fractionation and activity, assay as well as by immunofluorescent microscopy.

3) **Identification and characterization of molecular defects in the sialidase gene.**

We identified 9 new mutations in the sialidase gene by single-strand conformation polymorphism (SSCP), heteroduplex analysis, allele-specific oligonucleotide hybridization (ASO) analysis and direct sequencing. To characterize the mutations, we introduced them by site-directed mutagenesis in the pCMV expression vector containing the sialidase cDNA. We expressed the mutant proteins in COS-7 or fibroblasts cells and evaluated their biosyntheses, intracellular localization, as well as maturation, stability and ability to associate with the multienzyme lysosomal complex.

4) **Homology modeling of human lysosomal sialidase**

To determine the impact of mutations on the structure of sialidase, we generated structural models of the enzyme based on the atomic coordinates of homologous bacterial sialidases. Our results allowed to predict the effects of specific mutations and to speculate on how some of them affect the integrity of the 1.27 MDa multienzyme complex.

CHAPTER 2

Sialidase deficiency in galactosialidosis

Foreword

As reviewed in the introduction, CathA forms a 1.27 MDa lysosomal multienzyme complex with SIAL, GAL and GALNS, protecting these enzymes against excessive proteolytic degradation and contributing to their stability and lysosomal activity. In the neurodegenerative lysosomal storage disease, GS, a primary defect of CathA results in the combined secondary deficiencies of both SIAL and GAL. Studies investigating molecular mechanism of GAL deficiency in galactosialidosis indicated that GAL precursor is incorrectly processed and rapidly degraded. The rapid degradation was due to mutations in the CathA gene that prevented the interaction and hence protection of GAL (Hoogeveen et al., 1982; Galjart et al., 1992; Okamura-Oho, 1996).

In this article we have characterized the molecular mechanism of SIAL deficiency in galactosialidosis by studying the biogenesis of the enzyme in GS fibroblast cell lines. We have raised anti-sialidase antibodies and used them to characterize the glycosylation and processing of the enzyme by western blotting. The same antibodies were used for metabolic labeling and immunofluoresncent and immunoelectron microscopy to characterize the processing, targeting and stability of the SIAL in GS cells. Our results demonstrate that in GS cells, mature SIAL is rapidly degraded to catalytically inactive forms and that the expressed protein is localized both in the lysosomes and on plasma membranes.

Article1

Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation

Biochem. J. (1998) 330, 641–650 (Printed in Great Britain)

Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation

Maia V. VINOGRADOVA*[1], Lorraine MICHAUD*, Alexander V. MEZENTSEV*, Kiven E. LUKONG*, Mohamed EL-ALFY†, Carlos R. MORALES†, Michel POTIER* and Alexey V. PSHEZHETSKY*[2]

*Université de Montréal, Service de Génétique Médicale, Département de Pédiatrie, Hôpital Sainte-Justine, Montréal, Québec, H3T 1C5 Canada, and †Department of Anatomy and Cell Biology, McGill University, Montréal, Québec, Canada

Galactosialidosis is an inherited lysosomal storage disease caused by the combined deficiency of lysosomal sialidase and β-galactosidase secondary to the deficiency of cathepsin A/protective protein, which is associated with sialidase and β-galactosidase in a high-molecular weight (1.27 MDa) complex. Clinical phenotypes of patients as well as the composition of compounds which are stored in patient's tissues implicate sialidase deficiency as the underlying pathogenic defect. The recent cloning and sequencing of lysosomal sialidase [Pshezhetsky, Richard, Michaud, Igdoura, Wang, Elsliger, Qu, Leclerc, Gravel, Dallaire and Potier (1997), Nature Genet. **15**, 316–320] allowed us to study the molecular mechanism of sialidase deficiency in galactosialidosis. By Western blotting, using antibodies against the recombinant human enzyme, and by NH_2-terminal sequencing, we showed that sialidase is synthesized as a 45.5 kDa precursor and after the cleavage of the 47-amino acid signal peptide and glycosylation becomes a 48.3 kDa mature active enzyme present in the 1.27 kDa complex. Transgenic expression of sialidase in cultured skin fibroblasts from normal controls and from galactosialidosis patients, followed by immunofluorescent and immunoelectron microscopy showed that in both normal and affected cells the expressed sialidase was localized on lysosomal and plasma membranes, but the amount of sialidase found in galactosialidosis cells was ~ 5-fold reduced. Metabolic labelling studies demonstrated that the 48.3 kDa mature active form of sialidase was stable in normal fibroblasts (half-life ~ 2.7 h), whereas in galactosialidosis fibroblasts the enzyme was rapidly converted (half-life ~ 30 min) into 38.7 and 24 kDa catalytically inactive forms. Altogether our data provide evidence that the molecular mechanism of sialidase deficiency in galactosialidosis is associated with abnormal proteolytic cleavage and fast degradation.

INTRODUCTION

Sialidase (acetylneuraminyl hydrolase or neuraminidase, EC 3.2.1.18) catalyses the hydrolysis of terminal sialic acid residues of oligosaccharides, glycoproteins and glycolipids. Lysosomal sialidase is associated with cathepsin A (CathA, EC 3.4.16.1, also named 'protective protein'), β-galactosidase (GAL, EC 3.2.1.23) and *N*-acetylgalactosamine-6-sulphate sulphatase (GALNS, EC 3.1.6.4) in a 1.27 MDa multienzyme complex [1–5]. This complex is essential for the expression of sialidase and GAL activities in the lysosome [1–3] as confirmed by an autosomal recessive disease, galactosialidosis, characterized by the combined deficiency of GAL and sialidase secondary to CathA deficiency [6,7]. Clinical phenotypes are different for the patients with early infantile, late infantile and juvenile/adult onsets of galactosialidosis, but generally involve cherry-red macular spots, corneal clouding, skeletal dysplasia, hepatosplenomegaly, growth retardation, neurologic and mental deteriorations. These features as well as the composition of the lysosomal storage compounds (mostly sialylated gangliosides, oligosaccharides and glycopeptides), which are accumulated in patient's tissues and excreted in urine resemble those of patients with the primary sialidase genetic defect, sialidosis [8], and implicate sialidase deficiency as the major pathogenic defect underlying galactosialidosis.

Previous studies showed that in galactosialidosis cells the 85 kDa GAL precursor is incorrectly processed and rapidly degraded [2,9]. The characterization of various galactosialidosis mutations in CathA [10–13] as well as inactivation of CathA by site-directed mutagenesis [14] demonstrated that rapid degradation of GAL in galactosialidosis results from the absence of its interaction with CathA. On the basis of the structural modelling we suggested that in the complex, CathA monomers cover most of the GAL surface thus preventing the intralysosomal proteolysis of GAL [15]. Recently we showed that in the lysosome CathA may also protect GALNS, another component of the 1.27 MDa complex, since both GALNS activity and cross-reacting material were reduced in the fibroblasts of patients affected with galactosialidosis [5].

The molecular mechanism of sialidase deficiency in galactosialidosis has never been characterized since until recently the lysosomal sialidase has not been cloned or purified. The sialidase activity has always been associated with the 1.27 MDa complex in the process of purification from tissues [16–20]. The dissociation of the complex resulted in the complete inactivation of sialidase. However, the activity could be restored after the reassociation of the complex *in vitro* [3]. These results led to the hypothesis that the association with the complex is required for sialidase to adopt the catalytically active conformation [3] but direct experimental evidence supporting this mechanism was not obtained.

Recently we have identified a cDNA coding for human lysosomal sialidase and characterized molecular defects in several

Abbreviations used: CathA, cathepsin A/protective protein; GAL, β-galactosidase; HEX, β-hexosaminidase; GALNS, *N*-acetyl-galactosamine-6-sulphate sulphatase; PATGAL, *p*-aminophenyl-β-D-thiogalactopyranoside; FPLC, fast protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; GST, glutathione transferase; HBSS, Hank's Balanced Salt Solution; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-HCl-buffered saline; LAMP, lysosomal integral membrane protein; PAGE, polyacrylamide gel electrophoresis; EMEM, Eagle's Minimal Essential Medium, 4 MU α-neur, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid.

[1] Present address: A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119899, Russia.

[2] To whom correspondence should be addressed.

sialidosis patients [21]. In the present work in order to understand the molecular mechanism of sialidase deficiency in galactosialidosis we studied the biogenesis and intracellular localization of the enzyme in cultured skin fibroblasts of normal controls and of galactosialidosis patients, using the antibodies raised against the recombinant human sialidase. We demonstrated that in both types of cells the sialidase is targeted to lysosomes, but in galactosialidosis fibroblasts it is abnormally processed and further rapidly degraded.

MATERIALS AND METHODS

Cell culturing

Human skin fibroblasts from galactosialidosis and sialidosis patients were obtained from NIGMS Human Genetic Mutant Cell Repository (GM05076, GM02438A), the Montreal Children's Hospital Cell Repository (WG544) and Sainte-Justine Hospital Cell Repository (89.40.11, 83.29.34). Cells were cultured to confluency in Eagle's Minimal Essential Medium (EMEM, Mediatech, Washington DC), supplemented with 10% (v/v) fetal calf serum (MultiCell) and antibiotics.

Purification of lysosomal high-molecular-weight complex

The 1.27 MDa lysosomal complex was purified from human placenta extract by affinity chromatography on a concanavalin A-Sepharose column and a *p*-aminophenyl-β-D-thiogalactopyranoside (PATGAL)-agarose column as previously described [22]. Prior to the PATGAL-agarose chromatography, the crude glycoprotein fraction from human placenta extract was concentrated to 40 mg/ml of protein, dialysed against 20 mM sodium acetate buffer, pH 4.75, containing 50 mM EDTA, and incubated for 120 min at 37 °C to activate and stabilize lysosomal sialidase [3,17]. After affinity purification the preparation was dialysed against 20 mM sodium acetate buffer, pH 5.2, containing 0.15 M NaCl, 0.02% (w/v) NaN_3 and 50 mM EDTA, concentrated to 3 mg/ml of protein, applied to a FPLC Superose™ 6 column (Pharmacia) and eluted with the same buffer at a flow rate of 0.4 ml/min. Collected fractions were analysed for sialidase, GAL, and CathA activities [5] as well as by SDS/PAGE and Western blotting as described below. The M_r values of the eluted enzymes were determined using the calibration curve obtained with the following M_r standards (Pharmacia): blue dextran (M_r ~2000 kDa), thyroglobulin (M_r 669 kDa), apoferritin (M_r 440 kDa), catalase (M_r 232 kDa), aldolase (M_r 158 kDa), chymotrypsinogen (M_r 25 kDa), and ribonuclease A (M_r 13.7 kDa). The deglycosylation of sialidase was performed by treatment of 1.27 MDa complex (20 μg) by 2 U of endoglycosidase F (Sigma) in a 20 mM sodium phosphate buffer, pH 7.0, containing 0.1% (w/v) SDS, 0.2% (w/v) DTT and 0.5% (v/v) Triton X-100 for 17 h at 37 °C.

Antibodies

Rabbit polyclonal antiserum against human lysosomal hexosaminidase A was a generous gift of Dr. Roy A. Gravel (McGill University, Montreal). Rabbit polyclonal antibodies against recombinant human sialidase were prepared as follows. A 755 kb *Bst*EII/*Tag*I fragment of sialidase cDNA, encoding amino acids 58–308 of the sialidase protein, was treated with Klenow DNA polymerase and inserted into pGEX-2T vector (Pharmacia), restricted with *Eco*RI and treated with Klenow DNA polymerase. The plasmid was expressed in *E. coli* to produce glutathione transferase (GST)-sialidase fusion protein. The fusion protein was purified from the bacteria homogenate by affinity chromatography using glutathione-Sepharose (Pharmacia) followed by the FPLC anion-exchange chromatography on Mono Q column (Pharmacia). Purified fusion protein (1 mg), homogeneous by SDS/PAGE analysis, was used to immunize a rabbit. IgG fraction purified from the obtained antiserum by ammonium sulfate fractionation was passed through a recombinant GST-Sepharose column to absorb the anti-GST-specific antibodies. The resulting antibody preparation was used in a dilution of 1:2500 or 1:5000 for Western blotting, 1:500 – for immunoelectron and 1:200 – for immunofluorescent microscopy.

Table 1 Immunoprecipitation of lysosomal, cytosolic and plasma membrane sialidase, GAL and HEX in human liver extract by anti-recombinant human lysosomal sialidase antibodies

	Enzyme activity (% to control)				
Antibodies added (μg)	Lysosomal sialidase	Cytosolic sialidase	Plasma membrane sialidase	HEX	GAL
9	100	100	100	100	100
2	65	98.2	98	105	97.6
5	13.4	98.5	98	105	96.9
10	4.1	95.5	95	102	95.1
20	1.0	94	103	105	92.0
50	2.0	96	98	105	92.1

Immunotitration

1 g of human liver tissue obtained from autopsy materials was homogenized in 50 mM sodium phosphate buffer, pH 6.0, containing 100 mM NaCl and 0.5% (w/v) of sodium deoxycholate. The homogenate was centrifuged at 35000 *g* for 30 min. 200 μl of homogenate supernatant were then mixed with 100 μl of 50 mM sodium phosphate buffer, pH 6.0, containing 100 mM NaCl and 10 mg/ml of BSA and increasing amounts (1–50 μg) of anti-human sialidase antibodies or of IgG fraction from preimmune rabbit serum. In control experiments instead of liver extract we used 10 μg of 680 kDa placental GAL-CathA complex [22] dissolved in 200 μl of phosphate buffer mentioned above. After 2 h of incubation at 4 °C, the pellet from 300 μl of Pansorbin Cells (Calbiochem) was added. The samples were incubated for 2 h at 4 °C with constant shaking and then centrifuged at 13000 *g* for 10 min. The supernatants were assayed for GAL and β-hexosaminidase (HEX) activities as described [5]. Activity of lysosomal and cytosolic sialidase was assayed with 4 MU α-neur at pH 4.2 and 6.5, respectively [23]. Plasma membrane sialidase was assayed with GM2-ganglioside in the presence of 0.2% Triton-X100 as described [23,24]. The concentration of released sialic acid was measured by phenobarbituric method [25].

The immunotitration results (Table 1) showed that antibodies raised against recombinant lysosomal sialidase precipitated all activity of lysosomal sialidase in human liver extract, but not of cytosolic or plasma membrane sialidases, thus confirming that the antibodies are specific against the lysosomal enzyme and that the lysosomal, plasma membrane and cytosolic sialidases do not have common antigenic determinants, as has been suggested [26]. The anti-sialidase antibodies did not precipitate purified GAL (not shown) but precipitated up to 8% of GAL activity in liver extract, which probably represents the fraction of GAL associated with the lysosomal sialidase in the 1.27 MDa complex. The activity of HEX which is not part of the complex was not changed by anti-sialidase antibodies.

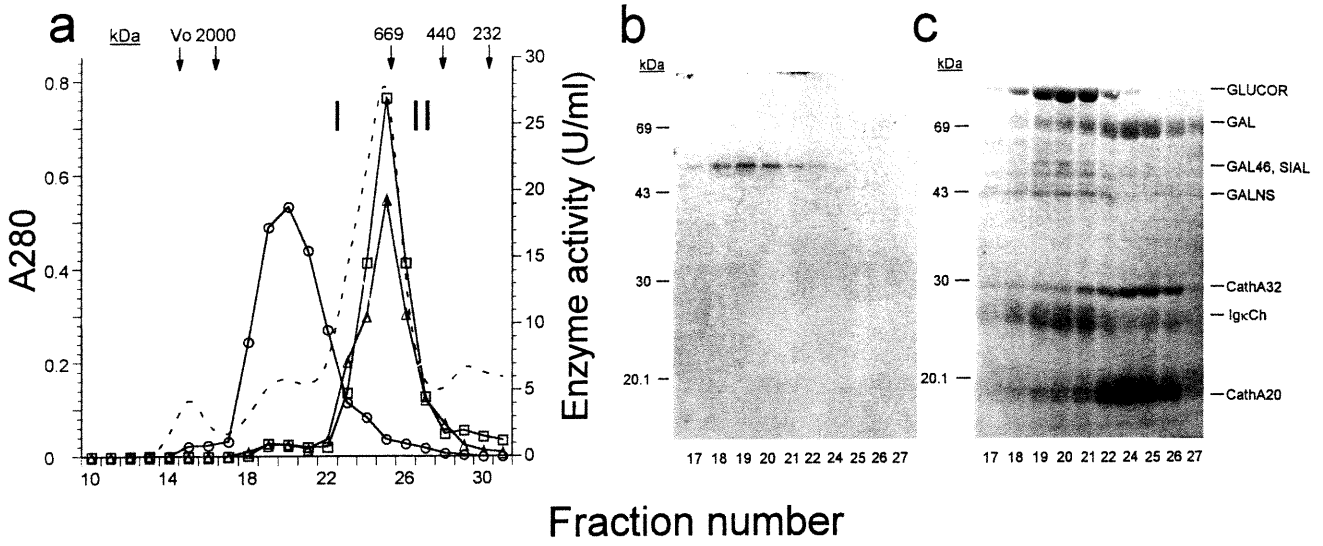

Figure 1 FPLC gel-filtration (a), Western blotting using anti-sialidase antibodies (b) and SDS–PAGE analysis (c) of the 1.27 MDa complex from human placenta

The complex (3 mg) was analysed by FPLC gel-filtration on Superose 6 HR column. (---), Absorbance at 280 nm; (□), GAL activity; (△), CathA activity ($\times 10^{-1}$); (○), sialidase activity ($\times 10^{2}$). The positions of the elution peaks of the M_r standards and void volume (V_0) are shown by arrows. The indicated gel-filtration fractions were analysed by SDS/PAGE and Western blot, as described. The protein bands are identified on the right side of the gel as follows: GLUCOR, β-glucuronidase; GAL, 64 kDa subunit of β-galactosidase; GAL46, a 46 kDa product of C-terminal processing of β-galactosidase; SIAL, sialidase; GALNS, 40 kDa subunit of *N*-acetylgalactosamine-6-sulphate sulphatase; CathA32 and CathA20 are 32 kDa and 20 kDa subunits of cathepsin A; IgκCh, immunoglobulin κ-chain.

Western blotting

FPLC gel-filtration fractions (25 μl), 1.27 MDa complex preparation (5 μg), treated or not treated with endoglycosidase F, concentrated crude glycoprotein fraction from human placenta (150 μg) and fibroblast homogenates (50 μg) were subjected to SDS/PAGE and electrotransferred to NITRO ME nitrocellulose membrane (Micron Separations Inc, Westboro, MA) as described [4]. The detection of protein bands, cross-reacting with anti-sialidase antibodies was performed using the BM Chemiluminiscence kit (Boehringer Mannheim) in accordance with the manufacturer's protocol.

Expression of sialidase cDNA in sialidosis fibroblasts

Full-length human sialidase cDNA was obtained as an *Xma*III fragment from pCRII vector and cloned into NotI site of pCMV expression vector [27] kindly provided by Dr. S. G. MacGregor and Dr. T. Caskey (Baylor College of Medicine, Houston).

Skin fibroblasts of galactosialidosis patients and of normal controls were transfected with pCMV-sialidase expression vector using Lipofectamine reagent (Life Technologies Inc, Gaithersburg, MD) in accordance with the manufacturer's protocol. 24 h after the transfection, sialidase and hexosaminidase activities were assayed in cell homogenates using the corresponding fluorogenic 4-methylumbelliferyl glycoside substrates [28,29]. One unit of enzyme activity (U) was defined as the conversion of 1 μmol of substrate per min. Proteins were assayed according to Bradford [30] with BSA (Sigma) as a standard.

Immunofluorescent microscopy

24 h after the transfection, fibroblasts were fixed on the glass slides with acetone/methanol (4:1) at −20 °C, washed in PBS, blocked for 1 h with 2 % (w/v) BSA in PBS, incubated with anti-sialidase or anti-hexosaminidase A antibodies at a final dilution of 1/200 for 1 h at room temperature, washed with PBS and further incubated for 30 min with rhodamine-conjugated goat anti-rabbit IgG at a dilution of 1/100 [81]. Epifluorescent microscopy was performed using Zeiss Axioskop microscope.

Immunoelectron microscopy

24 h after transfection with pCMV-sialidase or pCMV expression vectors cultured fibroblasts were detached from the culture dishes with a rubber policeman, washed with Hank's Balanced Salt Solution (HBSS), and fixed in 4 % paraformaldehyde/0.5 % glutaraldehyde in 50 mM phosphate buffer, pH 7.5. The cell pellets were dehydrated in methanol and embedded in Lowicryl K4M as described previously [32,33].

Ultrathin Lowicryl sections were mounted on 300-mesh Formvar-coated nickel grids (Polysciences, Inc., Warrington, PA). Each section was incubated for 15 min in 20 mM Tris/HCl-buffered saline (TBS), containing 0.1 % (v/v) Tween 20 and 15 % (v/v) goat serum and for 30 min in anti-sialidase antibodies diluted 1:500 in TBS. The sections were then washed four times with TBS containing 0.05 % (v/v) Tween 20, incubated for 15 min in TBS containing 15 % (v/v) goat serum, and for 30 min – in colloidal gold (10 nm)-conjugated goat anti-rabbit IgG (Zollinger Incorporated, Montréal, Québec, Canada). The sections were washed twice with TBS containing 0.05 % (v/v) Tween 20, twice with distilled water and counterstained with uranyl acetate followed by lead citrate [32,33]. Normal rabbit serum was used as a control. Electron micrographs were taken on a Philips 400 electron microscope (Philips Electronics, Toronto, Ontario). The number of colloidal gold particles per μm^2 was counted in the lysosomes of five normal and five galactosialidosis fibroblasts both transfected with the sialidase expression vector. The area of the lysosomes (vesicles with a diameter of 250–300 nm containing intraluminal electrondense

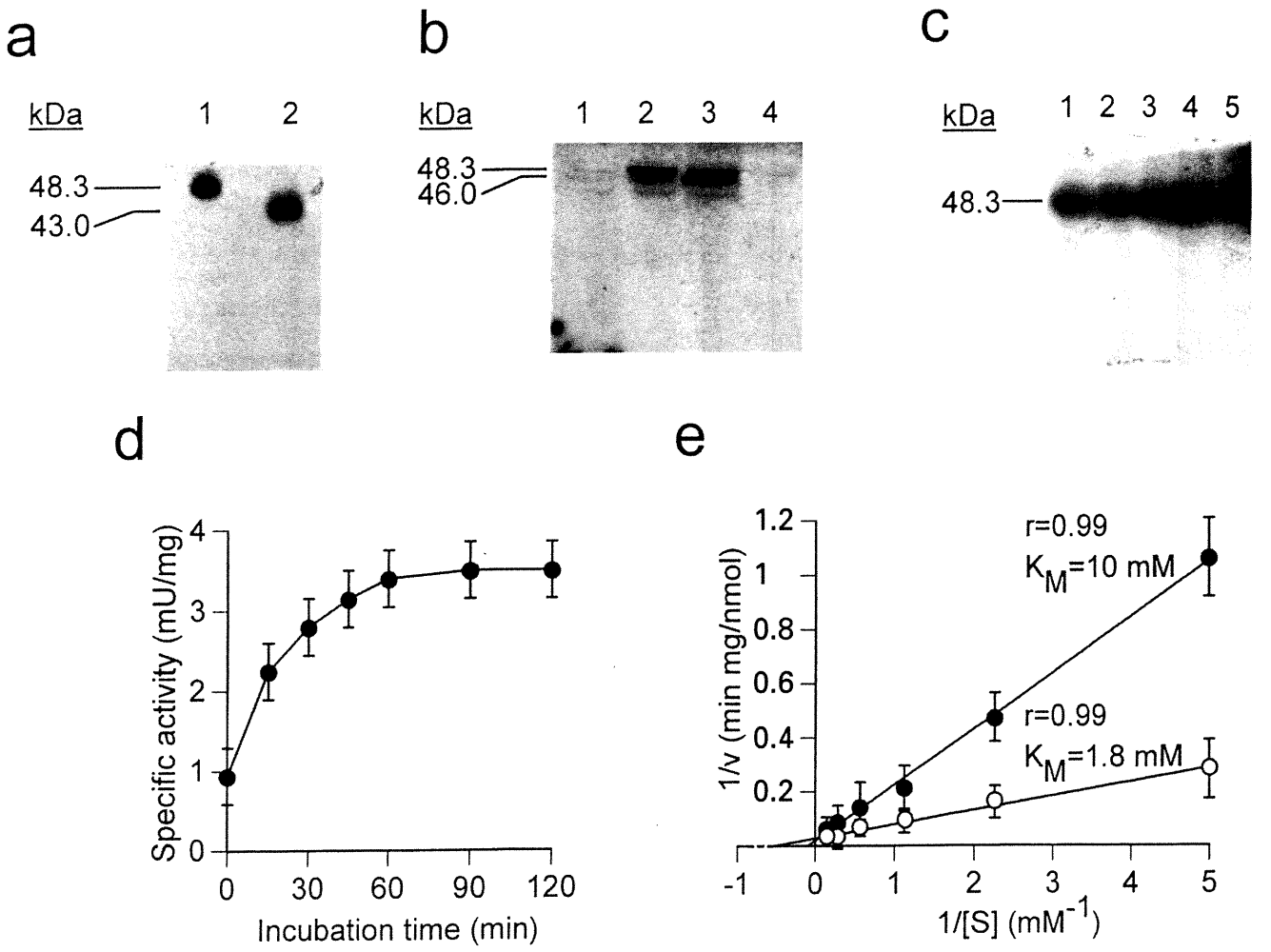

Figure 2 Immunoblotting of sialidase

(**a**) 1.27 MDa complex before (lane 1) or after treatment with endoglycosidase F (lane 2). (**b**) Sialidosis (cell line WG544) fibroblast homogenates: non transfected (lane 1), transfected with pCMV-sialidase (lanes 2 and 3) or with pCMV (lane 3) expression vectors. (**c**) Concentrated crude glycoprotein fraction from human placenta: non-treated (lane 1) or incubated for 30 (lane 2), 60 (lane 3), 90 (lane 4) and 120 (lane 5) min at 37 °C. Protein samples were subjected to SDS/PAGE, transferred to a nitrocellulose membrane and stained with rabbit anti-sialidase antibodies as described. (**d**) Activation of sialidase in concentrated crude glycoprotein fraction during the 120 min incubation at 37 °C. The steady state rate of 4 MU α-neur hydrolysis was measured at 0.2 mM substrate concentration. (**e**) Lineweaver–Burk plot of the substrate dependence of the initial rate of 4 MU α-neur hydrolysis catalysed by sialidase in concentrated crude glycoprotein faction before (○) or after (●) the 120 min incubation at 37 °C. K_m values and the r values for the regression lines are shown.

material) was measured with a digital analyser MOP-3 (Carl Zeiss, Berlin, Germany).

Metabolic labelling

Human fibroblasts, grown to confluency in 75 cm^2 culture flasks ($\sim 10^6$ cells), were washed twice with HBSS, then incubated for 2 h in methionine-free Dulbecco's Modified Eagles Medium (D-MEM, Gibco-BRL) supplemented with L-glutamine and sodium pyruvate, and for 40 min – in 5 ml of the same medium supplemented with [^{35}S]methionine (Dupont), 0.1 mCi/ml. The radioactive medium was then removed, and the cells were washed twice with HBSS and chased at 37 °C in EMEM supplemented with 20 % (v/v) fetal calf serum.

At the time indicated in the figures, the cells were placed on ice, washed twice with ice-cold PBS then lysed for 30 min on ice in 1 ml of radioimmunoprecipitation assay (RIPA) buffer, containing 50 mM Tris/HCl, pH 8.0; 150 mM NaCl; 1 % (v/v) NP-40; 0.5 % (w/v) sodium deoxycholate; 0.1 % (w/v) SDS; 5 μg/ml leupeptin and 0.1 mM PMSF. The lysate was collected and centrifuged at 13000 g for 10 min to remove the cell debris.

Immunoprecipitation, electrophoresis and quantitation of sialidase

1.0 ml of lysate was incubated for 4 h with preimmune serum at a final dilution of 1/20. Then the pellet obtained from 300 μl of Pansorbin Cells (Calbiochem) was added and the resulting suspension was incubated for 2 h at 4 °C, followed by centrifugation for 10 min at 13000 g. Supernatants were incubated overnight with the anti-sialidase antibodies in a 1/100 final dilution, then for 2 h at 4 °C with the pellet from 100 μl of Pansorbin Cells and precipitated as above. The pellet was washed three times with 1 ml of RIPA buffer. The antigens were eluted from the pellet by the addition of 100 μl of a buffer containing 0.1 M Tris/HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 0.2 M DTT and 0.02 % (w/v) bromphenol blue. The proteins

were denatured by boiling for 5 min and 50 μl of each sample were subjected to SDS/PAGE according to Laemmli [34]. The molecular weights were determined with [^{14}C]-labelled protein markers (Amersham). The gels were fixed in acetic acid/isopropanol/water (10/50/40), soaked for 30 min in Amplify™ solution (Amersham), vacuum dried at 60 °C and analysed either by autoradiography and scanning densitometry or by quantitative fluorometry on a PhosphorImager SI analysis screen (Molecular Dynamics) using the software supplied by the manufacturer. The half-lives of sialidase in normal and galactosialidosis cells were calculated from the slopes of regression lines in semilogarithmic plots (e.g. Figure 6c).

RESULTS

Previous studies [3,5] demonstrated that active sialidase can be purified from human tissues only as a part of the 1.27 MDa complex, which has multiple protein components. Therefore to provide the basis for studies of sialidase biogenesis we first had to ensure which protein component of the 1.27 MDa complex represents the mature active sialidase. The 1.27 MDa multienzyme lysosomal complex was purified from human placenta as described previously [5] using PATGAL-agarose affinity column followed by FPLC gel-filtration on Superose 6 column (Figure 1a). On the last step the 1.27 MDa complex [4,5] containing all sialidase and about 2% of total GAL and CathA activities (peak I in Figure 1a) was separated from a 680 kDa GAL-CathA complex which contained most of GAL and CathA but no sialidase activity (peak II in Figure 1a). On Western blots of the gel-filtration fractions anti-sialidase antibodies labelled only the 48.3 kDa band which elution profile matched that of sialidase activity (Figure 1b). The observed molecular mass also corresponded to that predicted from the deduced amino acid sequence of sialidase, in which the potential signal peptide (amino acids 1–47) is removed and three putative sites [21] are *N*-glycosylated. Indeed, the treatment of the preparation with endoglycosidase F prior to Western blotting (Figure 2a) reduced the molecular mass of the enzyme to 43 kDa, close to that predicted for the mature deglycosylated enzyme, 42.5 kDa, and similar to that reported by Bonten et al. [35] and Milner et al. [36]. The comparison of the Western blot with the silver-stained SDS/PAGE gel (Figure 1c vs. Figure 1b) indicated that anti-sialidase antibodies reacted with a component of 46–48 kDa diffuse protein band. Sequencing of the NH_2-terminal of the 46–48 kDa protein yielded both the major amino acid sequence, QRMFEIDYSRD, corresponding to that of human GAL [37] and also a minor sequence, ENDFXLVQ, corresponding to the amino acids 48 to 55 of sialidase sequence [21]. Therefore, the 46–48 kDa diffuse band contained 48.3 kDa mature sialidase which co-migrated on gel with the previously described 46 kDa product of GAL proteolytic digestion [38]. These data also confirmed that the signal peptide (amino acids 1–47) is cleaved in the active sialidase.

Previously [21] we have described the sialidosis fibroblast line, WG544, characterized by 10-fold reduced mRNA level and negligible activity of sialidase. In the homogenates of these cells

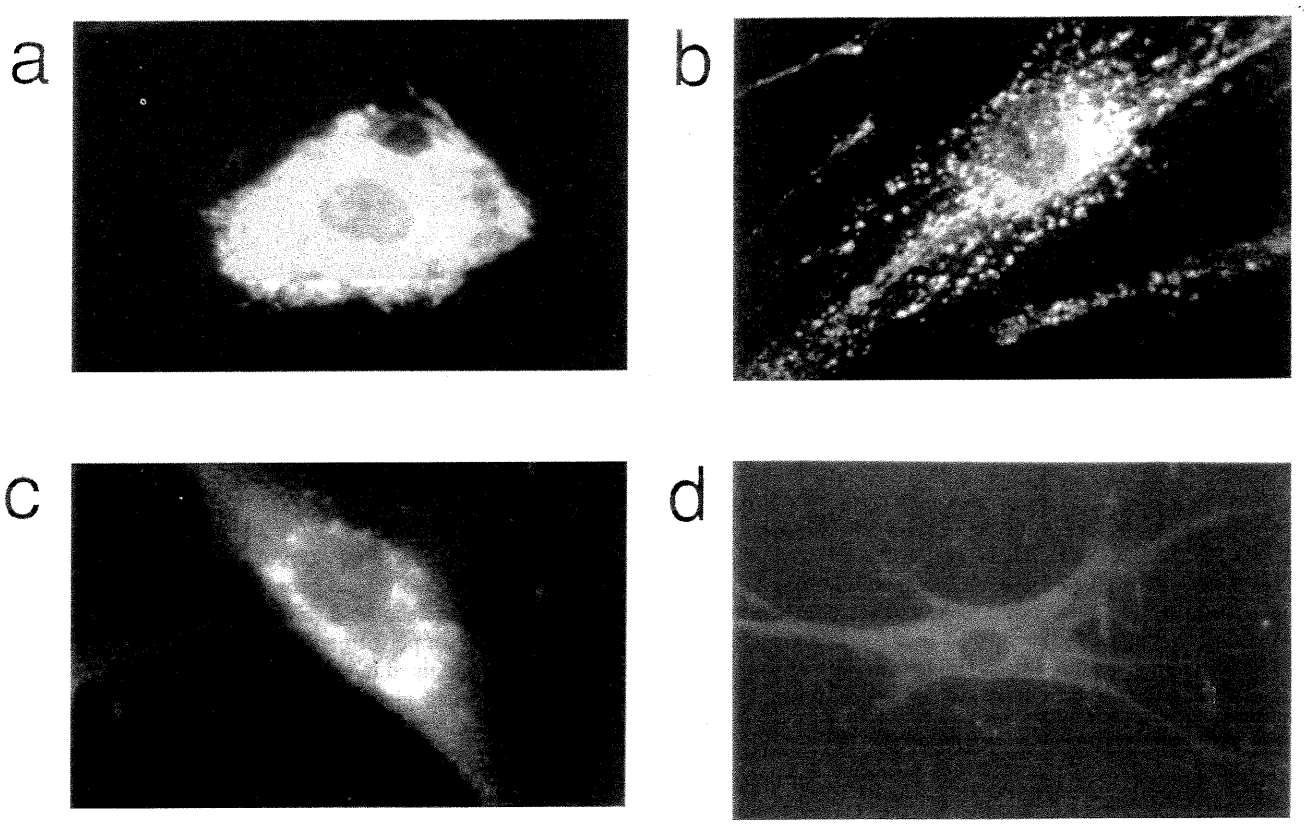

Figure 3 Immunofluorescence of cultured human skin fibroblasts of normal controls (a, c) and galactosialidosis patient (b, d)

24 h after the transfection with pCMV-sialidase (**a**, **b**, **c**) or with pCMV (**d**) expression vectors fibroblasts were fixed with cold acetone/methanol (4:1) and stained with anti-sialidase (**a**, **b**, **d**) or anti-lysosomal hexosaminidase A (**c**) rabbit antibodies, followed by staining with rhodamine-conjugated goat anti-rabbit IgG antibodies. Magnification × 400.

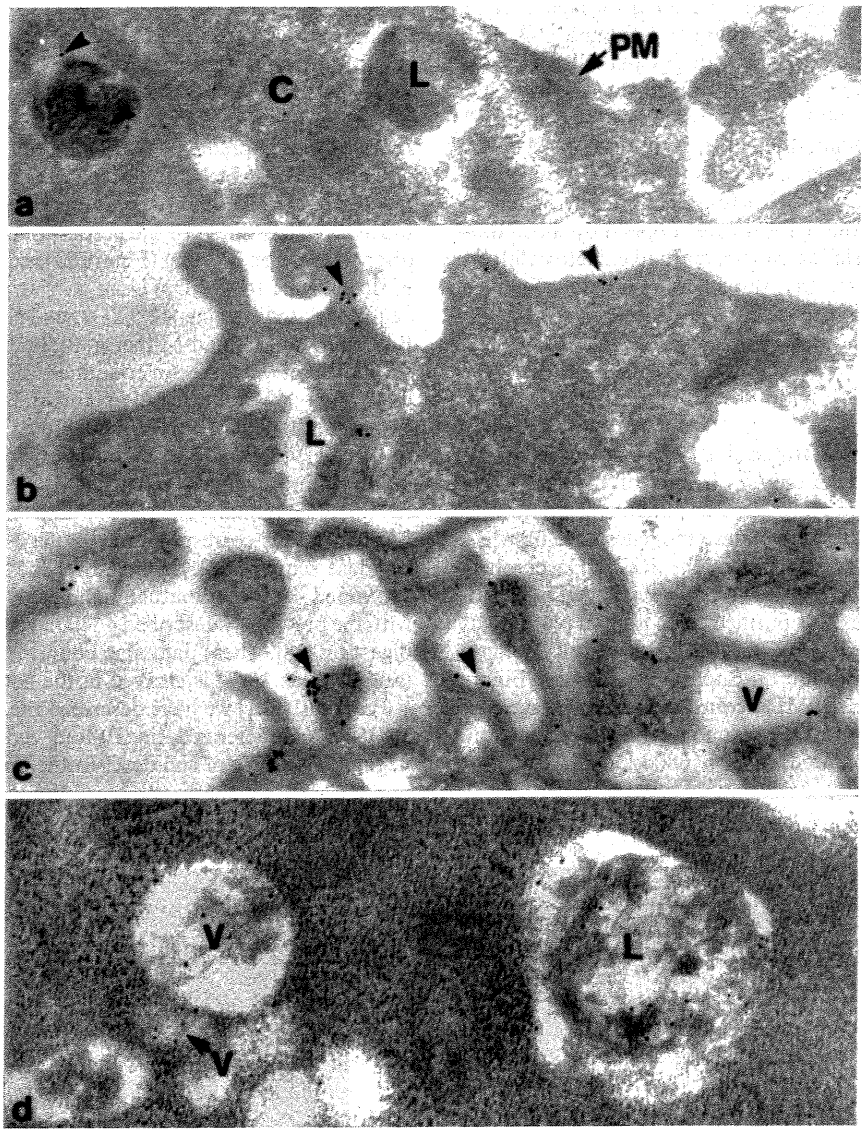

Figure 4 Electron micrograph of normal fibroblasts transfected with pCMV (a) or with pCMV-sialidase (b–d) expression vectors, immunogold-labelled with anti-sialidase antibodies

(**a**) Except for the presence of a few grains (arrowheads) in a lysosome (L), the cytoplasm (C) and plasma membrane (PM) of this cell is unlabelled. (**b–d**) Transfection induced a strong immunogold labelling (arrowheads) of the plasma membrane, vesicles (V) and lysosomes (L). Magnification: × 60 000 (a–c) or × 65 000 (d).

transfected with pCMV-sialidase expression vector, which increased 15-fold the specific sialidase activity [21], two 48.3 and 46 kDa bands were detected (Figure 2b, lanes 2 and 3). Only the major, 48.3 kDa band was found to be associated with the 1.27 MDa complex (Figure 1b) or present in the crude glycoprotein fraction of human placenta extract (Figure 2c). Since this fraction contained all sialidase activity in tissue, we proposed that the 48.3 kDa form corresponded to the active sialidase, whereas the 46 kDa form represented a catalytically inactive, partially degraded or alternatively glycosylated sialidase. In a control experiment we did not detect any bands cross-reacting with lysosomal sialidase in untransfected cells or in the cells transfected with pCMV vector (Figure 2b, lanes 1 and 4).

Verheijen et al. [17] reported that sialidase can be activated by 90–120 min incubation of the concentrated crude glycoprotein fraction at 37 °C and suggested that the activation of sialidase results from the formation of the sialidase-CathA complex. In contrast other researchers hypothesized that activation of sialidase *in vitro* involves its proteolytic processing [39,40]. To understand the molecular mechanism associated with the activation process we performed the Western blotting of sialidase in the samples obtained in the process of the 120 min incubation of the concentrated glycoprotein fraction at 37 °C (Figure 2c), which increased the specific activity of sialidase from 0.94 mU/mg to 3.5 mU/mg (Figure 2d). We could not detect any changes in the molecular mass of sialidase, which indicated that the activation process cannot be attributed to any detectable proteolytic modification of the protein. Besides, kinetic studies (Figure 2e) demonstrated that the 'activation procedure' increased the affinity of the enzyme towards the substrate ($K_m = 1.8 \pm 0.7$ mM after the activation versus $K_m = 10 \pm 1.3$ before the activation) but did not change the maximal reaction

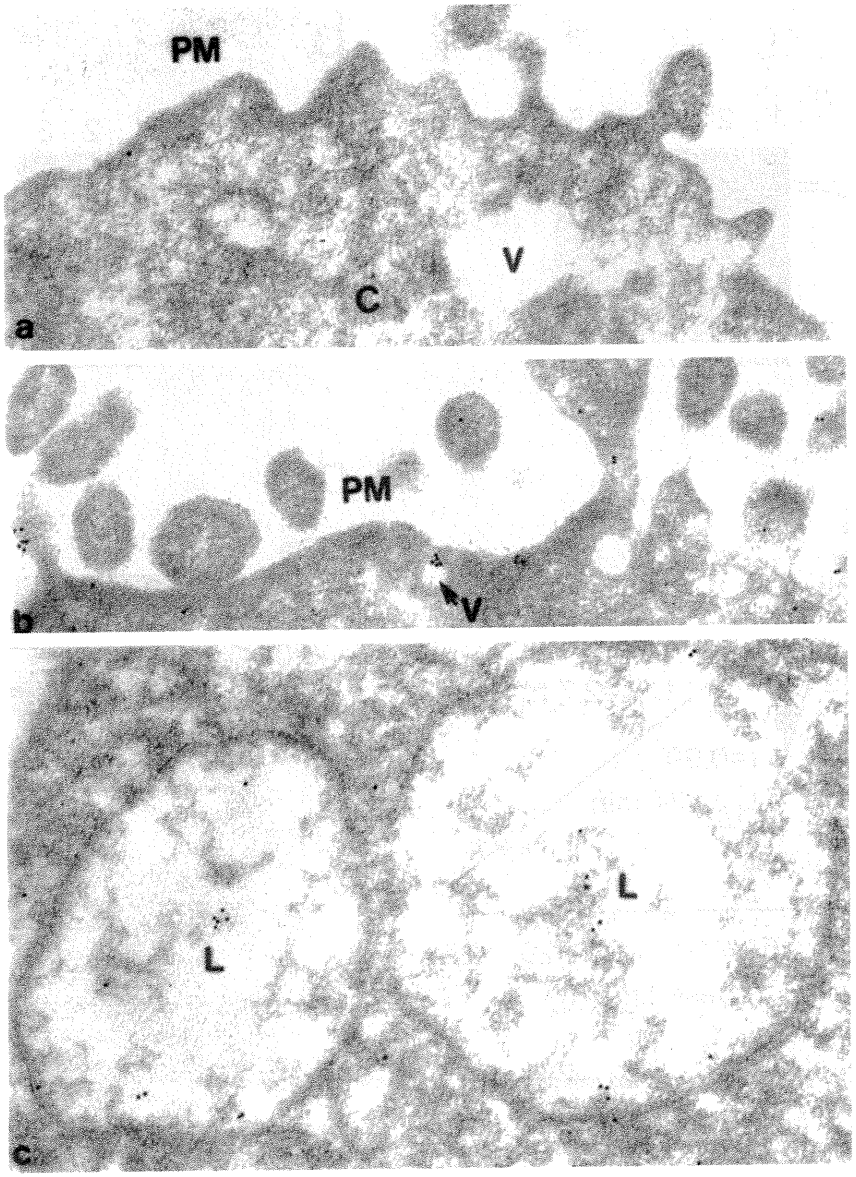

Figure 5 Fibroblasts from galactosialidosis patient transfected with pCMV (a) or with pCMV-sialidase (b–c) expression vectors, immunogold-labelled with anti-sialidase antibodies

(**a**) Plasma membrane (PM), cytoplasm (C) and vesicles (V) are unlabelled. (**b–c**) Plasma membrane (PM) and vesicles (arrowheads) are moderately labelled. Large vesicles reminiscent of lysosomes (L) with a dispersed electron dense content also show a moderate labelling. Magnification: × 60 000.

rate. These results, similar to those previously described for β-galactosidase [15], suggest that activation of sialidase in the complex may result from the conformational change of the enzyme. The studies of the tertiary structure of sialidase necessary to understand the molecular mechanism of this phenomenon are in progress in our laboratory.

To study the biogenesis of the lysosomal sialidase we expressed it in cultured human fibroblasts from normal controls and galactosialidosis patients using the previously described pCMV-sialidase vector [21] and characterized the activity, intracellular localization and processing of the expressed enzyme. We found a three-fold increase of sialidase activity in normal fibroblasts transfected with pCMV-sialidase (2.3 ± 0.03 mU/mg) as compared to untransfected cells or to the cells transfected with pCMV vector which did not contain the sialidase insert (0.82 ± 0.02 mU/mg). In contrast the sialidase activity in all four fibroblast lines of galactosialidosis patients transfected with pCMV-sialidase vector (0.04 ± 0.01 mU/mg) did not differ from that of untransfected cells or of the cells transfected with pCMV (0.05 ± 0.02 mU/mg) which again confirmed that sialidase requires an association with CathA for the expression of enzymatic activity [3].

To understand if in both normal control and galactosialidosis cells the expressed sialidase is targeted to lysosomes we studied its intracellular localization by immunofluorescent and immunoelectron microscopy. Fluorescent microscopy (Figure 3a,b) revealed in both control and galactosialidosis fibroblasts transfected with pCMV-sialidase a perinuclear punctate pattern

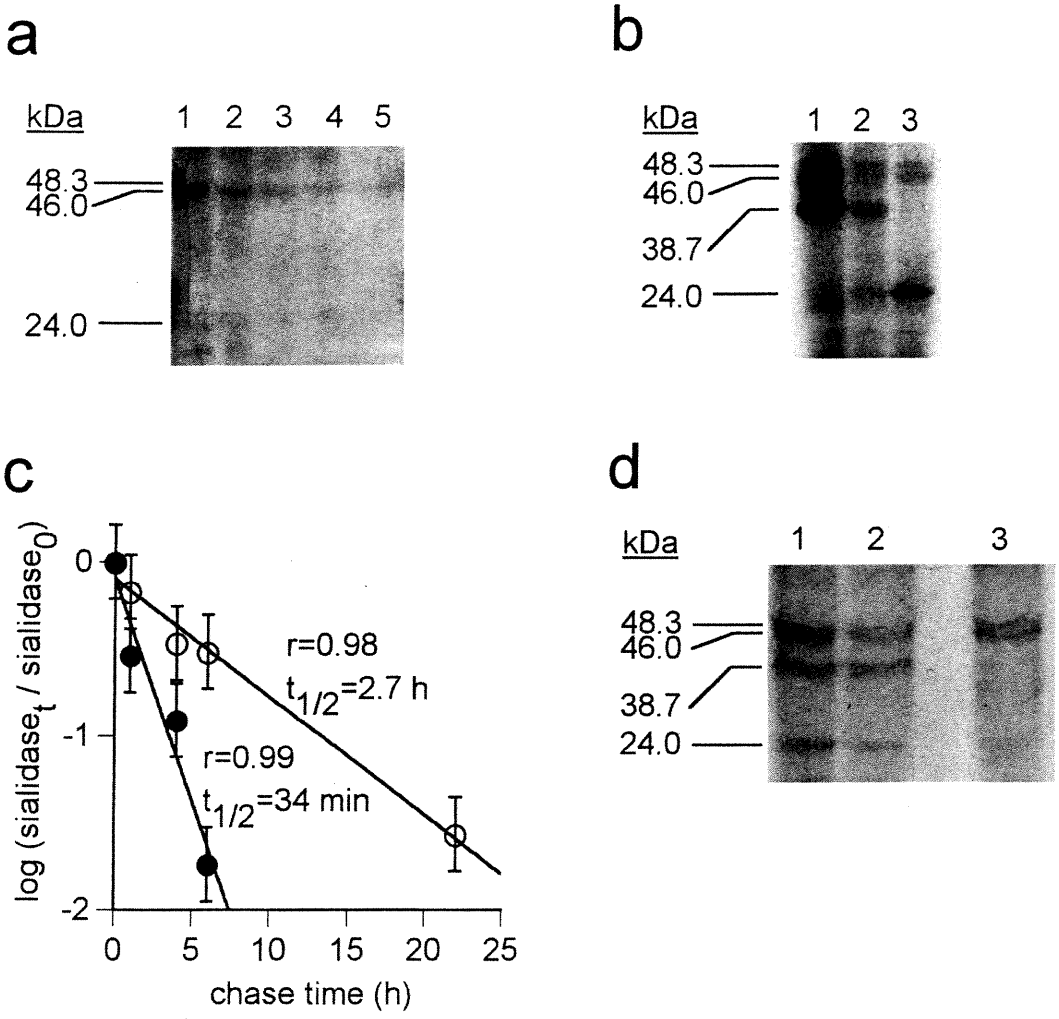

Figure 6 Metabolic labelling of sialidase in normal and galactosialidosis fibroblasts

(**a**, **b**) Fluorographs of pulse-labelling and chase of sialidase in normal and galactosialidosis (line 83.29.34) fibroblasts 24 h after the transfection with pCMV-sialidase expression vector. Lanes 1, pulse; lanes 2–5, chase for 1, 4, 6 and 22 h, respectively. M_r values of two forms of mature sialidase (48.3 and 46 kDa) and its degradation products (38.7 and 24 kDa) are shown. (**c**) Semilogarithmic plots of the relative amount of mature sialidase remaining in the normal (○) and galactosialidosis (●) fibroblasts versus the chase time. The half-life ($t_{1/2}$) values and the r values for the regression lines are shown. (**d**) Fluorograph of the sialidase labelled for 4 h in galactosialidosis (lane 1, cell line GM05076; lane 2, cell line GM02438A) and normal control (lane 3) fibroblasts. The patterns similar to those presented in (**a**), (**b**) and (**d**) were observed in three experiments performed on different occasions.

similar to that obtained with the anti-lysosomal hexosaminidase A antibodies (Figure 3c) and consistent with the localization of the expressed sialidase in a lysosomal compartment. However, the galactosialidosis fibroblasts showed less intense fluorescent staining, suggesting the instability of the expressed sialidase.

Immunoelectron microscopy (Figures 4 and 5) confirmed the intralysosomal localization of the expressed sialidase. Non-transfected normal control fibroblasts incubated with the anti-sialidase antibodies showed occasional immunogold labelling of lysosomes (Figure 4a). Normal fibroblasts transfected with the pCMV-sialidase expression vector demonstrated an intense immunogold labelling of lysosomes, the plasma membrane and some intracellular vesicles (Figure 4b–d). Control serum did not yield any immunogold labelling in either type of cells, indicating that the observed immunoreactivity was specific (not shown).

Non-transfected fibroblasts obtained from the galactosialidosis patients incubated with anti-sialidase antibodies did not yield immunogold labelling of the plasma membrane or of any type of intracellular vesicles including the lysosomes (Figure 5a), whereas the fibroblasts transfected with the pCMV-sialidase expression vector showed an immunogold labelling of the plasma membrane and of intracellular vesicles (Figure 5b). Large vacuoles reminiscent of lysosomes containing the storage materials were moderately labelled (Figure 5c). The number of gold particles per μm^2 was five-fold higher in lysosomes of the normal cells (59 ± 9) than in those of galactosialidosis fibroblasts (10 ± 5).

Figure 6 shows the results of 24-h pulse-chase experiments with normal and galactosialidosis fibroblasts. Two fragments ($M_r \sim 48.3$ and ~ 46 kDa) similar to those previously observed by Western blotting (Figure 2) were precipitated by anti-sialidase antibodies in normal fibroblast homogenates transfected with pCMV-sialidase vector (Figure 6a). The ratio between the 48.3 and 46 kDa forms of mature sialidase appeared to be constant and the intensities of both fragments decreased proportionally with chase time. In contrast, in galactosialidosis fibroblasts (Figure 6b), the intense abnormal 38.7 kDa sialidase fragment

became detectable after 40 min of pulse, whereas the amounts of both the 48.3 and 46 kDa forms of mature sialidase were remarkably reduced as compared to normal cells. During the chase in galactosialidosis fibroblasts both 38.7 kDa fragment and mature sialidase were rapidly degraded into a 24 kDa product. In Figure 6c these results are shown quantitatively for both control and galactosialidosis cells. In normal fibroblasts mature sialidase is reduced to 5–10 % of total by ~ 15 h, whereas in galactosialidosis fibroblasts, sialidase is nearly undetectable after only 4 h of chase. The kinetic curve for mature sialidase in normal human fibroblasts represents a slow monophasic decline with a half-life of ~ 2.7 h. The same plot for galactosialidosis fibroblasts is characterized by a much lower half-life of ~ 30 min (Figure 6c). Fast degradation of mature sialidase and the presence of the abnormal 38.7 and 24 kDa sialidase fragments were also observed in the three other galactosialidosis cell lines both when transfected with pCMV-sialidase (not shown) and when the metabolic labelling and immunoprecipitation were performed for the endogenous sialidase (Figure 6d).

DISCUSSION

Numerous attempts to purify and characterize the lysosomal sialidase from human, porcine and bovine tissues resulted in the isolation of the 1.27 MDa lysosomal complex [3,5,20], but further purification of sialidase was complicated by its low tissue content, instability and complete inactivation after the dissociation from the 1.27 MDa complex [3]. The 1.27 MDa complex in addition to 64 kDa GAL, 20 and 30 kDa CathA subunits contains five other proteins as demonstrated by SDS/PAGE analysis (see Figure 1c). These proteins with M_r ranging from 78 to 40 kDa were suggested as the candidate sialidase peptides on the basis of immunotitration of sialidase activity and photoaffinity labelling experiments [3,20,41,42]. However, further analysis of these peptides by the NH_2-terminal sequencing demonstrated that they represented other previously identified proteins [42]. This discrepancy is explained by the results obtained in this work. Using antibodies against recombinant sialidase peptide, we mapped a 46–48 kDa protein band on Western blots of the 1.27 MDa complex. This band contained a 46 kDa fragment of GAL as a major component and a 48.3 kDa sialidase peptide as a minor component which yielded a weak signal probably undetected or ignored in previous studies in the process of NH_2-terminal sequence analysis. Our data are consistent with the hypothesis that sialidase is synthesized as a 45.5 kDa precursor which after the cleavage of the signal peptide (first 47 amino acid) and glycosylation becomes a 48.3 kDa active mature enzyme. An alternatively glycosylated or processed 46 kDa form, which can be found in fibroblast homogenates is not present in the 1.27 MDa complex and probably has no enzymatic activity.

The immunofluorescent and immunoelectron microscopy studies clearly demonstrated the intralysosomal localization of sialidase in both normal and galactosialidosis cells, suggesting that the intralysosomal targeting of sialidase is independent of the presence of CathA, and that the deficiency of sialidase in galactosialidosis cannot be explained by its mistargeting. Previously [21] we reported that sialidase contains a C-terminal 'Tyr-X-X-hydrophobic residue' motif, common to several lysosomal integral membrane proteins [43–47], which are first targeted to the plasma membrane and then internalized by endocytosis. The hypothesis that sialidase is transported to the lysosome by a similar mechanism is supported by the results of our immunoelectron microscopy experiments. In all cells studied we observed not only the labelling of the inner side of the lysosomal membrane and lysosomal content but also of the plasma membrane and intracellular (possibly endocytic) vesicles. Alternatively, sialidase can be targeted to the lysosome by the mannose-6-phosphate receptors, as for most of the soluble lysosomal enzymes, and then anchored to the lysosomal membrane via a second protein. The mechanism of sialidase lysosomal targeting is currently under investigation in our laboratory.

The unique feature that distinguishes lysosomal sialidase from other sialidases is its activation by the formation of the 1.27 MDa complex with CathA. We found that two molecular mechanisms underlay this process. First, the association of sialidase with CathA in 1.27 MDa complex is required for this enzyme to obtain the catalytically active conformation. This hypothesis was supported by our finding that *in vitro* the activation of sialidase in the concentrated crude glycoprotein fraction did not involve any change of the enzyme molecular mass but increased the affinity of the enzyme for the substrate. Second, metabolic labelling of sialidase in the fibroblasts of galactosialidosis patients demonstrated that sialidase is not stable in the absence of CathA and undergoes abnormal processing as indicated by the rapid disappearance of active mature sialidase (half-time ~ 30 min vs. ~ 2.7 h in control cells) which is degraded into a 38.7 kDa and further into a 24 kDa protein. Therefore, we conclude that the molecular mechanism of sialidase deficiency in galactosialidosis is associated with its abnormal intralysosomal processing and rapid proteolytic degradation.

This work has been supported in part by grants from the Medical Research Council of Canada to A.V.P. and C.R.M. C.R.M. is a recipient of a fellowship from the Fonds de La Recherche en Santé du Québec and A.V.P. from l'Hôpital Sainte-Justine Foundation. The authors are grateful to Dr. R. A. Gravel for the anti-human β-hexosaminidase A antibodies and stimulating discussions, to Dr. R. Kenigsberg for the help in immunocytochemical studies, to Dr. L. Ashmarina for helpful advice and to M. Patenaude for excellent secretarial assistance.

REFERENCES

1 d'Azzo, A., Hoogeveen, A., Reuser, A. J., Robinson, D. and Galjaard, H. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 4535–4539
2 Hoogeveen, A. T., Verheijen, F. W. and Galjaard, H. (1983) J. Biol. Chem. **258**, 12143–12146
3 Van der Horst, G. J., Galjart, N. J., d'Azzo, A., Galjaard, H. and Verheijen, F. W. (1989) J. Biol. Chem. **264**, 1317–1322
4 Pshezhetsky, A. V. and Potier, M. (1994) Arch. Biochem. Biophys. **313**, 64–70
5 Pshezhetsky, A. V. and Potier, M. (1996) J. Biol. Chem. **271**, 28359–28365
6 d'Azzo, A., Andria, G., Strisciuglio, P. and Galijaard, H. (1995) in Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 2835–2837, McGraw-Hill, New York
7 Okamura-Oho, Y., Zhang, S. and Callahan, J. W. (1994) Biochim. Biophys. Acta **1225**, 244–254
8 Thomas, G. H. and Beaudet, A. L. (1995) in The Metabolic and Molecular Bases of Inherited Disease (Scriber, C. R., Beaudt, A. L., Sly, W. S. and Valle, D., eds.), pp. 2542–2545, McGraw-Hill, New York
9 Okamura-Oho, Y., Zhang, S., Hilson, W., Hinek, A. and Callan, J. W. (1996) Biochem. J. **313**, 787–794
10 Shimmoto, M., Takano, T., Fukuhara, Y., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) Proc. Jpn. Acad. **66B**, 217–222
11 Zhou, X. Y., Galjart, N. J., Willemsen, R., Gillemans, N., Galjaard, H. and d'Azzo, A. (1991) EMBO J. **10**, 4041–4048
12 Fukuhara, Y., Takano, T., Shimmoto, M., Oshima, A., Takeda, E., Kuroda, Y., Sakuraba, H. and Suzuki, Y. (1992) Brain Dysfunct. **5**, 319–325
13 Shimmoto, M., Fukuhara, Y., Itoh, K., Oshima, A., Sakuraba, H. and Suzuki, Y. (1993) J. Clin. Invest. **91**, 2393–2398
14 Galjart, N. J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E. J. and d'Azzo, A. (1991) J. Biol. Chem. **266**, 14754–14762
15 Pshezhetsky, A. V., Elsliger, M.-A., Vinogradova, M. V. and Potier, M. (1995) Biochemistry **34**, 2431–2440
16 Verheijen, F., Brossmer, R. and Galjaard, H. (1982) Biochem. Biophys. Res. Commun. **108**, 868–875
17 Verheijen, F., Palmeri, S., Hoogeveen, A. T. and Galjaard, H. (1985) Eur. J. Biochem. **149**, 315–321
18 Verheijen, F., Palmeri, S. and Galjaard, H. (1987) Eur. J. Biochem. **162**, 63–67

19 Yamamoto, Y. and Nishimura, K. (1987) Int. J. Biochem. **19**, 435–442
20 Hiraiwa, M., Nishizawa, M., Uda, Y., Nakajima, T. and Miyatake, T. (1988) J. Biochem. (Tokyo) **103**, 86–90
21 Pshezhetsky, A. V., Richard, C., Michaud, L., Igdoura, S., Wang, S. P., Elsliger, M.-A., Qu, J. Y., Leclerc, D., Gravel, R., Dallaire, L. and Potier, M. (1997) Nature Genet. **15**, 316–320
22 Pshezhetsky, A. V. and Potier, M. (1993) Biochem. Biophys. Res. Commun. **195**, 354–362
23 Miyagi, T., Hata, K., Hasegawa, A. and Aoyagi, T. (1993) Glycoconjugate J. **10**, 45–49
24 Schneider-Jacob, H. R. and Cantz, M. (1991) Biol. Chem. Hoppe-Seyler **372**, 443–450
25 Thiobarbituric assay for sialic acids with spectrophotometric or HPLC detection, in Current Protocols in Molecular Biology, ed. Ford, N. (Cold Spring Harbor Lab. Press, 1989), Supp. 3
26 Miyagi, T., Sagawa, J., Konna, K. and Tsuiki, S. (1990) J. Biochem. (Tokyo) **107**, 794–798
27 MacGregor, G. R. and Caskey, C. T. (1989) Nucleic Acids Res. **17**, 2365
28 Potier, M., Mameli, L., Belisle, M., Dallaire, L. and Melançon, S. B. (1979) Anal. Biochem. **94**, 287–296
29 Rome, L. H., Garvin, A. J., Allietta, M. M. and Neufeld, E. F. (1979) Cell **17**, 143–153
30 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
31 Baker, R. E., Jefferson, L. S. and Shiman, R. (1981) J. Cell. Biol. **90**, 145–152
32 Sylvester, S. R., Morales, C. R., Oko, R. and Griswold, M. D. (1989) Biol. Reprod. **41**, 941–948
33 Hermo, L., Wright, J., Oko, R. and Morales, C. R. (1991) Biol. Reprod. **44**, 1113–1131
34 Laemmli, U. K. (1970) Nature **227**, 680–685
35 Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G. and d'Azzo, A. (1996) Genes Dev. **10**, 3156–3169
36 Milner, C. M., Smith, S. V., Belen Carillo, M., Taylor, G. L., Hollinshead, M. and Campbell, R. D. (1997) J. Biol. Chem. **272**, 4549–4558
37 Morreau, H., Galjart, N. J., Gillemans, N., Willemsen, R., van der Horst, G. T. and d'Azzo, A. J. (1989) Biol. Chem. **264**, 20655–20663
38 Okamura-Oho, Y., Zhang, S. Q., Hilson, W., Hinek, A. and Callahan, J. W. (1996) Biochem. J. **313**, 787–794
39 D'Agrosa, R. M. and Callahan, J. W. (1988) Biochem. Biophys. Res. Commun. **157**, 770–775
40 Hiraiwa, M., Yamauchi, T., Tsuji, S., Nishizawa, M., Miyatake, T., Oyanagi, K., Kuta, F. and Uda, Y. (1993) J. Biochem. (Tokyo) **114**, 901–905
41 Van der Horst, G. T., Rose, U., Brossmer, R. and Verheijen, F. W. (1990) FEBS Lett. **277**, 42–44
42 Van der Horst, G. T. J. (1993) Ph.D. Thesis (Erasmus University Press, Amsterdam)
43 Aerts, J. M., Schram, A. W., Strijland, A., van Weely, S., Jonsson, L. M., Tager, J. M., Sorrell, S. H., Ginns, E. I., Barranger, J. A. and Murray, G. J. (1988) Biochim. Biophys. Acta **964**, 303–308
44 Jadot, M., Canfield, W. M., Gregory, W. and Kornfeld, S. (1992) J. Biol. Chem. **267**, 11069–11077
45 Guarnieri, F. G., Arterburn, L. M., Penno, M. B., Cha, Y. and August, J. T. (1993) J. Biol. Chem. **268**, 1941–1946
46 Sosa, M. A., Schmidt, B., von Figura, K. and Hille-Rehfeld, A. (1993) J. Biol. Chem. **268**, 12537–12543
47 Akasaki, K., Michihara, A., Fukuzawa, M., Kinoshita, H. and Tsuji, H. (1994) J. Biochem. **116**, 670–676

Received 25 July 1997/13 October 1997; accepted 22 October 1997

CHAPTER 3

Characterization of molecular defects in sialidase gene

Foreword

The isolation and characterization of the human sialidase gene (Bonten et al., 1996; Pshezhetsky et al., 1997; Milner et al., 1997) enabled the study of the pathophysiological basis of sialidosis, another inherited disorder caused by sialidase deficiency. The disease is characterized by a progressive neurodegeneration in affected infants and children and associated with macular cherry-red spots, dysostosis multiplex and hepatosplenomegaly.

In order to develop the DNA-based method to characterize the molecular defects in SIAL, we have characterized the structure and sequence of the SIAL gene. Altogether, we have found a frameshift mutation and 6 missense mutations in the genomic DNA of 9 unrelated patients. To predict impact of the point mutations on sialidase function and structure, we have modeled the tertiary structure of sialidase using the atomic coordinates of crystallized bacterial sialidases. We have also obtained the constructs for the expression of sialidase mutants in COS-7 cells. The expression studies enabled us to correlate the residual enzymatic activity of the mutants to patients' phenotype and also to study the biosynthesis and processing of the mutant proteins by western blot analysis.

Article 2

Characterization of the sialidase molecular defects in sialidosis patients suggests the structural organization of the lysosomal multienzyme complex

Human Molecular Genetics, 2000, Vol. 9, No. 7 1075–1085

Characterization of the sialidase molecular defects in sialidosis patients suggests the structural organization of the lysosomal multienzyme complex

Kiven E. Lukong, Marc-André Elsliger+, Yuan Chang, Catherine Richard, George Thomas[1], William Carey[2], Anna Tylki-Szymanska[3], Barbara Czartoryska[4], Tina Buchholz[5], German Rodríguez Criado[6], Silvia Palmeri[7] and Alexey V. Pshezhetsky§

Service de Génétique Médicale, Hôpital Sainte-Justine, 3175 Côte Ste-Catherine and Département de Pédiatrie, Faculté de Médicine, Université de Montréal, Montréal, Quebec H3T 1C5, Canada, [1]John Hopkins University School of Medicine, USA, [2]Women's and Children's Hospital, North Adelaide, Australia, [3]Metabolic Diseases Department, Child Health Center, Warsaw, Poland, [4]Institute of Psychiatry and Neurology, Warsaw, Poland, [5]Klinikum Grosshadern, Munich. Germany, [6]Unidad de Dismorfología, Hospital Virgen del Rocío, Sevilla, Spain and [7]Institute of Neurological Sciences, University of Siena, Italy

Received 22 November 1999; Revised and Accepted 28 January 2000

Sialidosis is an autosomal recessive disease caused by the genetic deficiency of lysosomal sialidase, which catalyzes the hydrolysis of sialoglycoconjugates. The disease is associated with progressive impaired vision, macular cherry-red spots and myoclonus (sialidosis type I) or with skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation and hepatosplenomegaly (sialidosis type II). We have analyzed the genomic DNA from nine sialidosis patients of multiple ethnic origin in order to find mutations responsible for the enzyme deficiency. The activity of the identified variants was studied by transgenic expression. One patient had a frameshift mutation (G623delG deletion), which introduced a stop codon, truncating 113 amino acids. All others had missense mutations: G679G→A (Gly227Arg), C893C→T (Ala298Val), G203G→T (Gly68Val), A544A→G (Ser182Gly) C808C→T (Leu270Phe) and G982G→A (Gly328Ser). We have modeled the three-dimensional structure of sialidase based on the atomic coordinates of the homologous bacterial sialidases, located the positions of mutations and estimated their potential effect. This analysis showed that five mutations are clustered in one region on the surface of the sialidase molecule. These mutations dramatically reduce the enzyme activity and cause a rapid intralysosomal degradation of the expressed protein. We hypothesize that this region may be involved in the interface of sialidase binding with lysosomal cathepsin A and/or β-galactosidase in their high-molecular-weight complex required for the expression of sialidase activity in the lysosome.

INTRODUCTION

Sialidosis (also called mucolipidosis I and cherry-red spot myoclonus syndrome) is an autosomal recessive lysosomal storage disease caused by the genetic deficiency of lysosomal sialidase activity (reviewed in refs 1–3). It is characterized by tissue accumulation and urinary excretion of sialylated oligosaccharides and glycoproteins (1). Sialidosis is subdivided into two main clinical variants with different age of onset and severity. Sialidosis type I or non-dysmorphic type is a late-onset mild form, characterized by bilateral macular cherry-red spots, progressive impaired vision and myoclonus syndrome (4–8). Sialidosis type II or dysmorphic type is the infantile-onset form, which is also associated with skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation and hepatosplenomegaly (9–12). A severe form of the disease manifests prenatally and is associated with ascites and hydrops fetalis (13–15). The age of onset and severity of the clinical manifestations correlate with the amount of residual sialidase activity, suggesting the existence of considerable genetic heterogeneity (1–3).

An understanding of the molecular defects and biochemical mechanism of sialidosis became possible after the recent cloning of the sialidase gene and characterization of the protein in our laboratory and others (16–19). Previously, we have identified a frameshift mutation caused by an ACTG duplication after nucleotide 7 (7insACTG) and two missense mutations, 779T→A (Phe260Tyr) and 1088T→C (Leu363Pro), in sialidosis type II patients (17). Bonten *et al.* (18) identified a 1258G→T transversion, which introduced a premature stop codon and the C-terminal truncation of 38 amino acids in two

+Present address: The Scripps Research Institute, La Jolla, CA 92037, USA
§To whom correspondence should be addressed. Tel: +1 514 345 4931/2736; Fax: +1 514 345 4801; Email: alex@justine.umontreal.ca

siblings with type I sialidosis. They also described a 401T→G transversion (Leu91Arg) and a 1337delG deletion that caused a frameshift and extended the protein by 69 amino acids in a type II patient. In addition to mutations identified in humans, a 625C→A (Leu209Ile) change in the sialidase gene was reported in the SM/J mouse strain, characterized by reduced sialidase activity in selected tissues (20,21). The biochemical consequences of some of the identified missense mutations (like Phe260Tyr or Leu209Ile) are difficult to explain since they do not affect the putative active site residues of the enzyme and are not expected to introduce significant change in the conformation of the enzyme.

We screened the genomic DNA of nine patients of diverse ethnic origin affected by type I and type II forms of sialidosis for the mutations in the sialidase gene. To prove that the identified changes cause the deficiency of sialidase, we expressed the variants in COS-7 cells. We have identified six new missense mutations and localized them in the modeled tertiary structure of sialidase. We found that most of the mutations causing a severe type II sialidosis are clustered in one region of the sialidase molecule, suggesting that this region may be involved in the sialidase binding interface with the lysosomal multienzyme complex, also containing β-galactosidase and cathepsin A.

RESULTS

Sequence of the sialidase gene

Although the structures of the human and mouse sialidase genes were reported previously (19,21,22), the sequences of the splice junctions did not extend far enough into the introns to provide the selection of the oligonucleotide primers suitable for amplification of sialidase exons. To determine the intronic sequence of the sialidase gene, we amplified the entire gene in four overlapping fragments using genomic human DNA and cDNA primers complementary to exon sequences (Fig. 1). Those were subcloned into pCR2.1 vector and sequenced. We found that intron 1 (424 bp) starts after nucleotide 159 in the cDNA, intron 2 (547 bp) after nucleotide 352, intron 3 (564 bp) after nucleotide 615, intron 4 (174 bp) after nucleotide 798 and intron 5 (96 bp) after nucleotide 1021 (Fig. 1). The total length of the gene from the initiating to the stop codon is 3.051 kb. The sequences of the intron–exon junctions were in agreement with those reported by Milner *et al.* (19). The positions and sequences of the oligonucleotide primers chosen to amplify each of six exons of the sialidase gene and the flanking intron sequences are shown in Figure 1.

Identification of mutations in the sialidase gene

DNA of nine sialidosis patients (Table 1), representing a total of 18 disease alleles, was analyzed. Seven patients were diagnosed with severe, infantile-onset type of sialidosis (type II) and two patients had the mild juvenile-onset type of disease (type I) (23–28). For the mutation analysis, the exons of the sialidase gene and flanking intron regions were amplified by polymerase chain reaction (PCR). The analysis of the PCR products using agarose gel electrophoresis (data not shown) demonstrated products of the expected size in all patients. The fragments were studied by single-strand conformation polymorphism (SSCP) analyses (29). [^{35}S]dATP- or [^{35}P]dATP-labeled PCR fragments were denatured and separated on polyacrylamide gel with and without 10% glycerol. Band patterns were compared between normal and mutant DNA. Differences were observed in fragments containing exon 2 of sialidosis patient 6, exon 3 of patient 4, exon 4 of patients 1, 2 and 5, and exon 5 of patients 3, 7, 8 and 9 (data not shown). These fragments were sequenced (Fig. 2). The results of the mutation analysis are summarized in Table 2.

Sequencing of exon 4 from an infant of Turkish origin born of consanguineous parents (patient 5) revealed that the patient is homozygous for a frameshift mutation: a deletion after nucleotide 623 (623delG). This mutation results in a premature stop codon at nucleotide 904, and early truncation of sialidase protein at residue 301. To confirm the inheritance of the mutation, we studied parental DNA. Direct sequencing of the corresponding regions of the PCR-amplified fragments (data not shown) resulted in the appearance of ambiguous sequence following the frameshift site, suggesting that both parents are heterozygous for the 623delG mutation. This finding justifies the complete absence of sialidase activity in the patient's cultured fibroblasts.

In two sibs of Spanish origin (patients 7 and 8), we have identified an 808C→T homozygous nucleotide change resulting in Leu270Phe amino acid substitution. The 808C→T mutation removed an *Sal*I restriction site, which was used to confirm the mutation (Fig. 3, top right). The *Sal*I site was absent in both alleles of patients 7 and 8, in one allele of their parents, but not in any of 20 normal chromosomes tested.

Sequencing of exon 4 in patients 1 and 2, of Caucasian origin from Mexico and the USA, revealed that both are homozygous for a 679G→A nucleotide change resulting in Gly227Arg mutation. This change removes an *Mae*II restriction site in the genomic DNA PCR product of patients 1 and 2, but not of any of 20 normal subjects (Fig. 3, top left).

Exon 2 of patient 6 contained a homozygous G203T mutation substituting Gly68 for Val. Exon 3 of patient 4 contained a homozygous 544A→G (Ser182Gly) change and exon 5 of patient 3 contained a homozygous 893C→T (Ala298Val) change. Since all of these mutations did not alter any known restriction site, they were confirmed by allele-specific oligonucleotide hybridization (ASO). All 40 alleles from 20 unrelated normal controls were all negative for these nucleotide changes (Fig. 3, bottom).

Exon 5 of patient 9 contained one copy of a 982G→A substitution, which changes Gly328 to Ser. This change was also confirmed by ASO. Both the normal and mutant oligonucleotide probes hybridized with the exon 5 amplification product of patient 9, whereas only the normal primer hybridized with the exon 5 amplification product of 20 normal subjects (data not shown). A mutation in the second allele of this patient has not been identified so far.

Two different sialidosis-related diseases are presently listed in the Mendelian Inheritance in Man (MIM) database, whose relationships have, until now, been unclear. These are neuraminidase deficiency (MIM 256550) and nephrosialidosis (MIM 256150). In addition, Goldberg syndrome or galactosialidosis (MIM 256540) presents as a combined deficiency of sialidase and β-galactosidase activities. Patient 6 studied in this paper (27) appears to be a well documented case of nephrosialidosis, whereas all others were diagnosed as having type I, type II or

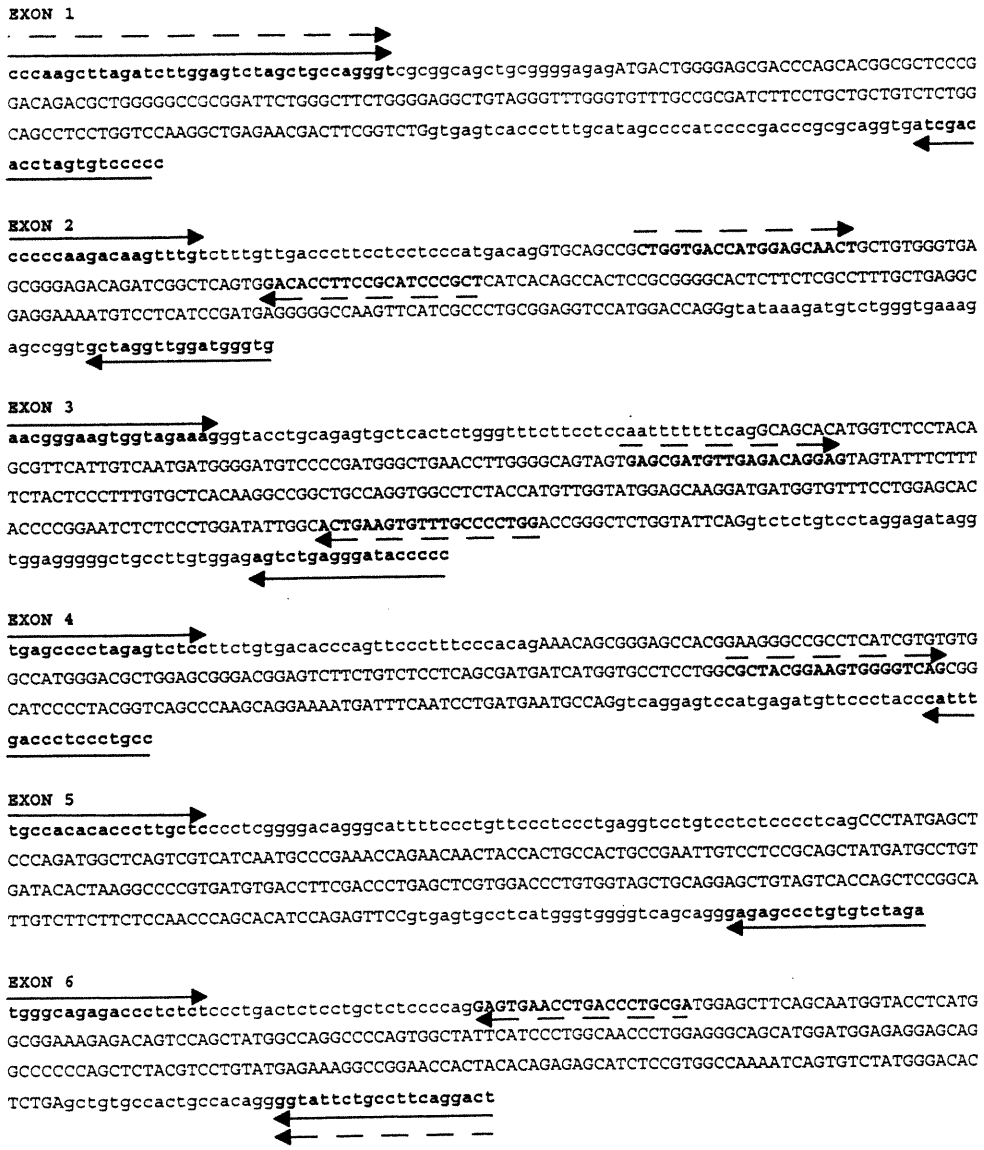

Figure 1. Nucleotide sequence of the intron/exon splice sites of human sialidase gene and their flanking regions. Exonic sequence is shown in uppercase letters and intronic sequence in lowercase letters. Positions of the oligonucleotide primers used to amplify the exons and genomic fragments of sialidase are shown by solid and dashed arrows, respectively, and their sequences are given in bold.

congenital sialidosis. The original patient of Goldberg, who was previously reported to have neuraminidase deficiency rather than galactosialidosis (23), is patient 1 in this report. Therefore, our data indicate that the various forms of isolated sialidase deficiencies, from the most severe to the mildest, are in fact allelic and are caused by the mutations in the lysosomal sialidase gene.

Expression analysis

The functional significance of new mutations was assayed by transient expression of the mutant cDNA. In addition to six point mutations identified in this study, we also expressed sialidase 779T→A (Phe260Tyr) and 1088T→C (Leu363Pro) mutants that we have described previously (17). Mutations were generated by site-directed mutagenesis in the pCMV-SIAL vector previously used for the expression of sialidase (17). Short restriction cassettes containing the mutations were then inserted into the parental pCMV-SIAL vector replacing the corresponding fragments of wild-type sialidase cDNA. The inserts and junction regions of the resulting constructs were verified by sequencing to ensure the correct introduction of mutations. Mutant or wild-type sialidase was co-expressed with human cathepsin A (CathA), which is necessary for the expression of sialidase activity. We used for transfection both COS-7 cells and papilloma virus-immortalized cultured skin fibroblasts of a sialidosis type II patient (line WG0544), characterized by very low sialidase mRNA level and activity (17). Forty-eight hours after transfection, the cell lysates were assayed for sialidase, CathA and control β-hexosaminidase activities.

The expression results are shown in Figure 4. All transfected cells had similar CathA activity, suggesting the same transfection efficiency for all cells. Five of the expressed mutants,

Table 1. Sialidosis patients

Patient	Ethnicity	Gender	Age at diagnosis (years)	Diagnosis	Sialidase activity in fibroblasts (% of normal)	Comment	Reference
1	Mexican	Male	20	Sialidosis type II	2–17	β-galactosidase, 200%; consanguineous ancestry; cherry-red spots; seizures, myoclonus; mental retardation	23
2	American (Caucasian)	Male	14	Sialidosis type II	12	Hurler-like face; skeletal abnormalities; mental retardation; cherry-red spots	24
3	Ashkenazi Jewish	Female	3 months	Sialidosis type II	0–3	β-galactosidase, normal; 4 sibs, all affected; seizures; hydrops fetalis; hepatosplenomegaly	25
4	Chinese	Male	24	Sialidosis type I	2–6	Myoclonus; mild dysphagia; normal leukocyte sialidase and oligosaccharide pattern	26
5	Turkish	Female	1	Sialidosis type II	0	β-galactosidase normal; consanguineous ancestry; hydrops fetalis; skin desquamation; healthy sister; died at 82 days	NA
6	Polish	Male	4.5	Sialidosis type II	7–8	β-galactosidase normal; consanguineous ancestry; hydrops fetalis; nephrosialidosis, first in Poland; cherry-red spots	27
7	Spanish	Male	10	Sialidosis type II	1–2	Brother of patient 8; consanguineous ancestry	NA
8	Spanish	Female	18 months	Sialidosis type II	0	Sister of patient 7; consanguineous ancestry; hydrops fetalis; died at 2 years	NA
9	Italian	Female	23	Sialidosis type I	2–6	β-galactosidase, normal; healthy until 17 years, ataxia; seizures, myoclonus; cherry-red spots; slight mental deterioration	(28)

NA, not applicable.

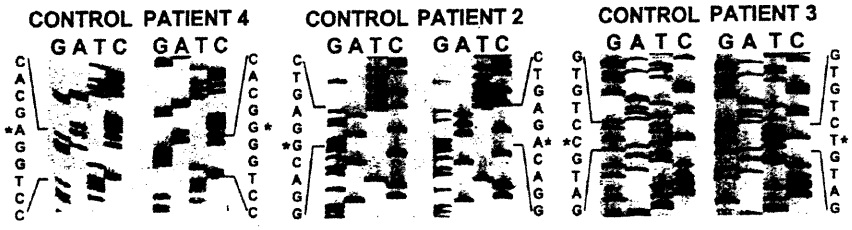

Figure 2. Examples of the mutation detection in the sialidase gene. Direct sequence of mutant and normal DNA, showing the presence of mutation 544A→G in exon 3 of the sialidosis patient 4 (left), 679G→A in exon 4 of patient 2 (middle) and 893C→T in exon 5 of patient 3 (right). Positions of mutations are indicated with asterisks.

Gly68Val, Gly227Arg, Ala298Val, Gly328Ser and Leu363Pro, had very low (<10% of normal) or absent sialidase activity (Fig. 4A). The activity of Phe260Tyr and Leu270Phe mutants was between 10 and 20% of normal, and that of the Ser182Gly mutant was between 20 and 40% of normal (Fig. 4A). Additional experiments showed that Leu270Phe and Phe260Tyr mutants were also significantly less stable than the wild-type sialidase. The half-life of their enzymatic activity in cellular lysates at 37°C was ~30 min as compared with the 2 h half-life of the wild-type enzyme.

The expressed sialidase protein was studied by western blotting (Fig. 4B). In the cells co-transfected with wild-type sialidase and CathA cDNA, we observed a double band of 48.3 and 46 kDa proteins, a product previously identified as a mature active sialidase (30). The same pattern was observed in the cells transfected with Ser182Gly mutant, suggesting that the protein reaches the lysosomes, and is correctly processed and stable. In contrast, in the cells transfected with the Gly227Arg and Leu363Pro mutants, the anti-sialidase antibodies reacted with two high molecular weight (62 and 70 kDa) bands, suggesting that most of the protein is retained in pre-lysosomal compartments. In the cells transfected with Gly328Ser, Ala298Val, Leu270Phe, Gly68Val or Phe260Tyr mutants, the antibodies detected both mature processed sialidase protein

Table 2. Mutations identified in the sialidase gene

Patient	Site	Nucleotide change[a]	Method of confirmation	Putative consequence
1 and 2	Exon 4, codon 227	679G→A	REA	Gly227Arg
3	Exon 5, codon 298	893C→T	ASO	Ala298Val
4	Exon 3, codon 182	544A→G	ASO	Ser182Gly
5	Exon 4, codon 208	623delG		Frameshift, Stop904
6	Exon 2, codon 68	203G→T	ASO	Gly68Val
7 and 8	Exon 5, codon 270	808C→T	REA	Leu270Phe
9	Exon 5 Codon 328	982G→A	ASO	Gly328Ser

[a]Patients 1–8 are homozygous with the mutations identified in heterozygous form in both parents. Patient 9 is a compound heterozygote.

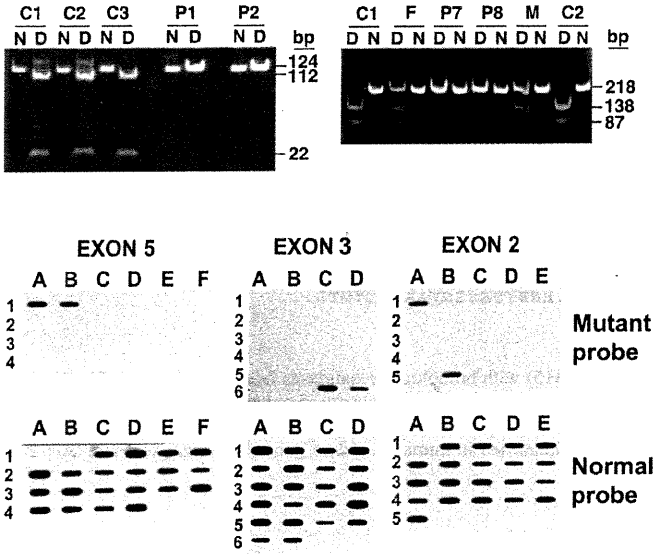

Figure 3. Examples of the mutation confirmation by REA or ASO. (Top left) 679G→A mutation destroyed an *Mae*II site in the genomic DNA PCR product of patients 1 and 2 (P1 and P2), but not of normal subjects (C1, C2 and C3). D, DNA sample digested; N, not digested with a restriction enzyme. (Top right) 808C→T mutation destroyed an *Sal*I site in one allele of the genomic DNA PCR product of both the mother and father of patients 7 and 8 (M and F), in both alleles of patients 7 and 8 (P7 and P8), but not in normal controls (C1 and C2). (Bottom) Only the oligonucleotide probes containing 893C→T, 544A→G and 203G→T mutations, respectively, hybridized with the exon 5 amplification product of patient 3 (left, positions A1, B1), exon 3 amplification product of patient 4 (middle, positions C6, D6) and exon 2 amplification product of patient 6 (right, positions A1, B5). Only the normal primers hybridized with the corresponding amplification products of 20 normal subjects (left, positions C1–D4; middle, positions A1–B6; right, positions B1–E5).

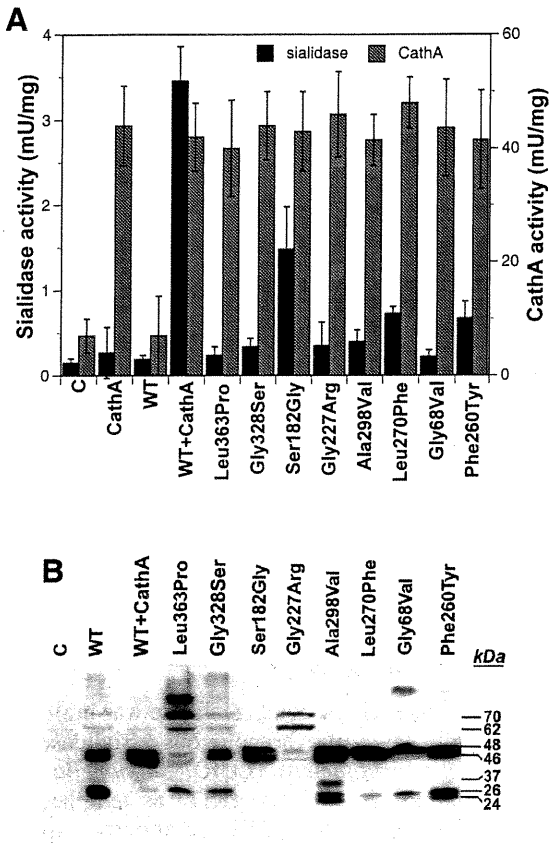

Figure 4. Expression of mutant sialidase in COS-7 cells. (**A**) Sialidase and CathA activities in cellular lysates of COS-7 cells measured 48 h after co-transfection with CathA and mutant sialidase cDNA. Cells were transfected and enzyme activities assayed as described in Materials and Methods. Values represent means ± SD of triplicate experiments. CathA, cells transfected with the CathA cDNA only; WT, cells transfected with the wild-type sialidase cDNA; WT + CathA, cells co-transfected with the wild-type sialidase and CathA cDNA; C, control COS-7 cells. Enzyme activities in the cell lysates were determined 48 h after transfection. Values represent means ± SD of five independent experiments. The expression studies performed with cultured skin fibroblasts of the sialidosis patient WG0544 produced similar results (data not shown). (**B**) Detection of sialidase in the cellular lysates of COS-7 cells co-transfected with CathA and mutant sialidase cDNA by western blotting. Lysate aliquots (20 μg of protein) were subjected to SDS–PAGE, transferred to a nitrocellulose membrane and stained with rabbit anti-sialidase antibodies as described. Samples are indicated as in (A).

and several proteins with lower molecular masses (37, 26 and 24 kDa). These proteins, also found in the cells transfected with wild-type sialidase in the absence of CathA (Fig. 4B), probably represent fragments of sialidase molecule, suggesting the rapid proteolytic degradation of mutant sialidase in the lysosome. Immunolabeling of the sialidase mutants expressed in COS-7 cells (data not shown) confirmed its intracellular localization suggested on the basis of the western blots. Leu363Pro and Gly227Arg mutants showed mostly a diffuse cytoplasmic localization, whereas in the case of all other mutants anti-sialidase immunofluorescence was observed in

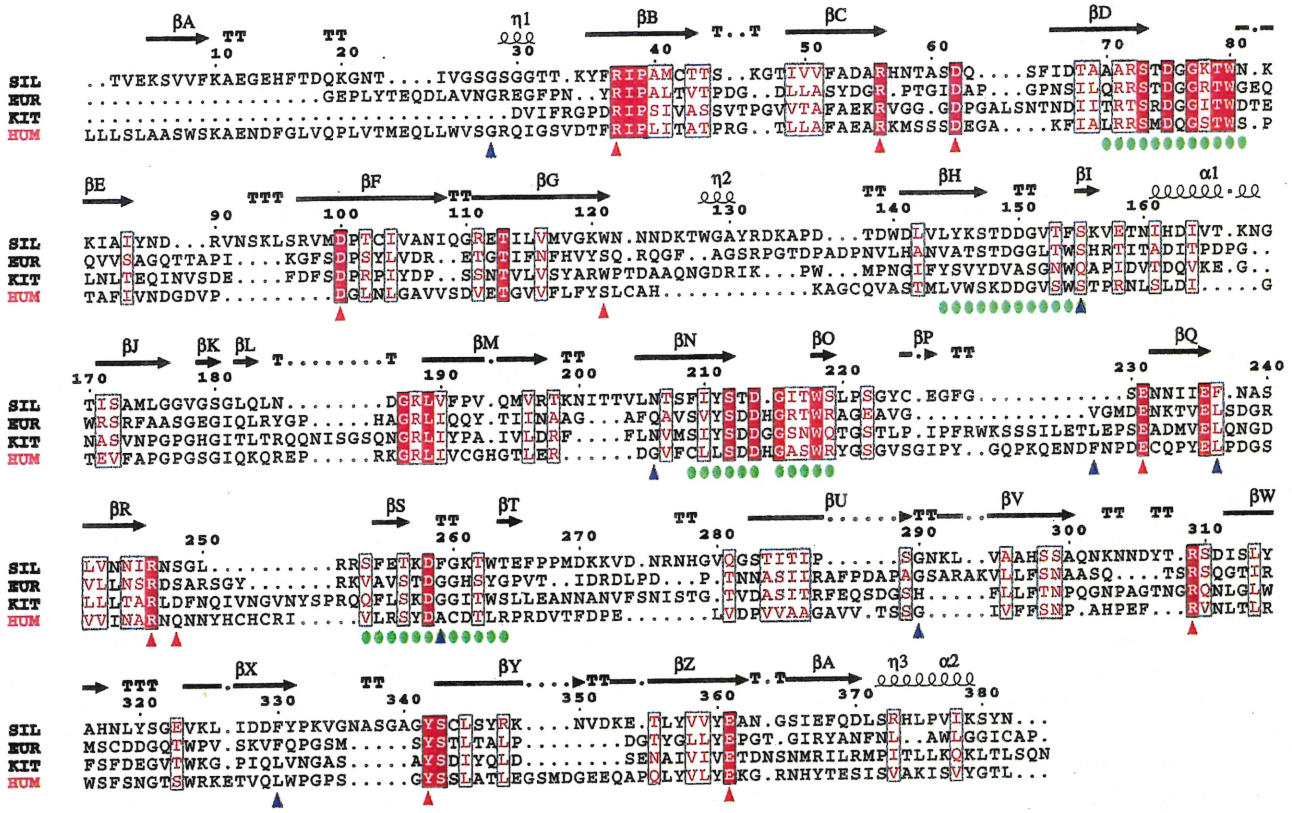

Figure 5. Amino acid sequence alignment of human lysosomal sialidase (HUM; residues 36–415) with homologous sialidases from *V.cholerae* (KIT), *M.viridifaciens* (EUR) and *S.typhimurium* (SIL). The identical residues are shown as white on red. Homologous residues are shown in red and boxed. Indicated above the alignment are the secondary structural elements of the structure of bacterial sialidases (33–35); α-helixes are indicated by 'spirals', β-sheets by arrows and turns by 'T'. Beneath the alignment, the positions of active site residues (red triangles), mutations identified in human sialidase gene (blue triangles) and 'Asp-box' repeats (green dots) are indicated.

perinuclear punctate structures co-localized with lysosomal marker LAMP2.

Structural model of sialidase

The structural model of lysosomal sialidase was built using the atomic coordinates of homologous sialidases from *Micromonospora viridifaciens* (EUR), *Salmonella typhimurium* (SIL) and *Vibrio cholerae* (KIT) as templates. These structures were superimposed to determine structurally conserved regions (SCRs). The sequence of human neuraminidase was then aligned with the SCRs (Fig. 5).

Analysis of the deduced structure (Fig. 6) indicates that human lysosomal sialidase shares the same fold as bacterial and viral sialidases. This fold consists of six four-stranded antiparallel β-sheets arranged as the blades of a propeller around a pseudo six-fold axis (31–33). Viral sialidases are tetramers of four identical β-propellers (31), whereas some bacterial sialidases contain additional domains built around the central canonical fold (34,35). These additional domains are usually involved in carbohydrate recognition (34,35). The model reveals that human lysosomal sialidase does not contain similar domains. This suggests that other components of the multienzyme lysosomal complex may be responsible for previously observed binding of the sialidase to lysosomal and plasmatic membranes (30).

Despite the low sequence identity (15% between the bacterial and viral sialidases, and ~30% between different bacterial sialidases), the topology of the catalytic domain and the active site residues are strictly conserved in these enzymes. The model indicates that this architecture of the active site is also conserved in human sialidase (Table 3). In particular, the Arg78 in human enzyme that is found in an RIP/RLP motive motif (REP in viral enzymes) is probably one of the residues responsible for binding the sialic acid carboxylate group. The other two Arg residues that stabilize the carboxylic group in the active site are potentially Arg280 and Arg341 (Table 3). Finally, the conserved Asp135 in the human enzyme is in the proper location to bind the *N*-acetyl-/*N*-glycolyl group of the substrate [Asp292, Asp131 and Asp100 in *V.cholerae*, *M.viridifaciens* and *S.typhimurium*, respectively (Table 3)]. This Asp residue is not conserved in the influenza enzyme, the substituted residue creates a pocket that can bind the guanidinium group of a 4-guanidino-Neu5Ac2en (36), a highly specific inhibitor of viral enzyme widely studied as a potential anti-influenza drug (reviewed in ref. 37). Our model indicates that this class of influenza sialidase inhibitors should not, therefore, affect the endogenous human lysosomal enzyme. The Glu394 residue that stabilizes the position of Arg78 through a hydrogen bond, as well as Tyr370 and Glu264 that are connected by a hydrogen bond and may donate a proton in

Table 3. Conserved active site residues between sialidases from *V.cholerae* (KIT), *M.viridifaciens* (EUR), *S.typhimurium* (SIL) and human lysosomal sialidase (HUM)

KIT	EUR	SIL	HUM
R224	R68	R37	R78
R245	R87	R56	R97
D250	D93	D62	D103
D292	D131	D100	D135
W311	S150	W121	S156
N318	–	T127	–
E619	E260	E231	E264
R635	R276	R246	R280
D637	S278	S248	Q282
R712	R342	R309	R341
Y740	Y370	Y342	Y370
E756	E386	E361	E394

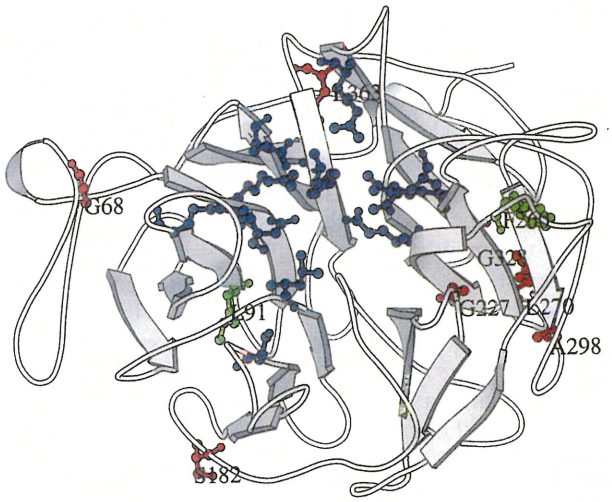

Figure 6. Ribbon drawing of the human lysosomal sialidase model showing the location of six new (red) and three previously identified (green) (17,18) point mutations. The deduced active site residue side chains are shown in blue.

the process of substrate hydrolysis (33–35), are also conserved. Asp103 is either a donor of the proton for the glycosidic bond or a stabilizer of the proton-donating water molecule.

The repeated so-called 'Asp-box' motifs (Ser/Thr-X-Asp-X-Gly-X-X-Trp/Phe) are also structurally conserved in human sialidase (Fig. 5). These repeats, found in all bacterial and mammalian sialidases, are always located between the third and the fourth β-strand at each sheet (βD and βE, βH and βI, βN and βO, and finally βS and βT). All Asp-boxes always have a similar arrangement with the aromatic residues packed into the hydrophobic core stabilizing the turn, whereas the hydrophilic Asp residues are solvent exposed. Gaskell *et al.* (34) have reported that similar motifs are also present at topologically conserved positions in eight-bladed β-propeller structure of bacterial methanol and methylamine dehydrogenases as well as seven-bladed fungal galactose and glyoxal oxidases, suggesting that these enzymes have evolved from the same four-bladed precursors through a gene duplication.

Potential effects of mutations and their correlation with enzymatic activity and patient phenotype

The Gly227Arg substitution was identified in two unrelated sialidosis type II male patients 1 and 2 of Caucasian origin from Mexico and the USA. Both patients were homozygous for this mutation and had a low residual level of sialidase activity in the cells. Our expression studies showed that the Gly227Arg mutant does not have enzymatic activity and lacks normal processing or lysosomal targeting. Gly227 is located in a βN strand of the sialidase molecule (second strand of the third sheet) and is not conserved in bacterial sialidases. Although such a substitution of a small neutral residue for a large charged one has the potential of greatly affecting the structure of the enzyme, we could not directly explain the clinical severity of the mutation with the model.

The Ala298Val (893C→T) mutation was found in both alleles of a sialidosis type II patient of Ashkenazi Jewish origin, whose severe phenotype and low residual cellular sialidase activity correlated with the expression results that showed a complete loss of the enzyme activity (Fig. 4). Our structural model indicates that Ala298 is situated in an 'Asp-box' repeat in the center of a turn loop between the third and the fourth strands of the fourth β-sheet (Fig. 6). The location of this mutation, in a surface loop, is consistent with expression studies which showed normal targeting and processing of the enzyme incompatible with a fold defect (Fig. 4).

Gly68Val substitution was found in both alleles of a patient of Polish origin who presented the severe clinical variant of sialidosis. In accordance with the clinical data, the Gly68Val sialidase mutant did not express any activity in COS-7 cells or in human fibroblasts (Fig. 4A). Gly68, which is conserved in the sialidases from *Micromonospora* and *Salmonella*, is a part of the short α-helix located between the first and the second β-strand. The low sequence homology in this region of the model did not permit us to draw conclusions on the possible structural effect of this mutation.

A Leu270Phe change was identified in two sibs of Spanish origin diagnosed with type II sialidosis. Both patients are homozygous for the mutation and have very low sialidase activity in their cells. Our expression studies showed that the Leu270Phe mutant is properly targeted and processed, has ~20% of the normal activity, but is significantly less stable than the wild-type enzyme (Fig. 4). Such low stability could result in almost complete sialidase deficiency in the cells of the affected patients and explains their severe clinical phenotype. In the human sialidase model, Leu270 is found at the end of the βQ strand. Although this Leu is conserved in both the *Micromonospora* and *Vibrio* enzymes, it was interesting to find a Phe residue in this position in *Salmonella*. Moreover, the CA of the Phe residue in *Salmonella* is shifted by 1.5 Å compared with the other two bacterial structures. Together, these results indicate that a Phe at this position can be accommodated, but with a significant structural shift which is consistent with the expression results and may explain the low stability of this mutant.

Previously, we have described a sialidosis type II patient (17), heterozygous for Phe260Tyr and Leu363Pro mutations.

The Phe260Tyr and Leu270Phe residue mutations are located at the opposite ends of the same βQ strand as a Leu270Phe mutation, but cause similar effects on the enzyme. The mutant products are normally targeted and processed, but are unstable and rapidly degrade in the lysosome. Phe260 is located in a surface 'loop' region in the model that does not exist in the other structures. Therefore, the only conclusion that may be deduced from the model is that this residue is probably accessible for binding other protein.

In contrast, the Leu363Pro mutant is retained in pre-lysosomal compartments and most probably lacks the proper fold. Leu363 is located in the center of the βX strand. A hydrophobic Leu (*Vibrio*) or Phe (*Micromonospora* and *Salmonella*) residue is found in this position in the bacterial structures. Substitution of this Leu for a Pro residue in either the model structure or in the *Vibrio* structures results in a steric clash with the backbone carboxylate of residue Leu344 or Leu715 located in the adjacent βW strand.

The Gly328Ser mutation found in one allele of a sialidosis type I patient of Italian origin is located in a highly variable turn loop region that bridges between the βU strand and the βV strand (the first and the second strands of the fifth β-sheet). Gly at this position is conserved in human, *Salmonella* and *Micromonospora* enzymes. The expressed product was targeted to the lysosomes and correctly processed, but showed <2% of the residual activity. It is tempting to speculate that the mutation in the second allele of the patient, which we were unable to identify, determines the mild phenotype with a late onset and a moderate clinical course.

The sialidosis type I patient of Chinese origin was homozygous for a Ser182Gly point mutation. Ser182 conserved in both sialidases from *M.viridifaciens* and *S.typhimurium* (Fig. 5) is located at the end of the second 'Asp-box' repeat. In our model, Ser182 is completely solvent accessible and located at the base in a long flexible surface loop. This may explain the relatively high (~40% of normal) activity expressed by the Ser182Gly sialidase mutant and correlates with the mild clinical phenotype of the patient.

DISCUSSION

Lysosomal sialidase has a unique feature, which distinguishes it from homologous non-lysosomal sialidases as well as from other lysosomal enzymes. Its functional activity absolutely depends on the integrity of the sialidase association with a multienzyme lysosomal complex containing cathepsin A/protective protein (CathA), β-galactosidase (GAL) and *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) (38–40). The complex protects sialidase and GAL against rapid proteolysis (30,38,41), and supports their catalytically active conformations (39,42). It was also reported that the association of CathA with Gal and sialidase provides proper targeting and processing of their precursors (43–45). In the autosomal recessive disease, galactosialidosis, a primary genetic defect of CathA (38,41) results in disruption of the complex and causes the combined deficiency of both GAL and sialidase activities. The clinical features and a composition of storage products in galactosialidosis resemble those in sialidosis (8,9,46). One may hypothesize that mutations in sialidase that destabilize its association with the complex can be 'lethal' for the enzyme even though they do not directly affect activity or stability of sialidase.

Results obtained in this study support this hypothesis. Six of nine sialidosis patients studied here had point mutations in the sialidase cDNA, leading to amino acid substitutions (Table 2). To understand the influence of these mutations on sialidase function, we have modeled the tertiary structure of human sialidase and located the identified mutations. None of the mutations directly affected the deduced active site residues or were found in the central core of the sialidase molecule, but all of them involved residues on the surface of the enzyme. Therefore, in most cases it is unlikely that these mutations would introduce electrostatic or steric clashes in the protein core leading to general folding defects of the sialidase and its retention in the ER/Golgi compartment as was observed for most of the mutations affecting CathA (47). Indeed, the expressed mutant products of sialidase were targeted to lysosomes, with the exception of the Gly227Arg and Leu363Pro mutants for which both western blotting and immunolabeling suggested misfolding and retention in the pre-lysosomal compartments. Moreover, structural analysis showed that five of the eight missense mutations, Gly227Arg, Ala298Val, Leu270Phe, Phe260Tyr and Gly328Ser, are clustered in one region on the surface of the sialidase molecule (Fig. 6). Western blot analysis demonstrated that Ala298Val, Leu270Phe, Phe260Tyr and Gly328Ser mutants expressed in COS-7 cells undergo normal intralysosomal processing, but then are rapidly degraded to smaller 37, 26 and 24 kDa fragments similar to those observed in COS-7 cells in which the wild-type sialidase was expressed in the absence of human CathA. We have previously observed exactly the same pattern of degradation products when we have expressed the wild-type human sialidase in the cells of a galactosialidosis patient, which lack CathA (30). Metabolic labeling studies (30) also demonstrated the dramatically reduced half-life of the 48.3 kDa active form of sialidase in galactosialidosis cells (30 min versus 2.7 h in normal cells), similar to that observed in this work (30). Together, these results permit us to speculate that the identified surface region where most mutations were found may represent part of the interface of sialidase binding with CathA and/or GAL in the lysosomal multienzyme complex (38,39). The data obtained will help to clarify the basic mechanism of the enzyme function in the lysosome and open the possibility to address directly the question of the supramolecular organization of the multienzyme lysosomal complex.

MATERIALS AND METHODS

Patients

All patients were clinically and biochemically diagnosed as having sialidosis. Available clinical data are presented in Table 1. Genomic DNA was purified either from the blood of patients or from their cultured skin fibroblasts as described previously (48).

Cloning of the sialidase gene and sequencing of sialidase introns

The sialidase gene was amplified in four overlapping fragments using genomic DNA from human placenta and oligo-

nucleotide primers complementary to exon sequences (Fig. 1). The amplified DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen, Mississauga, Ontario) and cloned into pCR2.1 vector using the TA cloning kit (InVitrogen, San Diego, CA) according to the manufacturer's protocol. Plasmid DNA was extracted using alkaline lysis (48) and automatically sequenced using the PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit on an Applied Biosystems 373A automated sequencer.

Amplification of sialidase exons

The oligonucleotide primers for the amplification of sialidase exons and adjacent splicing regions were selected at least 40 bp upstream of the acceptor splice sites and at least 40 bp downstream of donor sites. For some exons, more than one primer combination was tested to find one that produced a specific amplification of the exon. Sequences and positions of the primers used to amplify the exons are shown in Figure 1. The amplification conditions were: denaturation at 95°C for 2 min, 32 cycles, each consisting of 30 s at 95°C; 30 s at 54°C for exons 2 and 3, 56°C for exon 5 and 58°C for exons 1, 4 and 6, respectively; 1 min at 72°C followed by 10 min at 72°C.

Screening for previously undescribed mutations

Because of the small size of the exons, they were directly analyzed by SSCP as described (29,49). Typically, a 5–10 µl aliquot of the labeled PCR product was mixed with an equal volume of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene-cyanol FF. The sample was denatured by heating at 95°C for 3 min, rapidly cooled in an ice bath for 2 min and immediately loaded onto 0.4 mm × 31 cm × 38 cm 6% polyacrylamide gels containing 10 or 0% glycerol. Gels were run overnight at 10°C and 6 W constant power, or for ~3 h at 25°C and 30 W constant power, vacuum dried at 80°C and analyzed by autoradiography. Non-denatured samples were run beside denatured samples.

Exons and adjacent splicing regions for which the SSCP analysis produced band shifts or gain or loss of bands compared with normal controls, suggesting the presence of mutations, were manually sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Cleveland, OH).

Confirmation of mutations

For previously undescribed mutations, the nucleotide changes were confirmed directly in PCR-amplified products from genomic DNA. If the mutation created or destroyed a restriction enzyme site, restriction enzyme digestion was used to confirm the presence of the mutation. A 10–12 ng aliquot of the normal and mutant amplification product was digested with the appropriate restriction enzyme (*Sac*I for exon 5 of patients 7 and 8 or *Mae*II for exon 4 of patients 1 and 2) in a 15 µl volume for 1–2 h and analyzed by electrophoresis in 8% polyacrylamide gel containing ethidium bromide as described previously (48).

Mutations that did not create any changes in restriction enzyme sites were confirmed by ASO hybridization (48). The fragments surrounding mutations were amplified from genomic DNA of the patients and of 20 unrelated normal individuals, blotted on Zeta-Probe membrane (Bio-Rad, Hercules, CA) and hybridized with oligonucleotides complementary to normal and mutant DNA sequence, respectively (Table 4). The hybridization mixture typically contained 5 pmol of ^{32}P-labeled mutant probe and 50 pmol of normal unlabeled oligonucleotide or vice versa.

Table 4. Mutagenic and selection oligonucleotides used for site-directed mutagenesis

Mutation	Mutagenic oligonucleotide
G679A	5′-CTG GAG CGG GAC AGA GTC TTC TCT-3′
C893T	5′-AGC TAT GAT GTC TGT GAT ACA-3′
A544G	5′-GTT TCC TGG GGC ACA CCC CGG-3′
G203T	5′-GTG GGT GAG CGT GAG ACA GAT CGG-3′
C808T	5′-GCC CTA TGA GTT CCC AGA TGG-3′
G982A	5′-CAC CAG CTC CAG CAT TGT CTT C-3′
T779A	5′-AGG AAA ATG ATT ACA ATC CTG ATG-3′
T1088C	5′-AGA CAG TCC AGC CAT GGC CAG GCC-3′
Selection oligonucleotide	5′-GTG ACT GGT GAG GCC TCA ACC AAG TC-3′

The underlined nucleotides represent the bases changed during mutagenesis.

Construction of mutant sialidase expression vector

Site-directed mutagenesis was performed using a Transformer Site-Directed mutagenesis kit (Clontech, Palo Alto, CA), previously described pCMV-SIAL expression vector, mutagenic primers corresponding to mutant sialidase sequences and a selection primer used to eliminate a unique *Sca*I restriction site in the vector (Table 4), according to the supplier's protocols. Briefly, all primers were phosphorylated enzymatically and, for each mutant, the corresponding mutagenic primer and the selection primer were annealed to heat-denatured pCMV-SIAL plasmid. After elongation by T4 DNA polymerase, ligation and primary digestion with *Sca*I restriction enzyme to linearize all non-mutated DNA, the plasmid pool was used to transform the *mutS* strain of BMH71-18. Plasmid DNA obtained from the pool of ampicillin-resistant transformants was subjected to a second *Sca*I digestion and transformed into *Escherichia coli* DH5α. Positive clones were selected after a final *Sca*I restriction analysis and the entire sialidase cDNA sequenced. Up to 80% of transformants contained the desired mutation. DNA fragments of between 300 and 600 bp containing the introduced mutations were obtained from the mutant pCMV-SIAL plasmids by double digestion with either *Bst*EII–*Nae*I, *Nae*I–*Kpn*I or *Kpn*I–*Eco*RV and subcloned into the parental pCMV-SIAL plasmid. The final constructs were verified by sequencing.

Expression of sialidase in COS-7 cells and sialidosis fibroblasts

COS-7 cells or cultured skin fibroblasts of the sialidosis patient WG0544 (17) were transfected with pCMV-SIAL and pCMV-CathA expression vectors using Lipofectamine Plus reagent (Life Technologies, Gaithersburg, MD) in accordance with the manufacturer's protocol. Forty-eight hours after transfection,

sialidase and control *N*-acetyl-β-glucosaminidase activities were assayed in cellular homogenates using the corresponding fluorogenic 4-methylumbelliferyl-glycoside substrates as described (50–52). The CathA activity was determined with CBZ-Phe-Leu (53). One unit of enzyme activity (U) is defined as the conversion of 1 μmol of substrate per minute. Proteins were assayed according to Bradford (54) with bovine serum albumin (Sigma, St Louis, MO) as standard. To measure the stability of the expressed sialidase, the cellular homogenate was incubated at 37°C for 30 min, 1, 2 and 3 h before the assay of sialidase activity.

Western blotting

SDS–PAGE of proteins in cellular homogenates was performed under reducing conditions according to the method of Laemmli (55). After electrophoresis, the proteins were electrotransferred to NITRO ME nitrocellulose membrane (Micron Separations, Westboro, MA). The sialidase detection on western blots was performed with anti-sialidase rabbit antibodies as described previously (30) using the BM Chemiluminescence kit (Boehringer Mannheim, Mannheim, Germany) in accordance with the manufacturer's protocol.

Immunofluorescent microscopy

COS-7 cells overexpressing wild-type and mutant sialidase were double stained with rabbit polyclonal anti-sialidase antibodies and monoclonal antibodies against lysosomal membrane marker LAMP2 (Washington Biotechnology, Baltimore, MD) and studied on a Zeiss LSM410 inverted confocal microscope (Carl Zeiss, Thornwood, NY) as described previously (22).

Modeling of sialidase tertiary structure

Modeling was performed using the structures of homologous sialidases from *M.viridifaciens* (32, PDB file 1eur.pdb), *S.typhimurium* (31, PDB file 2sil.pdb) and *V.cholerae* (33, PDB file 1kit.pdb) as templates. These structures were superimposed with ProSup King's Beech Biosoftware Solutions to determine SCRs. The sequence of human sialidase was manually aligned with the sequences of SCRs. The modeling was then carried out with Modeler 4 software (Andrej Sali, The Rockefeller University, New York, NY).

ACKNOWLEDGEMENTS

The authors thank Dr Roy Gravel for the help with DNA analysis and stimulating discussions, and Dr Mila Ashmarina for critical reading of the manuscript. This work was supported by the operating grants from the Medical Research Council of Canada, Fonds de La Recherche en Santé du Québec and Vaincre les Maladies Lysosomales Foundation (France) to A.V.P., and from NICHD Mental Retardation Research Center to G.T.

NOTE ADDED IN PROOF

The 808C→T (Leu270Phe) mutation was also found in two other unrelated sialidosis type II patients from the same region of Spain.

REFERENCES

1. Thomas, G.H. and Beaudet, A.L. (1995) Disorders of glycoprotein degradation and structure: α-mannosidosis, β-mannosidosis, fucosidosis, sialidosis, aspartylglucosaminuria and carbohydrate-deficient glycoprotein syndrome. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Vallee, D. (eds), *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill, New York, NY, pp. 2529–2561.
2. Federico, A., Battistini, S., Ciacci, G., de Stefano, N., Gatti, R., Durand, P. and Guazzi, G.C. (1991) Cherry-red spot myoclonus syndrome (type I sialidosis). *Dev. Neurosci.*, **13**, 320–326.
3. Cantz, M. and Ulrich-Bott, B. (1990) Disorders of glycoprotein degradation. *J. Inherit. Metab. Dis.*, **13**, 523–537.
4. Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J.C. and Strecker, C. (1977) Sialidosis (mucolipidosis I). *Helv. Paediatr. Acta*, **32**, 391–400.
5. Sogg, R.L., Steinman, L., Rathjen, B., Tharp, B.R., O'Brien, J.S. and Kenyon, K.R. (1979) Cherry-red spot-myoclonus syndrome. *Ophthalmology*, **86**, 1861–1874.
6. Rapin, I., Goldfischer, S., Katzman, R., Engel Jr, J., and O'Brien, J.S. (1978) The cherry-red spot-myoclonus syndrome. *Ann. Neurol.*, **3**, 234–242.
7. O'Brien, J.S. (1979) The cherry red spot-myoclonus syndrome: a newly recognized inherited lysosomal storage disease due to acid neuraminidase deficiency. *Clin. Genet.*, **14**, 55–60.
8. Federico, A., Cecio, A., Battini, G.A., Michalski, J.C., Strecker, G. and Guazzi, G.C. (1980) Macular cherry-red spot and myoclonus syndrome. Juvenile form of sialidosis *J. Neurol. Sci.*, **48**, 157–169.
9. Till, J.S, Roach, E.S. and Burton, B.K. (1987) Sialidosis (neuraminidase deficiency) types I and II: neuro-ophthalmic manifestations. *J. Clin. Neuro-Ophthalmol.*, **7**, 40–44.
10. Kelly, T.E. and Graetz, G. (1977) Isolated acid neuraminidase deficiency: a distinct lysosomal storage disease. *Am. J. Med. Genet.*, **1**, 31–46.
11. Oohira, T., Nagata, N., Akaboshi, I., Matsuda, I. and Naito, S. (1985) The infantile form of sialidosis type II associated with congenital adrenal hyperplasia: possible linkage between HLA and the neuraminidase deficiency gene. *Hum. Genet.*, **70**, 341–343.
12. Winter, R.M., Swallow, D.M., Baraitser, M. and Purkiss, P. (1980) Sialidosis type 2 (acid neuraminidase deficiency): clinical and biochemical features of a further case. *Clin. Genet.*, **18**, 203–210.
13. Aylsworth, A.S., Thomas, G.H., Hood, J.L., Malouf, N. and Libert, J. (1980) A severe infantile sialidosis: clinical, biochemical and microscopic features. *J. Pediatr.*, **96**, 662–668.
14. Johnson, W.G., Cohen, C.S., Miranda, A.F., Waran, S.P. and Chutorian, A.M. (1980) Alpha-locus hexosaminidase genetic compound with juvenile gangliosidosis phenotype: clinical, genetic and biochemical studies. *Am. J. Hum. Genet.*, **32**, 43A.
15. Beck, M., Bender, S.W., Reiter, H.L., Otto, W., Bassler, R., Dancygier, H. and Gehler, J. (1984) Neuraminidase deficiency presenting as non-immune hydrops fetalis. *Eur. J. Pediatr.*, **143**, 135–139.
16. Pshezhetsky, A.V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M.A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L. and Potier, M. (1996) Sialidosis: cloning, expression and chromosomal mapping of human lysosomal sialidase (neuraminidase). *Am. J. Hum. Genet.*, **59**, 4, A279.
17. Pshezhetsky, A.V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M.A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L. and Potier, M. (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nature Genet.*, **15**, 316–320.
18. Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G. and d'Azzo, A. (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes Dev.*, **10**, 3156–3168.
19. Milner, C.M., Smith, S.V., Carrillo, M.B., Taylor, G.L., Hollinshead, M. and Campbell R.D. (1997) Identification of a sialidase encoded in the human major histocompatibility complex. *J. Biol. Chem.*, **272**, 4549–4558.
20. Rottier, R.J., Bonten, E. and d'Azzo, A. (1998) A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. *Hum. Mol. Genet.*, **7**, 313–321.
21. Carrillo, M.B., Milner, C.N., Ball, S.T., Snock, M. and Campbell, R.D. (1997) Cloning and characterization of sialidase from the murine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele. *Glycobiology*, **7**, 975–986.

22. Igdoura, S.A., Gafuik, C., Mertineit, C., Saberi, F., Pshezhetsky, A.V., Potier, M., Trasier, J.M. and Gravel, R.A. (1998) Cloning of the cDNA and the gene encoding mouse lysosomal sialidase and correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts. *Hum. Mol. Genet.*, 7, 115–121.
23. Thomas, G.H., Goldberg, M.F., Miller, C.S. and Reynolds, L.W. (1979) Neuraminidase deficiency in the original patient with Goldberg Syndrome. *Clin. Genet.*, **16**, 323–330.
24. Cantz, M., Gehler, J. and Spranger, J. (1977) Mucolipidosis I: increased sialic acid content and deficiency of an α-*N*-acetylneuraminidase in cultured fibroblasts. *Biochem. Biophys. Res. Commun.*, **74**, 732–738.
25. Johnson, W.G., Thomas, G.H., Miranda, A.F., Driscoll, J.M., Wigger, J.H., Yeh, M.N., Schwartz, R.C., Cohen, C.S., Berdon, W.E. and Koenigsberger, M.R. (1980) Congenital sialidosis: Biochemical studies; clinical spectrum in four sibs; two successful prenatal diagnoses. *Am. J. Hum. Genet.*, **32**, 43A.
26. Carey, W.F., Fletcher, J.M. and Wong, M.C. (1997) A case of sialidosis with unusual results. In *Proceedings of the 7th International Congress of Inborn Errors of Metabolism*. Vienna, Austria, p. 104.
27. Tylki-Szymanska, A., Lugowska, A. and Czartoryska, B. (1996) Neuraminidase deficiency presenting as a nephrosialidosis: The first case detected in Poland. *Acta Paediatr. Jpn*, **38**, 529–532.
28. Palmeri, S., Villanova, M., Malandrini, A., van Diggelen, O., Huijmans, J., Ceuterick, C., Rufa, A., DeFalco, D., Ciacci, G., Martin, J. and Guazzi, G. (2000) Type I sialidosis: a clinical, biochemical and neuroradiological study. *Eur. Neurol.*, **43**, 88–94.
29. Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K. (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics*, **5**, 874–879.
30. Vinogradova, M.V., Michaud, L., Mezentsev, A.V., Lukong, K.E., El-Alfy, M., Morales, C.R., Potier, M. and Pshezhetsky, A.V. (1998) Molecular mechanism of lysosomal sialidase defiency in galactosialidosis involves its rapid degradation. *Biochem. J.*, **330**, 641–650.
31. Varghese, J.N., Laver, W.G. and Colman, P.M. (1983) Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. *Nature*, **303**, 35–40.
32. Burmeister, W.P., Ruigrok, R.W.H. and Cusack, S. (1992) The 2.2 Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.*, **11**, 49–56.
33. Crennell, S.J., Garman, E.F., Laver, W.G., Vimr, E.R. and Taylor, G. (1993) Crystal structure of a bacterial sialidase (from *Salmonela typhimurium* LT2) shows the same fold as an influenza virus neuraminidase. *Proc. Natl Acad. Sci. USA*, **90**, 9852–9856.
34. Gaskell, A., Crennell, S. and Taylor, G. (1995) The three domains of a bacterial sialidase: a β-propeller, an immunoglobulin module and a galactose-binding jelly-roll. *Structure*, **3**, 1197–1205.
35. Crennell, S.J., Garman, E.F., Laver, W.G., Vimr, E.R. and Taylor, G. (1994) Crystal structure of a *Vibrio cholerae* sialidase reveals dual lectin-like domains in addition to the catalytic domain. *Structure*, **2**, 535–544.
36. Von Itzstein, M., Wu, W.Y., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B., Phan, T.V., Smythe, M.L., White, H.F., Oliver, S.W. *et al.* (1993) Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature*, **363**, 418–423.
37. Taylor, G. (1996) Sialidases: structures, biological significance and therapeutic potential. *Curr. Opin. Struct. Biol.*, **6**, 830–837.
38. d'Azzo, A., Hoogeveen, A., Reuser, A.J., Robinson, D. and Galjaard, H. (1982) Molecular defect in combined β-galactosidase and neuraminidase deficiency in man. *Proc. Natl Acad. Sci. USA*, **79**, 4535–4539.
39. Van der Horst, G.J., Galjart, N.J., d'Azzo, A., Galjaard, H. and Verheijen, F.W. (1989) Identification and in vitro reconstitution of lysosomal neuraminidase from human placenta. *J. Biol. Chem.*, **264**, 1317–1322.
40. Pshezhetsky, A.V. and Potier, M. (1996) Association of *N*-acetylgalactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of β-galactosidase, cathepsin A and neuraminidase. *J. Biol. Chem.*, **271**, 28359–28365.
41. Hoogeveen, A.T., Verheijen, F.W. and Galjaard, H. (1983) The relation between human lysosomal β-galactosidase and its protective protein. *J. Biol. Chem.*, **258**, 12143–12146.
42. Pshezhetsky, A.V., Elsliger, M.-A., Vinogradova, M. and Potier, M. (1995) Human lysosomal β-galactosidase–cathepsin A complex: definition of the β-galactosidase-binding interface on cathepsin A. *Biochemistry*, **34**, 2431–2440.
43. D'Agrosa, R.M., Hubbes, M., Zhang, S., Shankaran, R. and Callahan, J.W. (1992) Characterization of the β-galactosidase–carboxypeptidase complex in GM1-gangliosidosis and β-galactosialidosis fibroblasts. *Biochem. J.*, **285**, 833–838.
44. Okamura-Oho, Y., Zhang, S., Hilson, W., Hinek, A. and Callahan, J. (1996) Early proteolytic cleavage with loss of a C-terminal fragment underlies altered processing of the β-galactosidase precursor in galactosialidosis. *Biochem. J.*, **313**, 787–794.
45. van der Spoel, A., Bonten, E. and Azzo, A. (1998) Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. *EMBO J.*, **17**, 1588–1597.
46. d'Azzo, A., Andria, G., Strisciuglio, P. and Galijaard, H. (1995) Galactosialidosis. In Scriver, C.R., Beaudet, A.L., Sly, W.S. and Vallee, D. (eds), *Metabolic and Molecular Bases of Inherited Disease*, Vol. 2. McGraw-Hill, New York, NY, pp. 2835–2837.
47. Rudenko, G., Bonten, E., Hol, W.G.H. and d'Azzo, A (1998) The atomic model of the human protective protein/cathepsin A suggests a structural basis for galactosialidosis. *Proc. Natl Acad. Sci. USA*, **95**, 621–615.
48. Maniatis, T., Fritsch, E.F. and Sambrook, J. (eds) (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
49. Triggs-Raine, B.L., Akerman, B.R., Clarke, J.T. and Gravel, R.A. (1991) Sequence of DNA flanking the exons of the HEXA gene and identification of mutations in Tay–Sachs disease. *Am. J. Hum. Genet.*, **49**, 1041–1054.
50. Okada, S. and O'Brien, J.S. (1968) Generalized gangliosidosis: β-galactosidase deficiency. *Science*, **160**, 1002–1004.
51. Potier, M., Mameli, L., Belisle, M., Dallaire, L. and Melançon, S.B. (1979) Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-*N*-acetylneuraminate) substrate. *Anal. Biochem.*, **94**, 287–296.
52. Rome, L.H., Garvin, A.J., Allietta, M.M. and Neufeld, E.F. (1979) Two species of lysosomal organelles in cultured human fibroblasts. *Cell*, **17**, 143–153.
53. Pshezhetsky, A.V., Vinogradova, M.V., Elsliger, M.-A., El-Zein, F., Svedas, V.K. and Potier, M. (1995) Continuous spectrophotometric assay of human lysosomal cathepsin A/protective protein in normal and galactosialidosis cells. *Anal. Biochem.*, **230**, 303–307.
54. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72**, 248–254.
55. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Foreword

Sialidosis like other LSD including galactosialidosis has a higher frequency in the Japanese population. To understand if the spectrum of SIAL mutations in Japanese patients is different from that in other populations, we collaborated with our colleagues from the Tokyo Metropolitan Institute of Medical Science to study the pathogenesis of sialidosis in patients they diagnosed.

We identified and characterized two new compound heterozygous mutations in two unrelated Japanese patients affected with sialidosis type I. Both mutations, 649G→A (Val217Met) and 727G→A (Gly243Arg), occurred in CpG dinucleotides, which are recognized as mutational hot spots (Cooper and Youssoufian, 1988).

We used transient expression, enzyme assay, immunoblot and immunocytochemistry analyses, as well as structural modeling to characterize the biochemical defects in the mutant sialidase protein. The Val217Met mutation resulted in a mild structural change while the Gly243Arg, which completely abolished enzyme activity, had a significant impact on the conformation of the protein.

The expression, enzyme assay and immunoblots were conducted by Kiven Erique Lukong.

Article 3

Molecular and structural studies of Japanese patients with sialidosis type I

J Hum Genet (2000) 45:241–249

ORIGINAL ARTICLE

Yasunori Naganawa · Kohji Itoh · Michie Shimmoto
Kyoko Takiguchi · Hirofumi Doi · Yuzuru Nishizawa
Takayoshi Kobayashi · Sachiko Kamei · Kiven E. Lukong
Alexey V. Pshezhetsky · Hitoshi Sakuraba

Molecular and structural studies of Japanese patients with sialidosis type 1

Received: March 21, 2000 / Accepted: May 10, 2000

Abstract To gain insight into the pathogenesis of sialidosis type 1, we performed molecular investigations of two unrelated Japanese patients. Both of them are compound heterozygotes for base substitutions of ^{649}G-to-A and ^{727}G-to-A, which result in amino acid alterations V217M and G243R, respectively. Using homology modeling, the structure of human lysosomal neuraminidase was constructed and the structural changes caused by these missense mutations were deduced. The predicted change due to V217M was smaller than that caused by G243R, the latter resulting in a drastic, widespread alteration. The overexpressed gene products containing these mutations had the same molecular weight as that of the wild type, although the amounts of the products were moderately decreased. A biochemical study demonstrated that the expressed neuraminidase containing a V217M mutation was partly transported to lysosomes and showed residual enzyme activity, although a G243R mutant was retained in the endoplasmic reticulum/Golgi area and had completely lost the enzyme activity. Considering the data, we surmise that the V217M substitution may be closely associated with the phenotype of sialidosis type 1 with a late onset and moderate clinical course.

Key words Sialidosis · Lysosomal neuraminidase · Homology modeling · Protective protein / cathepsin A

Y. Naganawa · K. Itoh · M. Shimmoto · H. Sakuraba (✉)
Department of Clinical Genetics, The Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan
Tel. +81-3-3823-2101, ext. 5364; Fax +81-3-3823-6008
e-mail: sakuraba@rinshoken.or.jp

K. Takiguchi · H. Doi
Fujitsu Laboratories Ltd., Chiba, Japan

Y. Nishizawa
Second Department of Internal Medicine, Nippon Medical School, Tokyo, Japan

T. Kobayashi
Department of Neurology, Faculty of Medicine, Tokyo Medical and Dental University, Tokyo, Japan

S. Kamei · Y. Naganawa
School of Allied Health Science, Faculty of Medicine, Tokyo Medical and Dental University, Tokyo, Japan

K.E. Lukong · A.V. Pshezhetsky
Service de Génétique Médicale, Département de Pédiatrie, Hôspital Sainte-Justine, Université de Montréal, Montréal, Canada

Introduction

Lysosomal neuraminidase (sialidase, EC 3. 2. 1. 18) is an acid glycosidase that catalyzes the removal of the terminal sialic acid residues of glycoconjugates (Verheijen et al. 1982, 1985, 1987; Hiraiwa et al. 1988; van der Horst et al. 1989). Mammalian lysosomal neuraminidase is associated with protective protein/cathepsin A (PPCA; EC 3. 4. 16. 1) and β-galactosidase (EC 3. 2. 1. 23), and essentially requires PPCA for its activation (d'Azzo et al. 1982; van der Spoel et al. 1998). Defects in the *PPCA* gene result in a human metabolic disease, galactosialidosis, associated with secondary deficiencies of lysosomal neuraminidase and β-galactosidase (reviewed by d'Azzo et al. 1995).

The primary defect of the lysosomal neuraminidase causes another autosomal recessive genetic disorder, sialidosis (reviewed by Thomas et al. 1995). The disease is clinically divided into two groups: late-onset sialidosis type 1, characterized by macular cherry-red spots and myoclonus, and early-onset sialidosis type 2, distinguished from type 1 by the presence of dysmorphic features besides neurological symptoms and cherry-red spots. The molecular basis of the disease has remained obscure. Recently, human lysosomal neuraminidase cDNA was isolated (Bonten et al. 1996; Pshezhetsky et al. 1997; Milner et al. 1997), and studies for clarifying the pathogenesis of sialidosis were started. So far, six different mutations in the neuraminidase gene have been reported (Bonten et al. 1996; Pshezhetsky et al. 1997). However, little has been elucidated regarding the enzyme proteins caused by the mutant genes.

In this study, we characterized the molecular defects in lysosomal neuraminidase caused by gene mutations, which

were newly identified in two unrelated Japanese patients with sialidosis type 1.

Patients and methods

Patients

Patient 1. This patient is a 25-year-old female offspring of nonconsanguineous parents. At the age of 17 years, she developed an ataxic gait, and speech disturbance. There were subsequent episodes of generalized convulsions and visual disturbance. Ophthalmological examination disclosed macular cherry-red spots. Her neurological symptoms rapidly progressed, and she could not stand without support at the age of 18 years, and is bed-ridden at present. Her younger sister exhibited a similar clinical course, although her brother had a milder clinical course (Mitoma et al. 1993). Lysosomal enzyme assay revealed an isolated neuraminidase deficiency, but β-galactosidase activity was normal in these three siblings (Yamamoto et al. 1995).

Patient 2. This patient is a 42-year-old man. He was born to nonconsanguineous parents. He developed normally after birth until the age of 32 years, when involuntary movements began in the upper extremities. Then, he developed gait disturbance and slurred speech. Physical examination at age 41 years revealed dysarthria, action myoclonus, and hyperactive deep tendon reflexes. Lens opacities and macular cherry-red spots were detected. Neither computed tomography nor magnetic resonance imaging of the head revealed any abnormality. Lysosomal enzyme assay disclosed a neuraminidase deficiency. Other enzyme activities, including those of β-galactosidase and cathepsin A, were normal. His brother has shown similar clinical manifestations, but detailed data for the brother, including neuraminidase activity, are not available.

Materials

1,2-Dimyristyloxypropyl-3-dimethyl-hydroxy ethylammonium bromide (DMRIE)-C reagent for transfection was purchased from GIBCO/BRL (Grand Island, NY, USA). We purchased leupeptin from the Peptide Institute (Osaka, Japan); N-benzyloxycarbonyl-L-phenylalanyl-L-leucine (Z-Phe-Leu) from Sigma (St. Louis, MO, USA); and 1,4-diazabicyclo-[2,2,2]octane (DABCO) from Wako Pure Chemical Industries (Osaka, Japan). Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA).

Cell culture

Human skin fibroblasts derived from healthy Japanese subjects and patients with sialidosis type 1 were maintained in Ham's F-10 medium supplemented with 10% fetal calf serum (FCS) and antibiotics, as described previously (Sakuraba et al. 1999). For transient expression of the wild-type and mutant lysosomal neuraminidase cDNAs, a fibroblast cell line derived from a galactosialidosis patient (ASVGS-1) (Shimmoto et al. 1993; Gluzman et al. 1981) or COS-1 cells (kindly supplied by Dr. Y. Nabeshima, National Institute of Neuroscience, NCNP, Tokyo, Japan) were cultured in the same medium.

Gene analysis

Total RNA and genomic DNA were isolated from the cultured fibroblasts, as described previously (Davis et al. 1986). Lysosomal neuraminidase cDNA, including the entire protein coding sequence, was obtained by reverse transcription (RT)-polymerase chain reaction (PCR) (Kawasaki 1990), using an oligo(dT) primer and an RT-PCR kit (Amersham, Buckinghamshire, England), followed by cDNA amplification, using a pair of primers (sense, 5′-AGTCTAGCTGC CAGGGTCGC-3′ and antisense, 5′-AGTCCTGAAGGC AGAATACC-3′). The amplified lysosomal neuraminidase cDNA was subcloned into the pGEM-T vector (Promega, Madison, WI, USA). Nucleotide sequencing was performed with a DyeDeoxy Terminator Cycle Sequencing Kit and an automated sequencer (Applied Biosystems, Foster City, CA, USA). As described under "Results", ^{649}G-to-A (V217M) and ^{727}G-to-A (G243R) substitutions were detected in both the patients. These base substitutions are located in exon 4 of the lysosomal neuraminidase gene. To confirm the presence of these mutations, genomic DNA fragments, including exon 4, were amplified by PCR, using sense (5′-TGAGCCCCTAGAGTCTCC-3′) and antisense (5′-GGCAGGGAGGGTCAAATG-3′) primers. The amplified DNA fragment was analyzed by direct sequencing.

Transient expression of lysosomal neuraminidase cDNA

An expression vector for the wild-type neuraminidase was prepared by inserting the full-length human lysosomal neuraminidase cDNA into the *Not* I site of the pCMV expression vector (MacGregor and Caskey 1989), and designating this as pCMV-sialidase, as previously described (Vinogradova et al. 1998). For expression of the mutant lysosomal neuraminidase cDNA, the DNA fragment obtained from the mutant cDNA subclone, containing either ^{649}G-to-A (V217M) or ^{727}G-to-A (G243R), was double-digested with *Bst*EII and *Kpn* I, and substituted for the pCMV-sialidase plasmid. The cloned plasmids were designated pCMV-sialidase[V217M] and pCMV-sialidase[G243R], respectively.

For enzyme assay and immunoblotting, ASVGS-1 and COS-1 cells were seeded onto 60-mm dishes (2×10^5 cells), 1 day before transfection. For immunocytochemistry, COS-1 cells were seeded onto two-well chamber slides (Lab-Tek; Nunc, Naperville, IL, USA) (1×10^5 cells). The transfection was performed using DMRIE-C reagent (GIBCO/BRL) according to the manufacturer's protocol. Briefly, a mixture comprising the plasmid DNA (5μg) and DMRIE-C reagent (10μl) was added to cells cultured in FCS-free Ham's F-10 medium, followed by incubation for 5h. Then, the culture

medium was replaced with Ham's F-10 medium containing 10% FCS, and the cells were cultured for 4 days. As for ASVGS-1 cells for enzyme assay and COS-1 cells for immunocytochemical analysis, a pCAGGS vector (Miyazaki et al. 1991) containing human wild-type PPCA cDNA (pCAGGS-PP, 5μg) (Shimmoto et al. 1993) was co-transfected with the wild-type or mutant lysosomal neuraminidase cDNA.

Enzyme assay

As mentioned in the "Introduction", a sufficient amount of PPCA is required for the activation of lysosomal neuraminidase, and ASVGS-1 cells co-transfected with PPCA cDNA were used as host cells in this analysis. The transfected ASVGS-1 cells were washed with phosphate-buffered saline (PBS), harvested by scraping, and then suspended in distilled water containing 0.1 mM leupeptin. A homogenate was prepared by pipetting, and then neuraminidase activity was measured immediately, according to a method reported previously (Suzuki et al. 1981). The rest of the homogenate was sonicated and used for other lysosomal enzyme assays and protein determination. Glycosidase activities, including those of neuraminidase, β-galactosidase, and β-hexosaminidase, were assayed fluorometrically, using 4-methylumbelliferyl glycosides as substrates (Suzuki 1987). Cathepsin A activity was measured at pH 5.6, using Z-Phe-Leu as a substrate (Itoh et al. 1991). Protein determination was performed with a dye-binding assay kit (Bio-Rad, Richmond, CA, USA), using bovine serum albumin (BSA) as a standard.

Polyacrylamide gel electrophoresis and immunoblotting

After transfection, COS-1 cells were harvested, sonicated in distilled water containing 0.1 mM leupeptin, and then centrifuged at 10,000 × *g* for 15 min at 4°C. Aliquots of the resultant supernatant, after reduction with 25 mM mercaptoethanol, were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (1970), on a 10% acrylamide gel (Realgel Plate; Biocraft, Tokyo, Japan). Proteins were visualized by immunostaining, using an anti-neuraminidase antibody (Vinogradova et al. 1998) and a Super Signal Ultra Chemiluminescence Kit (Pierce, Rockford, IL, USA). Prestained SDS-PAGE standards (Bio-Rad, Hercules, CA, USA) were used as molecular mass standard proteins: phosphorylase b, 110 kDa; BSA, 84 kDa; ovalbumin, 50 kDa; carbonic anhydrase, 33 kDa; soybean trypsin inhibitor, 24 kDa; and lysozyme, 16 kDa.

Immunocytochemical analysis

The transfected COS-1 cells were fixed with ice-cold 4% paraformaldehyde/PBS (v/v) and then with 80% methanol/PBS (v/v). After nonspecific binding was blocked with 5% goat serum and 1% BSA in PBS, the cells were treated with the anti-neuraminidase antibody (Vinogradova et al. 1998) overnight at 4°C. Then the cells were washed with PBS, followed by treatment with fluorescein-isothiocyanate (FITC)-labeled F $(ab')_2$ fragments of goat anti-rabbit IgG (BioSource, Camarillo, CA, USA). After being washed with PBS, the cells were mounted with 55% glycerol/PBS (v/v) containing 3.8% DABCO. The stained cells were examined under a fluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany).

Structural modeling of human wild-type and mutant lysosomal neuraminidases

The crystal structure of neuraminidase from *Salmonella typhimurium* LT2 was determined (Crennell et al. 1993) and deposited in the Protein Data Bank (PDB; Brookhaven National Laboratory, Upton, NY, USA) (Sussman et al. 1998). On the basis of this information (PDB file: 2sil), models of the human wild-type lysosomal neuraminidase and its mutants were built, using molecular modeling software (SYBYL/COMPOSER; TRIPOS, St. Louis, MO, USA), installed on a PowerIndigo2 R8000 workstation (Silicon Graphics, Mountain View, CA, USA). Sequence alignment of the human lysosomal neuraminidase and *Salmonella typhimurium* neuraminidase was performed for definition of the structurally conserved regions of both target and template proteins. A fragment derived from the homologue was used to model the backbone of each structurally conserved region, and the rule-based procedure was used to generate the side chain (Sutcliffe et al. 1987).

Loop regions were then constructed by selecting a fragment for modeling loop regions from the general protein database (Jones and Thirup 1986; Claessens et al. 1989). The force field for simulation of proteins (Weiner et al. 1984, 1986) was employed in the minimization steps to optimize the geometry of a molecule. The maximum number of iterations to be performed during the minimization was set as 1000. The minimization procedure was performed to optimize the side chain rotamers, with the backbone and the conserved side chains fixed, and then to optimize the conformation of loops, with the structurally conserved regions fixed. The mutant model was built in the same way as for the wild-type, but based on the primary structure with the amino acid replacement. To determine the influence of the replacement on the model structure, the mutant model was superimposed on the wild-type model by the least-square-mean fitting method (McLachlan 1979). We defined that the structure was influenced by the amino acid replacement when the position of an atom of the mutant differed from that of the wild-type by more than the total root-mean-square distance value.

Results

Gene analysis

The PCR products of the entire cDNA coding sequence for lysosomal neuraminidase were subcloned and sequenced.

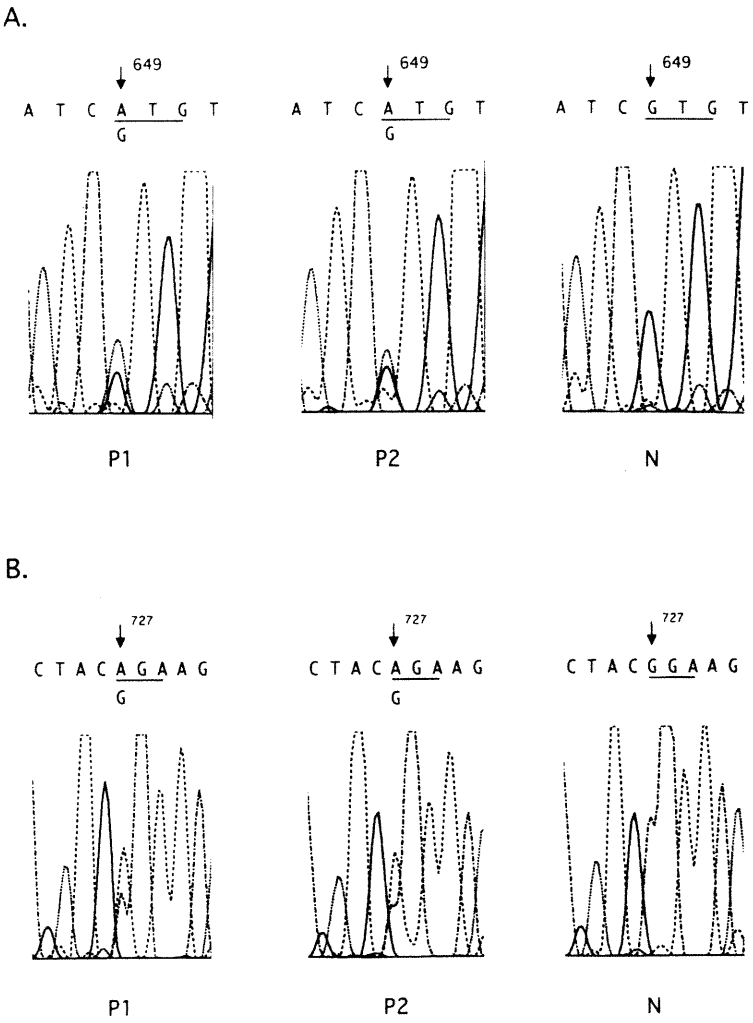

Fig. 1A,B. Direct sequencing of genomic DNA. The results show that both patients are heterozygous for **A** the ^{649}G-to-A and **B** the ^{727}G-to-A substitutions. The mutation sites are indicated by *arrows*. *P1*, Patient 1; *P2*, patient 2; *N*, a normal subject

Table 1. Transient expression of lysosomal neuraminidase cDNA

Plasmid	Neuraminidase (nmol/h per mg protein)	Cathepsin A (μmol/h per mg protein)	β-Galactosidase (nmol/h per mg protein)	β-Hexosaminidase (μmol/h per mg protein)
None	<1	<0.1	12	2.2
[a]pCAGGS-PP	28	5.4	43	1.6
[b]pCMV-sialidase and pCAGGS-PP	183	3.7	39	1.5
[c]pCMV-sialidase[V217M] and pCAGGS-PP	55	3.1	36	1.2
[d]pCMV-sialidase[G243R] and pCAGGS-PP	27	5.0	45	1.6

Lysosomal enzyme activities in a galactosialidosis fibroblast cell line (ASVGS-1 cells) transfected with plasmids, described under "Methods". Values are the means of two independent measurements

[a] pCAGGS-PP, a pCAGGS vector including PPCA cDNA

[b] pCMV-sialidase and pCAGGS-PP, a pCMV vector including the wild-type lysosomal neuraminidase cDNA and a pCAGGS vector including PPCA cDNA

[c] pCMV-sialidase[V217M] and pCAGGS-PP, a pCMV vector including mutant lysosomal neuraminidase cDNA with V217M and a pCAGGS vector including PPCA cDNA

[d] pCMV-sialidase[G243R] and pCAGGS-PP, a pCMV vector including mutant neuraminidase cDNA with G243M and a pCAGGS vector including PPCA cDNA

Two types of cDNA clones were detected in both patients, the first having a ^{649}G-to-A substitution and the other, a ^{727}G-to-A transition (data not shown). Because both changes were located in exon 4, the genomic DNA fragment including exon 4 was amplified and directly sequenced. The results showed that both patients were heterozygous for the ^{649}G-to-A and ^{727}G-to-A substitutions (Fig. 1). The results of the cDNA and genomic DNA analyses show that both patients have one allele carrying a G-to-A transition at nucleotide number 649, resulting in the

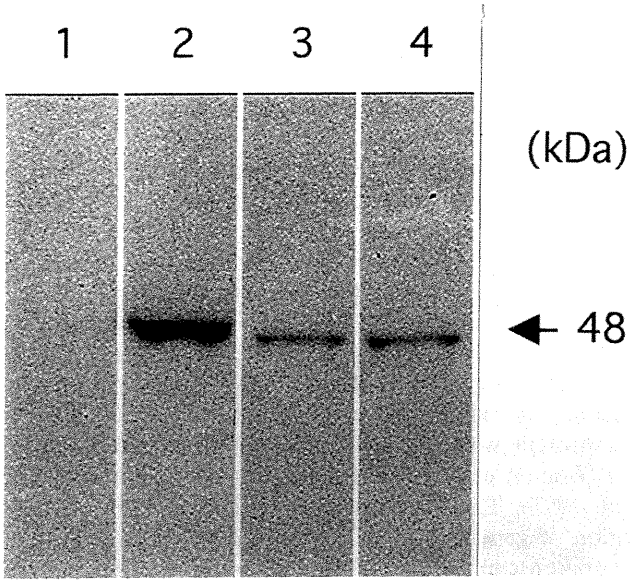

Fig. 2. Immunoblot analysis of lysosomal neuraminidase expressed in COS-1 cells. COS-1 cells were transfected with the wild-type lysosomal neuraminidase cDNA (*lane 2*), mutant neuraminidase cDNA with a V217M mutation (*lane 3*), and that with a G243R mutation (*lane 4*). Non-transfected cells were used as a control (*lane 1*). The cell extracts were subjected to sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and then immunoblotted with an anti-neuraminidase antibody, as described under "Methods"

amino acid substitution of methionine for valine (V217M), and the other allele bearing a G-to-A transition at nucleotide number 727, resulting in the substitution of arginine for glycine (G243R).

Transient expression of lysosomal neuraminidase cDNA

To characterize the effect of the amino acid substitutions, V217M and G243R, on lysosomal neuraminidase activity, the wild-type and mutant lysosomal neuraminidase cDNAs were transiently co-expressed with the human PPCA cDNA in a galactosialidosis-derived fibroblast cell line (ASVGS-1 cells). Table 1 summarizes the results. In ASVGS-1 cells, neuraminidase activity, as well as cathepsin A and β-galactosidase activities, were markedly decreased by a genetic defect of PPCA, which is essential for the expression of lysosomal neuraminidase activity. Mock transfection with the PPCA cDNA alone restored the neuraminidase, β-galactosidase, and cathepsin A activities in the cells. Co-transfection of the wild-type lysosomal neuraminidase cDNA and PPCA cDNA caused a further seven-fold increase in neuraminidase activity compared with that in the mock-transfected cells, whereas the activity of β-hexosaminidase, as a control enzyme, did not change significantly. Co-transfection of the mutant lysosomal neuraminidase cDNA containing the ^{727}G-to-A substitution (G243R) and PPCA cDNA did not cause any increase in neuraminidase activity. In contrast, a moderate increase in the enzyme activity was observed when the mutant neuraminidase cDNA containing the ^{649}G-to-A substitution

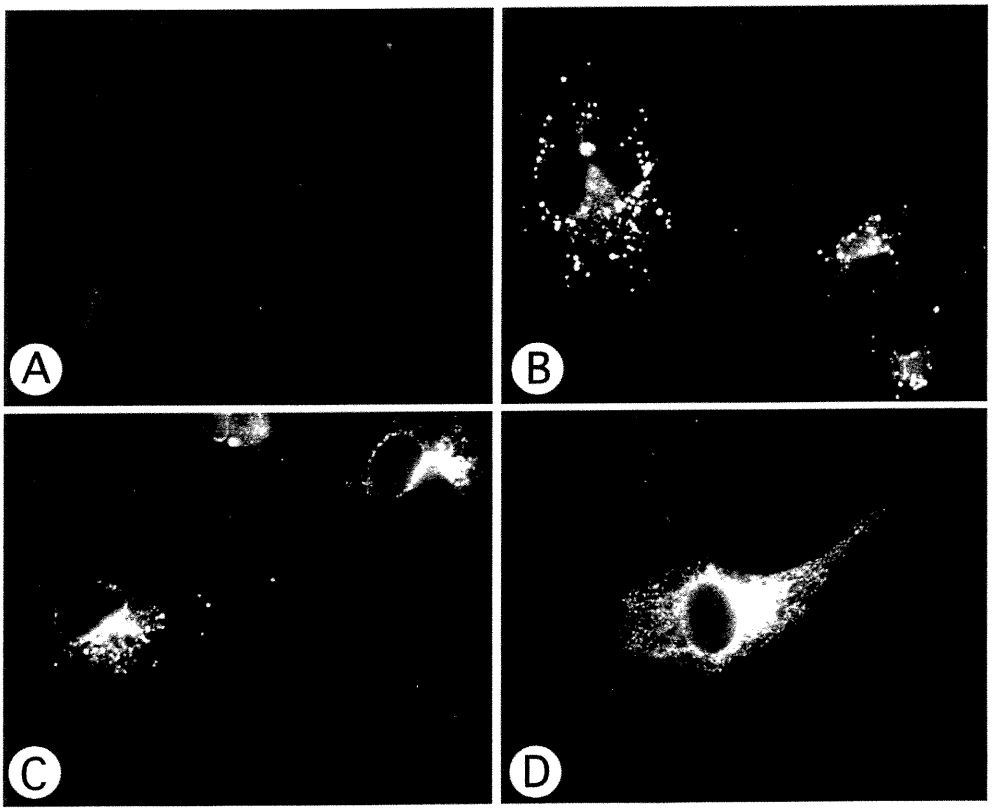

Fig. 3A–D. Immunocytochemical analysis of lysosomal neuraminidase expressed in transformed galactosialidosis cell lines. **A** Mock transfected with protective protein cathepsin A (PPCA) cDNA; **B** co-transfected with the wild-type lysosomal neuraminidase cDNA and PPCA cDNA; **C** co-transfected with the neuraminidase cDNA containing a Y217M mutation and PPCA cDNA; **D** co-transfected with the neuraminidase cDNA containing a G243R mutation and PPCA cDNA. ×400

(V217M) was transfected with PPCA cDNA. In this case, neuraminidase activity was found to be restored to 17% of that in the cells co-transfected with the wild-type lysosomal neuraminidase cDNA and PPCA cDNA.

Immunoblot analysis

As shown in Fig. 2, the wild-type and mutant lysosomal neuraminidase cDNA products expressed in COS-1 cells were analyzed by immunoblotting. We used a polyclonal antibody against a recombinant human lysosomal neuraminidase fragment produced by a bacterial gene expression system that has been reported previously (Vinogradova et al. 1998). For COS-1 cells transfected with the wild-type lysosomal neuraminidase cDNA, a single 48-kDa band was observed (Fig. 2, lane 2). The cells transfected with the mutant V217M and G243R cDNAs produced a gene product of the same size as those transfected with the wild-type cDNA (Fig. 2, lanes 3 and 4), although the intensity of the band was moderately decreased in both these cases. No other immunoreactive bands were observed.

Immunocytochemical analysis

The results of immunocytochemical analysis with the anti-neuraminidase antibody are shown in Fig. 3. Fluorescence microscopy revealed typical lysosomal granular staining in the cells co-transfected with the wild-type lysosomal neuraminidase cDNA and PPCA cDNA (Fig. 3B). In contrast, the expression product of lysosomal neuraminidase cDNA containing a G243R mutation showed a reticular staining pattern (Fig. 3D). On the other hand, both perinuclear reticular and partial punctate cytoplasmic staining were observed in the expression of the mutant with Y217M (Fig. 3C).

Structural modeling of wild-type and mutant lysosomal neuraminidases

According to the sequence alignment (Milner et al. 1997), we built a model of the human lysosomal neuraminidase without the N-terminal 47 residues, corresponding to the signal peptide (Milner et al. 1997; Vinogradova et al. 1998). The model was, thus, composed of the 368 amino acids from residue 48 to the terminal residue 415, the amino acid sequence homology with the aligned fragment of *Salmonella typhimurium* LT2 being 39.7%. The modeled lysosomal neuraminidase retains a basic structure composed of six four-stranded antiparallel β-sheets, and resembles a six-bladed propeller with the predicted active site cleft in the middle (Fig. 4). Both V217M and G243R are located in the β-sheet of the third β-sheet-loop unit (Fig. 4). The V217M transversion was deduced to alter the arrangement of 11 amino acid residues around the mutation site (Fig. 5). As to the G243R transversion, it was predicted to cause a drastic structural change with a wide range of up to 38 residues (Fig. 6). Neither mutation affected the residues involved in the predicted active site (R78, R97, D103, D135, R280, and R347) (Pshezhetsky et al. 1997; Crennell et al. 1993).

Discussion

Few reports on human lysosomal neuraminidase have been published, because the enzyme has low stability during purification and requires PPCA for the expression of its activity (Verheijen et al. 1982, 1985, 1987; Hiraiwa et al. 1988; van der Horst et al. 1989). Recent cDNA cloning for human lysosomal neuraminidase has revealed that it encodes a 45-kDa protein with three potential N-linked glycosylation sites (Bonten et al. 1996; Pshezhetsky et al. 1997; Milner et al. 1997). This information has facilitated the identification of gene mutations causing sialidosis. So far, only one nonsense mutation, E377X, has been found in a patient with sialidosis type 1 (Bonten et al. 1996). In sialidosis type 2, three missense mutations, L91R (Bonten et al. 1996), F260Y (Pshezhetsky et al. 1997), and L363P (Pshezhetsky et al. 1997); a four-base duplication causing a frameshift (Pshezhetsky et al. 1997); and a single-base deletion, resulting in a frameshift and extending the protein by 69 amino acid residues (Bonten et al. 1996), have been reported.

In this study, we identified two novel gene mutations in two Japanese patients with sialidosis type 1. Although these patients are unrelated according to our investigations, they are both compound heterozygotes for the ^{649}G-to-A and ^{727}G-to-A alterations. The base substitutions are found at the CpG site, which is known as a mutation hot spot (Cooper and Youssoufian 1988).

Using the information regarding the tertiary structure of bacterial neuraminidase, we modeled the structure of human lysosomal neuraminidase, and deduced the structural changes caused by the identified mutations. Both the V217M and G243R mutations were distant from the deduced active residues. The simulation suggests that the G243R replacement can cause a drastic widespread change in the protein structure. The biochemical study showed that the G243R mutant expressed low amounts of a protein with the same molecular size as in that in the wild type, but it could not be transported to the lysosomal compartment. The enzyme activity of the expressed product with the G243R mutation was completely abolished. The structural change is likely to cause a folding defect, which results in a transport defect and intracellular degradation and/or a defect of activation. On the other hand, the deduced structural change caused by V217M is smaller than that caused by G243R. The V217M mutant was partly transported to lysosomes, and showed residual enzyme activity.

Both patients described here are compound heterozygotes for V217M and G243M. Considering the results of this study, we surmise that the V217M mutation causes a mild change in the protein structure compatible with the presence of residual enzyme activity. Therefore, this mutation may be closely related to the phenotype with a late onset and moderate clinical course.

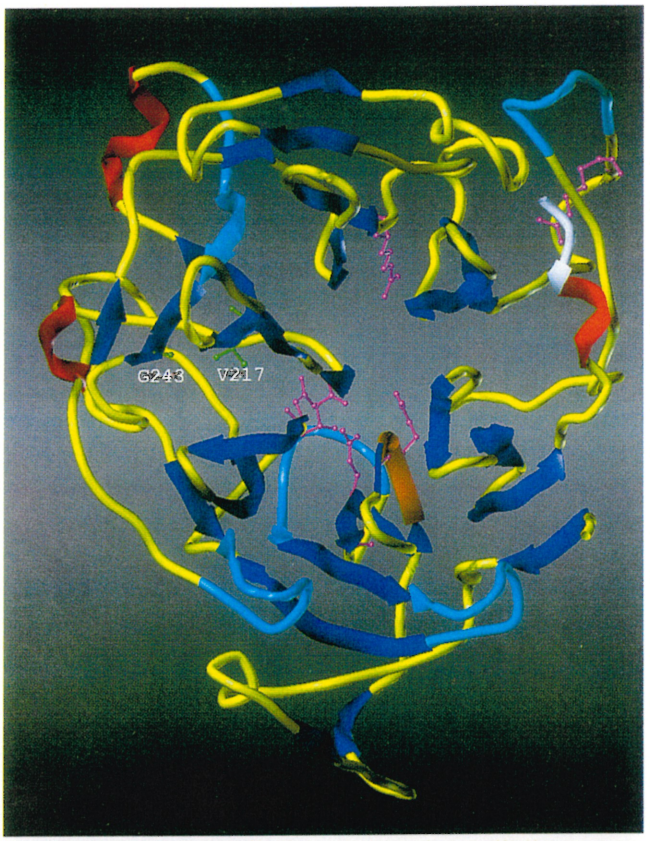

Fig. 4. Structural model of lysosomal neuraminidase and the locations of the mutations identified in two Japanese sialidosis patients. The backbone is colored according to secondary structures; α-helix (*red*), β-strand (*blue*), and coil (*yellow*). The sequence motifs conserved in the sialidase family, five Asp-boxes (Ser/Thr-X-Asp-X-Gly-X-X-Trp/Phe), a Phe-Arg-Ile-Pro sequence motif, and a C-terminal, Tyr-X-X-hydrophobic residue motif are colored *cyan*, *orange*, and *white*, respectively. The side chains of the predicted active residues are colored *magenta*. The residues of V217 and G243, where the amino acid residue replacements will occur in the patients, are colored *green*

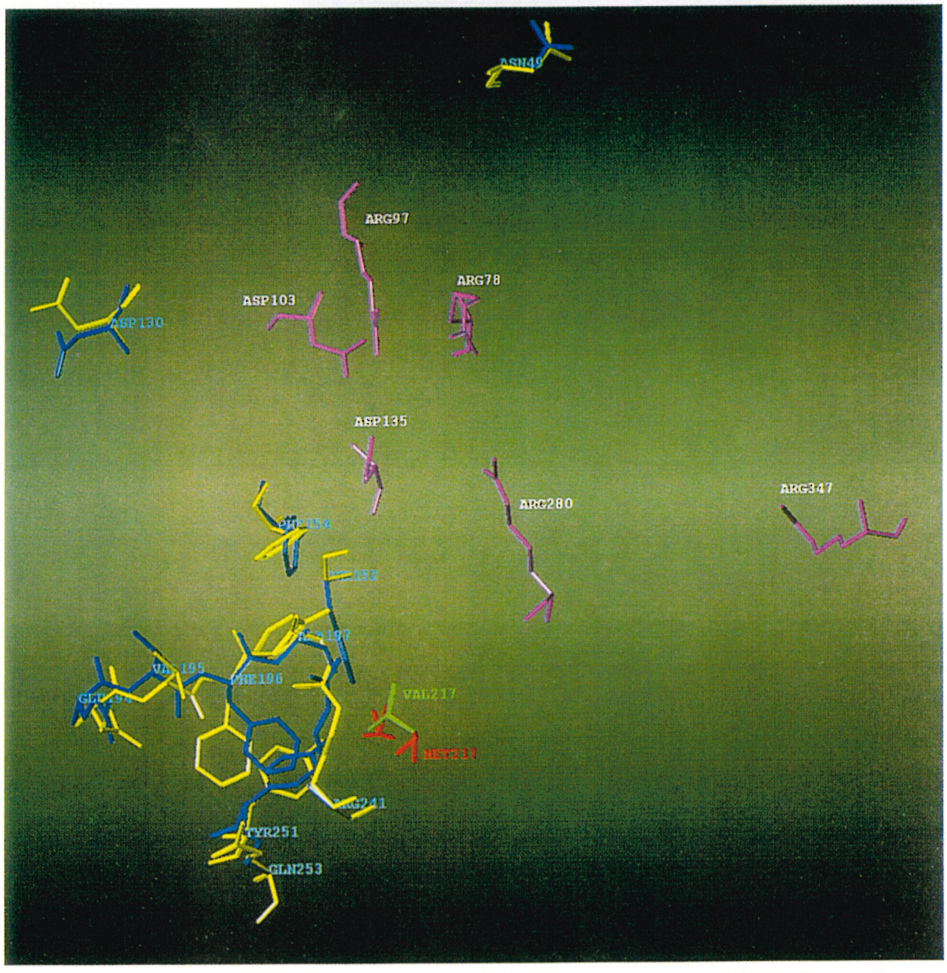

Fig. 5. Conformational change in lysosomal neuraminidase caused by V217M mutation. The amino acid residues influenced by the change from V217 (*green*) to M217 (*red*) are shown; those colored *blue* are the wild-type residues and those colored *yellow* are the mutant residues. The active site residues are colored *magenta*

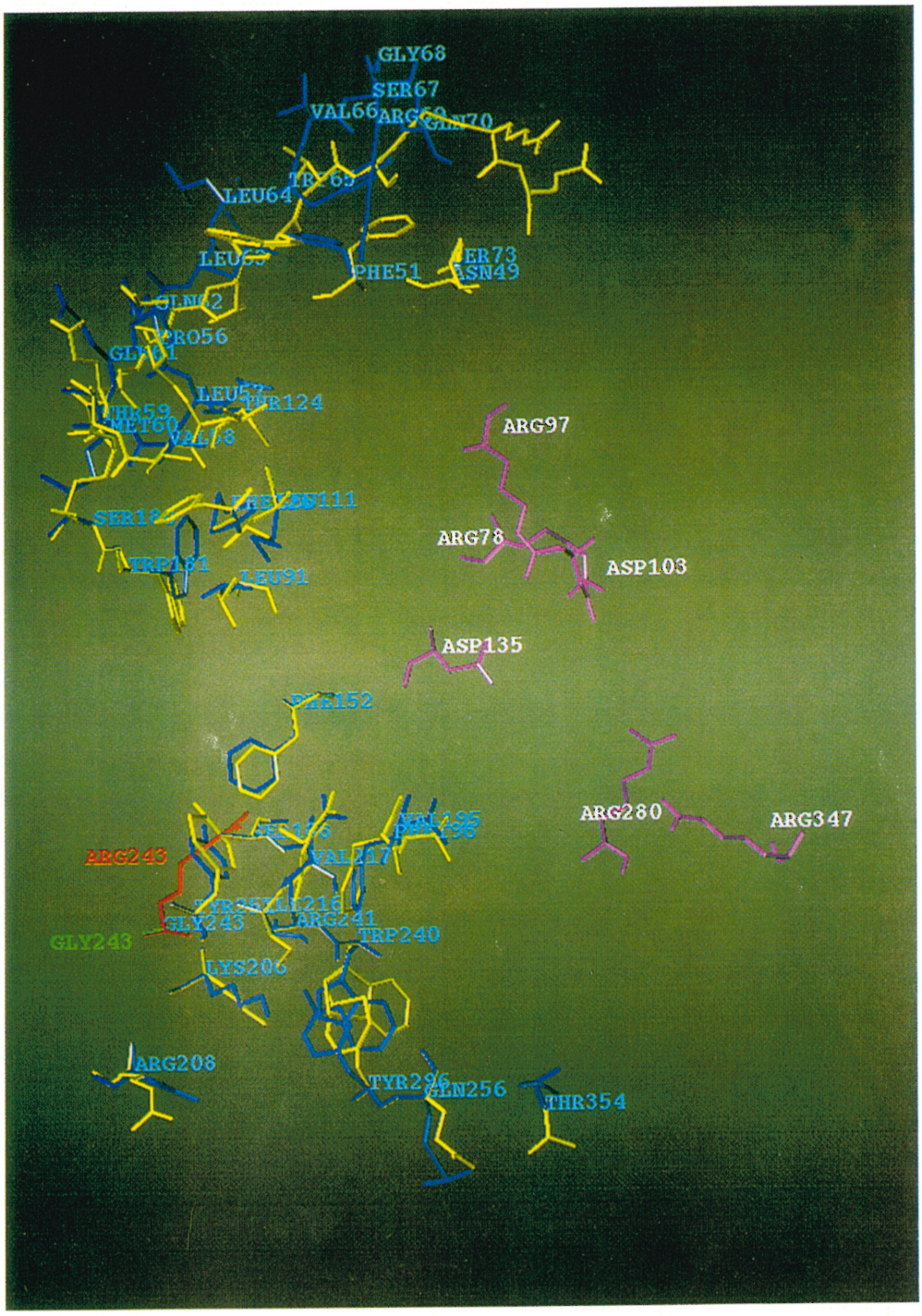

Fig. 6. Conformational change in lysosomal neuraminidase caused by G243R mutation. The amino acid residues influenced by the change from G243 (*green*) to R243 (*red*) are shown; those colored *blue* are the wild-type residues and those colored *yellow* are the mutant residues. The active residues are colored *magenta*

In conclusion, we identified two novel gene mutations in Japanese patients with sialidosis type 1, and characterized the structural and functional defects caused by these missense mutations. Molecular and structural studies will facilitate clarification of the complicated pathophysiology of sialidosis.

Acknowledgments We thank Dr. Michel Potier for helpful advice.

This work was partly supported by grants from the Tokyo Metropolitan Government, the Ministry of Education, Science, Sports and Culture of Japan, and the Ministry of Health and Welfare of Japan.

References

Bonten E, van der Spoel A, Fornerod M, Grosveld G, d'Azzo A (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. Genes Dev 10:3156–3169

Claessens M, van Cutsem E, Lasters I, Wodak S (1989) Modeling the polypeptide backbone with "spare parts" from known protein structures. Protein Eng 2:335–345

Cooper DN, Youssoufian H (1988) The CpG dinucleotide and human genetic disease. Hum Genet 78:151–155

Crennell SJ, Garman EF, Laver WG, Vimr ER, Taylor GL (1993) Crystal structure of a bacterial sialidase (from *Salmonella typhimurium* LT2) shows the same fold as an influenza virus neuraminidase. Proc Natl Acad Sci USA 90:9852–9856

d'Azzo A, Hoogeveen A, Reuser AJ, Robinson D, Galjaard H (1982) Molecular defect in combined beta-galactosidase and neuraminidase deficiency in man. Proc Natl Acad Sci USA 79:4535–4539

d'Azzo A, Andria G, Strisciuglio P, Galjaard H (1995) Galactosialidosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 7th edn. McGraw-Hill, New York, pp 2825–2837

Davis LG, Dibner MD, Battey JF (1986) Basic methods in molecular biology. Elsevier, New York

Gluzman Y (1981) SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23:175–182

Hiraiwa M, Nishizawa M, Uda Y, Nakajima T, Miyatake T (1988) Human placental sialidase: further purification and characterization. J Biochem 103:86–90

Itoh K, Takiyama N, Nagao Y, Oshima A, Sakuraba H, Potier M, Suzuki Y (1991) Acid carboxypeptidase deficiency in galactosialidosis. Jpn J Hum Genet 36:171–178

Jones TA, Thirup S (1986) Using known substructures in protein model building and crystallography. EMBO J 5:819–822

Kawasaki ES (1990) Amplification of RNA. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. A guide to methods and applications. Academic Press, San Diego, pp 21–27

Laemmli UK (1970) Cleavage of structure proteins during the assembly of the head bacteriophage T4. Nature 227:680–685

MacGregor GR, Caskey CT (1989) Construction of plasmids that express *E. coli* β-galactosidase in mammalian cells. Nucleic Acids Res 17:2365

McLachlan AD (1979) Gene duplications in the structural evolution of chymotrypsin. J Mol Biol 128:49–79

Milner CM, Smith SV, Carrillo MB, Taylor GL, Hollinshead M, Campbell RD (1997) Identification of a sialidase encoded in the human major histocompatibility complex. J Biol Chem 272:4549–4558

Mitoma H, Uchiyama T, Yokota T, Furukawa T, Tsukagoshi H (1993) Action myoclonus induced guided movement. J Neurol 241:92–95

Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K, Yamamura K (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–200

Pshezhetsky AV, Richard C, Michaud L, Igdloura S, Wang S, Elsliger M-A, Qu J, Leclerc D, Dallaire L, Potier M (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. Nature Genet 15:316–320

Sakuraba H, Itoh K, Shimmoto M, Utsumi K, Kase R, Hashimoto Y, Ozawa T, Ohwada Y, Imataka G, Eguchi M, Furukawa T, Schepers U, Sandhoff K (1999) GM2 gangliosidosis AB variant. Clinical and biochemical studies of a Japanese patient. Neurology 52:372–377

Shimmoto M, Sakuraba H, Suzuki Y (1993) Protective protein gene mutations in galactosialidosis. J Clin Invest 91:2393–2398

Sussman JL, Lin D, Jiang J, Manning NO, Prilusky J, Ritter O, Abola EE (1998) Protein data bank (PDB): database of three-dimensional structural information of biological macromolecules. Acta Crystallogr D54:1078–1084

Sutcliffe MJ, Hayes FRF, Blundell TL (1987) Knowledge based modeling of homologous proteins, part II: rules for the conformations of substituted side chains. Protein Eng 1:385–392

Suzuki K (1987) Enzymatic diagnosis of sphingolipidoses. In: Ginsbury V(ed) Methods in enzymology, vol 138. Academic Press, New York, pp 727–762

Suzuki Y, Sakuraba H, Potier M, Akagi M, Sakai M, Beppu H (1981) β-Galactosidase-neuraminidase deficiency in adults: deficiency of a freeze-labile neuraminidase in leukocytes and fibroblasts. Hum Genet 58:387–389

Thomas GH, Beaudet AL (1995) Disorders of glycoprotein degradation and structure: α-mannosidosis, β-mannosidosis, fucosidosis, sialidosis, aspartylglucosaminuria, and carbohydrate-deficient glycoprotein syndrome. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 7th edn. McGraw-Hill, New York, pp 2529–2561

van der Horst G, Galjart A, d'Azzo A, Galjaard H, Verheijen FW (1989) Indentification and in vitro reconstitution of lysosomal neuraminidase from human placenta. J Biol Chem 264:1317–1322

van der Spoel A, Bonten E, d'Azzo A (1998) Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. EMBO J 17:1588–1597

Verheijen FW, Brossmer R, Galjaard H (1982) Purification of acid β-galactosidase and acid neuraminidase from bovine testis: evidence for an enzyme complex. Biochem Biophys Res Commun 108:868–875

Verheijen FW, Palmeri S, Hoogeveen AT, Galjaard H (1985) Human placental neuraminidase. Activation, stabilization and association with β-galactosidase and its protective protein. Eur J Biochem 149:315–321

Verheijen FW, Palmeri S, Galjaard H (1987) Purification and partial characterization of lysosomal neuraminidase from human placenta. Eur J Biochem 162:63–67

Vinogradova MV, Michaudo L, Mezentsev AV, Lukong KE, El-Alfy M, Morales CR, Potier M, Pshezhetsky AV (1998) Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation. Biochem J 330:641–650

Weiner SJ, Kollman PA, Case DA, Singh UC, Ghio C, Alagona G, Profeta S Jr, Weiner P (1984) A new force field for molecular mechanical simulation of nucleic acids and proteins. J Am Chem Soc 106:765–784

Weiner SJ, Kollman PA, Nguyen DT, Case DA (1986) An all atom force field for simulations of proteins and nucleic acids. J Comput Chem 7:230–252

Yamamoto M, Yamauchi T, Yamamoto K, Kobayashi T (1995) Activated sialidase activity in transformed lymphocytes by Epstein-Barr (EB) virus of sialidosis type I (cherry-red spot-myoclonus syndrome). J Neurol Sci 131:105–107

Foreword

Previous works have described three clinical types of sialidosis distinguished mainly by their age of onset, severity and dysmorphic features (see Introduction). They include sialidosis type I, a late onset characterized amongst others by normomorphic features; sialidosis type II, an early infantile sialidosis in which patients usually have bone abnormalities and nephrosialidosis (congenital sialidosis), a phenotypic variant of sialidosis type II presenting severe hydrops fetalis, ascites and proteinuria.

This article presents a complete case report of a patient of Turkish origin with congenital sialidosis. We have shown that this patient is homozygous for a frameshift mutation reported previously in Article 2. The parents of the patient are both heterozygous for the same mutation. Interestingly, the patient lacked some of the symptoms of sialidosis type II such as bone abnormalities and presented uncommon features, which include excessive retinal tortuosity and severely dilated coronary arteries.

The mutational analysis were performed by Kiven Erique Lukong.

Article 4

Clinical presentation of congenital sialidosis in a patient with a neuraminidase gene frameshift mutation.

(Revised and accepted by the *European Journal of Pediatrics*, November, 2000)

Clinical presentation of congenital sialidosis in a patient with a neuraminidase gene frameshift mutation.

Tina Buchholz *[1], Guy Molitor [1], Kiven E. Lukong [3], Manfred Praun [1], Orsolya Genzel-Boroviczény [1], Matthias Freund [2], Alexey V. Pshezhetsky [3], Andreas Schulze [1].

[1] Division of Neonatology, Department of Obstetrics and Gynecology; [2] Division of Pediatric Critical Care and Cardiology; Klinikum Grosshadern, Ludwig-Maximilian-University of Munich, Germany, [3] Service de Génétique Medicale, Hopital Sainte-Justine, Département de Pédiatrie, Faculté de Medicine, Université de Montréal, Canada

* Address for correspondence:

Tina Buchholz, M.D.
Klinikum Grosshadern - LMU
Department of Obstetrics and Gynecology
81377 München - Germany
phone X49-89-7095-6810
fax X49-89-7095-6840
email: tina.buchholz@t-online.de

Abstract

Congenital sialidosis is a rare lysosomal storage disease, caused by a primary neuraminidase deficiency which results from defects in the neuraminidase gene on chromosome 6p. The inheritance is autosomal recessive. Patients exhibit excessive urine excretion of bound sialic acid and decreased or undetectable amounts of neuraminidase activity in various tissues. The clinical expression is variable, but ascites and hepatosplenomegaly are hallmarks of the disease. Skeletal abnormalities, facial dysmorphism and inguinal hernias have been described in most of the few reported cases. We describe a baby girl with biochemically proven sialidosis, who in addition to the above clinical features had severely dilated coronary arteries, excessive retinal vascular tortuosity and an erythematous, macular rash. Homozygosity for a frameshift mutation at residue 623 of neuraminidase cDNA was found. We speculate that the additional features found in our patient might be associated with the here described genotype of congenital sialidosis.

Key Words

nonimmune hydrops, congenital anomalies, dilated coronary arteries, hepatosplenomegaly, neuraminidase deficiency.

Introduction

Neuraminidase (sialidase) is an important lysosomal enzyme that removes tertiary sialic acid residues from sialoglycoconjugates. The inherited deficiency of neuraminidase results in a lysosomal storage disease called sialidosis that is characterized by the excessive excretion of bound sialic acid in the urine [16]. After the first clinical reports in the late seventies two major subtypes of sialidosis were distinguished based on additional dysmorphic features and the age of onset. Sialidosis type I affects normomorphic individuals in their second decade of life and is also known as the cherry red spot - myoclonus syndrome. Sialidosis type II (mucolipidosis I) is a dysmorphic syndrome with an infantile and a juvenile form [12]. Other clinical subtypes have also been described [7, 25, 26]. Lysosomal storage disorders represent a group of more than 40 genetically distinct, biochemically related, inherited diseases [15]. Congenital sialidosis is an autosomal recessive disorder and is caused by mutations in the neuraminidase gene on chromosome 6p [5]. Different mutations have been identified in a few patients [5, 13, 18].

Sialidosis can be diagnosed biochemically by detecting elevated urine excretion of bound sialic acids together with the deficiency of neuraminidase in tissues, for example, in lymphocytes or cultured fibroblasts, in the presence of normal β-galactosidase activity [17]. Early clinical diagnosis however is usually difficult because of the nonspecific nature of the symptoms. The leading clinical features are congenital ascites and hepatosplenomegaly [4, 6]. Facial dysmorphism such as a coarse facies, skeletal dysplasia and inguinal herniae

have been also described [1, 8, 11]. Patients suffer from failure to thrive and from recurrent respiratory infections. Most patients reported in the literature died before the age of one year [2].

We report an infant with neuraminidase deficiency and a homozygous mutation in the neuraminidase gene.Besides the clinical hallmarks of the disease, she had additional uncommon clinical symptoms that may also be linked to sialidosis.

Case report

A baby girl was born at 38 weeks gestation as the second child to a 26-year old mother by spontanous vaginal delivery. The Turkish parents were first cousins with one healthy eight-year old daughter.

Intrauterine growth retardation, ascites and oligohydramnios became evident at 34 weeks of gestation on prenatal ultrasound scans. A TORCH screen was negative, chromosomal aberration or blood type incompatibility between mother and fetus were not present.

At birth the infant was pale and floppy without spontaneous respiratory efforts. APGAR values were 1, 6, 8 at 1, 5 and 10 minutes respectively. The umbilical arterial blood pH was 7.27. The birth weight was 2480 g (below 10^{th} percentile), length 46 cm (3^{rd} percentile) and head circumference 33 cm (25^{th} percentile). The amniotic fluid and membranes were meconium stained and fetid.

On physical examination we found mild dysmorphic facial features with a broad nasal bridge, low set ears and a coarse facies (Figure 1). The infant was hydropic, the abdomen was prominent with an enlarged liver and spleen (4 cm and 3 cm below the costal margin, respectively) and ascites. There was an erythematous, macular rash with petechiae covering the entire trunk and abdomen (Figure 2). The infant showed severe muscular hypotonia.

The initial blood tests including coagulation tests, electrolytes and serum bilirubin levels were unremarkable, except for an elevated C-reactive protein (3.1 mg/dL), reduced platelets (99.000/µL) and slightly elevated liver enzymes (glutamic oxalacetic transaminase 51 U/L, glutamic pyruvic transaminase 17 U/L, gamma glutamyl transpeptidase 85 U/L). Cerebrospinal fluid (CSF) and urine samples were unremarkable. Blood smear revealed vacuolated lymphocytes. Bacterial cultures, viral screening and serologic tests on blood, urine and CSF were negative.

The heart appeared structurally normal on echocardiography, but the right atrium and right ventricle were severely dilated with impaired right ventricular function. In addition the coronary arteries were dilated to more than 2 mm in diameter. The ratio of coronary artery diameter to aortic root diameter exceeded the normal range by more than 50% [29]. These findings subsided during the following week. There were no gross anatomical abnormalities on cranial ultrasound, however, small echogenic dot-shaped structures were noted in both thalami. Radiographies of the chest, head, and right femur during the first week of life did not show any abnormalities of the bones.

Ophthalmologic examination revealed excessive retinal vascular tortuosity on the left eye and severely congested veins. These findings receded during the following weeks. A skin biopsy on day six of life showed a mild predominantly lymphocytic inflammatory reaction. Finally, the histology of the placenta was unremarkable.

Metabolic residues on blood levels were normal. However oligosaccharide levels were excessively elevated in the urine samples examined by thin-layer chromatography. Further investigations identified free sialic acid in the normal range but 100-fold elevation of bound sialic acid in the urine as well as a complete deficiency of neuraminidase with normal levels of β-galactosidase (5,4 mU/min/mg) in cultured skin fibroblasts. The diagnosis was confirmed at the molecular level (see below).

The infant was extubated on her second day of life and started to gain weight at two weeks of age with bottlefeeding and later breastfeeding. She was discharged home at the age of four weeks. A respiratory infection at seven weeks of age required readmittance to a hospital and a ten-day course of antibiotics and supplemental oxygen. The infant died at home at the age 82 days.

Mutation analysis

Genomic DNA was purified from the blood of the patient and her parents as described previously [14]. Neuraminidase exons and adjacent splicing regions were amplified from the genomic DNA using the primers complementary to the sequence of neuraminidase introns as described in [13]. Because of the small size of the exons (159 to 263 bp) they were directly analyzed by single-strand conformation polymorphism assay (SSCP) as described [19]. This produced band shifts, gain or loss of bands, compared to normal controls for exon 4 and adjacent splicing regions, suggesting the presence of mutations (not shown).

Direct manual sequencing was performed using the Thermosequenase kit (Amersham Life Science). Sequencing of exon 4 revealed that the patient is homozygous for a frameshift mutation, a G deletion after nucleotide 622 (c623delG, Figure 3) resulting in a premature stop codon at nucleotide 904, and early truncation of neuraminidase protein at residue 301. The truncated protein lacks 207 residues of normal neuraminidase sequence, which is incompatible with the enzymatic activity. The rest of the neuraminidase DNA sequence from the patient did not differ from that of controls.

To confirm the inheritance of the mutation we studied parental DNA. Direct sequencing of the corresponding regions of the PCR-amplified fragments (data not shown) resulted in the appearance of ambiguous sequence following the frameshift site suggesting that both parents are heterozygous for c623delG mutation.

Discussion

We present a case of congenital sialidosis with complex signs and symptoms, some of which may represent hitherto unknown features of the disease.

Ascites, hepatosplenomegaly and coarse facial dysmorphism suggested a lysosomal storage disease in our patient. However, the infant lacked symptoms that were previously described as characteristic for sialidosis including skeletal abnormalities such as dysostosis multiplex and inguinal herniaes [16]. Kelly et al. reported an 8 months old female with coarse facies, hepatosplenomegaly, and dysostosis multiplex detected by radiography first performed at the age of 10 days but evolving over months [10]. Aylsworth`s patient described in 1980 was found to have signs of dysostosis multiplex first noted at a later age of 5 months [1]. One child of a family, where both parents showed reduced levels of enzyme activity in the lymphocytes, was presumably affected as she had clinical signs of hepatomegaly and foam cells in the placenta [11]. The histological report of the placenta of our patient did not mention any vacuolated or foamed cells. Sergi et al. recently described a Syrian newborn, who died 28 days after birth. Unfortunately genotypic correlation has not been investigated [24].

Ries et al. reported one patient with infantile sialidosis in whom cerebral sonography showed stripe-like intracerebral echogenicities in the region of the basal ganglia [20]. These findings might be due to substrate deposits in vessel

walls. Similar findings were recently reported in a congenital sialidosis patient by Schmidt et al. [22]. We also found small echogenic dot-shaped structures in both thalami in our patient.

Our case presented uncommon symptoms including an erythematous, macular rash with petechiae and teleangiectasia over the entire trunk, which persisted for several weeks as well as the retinal vascular tortuosity and engorged retinal vessels. Tortuosity of the conjunctival vessels and aneurysma-like formations were described in one patient with sialic acid storage disease, a lysosomal storage disorder with a different molecular background [28]. The authors related these alterations to involvement of the blood capillary endothelial cells. The dilated coronary arteries in our patient might be caused by the excessive high-output heart failure. However, one patient showed dilated ventricles of the heart plus additional features such as petechiae covering chest and abdomen as well as bilateral hip luxation [27].

Inborn errors of metabolisms account for only about 1% of congenital non-immune hydrops cases. Most of them are lysosomal storage diseases with congenital sialidosis being very rare [23]. To our knowledge this is the first report on detailed combined clinical and molecular investigations. The wide phenotypic variability of this disease [3] might be related to different mutations in the neuraminidase gene, of which a few have been already identified [5, 13, 18]. Detection of further mutations will aid to a phenotype - genotype correlation. Prenatal diagnosis in affected families has been established by screening amniotic fluid for elevated amounts of sialic acids [9, 21]. Alternatively mutation

analysis on chorionic tissue in families with an identified mutation will assist as an individual test.

Acknowledgement

We thank Dr. E. Paschke (University Childrens Hopital, Graz, Austria) and Dr. B. Rolinski (Dr. v.Hauner Childrens Hospital, LMU Munich, Germany) for the enzyme assays and for the biochemical confirmation of the diagnosis. A.V.P. acknowledges operating grants from Medical Research Council of Canada and Vaincre les Maladies Lysosomales Foundation. We also thank our patient's parents for their support in this work.

References

1. Aylsworth A, Thomas GH, Hood JL, Malouf N, Libert J (1980) A severe infantile sialidosis: clinical, biochemical, and microscopic features. J Pediatr 96:662-668.

2. Beaudet AL, Thomas GH (1989) Disorders of glycoprotein degration: mannosidosis, fucosidosis, sialidosis, and aspartylglycosaminuria. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Stanbury JB, Wyngaarden JB, Fredrickson DS (eds.) The metabolic basis of inherited disease. New York: Mc Graw-Hill Book Co, pp 1603-1621.

3. Beck M, Bender SW, Reiter HL, Otto W, Bassler R, Dancygier H, Gehler J (1984) Neuraminidase deficiency presenting as non-immune hydrops fetalis. Eur J Pediatr 143(2):135-9.

4. Bohnhorst B, Poets CF, Schweitzer S, Sewell AC (1996) Neonataler Aszites als Erstmanifestation der infantilen Sialinspeicherkrankheit. Monatsschr Kinderheilk 144:374-378.

5. Bonten E, van der Spoel A, Fornerod M, Grosveld G, d`Azzo A (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. Genes Dev 10:3156-3169.

6. Daneman A, Stringer D, Reilly BJ (1983) Neonatal ascites due to lysosomal storage disease. Radiology 149:463-467.

7. Durand P, Gatti R, Cavalieri S, Borrone C, Tondeur M, Michalski JC, Strecker G (1977) Sialidosis (mucolipidosis I). Helv Paediat Acta 32:391-400.

8. Gillan JE, Lowden JA, Gaskin K, Cutz E (1984) Congenital ascites as a presenting sign of lysosomal storage disease. J Pediatr 104:225-231.

9. Heyes H, Gehler J, Töllner U, Göltner E (1988) Pränatale Diagnostik der Sialidose, eines Defektes des lysosomalen Enzyms Neuraminidase. Geburtsh u Frauenheilk 48:814-815.

10. Kelly T, Graetz G (1977) Isolated acid neuraminidase deficiency: A distinct lysosomal storage disease. Am J Med Genet 1:31-46.

11. Laver J, Fried K, Beer SI, Iancu TC, Heyman E, Bach G, Zeigler M (1983) Infantile lethal neuraminidase deficiency (sialidosis). Clin Genet 23:97-101.

12. Lowdon JA, O`Brien JS (1979) Sialidosis: a review of human neuraminidase deficiency. Am J Hum Genet 31:1-18.

13. Lukong KE, Elsliger MA, Chang Y, Richard C, Thomas G, Carey W, Czartoryska B, Buchholz T, Criado G, Palmeri S, Pshezhetsky AV (2000) Charakterisation of the molecular defects of lysosomal sialidase in sialidosis patients defines the structural organization of the lysosomal multienzyme complex. Hum Mol Genet 9(7):1075-1085.

14. Maniatis T, Fritsch E, Sambrook J (1982) Maniatis T, Fritsch E, Sambrook J (eds.), Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

15. Meikle PJ, Hopwood JJ, Clague AE, Carey WF (1999) Prevalence of lysosomal storage disorders. JAMA 281(3):249-54.

16. Nakamura Y, Takahashi Y, Yamaguchi S, Omiya S, Orii T, Yara A, Gushiken M (1992) Severe infantile sialidosis -- the characteristics of oligosaccharides isolated from urine and the abdominal ascites. Tohoku J Exp Med 166:407-415.

17. O`Brien JS, Warner TG (1980) Sialidosis: delineation of subtypes by neuraminidase assay. Clin Genet 17:35-38.

18. Pshezhetsky A, Richard C, Michaud L, Igdoura S, Wang S, Elsliger M, Qu J, Leclerc D, Gravel R, Dallaire L, Potier M (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. Nature Genetics 15:316-320.

19. Richard C, Tranchemontagne J, Elsinger M, Mitchell G, Potier M, Pshezhetsky A (1998) Molecular pathology of galactosialidosis in a patient affected with two new frameshift mutations in the cathepin A/protective protein gene. Hum Mutat 11:461-469.

20. Ries M, Deeg KH, Wolfel D, Ibel H, Maier B, Buheitel G (1992) Colour doppler imaging of intracranial vasculopathy in severe infantile sialidosis. Pediatr Radiol 22:179-181.

21. Sasagasako N, Miyahara S, Saito N, Shinnoh N, Kobayashi T, Goto I (1993) Prenatal diagnosis of congenital sialidosis. Clin Genet 44:8-11.

22. Schmidt M, Fahnenstrich H, Haverkamp F, Platz H, Hansmann M, Bartmann P (1997) Sialidose und Galaktosialidose als Ursache des nicht-immunologischen Hydrops fetalis. Z Geburtsh Neonatol 201:177-180.

23. Scully R, Mark E, McNeely W, Ebeling S, Phillips L (1997) Presentation of case. N Engl J Med 337:260-267.

24. Sergi C, Beedgen B, Kopitz J, Zilow E, Zoubaa S, Otto HF, Cantz M, Linderkamp O (1999) Refractory congenital ascites as a manifestation of neonatal sialidosis: clinical, biochemical and morphological studies in a newborn Syrian male infant. Am J Perinatol 16(3):133-41.

25. Spranger J (1981) Mucolipidosis I: phenotype and nosology. Perspect Inherit Metab Dis 4:303-315.

26. Swallow D, Hoogeveen A, Verheijen F, Galjaard H (1981) Complementation analysis of human sialidase deficiency using natural substrates. Ann Hum Genet 45:105-112.

27. Tabardel Y, Soyeur D, Vivario E, Senterre J (1989) Primary neuraminidase deficiency with prenatal manifestations. Arch Fr Pediatr 46:737-740.

28. Tondeur M, Libert J, Vamos E, Van Hoof F, Thomas GH, Strecker G (1982) Infantile form of sialic acid storage disorder: Clinical, ultrastructural, and biochemical studies in two siblings. Eur J Pediatr 139:142-147.

29. Velvis H, Schmidt K, Silverman N, Turley K (1989) Diagnosis of coronary artery fistula by two-dimensional echocardiography, pulsed Doppler ultrasound and color flow imaging. J Am Coll Cardiol 14:968-976.

Legends for figures

Fig. 1: The sialidosis patient at 10 days of age.

Note mild dysmorphic facial features with a broad nasal bridge, low set ears and coarse facies.

Fig. 2: Erythematous, macular rash that covers the entire trunk.

Fig. 3. Partial sequence of exon 4 of the neuraminidase gene showing the deletion of a G residue within a stretch of three G residues (boxed) after the nucleotide 622.

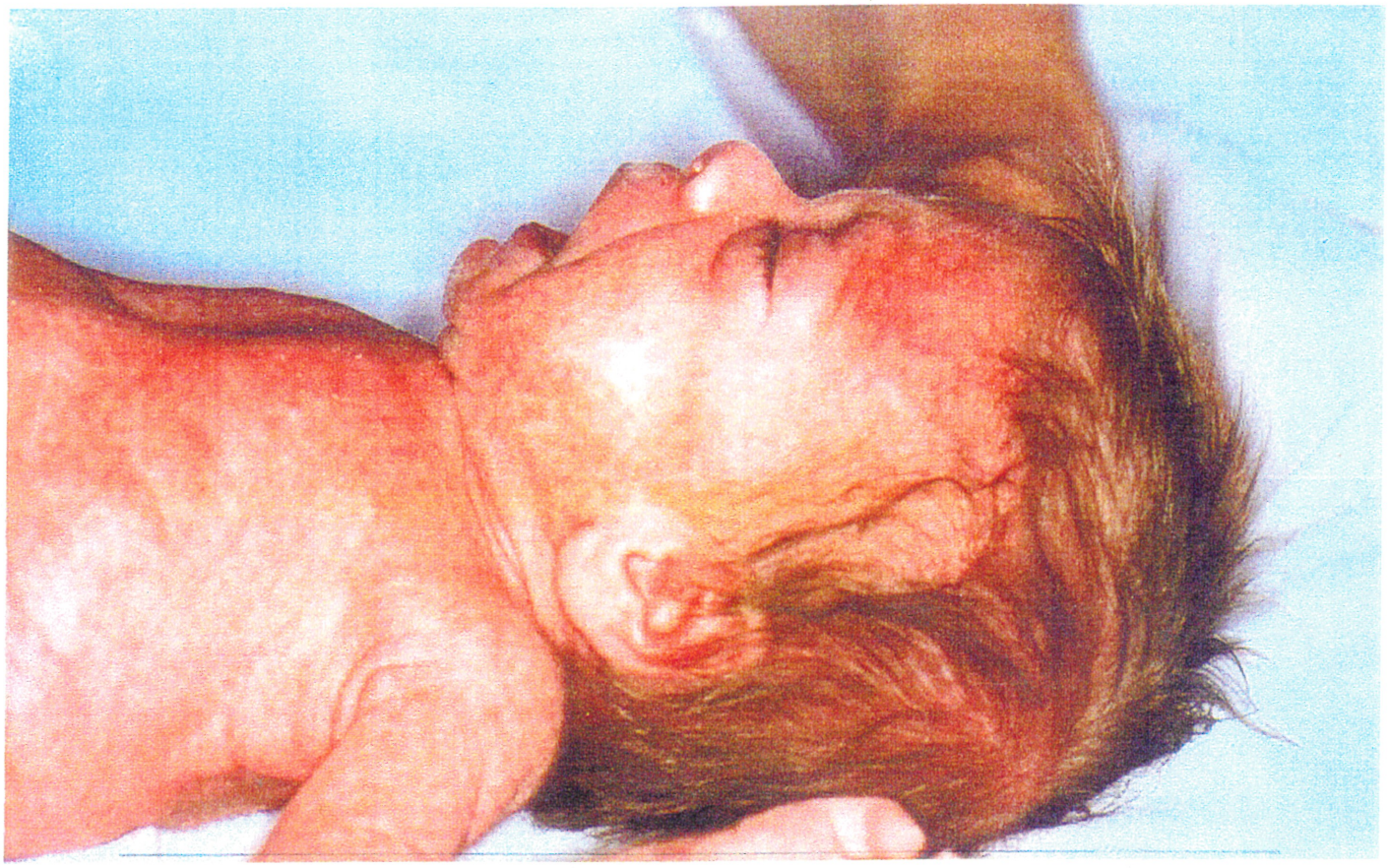

Figure 1

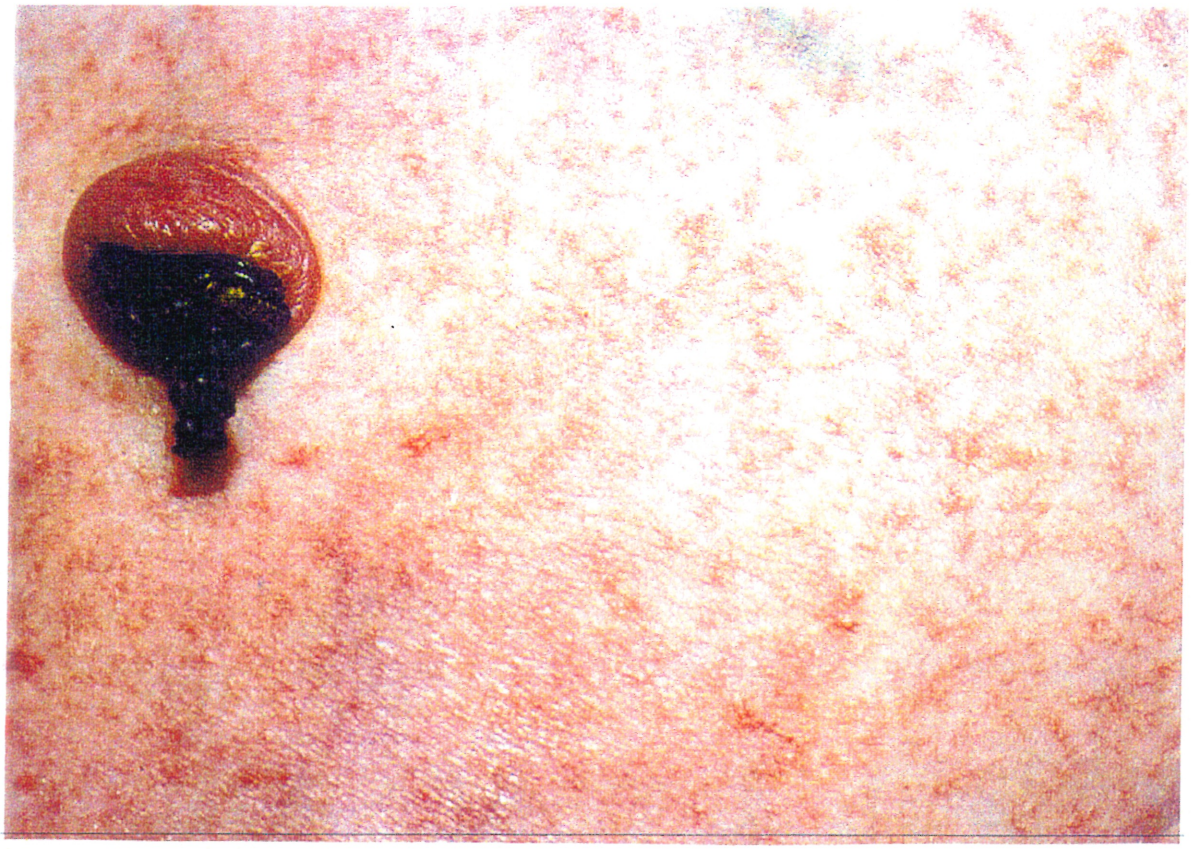

Figure 2

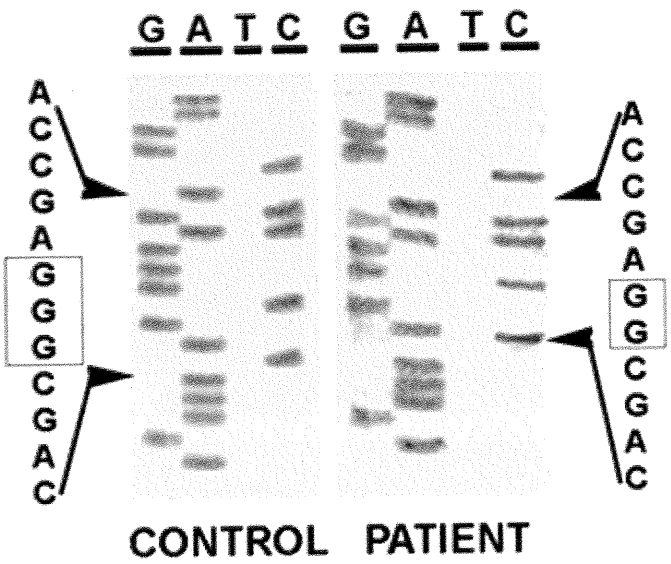

FIGURE 3

Foreword

In articles 2 and 3, we described the identification of a frameshift and eight missense mutations in the SIAL gene and their localization on the tertiary model of enzyme. Five of the missense mutations forming a cluster in the surface region of the SIAL structure were shown to cause drastically reduced enzyme activities. At the same time structural modeling predicted that none of the identified mutations is supposed to cause significant conformational changes to affect the active site residues. We hypothesized that this region may be involved in the SIAL binding with lysosomal CathA and/or GAL in their 1.27 MDa multienzyme complex.

To prove our hypothesis experimentally, we have expressed these mutants in COS-7 cells. To determine directly if the mutations affect the ability of SIAL to bind to the multienzyme complex, we performed analysis of the oligomeric organization of SIAL by density gradient centrifugation. As in Article 1, metabolic labeling and immunohystochemistry were used to study the biogenesis and localization of the mutant enzyme in the cell.

Article 5

Mutations in sialidosis impair sialidase binding to the lysosomal multienzyme complex.

Mutations in sialidosis impair sialidase binding to the lysosomal multienzyme complex.

Running title: **Molecular defects of sialidase in sialidosis**

Kiven E. Lukong[1], Karine Landry[1], Marc-André Elsliger[1†], Yuan Chang[1], Stephane Lefrancois[2], Carlos R. Morales[2] and Alexey V. Pshezhetsky[1]*

[1]Service de Génétique Médicale, Hôpital Sainte-Justine and Département de Pédiatrie, Faculté de Médicine, Université de Montréal, Montréal (Qc), H3T 1C5, Canada;

[2]Department of Anatomy and Cell Biology, McGill University, Montréal, (Qc), Canada

*- To whom correspondence should be addressed:

Service de génétique médicale

Hôpital Sainte-Justine

3175 Côte Ste-Catherine

Montréal (Qc) H3T 1C5 - Canada

Tel. (514) 345-4931/2736

Fax. (514) 345-4801

E.-Mail: alex@justine.umontreal.ca

[1] †- Present address: The Scripps Research Institute, La Jolla, CA 92037, USA.

SUMMARY

Sialidosis is an autosomal recessive disease caused by the genetic deficiency of lysosomal sialidase, which catalyzes the catabolism of sialoglycoconjugates. The disease is associated with progressive impaired vision, macular cherry-red spots, and myoclonus (sialidosis type I) or with skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation, and hepatosplenomegaly (sialidosis type II). We have analyzed the effect of missense mutations, Gly68Val, Ser182Gly, Gly227Arg, Phe260Tyr, Leu270Phe, Ala298Val, Gly328Ser and Leu363Pro, identified in the sialidosis patients on the activity, stability and intracellular distribution of the sialidase. We found that 3 mutations, Phe260Tyr, Leu270Phe and Ala298Val clustered in one region on the surface of sialidase molecule dramatically reduce the enzyme activity and cause a rapid intralysosomal degradation of the expressed protein. We suggested that this region may be involved in the sialidase binding with lysosomal cathepsin A and/or β-galactosidase in the multienzyme lysosomal complex required for the expression of sialidase activity. Transgenic expression of mutants followed by density gradient centrifugation of cellular extracts confirmed this hypothesis and showed that sialidase deficiency results from disruption of the lysosomal multienzyme complex.

INTRODUCTION

Sialidosis (also called mucolipidosis I and cherry-red spot myoclonus syndrome) is an autosomal recessive disease caused by the genetic deficiency of lysosomal sialidase (neuraminidase) activity (reviewed in 1-3). It is characterized by tissue accumulation and urinary excretion of sialylated oligosaccharides and glycoproteins (1). Sialidosis includes two main clinical variants with different age of onset and severity. Sialidosis type I or non-dysmorphic type is a late-onset mild form, characterized by bilateral macular cherry-red spots; progressive impaired vision and myoclonus syndrome (4-8). Sialidosis type II or dysmorphic type is the infantile-onset form, which is also associated with skeletal dysplasia, Hurler-like phenotype, dysostosis multiplex, mental retardation and hepatosplenomegaly (9-12). A severe form of the disease manifests prenatally and is associated with ascites and hydrops fetalis (13-15). The age of onset and severity of the clinical manifestations correlate with the amount of residual sialidase activity suggesting the existence of considerable genetic heterogeneity (1-3).

Although sialidosis from the moment of discovery was recognized as a deficiency of lysosomal sialidase (16) the molecular mechanism of this disorder has not been characterised for the following two decades because the identification and sequencing of sialidase has been hampered by low tissue content and instability of the enzyme. Several works showed that sialidase is a part of a multienzyme complex containing also other lysosomal enzymes, cathepsin A (protective protein), β-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase (17-19). The functional activity of sialidase completely depends on the integrity of its association with cathepsin A, so it was hypothesized that cathepsin A supports catalytically active conformation of this enzyme (18). In addition, the complex protects sialidase and β-galactosidase against rapid proteolysis (17,20,22) and may be also important for proper sorting and processing of their

precursors (22-25). In the autosomal recessive disease, galactosialidosis, a primary genetic defect of cathepsin A (17,20) results in disruption of the complex and causes the combined deficiency of both β-galactosidase and sialidase activities. The clinical features and a composition of storage products in galactosialidosis resemble those in sialidosis (8, 9, 26).

Recently the gene coding for sialidase was cloned and a series of mutations in sialidosis patients identified (27-31). In particular we have found 2 frameshift and 8 missense mutations in 9 sialidosis patients of multiple ethnic origin (28,31). To understand the effect of these mutations on sialidase we have modeled the tertiary structure of the enzyme and localized the identified amino acid substitutions (31). Surprisingly none of mutations directly affected the deduced active site residues or were found in the central core of the sialidase molecule, but all of them involved residues on the surface of the enzyme. Therefore, in most cases it was unlikely that these mutations would introduce electrostatic, or steric clashes in the protein core leading to general folding defects of sialidase and its retention in the ER/Golgi compartment as it was observed for the most of mutations affecting cathepsin A (32). Moreover, structural analysis showed that 4 of 8 missense mutations, causing a severe type II sialidosis are clustered in one region on the surface of the sialidase molecule (31).

In this paper we provide direct experimental evidence that 3 of these sialidase mutants expressed in COS-7 cells are correctly processed and sorted, but are not associated with the complex and rapidly degraded in the lysosome. These results permit us to speculate that the surface region containing these mutations may be involved in the sialidase binding interface with the lysosomal multienzyme complex, and that sialidase deficiency in some sialidosis patients may be secondary to the disruption of the lysosomal multienzyme complex.

EXPERIMENTAL PROCEDURES

Expression of sialidase mutants in COS-7 cells

Site-directed mutagenesis was performed using a TransformerTM Site-Directed mutagenesis kit (Clontech), previously described pCMV-SIAL expression vector, mutagenic primers corresponding to mutant sialidase sequences and a selection primer used to eliminate a unique *Sca*I restriction site in the vector, according to supplier's protocols. All primers were enzymatically phosphorylated and, for each mutant, the corresponding mutagenic primer and the selection primer were annealed to heat-denatured pCMV-SIAL plasmid. After elongation by T4 DNA polymerase, ligation and primary digestion with *Sca*I restriction enzyme to linearize all non-mutated DNA, the plasmid pool was used to transform the *mut S* strain of BMH71-18 bacteria. Plasmid DNA obtained from the pool of ampicillin-resistant transformants was subjected to a second *Sca*I digestion and transformed into *E. coli* DH5α. Positive clones were selected after a final *Sca*I restriction analysis and the entire sialidase cDNA sequenced. Up to 80% of transformants contained the desired mutation. DNA fragments of between 300 and 600 base pairs containing the introduced mutations were obtained from the mutant pCMV-SIAL plasmids by double-digestion with either *Bst*EII/*Nae*I, *Nae*I/*Kpn*I or *Kpn*I/*Eco*RV enzymes and subcloned into the parental pCMV-SIAL plasmid. The final constructs were verified by sequencing.

COS-7 cells seeded in T-25 flasks or 60 mm round dishes were co-transfected with pCMV-SIAL and pCMV-CathA expression vectors (28) using Lipofectamine Plus reagent (Life Technologies Inc, Gaithersburg, MD) in accordance with the manufacturer's protocol. 48 h after transfection, sialidase and control N-acetyl-ß-glucosaminidase activities were assayed in cellular homogenates using the corresponding fluorogenic 4-methylumbelliferyl-glycoside substrates as

described (33-35). The cathepsin A activity was determined with CBZ-Phe-Leu and FA-Phe-Leu substrates (36). One unit of enzyme activity (U) is defined as the conversion of 1 μmol of substrate/min. Proteins were assayed according to Bradford (37) with bovine serum albumin (Sigma) as standard. To measure the stability of the expressed sialidase, the cellular homogenate was incubated at 37^0 C for 0.5, 1, 2 and 3 h before the assay of sialidase activity.

Metabolic labeling

48 h after transfection with wild-type or mutant sialidase cDNA, COS-7 cells grown to confluency in 60 mm dishes, were washed twice with HBSS, then incubated for 2 h in cysteine and methionine-free Dubecco's Modified Eagles Medium (D-MEM, Gibco BRL) supplemented with L-glutamine and sodium pyruvate, and for 40 min - in 5 ml of the same medium supplemented with a mixture of ^{35}S-Cys and ^{35}S-Met (Trans^{35}S Label, ICN, 0.1 mCi/ml of medium). The radioactive medium was then removed, and the cells were washed twice with HBSS and chased at 37° C in EMEM supplemented with 20% (v/v) fetal calf serum.

At the time indicated in the figures, the cells were placed on ice, washed twice with ice-cold PBS then lysed for 30 min in 1 ml of radioimmunoprecipitation assay (RIPA) buffer, containing 50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% (v/v) NP-40; 0.5% (w/v) sodium deoxycholate; 0.1% (w/v) SDS; 5 μg/ml leupeptin and 0.1 mM α-toluensulfonyl fluoride (PMSF). The lysate was collected and centrifuged at 13,000 g for 10 min to remove the cell debris.

Immunoprecipitation, electrophoresis and quantitation of sialidase

1.0 ml of lysate was incubated for 4 h with preimmune serum at a final dilution of 1/20. Then the pellet obtained from 300 µl of Pansorbin Cells (Calbiochem) was added and the resulting suspension was incubated for 2 h at 4° C, followed by centrifugation for 10 min at 13,000 g. Supernatants were incubated overnight with the anti-sialidase antibodies at a 1/100 final dilution, then for 2 h at 4° C with the pellet from 100 µl of Pansorbin Cells and precipitated as above. The pellet was washed three times with 1 ml of RIPA buffer. The antigens were eluted from the pellet by the addition of 100 µl of a buffer containing 0.1 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2 M DTT and 0.02% (w/v) bromphenol blue. The proteins were denatured by boiling for 5 min and 50 µl of each sample were subjected to SDS-PAGE according to Laemmli (38). The molecular weights were determined with [^{14}C]-labeled protein markers (Amersham). The gels were fixed in acetic acid/isopropanol/water (10/50/40), soaked for 30 min in Amplify™ solution (Amersham), vacuum dried at 60° C and analyzed by quantitative fluorometry on a PhosphorImager SI analysis screen (Molecular Dynamics) using the software supplied by the manufacturer.

Immunofluorescent microscopy

48 h after transfection with wild-type or mutant sialidase, COS-7 cells were treated for 40 min with 75 nM LysoTracker Red DND-99 (Molecular Probes, Eugene, OR) dye, washed twice with ice-cold PBS and fixed in 3% paraformaldehyde in PBS for 40 min. Cells were permeabilized by incubating with 0.3% Triton X-100, washed twice with PBS and stained with rabbit polyclonal anti-sialidase antibodies and FITC-conjugated monoclonal antibodies against

rabbit IgG. Alternatively cells were double stained with rabbit polyclonal anti-sialidase antibodies and monoclonal antibodies against lysosomal membrane marker LAMP-2 (Washington Biotechnology Inc., Baltimore). Slides were studied on a Zeiss LSM410 inverted confocal microscope (Carl Zeiss Inc., Thornwood).

Density gradient centrifugation of cell extracts.

COS-7 cells grown to confluency in T-25 flasks harvested 48 hours after transfection with wild-type or mutant sialidase were solubilized in 0.2 ml of 0.15 M sodium acetate buffer, pH 5.2, containing 0.5 mg BSA/ml and 1% (w/v) Zwitterionic^{TM} detergent 3-12 (Calbiochem) as described (19) and centrifuged at 13,000 *g* for 15 min. The supernatants were applied on the top of the density gradient of 30% metrizamide (OptiPrep, Nycomed) preformed by 2 h ultracentrifugation at 45,000 rpm in a Beckman SW-55 Ti swinging bucket rotor. After application of the sample the centrifugation was continued for 17 more hours at the same speed. Immediately after centrifugation each tube was divided to 10 fractions, using a Beckman Tube slicer kit. Each fraction was essayed for activities of sialidase, β-galactosidase and cathepsin A as well as for the presence of human sialidase and cathepsin A protein by western blot as previously described (20). The activity of endogenous N-acetyl β-hexosaminidase in fractions was used as an internal control. The molecular masses of proteins were approximated using the following Mr standards (Pharmacia): thyroglobulin (Mr 669 kDa), catalase (Mr 232 kDa), and BSA (Mr 69 kDa). Thyroglobulin and BSA were covalently labelled with FITC to facilitate their detection in fractions containing proteins from COS-7 cell.

Modeling of sialidase tertiary structure.

The modeling was performed using the structures of homologous sialidases from *Micromonospora Viridifaciens* (39, PDB file 1eur.pdb), *Salmonella Typhimurium* (40, PDB file 2sil.pdb) and *Vibrio Cholerae* (41, PDB file 1kit.pdb) as templates. These structures were superimposed with ProSup King's Beech Biosoftware Solutions to determine structurally conserved regions (SCRs). The sequence of human sialidase was manually aligned with the sequences of SCRs. The modeling was then carried out with a Modeler 4 software (Andrej Sali, The Rockefeller University, New York).

RESULTS

Expression and intracellular targeting of sialidase mutants

The effect of sialidase mutations on the enzyme biogenesis was studied by the transient expression of the mutant cDNA. Mutations were generated by site directed mutagenesis in the pCMV-SIAL vector previously used for the expression of sialidase (28). Short restriction cassettes containing the mutations were then inserted into the parental pCMV-SIAL vector replacing the corresponding fragments of wild-type sialidase cDNA. The inserts and junction regions of the resulting constructs were verified by sequencing to ensure the correct introduction of mutations. Mutant or wild-type sialidase was co-expressed with human cathepsin A, which is necessary for the expression of sialidase activity. 48 hours after transfection the cell lysates were assayed for sialidase, cathepsin A and control β-hexosaminidase activities.

The expression results are shown in Table 1. All transfected cells had similar cathepsin A activity suggesting the same transfection efficiency for all cells. Four of the expressed mutants, Gly68Val, Gly227Arg, Ala298Val, and Leu363Pro had very low (< 10% of normal) sialidase activity. The activity of Phe260Tyr and Leu270Phe mutants was between 10 and 20% of normal, and that of Ser182Gly and Gly328Ser mutants was between 20 and 40% of normal. Additional experiments showed that, Phe260Tyr and Leu270Phe mutants were also significantly less stable than the wild-type sialidase. The half-life of their enzymatic activity in cellular lysates at 37^0 C was about 30 minutes as compared to 2-h half-life of the wild-type enzyme.

Using immunolabeling, we have studied the intracellular distribution of the sialidase mutants expressed in COS-7 cells. To identify the lysosomal-late endosomal compartment, prior to fixation and immunostaining with anti-sialidase antibodies the COS-7 cells were treated for 40 min with 75 mM LysoTracker Red DND-99 dye. Alternatively the cells were double-stained with

anti-sialidase antibodies and monoclonal antibodies against human LAMP-2. For the wild-type sialidase we have observed the complete co-localization of anti-sialidase immunostaining with lysosomal markers LysoTracker Red (Fig. 1) or LAMP-2 (not shown). The Gly68Val, Ser182Gly, Phe260Tyr, Leu270Phe, Ala298Val, and Gly328Ser mutants showed similar localization suggesting that mutant protein is able to reach the lysosomes. Although partial co-localization of anti-sialidase and LysoTracker staining was also detectable in the cells transfected with the Gly227Arg and Leu363Pro mutants, majority of anti-sialidase antibodies labeled distinct cellular areas suggesting that mutant protein is mostly retained in pre-lysosomal compartments. This finding is consistent with the results of structural modeling of sialidase mutants that suggested general folding defects and retention in the ER/Golgi compartment for both Leu363Pro and Gly227Arg substitutes (31).

Metabolic labeling of sialidase mutants

The results of sialidase activity assay in COS-7 cells expressing sialidase mutants have suggested that some of them, i.e. Leu270Phe, and Phe260Tyr mutants, had significantly lower stability in cellular homogenates than the wild-type enzyme. To address directly the stability of sialidase mutants in the cell we have performed pulse-chase experiments. The 46-48 kDa polypeptides similar to those previously observed by both immunoprecipitation and western blotting (20) were precipitated by anti-sialidase antibodies from homogenates of cells transfected with wild-type or mutant sialidase cDNA and pulsed for 40 min (Fig. 2). The intensities of both bands decreased proportionally with time of chase. By 4 h of chase normal wild-type sialidase was reduced to ~50% of total. In contrast, the degradation rate of Phe260Tyr, Leu270Phe, and Ala298Val mutants was remarkably increased so that for all these cells 46-48 kDa sialidase bands were nearly undetectable already after 4 h of chase.

Association of sialidase mutants with the multienzyme complex

The ability of sialidase mutants to associate with cathepsin A, forming a high molecular weight multienzyme complex was studied by the density gradient ultracentrifugation of the cell extracts (Fig. 3). In the extracts of COS-7 cells co-transfected with wild-type sialidase and cathepsin A, all sialidase activity is associated with the peak that sediments before thyroglobulin (Mr 669 kDa). This peak, which also contains almost all cathepsin A and majority of endogenous β-galactosidase activities, probably, represents the high molecular weight multienzyme complex (Fig. 3 a). A similar distribution of oligomers was observed in the extract of cells transfected with sialidase Gly328Ser or Ser182Gly mutants. Although about 3-fold less sialidase activity was detected in the collected fractions as compared to that of wild-type control, all activity was associated with the high molecular weight fraction. The distribution of sialidase protein detected by Western blot (Fig. 3 b) followed that of the enzyme activity. In contrast, in the cells transfected with Gly227Arg, Phe260Tyr, Leu270Phe, and Ala298Val mutants high molecular weight forms of sialidase were not detected. Both sialidase protein and the trace levels of sialidase activity were found in the peak that sedimented together with the low molecular weight marker, BSA (Mr 69 kDa) suggesting that the mutant enzyme does not associate with the multienzyme complex. Moreover although a substantial amount of sialidase cross-reacting protein was detected in these fractions for Phe260Tyr, Leu270Phe, and Ala298Val-ransfected cells, the sialidase activity was ~50-100-fold reduced as compared to wild-type enzyme, which is consistent with the inactivation of sialidase after the dissociation from the complex.

DISCUSSION

Comparative analysis of molecular defects in the cathepsin A gene, leading to galactosialidosis and in the sialidase gene leading to a clinically almost similar disorder, sialidosis, shows that the last disease has very distinct spectrum of mutations. Most sialidosis patients studied so far have had amino acid substitutions (21 of 27) and not frameshifts or splicing defects (27, 28, 30, 31, 42, Sakuraba, private communication). The localization of the missence mutations on the sialidase structural model (Fig. 4) suggested that several of them (shown in blue in Fig. 4) affect active site residues (Tyr370Cys) or may interfere with their correct positions (Leu91Arg with the active site residue R^{78}; Pro80Leu with R^{97}; dup399HisTyr with E^{394}; Pro316Ser with R^{280}; and Pro335Gln with R^{341}). Leu363Pro mutation is situated on a β-stand adjacent to that containing the active site residue Tyr^{370}. The Leu^{363} residue is probably necessary to anchor this β-stand to the one containing Y^{370}, so the Leu363Pro mutation can potentially also have an effect on the active site. However, in contrast to mutations in cathepsin A, which mostly affect the enzyme central core and cause unfolding of the protein (32), the majority of sialidase mutations involve residues on the surface of the enzyme, and are not predicted to result in significant structural changes. Moreover, the distribution of mutations on the sialidase surface is uneven. The region that contains majority of mutations resulting in complete or almost complete inactivation of the enzyme and causing severe sialidosis type II phenotype is easily detectable (shown in red in Fig. 4). In particular this region contains mutations Gly227Arg, Phe260Tyr, Leu270Phe, and Ala298Val (28,31), Arg294Ser, Leu231His, and Gly218Ala (30), Trp240Arg (Sakuraba, private communication), as well as Val217Met and Gly243Arg (42).

We have expressed 8 sialidase mutants 4 of which contained amino acid substitution in the defined surface patch (Gly227Arg, Phe260Tyr, Leu270Phe, and Ala298Val) and 4 at the opposite surface of the sialidase molecule (Gly68Val, Ser182Gly, Gly328Ser, and Leu363Pro) in COS-7 cells and studied sorting, activity and stability of the produced protein. We found that in two cases (Gly227Arg and Leu363Pro) majority of the mutant protein was not sorted to the lysosomes suggesting that these mutations can cause general folding defects and retention of the mutant in the pre-lysosomal compartments. All other expressed sialidase mutants were targeted to lysosomes and correctly processed.

Subsequent experiments revealed that the sialidase mutants, Phe260Tyr, Leu270Phe, and Ala298Val containing amino acid substitutions in surface patch of the fifth β-sheet have similar properties. First, they had very low or absent sialidase activity. Second, stability of sialidase mutants in cellular homogenates or their half-life in the cell estimated by metabolic labeling was significantly lower then that of the wild-type enzyme. In addition, previous analysis of COS-7 cells transfected with Phe260Tyr, Leu270Phe, and Ala298Val mutants by western blot (31) demonstrated the presence of 37 kDa, 26 kDa and 24 kDa fragments of sialidase protein similar to those observed in COS-7 cells in which wild-type sialidase was expressed in the absence of human cathepsin A. The same pattern of sialidase degradation products was also observed in the cells of a galactosialidosis patient, which lack functional cathepsin A (20). Metabolic labeling studies (20) also demonstrated the dramatically reduced half-life of wild-type sialidase expressed in galactosialidosis cells (30 min versus 2.7 h in normal cells); similar to that observed in this work. Together these data suggest that Phe260Tyr, Leu270Phe, and Ala298Val mutants are not protected by cathepsin A, although the same high amount of functional human cathepsin A was expressed by COS-7 cells in all cases. Indeed in the extracts of cells transfected with Phe260Tyr,

Leu270Phe, and Ala298Val mutants we could not detect high molecular weight complex of sialidase with cathepsin A. Instead we observed that sialidase protein precipitated during the density gradient centrifugation together with low molecular weight markers. We hypothesize that Phe260Tyr, Leu270Phe, and Ala298Val mutations in sialidase destabilize its association with cathepsin A in the complex, leading to the enzyme inactivation and rapid degradation.

The obtained data will help to clarify the basic mechanism of the enzyme function in the lysosome and open the possibility to address directly the question of the supramolecular organization of the multienzyme lysosomal complex.

ACKNOWLEDGMENTS

The authors thank our colleagues Drs. Alexandra d'Azzo and Hitoshi Sakuraba, who generously provided unpublished information. We also thank Dr. Mila Ashmarina for critical reading of the manuscript. This work was supported by the operating grants from Canadian Institutes of Health Research (MT-15079), Fonds de La Recherche en Santé du Québec and Vaincre les Maladies Lysosomales Foundation (France) to A.V.P.

REFERENCES

1. Thomas, G.H., and Beaudet, A.L. (1995) *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, N.Y., pp 2529-2561

2. Federico, A., Battistini, S., Ciacci, G., de Stefano, N., Gatti, R., Durand, P., and Guazzi, G.C. (1991). *Dev. Neurosci.* **13**, 320-326

3. Cantz, M., and Ulrich-Bott, B. (1990) *J. Inherit. Metab. Dis.* **13**, 523-537

4. Durand, P., Gatti, R., Cavalieri, S., Borrone, C., Tondeur, M., Michalski, J.C., and Strecker, C. (1977) *Helvetica Paediatrica Acta* **32**, 391-400

5. Sogg, R.L., Steinman, L., Rathjen, B., Tharp, B.R., O'Brien, J.S., and Kenyon, K.R. (1979) *Ophtalmology* **86**, 1861-1874

6. Rapin, I., Goldfischer, S., Katzman, R., Engel, J., Jr., and O'Brien, J.S. (1978) *Ann. Neurol.* **3**, 234-242

7. O'Brien, J.S. (1979) *Clin. Genet.* **14**, 55-60

8. Federico, A., Cecio, A., Battini, G.A., Michalski, J.C., Strecker, G., and Guazzi, G.C. (1980) *J. Neurol. Sci.* **48**, 157-169

9. Till, J.S, Roach, E.S., and Burton, B.K. (1987) *J. Clin. Neur-Ophtalmol.* **7**, 40-44

10. Kelly, T.E., and Graetz, G. (1977) *Am. J Med. Genet.* **1**, 31-46

11. Oohira, T., Nagata, N,. Akaboshi, I., Matsuda, I., and Naito, S. (1985) *Hum. Genet.* **70**, 341-343

12. Winter, R.M., Swallow, D.M., Baraitser, M., and Purkiss, P. (1980) *Clin. Gen.* **18**, 203-210

13. Aylsworth, A.S., Thomas, G.H., Hood, J.L., Malouf, N., and Libert, J. (1980) *J. Pediatr.* **96**, 662-668

14. Johnson, W.G., Cohen, C.S., Miranda, A.F., Waran, S.P., and Chutorian, A.M. (1980) *Am. J Hum. Genet.* **32**, 43A

15. Beck, M., Bender, S.W., Reiter, H.L., Otto, W., Bassler, R., Dancygier, H., and Gehler, J. (1984) *Eur. J Pediatr.* **143**, 135-139

16. Cantz, M., Gehler, J., and Spranger, J. (1977) *Biochem. Biophys. Res. Commun.***74,** 732-738

17. d'Azzo, A., Hoogeveen, A., Reuser, A.J., Robinson, D., Galjaard, H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4535-4539

18. Van der Horst, G.J., Galjart, N.J., d'Azzo, A., Galjaard, H., and Verheijen, F.W. (1989) *J. Biol. Chem.* **264,**1317-1322

19. Pshezhetsky, A.V., and Potier, M. (1996) *J. Biol. Chem.* **271**, 28359-28365

20. Vinogradova, M.V., Michaud, L., Mezentsev, A.V., Lukong, K.E., El-Alfy, M., Morales, C.R., Potier, M.. and Pshezhetsky, A.V. (1998) *Biochem. J.* **330**, 641-650

21. Hoogeveen, A.T., Verheijen, F.W., and Galjaard, H. (1983) *J. Biol. Chem.* **258**, 12143-12146

22. van der Spoel, A., Bonten, E., and Azzo, A. (1998) *EMBO J.*, **17**, 1588-1597

23. Morreau, H., N.J. Galjart, R. Willemsen, N. Gillemans, X.Y. Zhou, and A. d'Azzo. (1992) *J. Biol. Chem.* **267**, 17949-17956

24. D'Agrosa, R.M., Hubbes, M., Zhang, S., Shankaran, R., and Callahan, J.W. (1992) *Biochem. J.* **285**, 833-838

25. Okamura-Oho, Y., Zhang, S., Hilson, W., Hinek, A., and Callahan, J. (1996) *Biochem. J.* **313**, 787-794

26. d'Azzo, A., Andria, G., Strisciuglio, P., and Galijaard, H. (1995) *Metabolic and Molecular Bases of Inherited Disease.* New York: McGraw-Hill, Vol.2, pp 2835-2837

27. Bonten, E., van der Spoel, A., Fornerod, M., Grosveld, G., and d'Azzo, A. (1996). Genes and Devel. **10**, 3156-3168

28. Pshezhetsky, A.V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M. A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L., and Potier, M. (1997) *Nat. Genet.* **15**, 316-320

29. Milner, C.M., Smith, S.V., Carrillo, M.B., Taylor, G.L., Hollinshead, M., and Campbell R.D. (1997) *J. Biol. Chem.* **272**, 4549-4558

30. Bonten EJ, Arts WF, Beck M, Covanis A, Donati MA, Parini R, Zammarchi E, d'Azzo A. (2000) *Hum Mol Genet* Nov 1;9(18):2715-2725

31. Lukong KE, M.A. Elsliger, Y. Chang, C. Richard, G. Thomas, W. Carey, A. Tylki-Szymanska, B. Czartoryska, T. Buchholz, G.R. Criado, S. Palmeri, and A.V. Pshezhetsky. (2000) *Hum. Mol. Genet.* **9(7)**, 1075-1085.

32. Rudenko, G., Bonten, E., Hol, W.G.H., and d'Azzo, A (1998) *Proc. Natl. Acad. Sci. USA* **95**, 621-615

33. Okada, S., and O'Brien, J.S. (1968) *Science* **160**, 1002-1004

34. Potier, M., Mameli, L., Belisle, M., Dallaire, L., and Melançon, S.B. (1979) *Anal. Biochem.* **94**, 287-296

35. Rome, L.H., Garvin, A.J., Allietta, M.M., and Neufeld, E.F. (1979) *Cell* **17**,143-153

36. Pshezhetsky, A.V., Vinogradova, M.V., Elsliger, M-A., El-Zein, F., Svedas, V.K., and Potier, M. (1995) *Anal. Biochem.* **230,** 303-307

37. Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248-254

38. Laemmli, U.K. (1970) *Nature* **227**, 680-685

39. Burmeister, W.P., Ruigrok, R.W.H., and Cusack, S. (1992) *EMBO J* **11**, 49-56

40. Varghese, J.N., Laver, W.G., and Colman, P.M. (1983) *Nature* **303**, 35-40

41. Crennell, S.J., Garman, E.F., Laver, W.G., Vimr, E.R., and Taylor, G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9852-9856

42. Naganawa, Y., Itoh, K., Shimmoto, M., Takiguchi, K., Doi, H., Nishizawa,Y., Kobayashi, T., Kamei, S., Lukong, K.E., Pshezhetsky, A.V., and Sakuraba, H. (2000) *J. Hum. Genet.* **45(4)**, 241-249

TABLE 1. Sialidase and cathepsin A activities in cellular lysates of COS-7 cells measured 48 h after co-transfection with cathepsin A (CathA) and wild-type (WT) or mutant sialidase cDNA.

Plasmid	Enzyme activity (mU/mg)	
	Sialidase	**Cathepsin A**
No DNA	0,15±0,05	7±3
CathA	0,27±0,3	44±7
WT+CathA	3,46±0,4	42±6
Gly68Val+CathA	0,22±0,08	43,6±8,5
Ser182Gly+CathA	1,48±0,5	43±7
Gly227Arg+CathA	0,35±0,28	46±7,5
Phe260Tyr+CathA	0,67±0,2	41,5±8,7
Leu270Phe+CathA	0,73±0,08	48±4,5
Ala298Val+CathA	0,4±0,14	41,5±4,5
Gly328Ser+CathA	1,22±0,3	44±6
Leu363Pro+CathA	0,24±0,1	40±8,5

Cells were transfected and enzyme activities assayed as described under Experimental Procedures. Values represent means ± S.D. of triplicate experiments.

LEGENDS TO FIGURES

Figure 1: Immunohistochemical localization of sialidase mutants expressed in COS-7 cells.

COS-7 cells were probed with 75 nM of lysosomal marker, LysoTracker Red DND-99 (red) for 30 min at 37^0 C, 48 h post-transfection with cathepsin A and wild-type or mutant sialidase cDNAs, fixed and stained with rabbit polyclonal anti-sialidase antibodies (green). Slides were studied on a Zeiss LSM410 inverted confocal microscope. Co-localization of anti-sialidase antibodies and LysoTracker (yellow). Bar =10 μm.

Figure 2: Metabolic labeling of sialidase.

Non transfected COS-7 cells (**Control**) as well as the cells co-transfected with cathepsin A and wild-type sialidase (**WT**) or mutant sialidase cDNA as indicated on the figure were metabolically labeled with a mixture of ^{35}S-Cys and ^{35}S-Met for 40 min and chased at 37° C for 0 h, 1 h and 4 h in EMEM supplemented with 20% (v/v) fetal calf serum. The sialidase (**SIAL**) was immunoprecipitated from cell lysates with rabbit anti-sialidase antibodies and resolved on SDS-PAGE.

Figure 3 Density gradient centrifugation of cell extracts.

COS-7 cells were co-transfected with cathepsin A and wild-type sialidase (WT) or mutant sialidase cDNA as indicated on the figure. 48 h after transfection cellular extracts were analysed by density gradient centrifugation as described in Experimental Procedures.

a) The 0.5 ml fractions were collected and assayed for cathepsin A activity (Δ), sialidase activity (□) β-galactosidase activity (○)N-acetyl-β-glucosaminidase activity in fractions (not shown) was used as an internal control. Each curve represents the average of several independent experiments. The positions of the sedimentation peaks of the Mr standards described in Experimental Procedures are shown by arrows.

The indicated fractions with the peak sialidase and cathepsin A activities were analysed for the presence of sialidase **(b)** and cathepsin A **(c)** proteins by western blotting as described. The protein bands are indicated on the right side the blots: Cath30 and Cath20, 30- and 20-kDa polypeptide chains of cathepsin A; SIAL, sialidase.

Figure 4: Schematic diagram of sialidase model, showing the location of mutations identified in sialidosis patients. Mutations localised in putative sialidase-cathepsin A binding site are shown in red, mutations in the active site residues or those that may affect the positions of the active site residues, in blue; and mutations that are not predicted to cause obvious structural changes, in green.

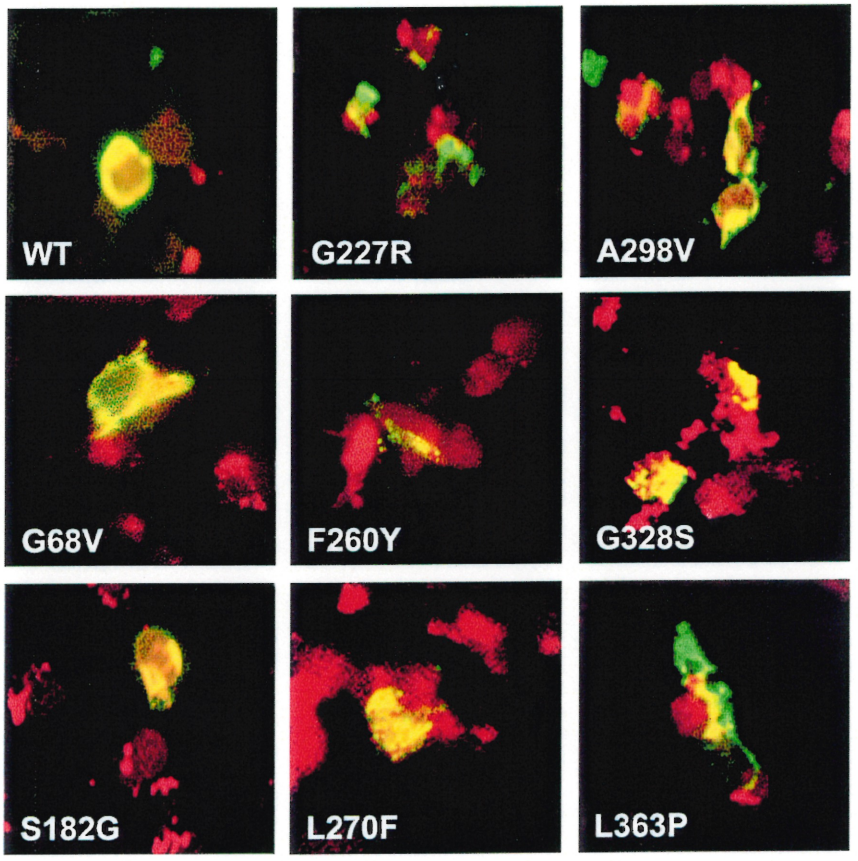

FIGURE 1

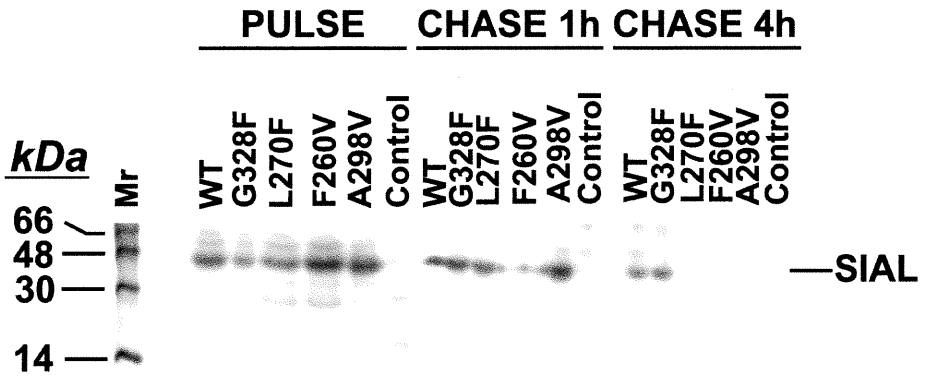
PULSE
CHASE 1h
CHASE 4h
kDa
Mr
66
48
30
14
WT
G328F
L270F
F260V
A298V
Control
WT
G328F
L270F
F260V
A298V
Control
WT
G328F
L270F
F260V
A298V
Control
—SIAL

FIGURE 2

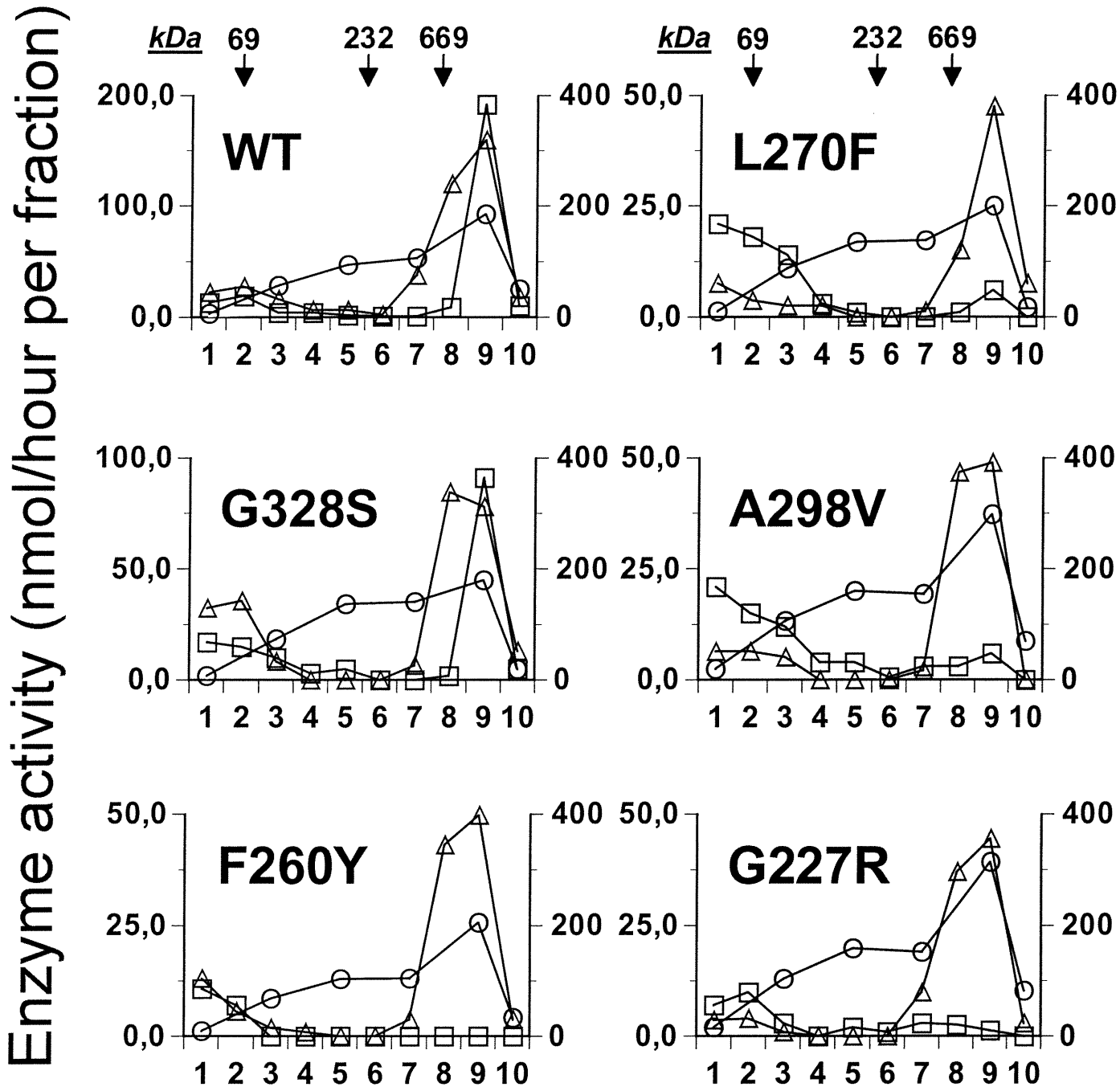
kDa
69
232
669
WT
L270F
G328S
A298V
F260Y
G227R
200,0
100,0
0,0
50,0
25,0
400
200
0
1 2 3 4 5 6 7 8 9 10
Enzyme activity (nmol/hour per fraction)
Fraction number

FIGURE 3a

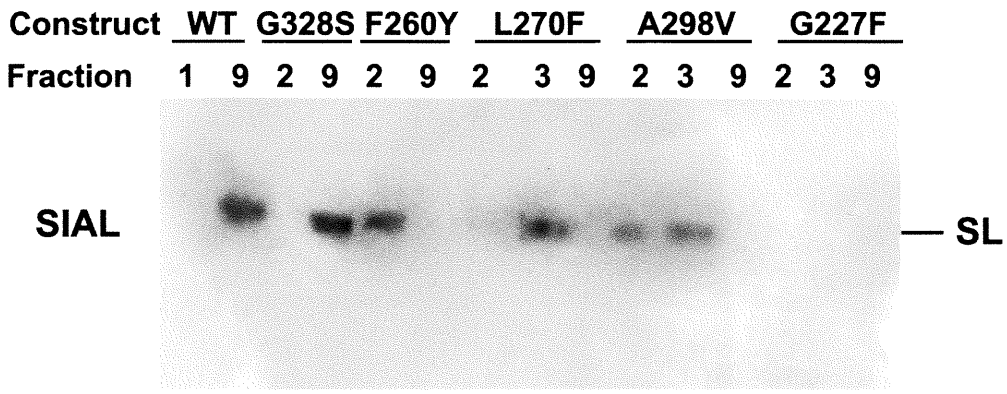
Construct WT G328S F260Y L270F A298V G227F
Fraction 1 9 2 9 2 9 2 3 9 2 3 9 2 3 9
SIAL
SL

FIGURE 3b

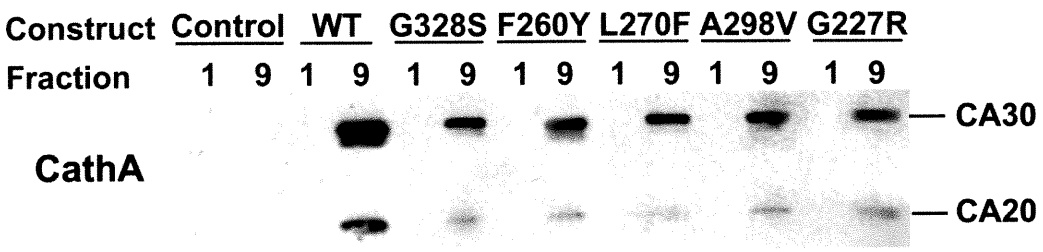
Construct Control WT G328S F260Y L270F A298V G227R
Fraction 1 9 1 9 1 9 1 9 1 9 1 9 1 9
CathA
CA30
CA20

FIGURE 3c

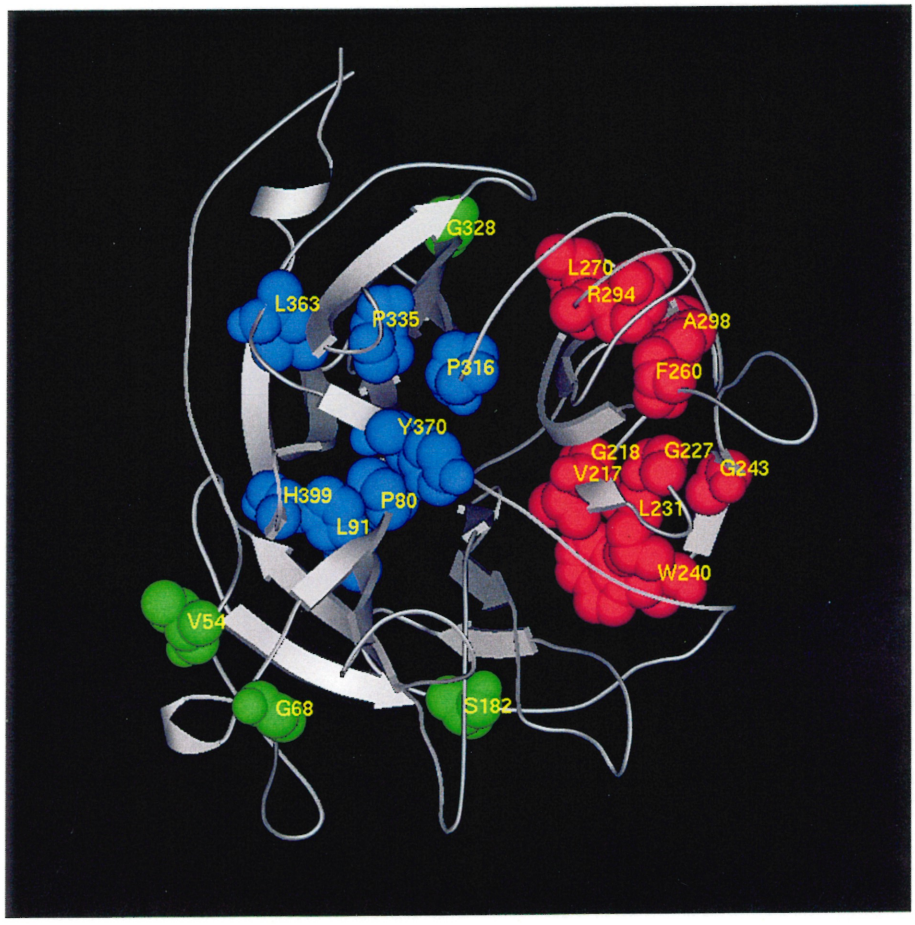
G328
L270
R294
L363
P335
A298
P316
F260
Y370
G218
G227
V217
G243
H399
P80
L91
L231
W240
V54
G68
S182

FIGURE 4

CHAPTER 4

Lysosomal sorting of sialidase

Foreword

Generally, proteins that reach the Golgi apparatus from the ER are distributed to the plasma membrane, lysosomes or secretory vesicles by specific mechanisms. Soluble lysosomal proteins, for example, carry unique mannose-6-phosphate groups added exclusively to N-linked oligosaccharides on their surface. These groups bind to specific mannose-6-phosphate receptors, which enable the targeting of lysosomal hydrolases to the lysosome.

Studies have indicated that plasma membrane proteins bear a consensus, Tyr-X-X-hydrophobic residues or Leu-Leu amino acid signals in their cytoplasmic tail. These signals associate with μ-subunits of adaptor complexes, enabling the sorting of the proteins to the lysosome.

In the following article, we proved the presence of the C-terminal tetrapeptide internalization signal in sialidase. We used site-directed mutagenesis to construct substitution mutants carrying mutations in the potential signal peptide, expressed them in COS-7 cells and localized the expressed products immunohistochemically. We also performed subcellular fractionation of the transfected cells and determined the distribution of the sialidase activity between the plasma membrane and the lysosome. Our results provide strong evidence that SIAL is internalized to the lysosome from the plasma membrane via the C-terminal targeting signal.

Article 6

Internalization signal in the cytoplasmic tail of lysosomal sialidase

Internalization signal in the cytoplasmic tail of lysosomal sialidase

Kiven E. Lukong[1], Karine Landry[1], Stéphane Lefrancois[2], Carlos R. Morales[2] and Alexey V. Pshezhetsky[1]*

[1]Service de Génétique Médicale, Hôpital Sainte-Justine and Département de Pédiatrie, Faculté de Médicine, Université de Montréal, Montréal (Qc), H3T 1C5, Canada;

[2]Department of Anatomy and Cell Biology, McGill University, Montréal, (Qc), Canada

*- To whom correspondence should be addressed:

Service de génétique médicale

Hôpital Sainte-Justine

3175 Côte Ste-Catherine

Montréal (Qc) H3T 1C5 - Canada

Tel. (514) 345-4931/2736

Fax. (514) 345-4801

E.-Mail: alex@justine.umontreal.ca

Running title: Internalization of lysosomal sialidase

ABSTRACT

Sialidase (neuraminidase) encoded by the NEU-1 gene in the MHC locus on human chromosome 6 is involved in the intralysosomal catabolism of sialylated glycoconjugates. Inherited deficiency of sialidase in humans results in two severe metabolic disorders: sialidosis and galactosialidosis, both associated with tissue accumulation and urinary excretion of sialylated oligosaccharides and glycoproteins. In addition, sialidase plays an important role in the cellular signalling and, in particular, it is required in activated T lymphocytes for the production of cytokine IL-4. Activation of T lymphocytes is associated with the increase of *neu-1*-encoded sialidase on the cell surface, suggesting the existence of the specific mechanism for sorting of the enzyme to the plasma membrane. We demonstrate that the C-terminus of sialidase contains internalization signal found in many lysosomal membrane proteins and endocytosed receptors, which are targeted to the endosomes from the plasma membrane via clathrin-coated pits. By studying the internalization of sialidase substitution mutants we show that the internalization signal consists of a tetrapeptide ^{412}YGTL^{415} and that Tyr412 and Leu415 residues are essential for rapid endocytosis of the enzyme. We speculate that the expression of sialidase on the surface of activated T cells may be controlled by the phosporylation of the essential tyrosine by T-lymphocyte associated Src kinase.

Key words

Sialidase/neuraminidase/T lymphocytes/lysosomal internalization signal/adaptor complex/ endocytosis/sialidosis/galactosialidosis.

INTRODUCTION

Lysosomal sialidase (neuraminidase, EC 3.2.1.18), encoded by the *neu-1* gene in the MHC locus on human chromosome 6 catalyzes the hydrolysis of terminal sialic acid residues of oligosaccharides, glycoproteins and glycolipids. In the lysosome, sialidase is associated with lysosomal carboxypeptidase A (EC 3.4.16.1, also named cathepsin A or protective protein), ß-galactosidase (EC 3.2.1.23) and N-acetylgalactosamine-6-sulfate sulfatase (EC 3.1.6.4) in a high molecular weight multienzyme complex (d'Azzo et al., 1982; Hoogeveen et al. 1983; van der Horst et al., 1989; Pshezhetsky and Potier 1996). The dissociation of the complex *in vitro* results in complete inactivation of sialidase, although, the activity can be restored after the reconstitution of the complex (van der Horst et al., 1989). These results suggested that the association with the complex is required for sialidase to adopt the catalytically active conformation, but direct structural data supporting this mechanism have not been obtained. Inherited mutations in cathepsin A result in the disruption of the complex and cause an autosomal recessive disease, galactosialidosis, characterized by the combined deficiency of sialidase, ß-galactosidase and cathepsin A (reviewed in d'Azzo et al., 1995; Okamura-Oho et al., 1994). Another autosomal recessive disease, sialidosis is caused by the mutations directly affecting the lysosomal sialidase gene (reviewed in Thomas, 1995).

Numerous data obtained during the biochemical purification of sialidase from different tissues demonstrated the existence in the cell of two pools of lysosomal sialidase, soluble and membrane associated. Both forms are absent in cultured cells of sialidosis patients and are, therefore encoded by the same gene (Miyagi et al., 1990, 1992, 1993; Verheijen et al, 1983). Using immunoelectron microscopy, we have directly

demonstrated the presence of sialidase on lysosomal membranes and lumen, as well as on plasma membranes of the transfected cells (Vinogradova et al., 1998). In addition, the activation of T lymphocytes is associated with a several-fold increase of sialidase activity on the cell surface (Landolfi et al., 1985; Naraparaju and Yamamoto, 1994). The sialidase expressed on the cell surface and lysosomal sialidase are the products of the same gene, since the activation does not happen in T-cells obtained from SM/J or SM/B10 mouse strains carrying a mutation in a *neu-1* gene coding for lysosomal sialidase (Carillo et al., 1997; Rottier et al., 1998). All these data suggest the existence of the mechanisms for sorting of a new-synthesized sialidase to both plasma membranes and lysosomes, as well as for the retention of sialidase on plasma membranes.

Previous studies have described several pathways for sorting the lysosomal membrane proteins and endocytosed receptors, transported between the Trans-Golgi network, plasma membrane and endosome. In all these proteins, amino acid motifs containing conserved tyrosine or dileucine residues are recognized by so-called adapter protein (AP) complexes, coat proteins, which form vesicles destined for the lysosome (reviewed in Peters and Figura, 1994, Pearse et al., 2000; Hirst and Robinson, 1998). Two adaptor complexes AP1 and AP2 are associated with clathrin-coated vesicles derived from Golgi and plasma membrames respectively. Both AP1 and AP2, contain μ-subunits (μ1 and μ2, respectively) that recognize and bind to the internalization signals of the cargo protein (Jarousse and Kelly, 2000).

In this study we obtained direct evidence that sialidase is synthesized and transported to the lysosomes as a membrane bound protein. We showed that the C-terminus of sialidase contains a tyrosine-containing internalization signal represented by

tetrapeptide ^{412}YGTL^{415}. The signal can be inactivated by mutating the essential Tyr412 and Leu415 residues. Alignment and analysis of the sialidase sequence predicted that the YGTL forms a tight β–turn structure similar to that of other internalization peptides, and is located close to a potential trans-membrane domain in the putative cytoplasmic tail of sialidase.

RESULTS

Distribution of lysosomal sialidase between "light" (late endosomal) and "dense" lysosomal pools resembles those of lysosomal membrane proteins that are internalized from plasma membrane via clathrin-coated pits

The purification of light mitochondrial fraction from liver homogenates performed according to the described protocols (Burnside and Schneider, 1982; Dobrota and Hinton, 1980) was followed by the ultracentrifugation in a self-forming gradient of metrizamide (Wattiaux et al., 1983). A typical distribution of the marker enzymes for lysosomes, microsomes, and mitochondria in fractions collected after the centrifugation is shown at Fig. 1a. As expected, lysosomal marker enzymes were found in two major peaks, previously described as "light" (fractions 3-5) and "heavy" (fractions 11-13) lysosomes. A pick of "light" lysosomes was well separated from both microsomes, and mitochondria, whereas a peak of "dense" lysosomes partially overlapped with the peaks of mitochondria and peroxisomes. Previous data showed that "light" lysosomes are identical to late endosomes, mannose 6-phosphate receptor-positive organelles, containing internal lipid vesicles and responsible in most cells for the majority of intracellular catabolism.

When we compared the distribution of soluble lysosomal enzymes that are known to be targeted by mannose 6-phosphate receptor-dependent pathway (β-galactosidase, cathepsin A, cathepsin B, N-acetyl β-hexosaminidase) we found that they are equally distributed between the "light" and "dense" lysosomes (Fig. 1b). Membrane lysosomal proteins (LAMP-2, β-glucocerebrosidase) as well as soluble lysosomal acid phosphatase, which are all endocytosed from the plasma membrane via clathrin-coated pits, are present

mostly in the "light" pool of lysosomes (Fig. 1b). Both sialidase activity assay (Fig. 1b) and western blotting using anti-sialidase antibodies (not shown) demonstrated that the distribution of sialidase between secondary endosomes and lysosomes is similar to that of lysosomal proteins internalized from plasma membrane.

C-terminal sialidase tetrapeptide YGTL has sequence and structure similarities to the internalization signals of lysosomal membrane proteins and endocytosed surface receptors.

The analysis of the deduced amino acid sequence of sialidase reveales that its C-terminal tetrapeptide, YGTL has similarity to the internalization signals previously determined in cytoplasmic domains of several internalized membrane proteins including LAMP-1, LAMP-2 and LAMP-3 (CD63) (Guarnieri et al., 1993), LGP-85 (Akasaki et al., 1994), or endocytosed receptors including those for LDL (Chen et al., 1990), transferrin (Collawn et al., 1990; 1991; 1993), asialoglycoprotein (Ozaki et al., 1993), polymeric immunoglobulin (Okamoto et al., 1992) and mannose 6-phosphate (Canfield et al., 1991; Jadot et al., 1992) as well as in adhesion receptor, P-selectin (Blagoveshchenskaya et al., 1998) and cystic fibrosis conductance regulator (Prince et al., 1999). All these proteins are transported to the lysosome via clathrin-coated vesicles by a mechanism, which involves the association of the internalization signal with the μ2 subunit of AP2 adaptor complex (Peters and Figura, 1994, Pearse et al., 2000; Hirst and Robinson, 1998). The same pathway is also used by lysosomal acid phosphatase (Lehmann et al., 1992), which is synthesized as a transmembrane protein but is cleaved inside the lysosome into a soluble form (Waheed et al., 1988; Gottschalk et al., 1989).

The consensus internalization signal is a short stretch of four or six amino acids, containing the essential tyrosine and a bulky hydrophobic amino acid (phenylalanine, valine, leucine or isoleucine), which is located three residues C-terminal from the essential tyrosine and is abbreviated Tyr-X-X-Φ (where Φ stands for hydrophobic residue). 2D-NMR studies of internalization peptides of lysosomal acid phosphatase, LDL receptor and transferrin receptor showed that in solution they adopt a tight β–turn structure necessary for their recognition and binding to a μ2-subunit of the AP2 adaptor complex (Collawn et al., 1990; Bansal and Gierash, 1991, Eberle et al., 1991).

Sequence alignment of the internalization peptides from 6 proteins (cation independent mannose 6-phosphate receptor, transferrin receptor, lysosomal acid phosphatase, LAMP-1, LAMP-2 and CD63) with the C-terminal fragment of human lysosomal sialidase (Fig. 2) shows that it contains both essential tyrosine (Tyr412) and a bulk hydrophobic amino acid residue located at a distance of two amino acids from tyrosine (Leu415). This tetrapeptide is found in sialidase sequence directly after the stretch of hydrophobic amino acids Ser403-Val411. Both structural alignment with homologous bacterial sialidases (Lukong et al., 2000), and empirical algorithms for prediction of the secondary structure suggest that this fragment may represent a trans-membrane α-helix. In this case the C-terminal Tyr412-Leu415 tail of sialidase is exposed in the cytoplasm, which is necessary for the interaction with the AP2 adaptor complex. In addition, several empirical algorithms predict tight β–turn structure for Tyr412-Leu415 peptide similar to that of other internalization peptides (Fig. 2).

Alanine substitution mutants identify C-terminal tetrapeptide YGTL as the sialidase internalization signal

To determine if the conserved Tyr412 and hydrophobic Leu415 on the C-terminus of sialidase are involved in the enzyme targeting to the endosome we have prepared sialidase mutants in which we changed codons for these amino acids to codons for alanine. In another mutant we have replaced alanine with the Gly413 residue, presumably not important for endocytosis of sialidase.

The mutants as well as wild-type sialidase were expressed in COS-7 cells together with human cathepsin A, which is necessary for the expression of sialidase activity (Lukong et al., 2000). 48 hours after transfection the cell lysates were assayed for sialidase, cathepsin A and control β-hexosaminidase activities. The expression results are shown on Fig. 3. All transfected cells had similar cathepsin A activity (not shown) suggesting the same transfection efficiency for all cells. Two of the expressed mutants, Gly413Ala and Leu415Ala had reduced (~ 30-45% of normal) sialidase activity (Fig. 3 a). The activity of Gly413Ala mutant was between 50 and 70% of normal (Fig. 3 a). The expressed sialidase protein was studied by western blot (Fig. 3, b): in the cells transfected with both wild-type and mutant sialidase, we observed a 48-46 kDa protein band, a product previously identified as a mature active sialidase (Vinogradova et al., 1998).

The internalization of wild-type and mutant sialidase was followed by immunohistochemical staining with anti-sialidase antibody. Immunolabeling of the wild

type sialidase expressed in COS-7 cells confirmed its localization in endosomal-lysosomal compartment: anti-sialidase immunofluorescence was observed in punctate structures co-localized with lysosomal markers LysoTracker Red dye or LAMP-2 (Fig. 4). Gly413Ala mutant showed similar localization suggesting that conservation of this residue is not essential for internalization of sialidase. In contrast both Tyr412Ala and Leu415Ala mutants showed a strong peripheral staining consistent with localization of the most of the expressed enzyme at the plasma membrane. In addition, in some cells, we were able to see the diffuse cytoplasmic staining partially overlapping with LysoTracker Red and possibly representing the Golgi compartment. Thus the presence of both Tyr412 and Leu415 in the C-terminal peptide of sialidase is sufficient for the internalization of this enzyme.

Quantification of the cellular localization of the expressed sialidase

To quantify the amount of sialidase mutants targeted to the cell surface and to the endosomal/lysosomal compartment we have assayed sialidase activity of purified lysosomal and plasma membranes. COS-7 cells harvested 48 hours after transfection with the wild-type and mutant sialidase cDNA were divided into two parts. From one part lysosomal membranes were purified by subcellular fractionation and density centrifugation in self-forming metrizamide (Nicodentz) gradient, and from the other plasma membranes were purified using polylysine-coated (Cytodex 1) beads as described in Materials and methods. The sialidase activity present on purified plasma membranes of COS-7 cells transfected with wild-type sialidase or sialidase Gly413Ala mutant was found to be very low (Fig. 5 a, lanes 2 and 3, black bars). In contrast a considerable

amount of sialidase activity was found on plasma membranes purified from the cells transfected Tyr412Ala and Leu415Ala mutants (Fig. 5 a, lanes 4 and 5, black bars). The activity of the control plasma membrane enzyme, alkaline phosphatase was similar for non-transfected cells and for the cells transfected with wild type and mutant sialidase (Fig. 5 a, open bars). An opposite effect was observed for lysosomal membranes. Transfection of COS-7 cells both with wild-type sialidase or sialidase Gly413Ala mutant increased ~3-fold the sialidase activity present on the lysosomal membranes (Fig. 5 b, lanes 2 and 3, black bars). The sialidase activity on lysosomal membranes purified from the cells transfected with Tyr412Ala and Leu415Ala mutants was similar to that present on the membranes purified from non-transfected control cells (Fig. 5 b, lanes 4 and 5, black bars). This result suggested that the increased amount of Tyr412Ala and Leu415Ala mutants on the plasma membranes resulted from their impaired endocytosis. As in the case of the plasma membranes, the activity of the endogenous membrane enzyme, lysosomal β-glucosidase was similar for all the cells studied.

A significant increase of the sialidase activity on the cell surface of COS-7 cells transfected with Tyr412Ala and Leu415Ala mutants as compared with non-transfected cells was observed when we incubated intact cells in the osmomolar buffer solution containing sialidase substrate. Small increase of the sialidase activity was also observed for the cells transfected with the wild type enzyme or Gly413Ala mutant. However, when the same assay was performed in the presence of 0.03% Triton X-100, which permeabilized the cell membranes (Fig. 6a, open bars) sialidase activity was increased 3-fold in the cells transfected with wild-type sialidase or Gly413Ala mutant, reflecting the amount of the enzyme localized inside the cells. In the case of Leu415Ala and

Tyr412Ala mutants the activity was the same in the absence or in the presence of Triton X-100, suggesting that all sialidase in the intact COS-7 cells transfected with Leu415Ala and Tyr412Ala mutants was available for the substrate. These results not only confirmed the effects, observed for the purified membranes, but also showed that sialidase is located on the outside surface of the cell wall. The activity of the control endogenous lysosomal enzyme N-acetyl β-hexosaminidase (not shown) demonstrated the same 90-95% latency for all the cells studied suggesting that the cells stayed intact during the assay and that detected sialidase activity did not result from the secretion of the expressed enzyme.

DISCUSSION

Like other lysosomal proteins sialidase is synthesized as a precursor and after the cleavage of 47-amino acid signal peptide and glycosylation becomes a 48.3 kDa mature active enzyme found in the lysosome and present in the multienzyme complex. However, the exact mechanism of sorting of the sialidase precursor until now remained unclear. Since sialidase contains three Asn-linked oligosaccharide side chains, it can be mannose 6-phosphorylated and targeted to the lysosome by the mannose 6-phosphate receptors as most of soluble lysosomal enzymes. However, the phosphorylation of sialidase is rather poor and is not sufficient for targeting the enzyme to the lysosomes (Van der Spoel et al., 1998). Comparing the intracellular distribution of human sialidase expressed in COS-1 cells transfected with sialidase cDNA alone or co-transfected with sialidase and human cathepsin A cDNA, Van der Spoel et al. (1998) suggested that the association of sialidase with cathepsin A may occur as early as in the ER and is required for the proper sorting of the enzyme. In this scenario, sialidase is transported to the lysosome via cathepsin A, which obtains a mannose 6-phosphate recognition signal and binds to mannose 6-phosphate receptor. In the absence of cathepsin A sialidase is partially secreted and partially segregates to endosomal compartment (Van der Spoel et al., 1998). This hypothesis is supported by the data that showed a significant increase of sialidase activity and immunoreactive material in the lysosomes of cells co-transfected with sialidase and cathepsin A plasmids as compared to the cells transfected with sialidase alone (Bonen et al., 1996). At the same time this mechanism is not consistent with previously observed partial sorting of sialidase to plasma membranes (Vinogradova et al., 1998). It also does not explain why the targeting of sialidase to the endosomal compartment was detected,

when the enzyme was expressed in cultured skin fibroblasts of galactosialidosis patients that lack cathepsin A (Vinogradova et al.; 1989). In addition, this study revealed that sialidase expressed in human fibroblasts is localized not only on the inner side of the lysosomal membrane and in lysosomal lumen but also on the plasma membrane and in small intracellular (possibly endocytic) vesicles (Vinogradova et al., 1989) suggesting that sialidase may be also transported by the mechanism involving its targeting to the plasma membrane and endocytosis via clathrin-coated pits.

We have demonstrated that the C-terminal tetrapeptide of sialidase ^{412}YGTL^{415} represents the tyrosine-containing lysosomal targeting signal, and that Tyr412 and Leu415 amino acid residues are essential for rapid endocytosis of sialidase by measuring the internalization of sialidase substitution mutants Tyr412Ala, Gly413Ala and Leu415Ala. The experiments showed that Tyr412Ala and Leu415Ala mutants are sorted to the plasma membrane but are not further internalized, suggesting that lysosomal sialidase is an integral membrane protein containing a single transmembrane domain and a short cytoplasmic tail carrying the internalization signal. It is possible that in the lysosome the transmembrane domain can be cleaved similarly to that of acid phosphatase resulting in the appearance of the soluble pool of the enzyme, but experimental evidences of that have to be obtained.

The identified mechanism of sialidase targeting not only provides an important information about its biogenesis but also extend our knowledge about several important physiological processes involving this enzyme. First, sialidase was reported to be a part of a plasma membrane complex containing also cathepsin A precursor and an alternatively spliced form of β-galactosidase, so-called elastin binding protein (EBP),

essential for the cell binding to the extracellular elastic fibres (reviewed in Hinek, 1996). After the synthesis EBP binds tropoelastin and excorts it through secretory pathways to the plasma membrane. Some of the protein stays on the plasma membrane associated with elastic fibers, but the majority of EBP after dissociation from tropoelastin is internalized, and sorted to the early and then to the recycling endosome, where it associates with newly- synthesized tropoelastin (Hinek, 1996). Since neither EBP itself nor cathepsin A precursor is able to bind the membrane, the sorting and functional activity of the EBP complex should depend on the presence of sialidase. Therefore, the observed failure of cells from sialidosis patients, that lack functional sialidase, to assemble elastic fibers (Callahan, 1999) is consistent with the suggestion that sialidase is an anchor that attaches the EBP complex to the endosomal or plasma membrane.

Multiple studies showed that *neu-1*-encoded sialidase in addition to its well established role in intralysosomal catabolism of sialylated glycoconjugates is also involved in cellular signalling during the immune response. In particular, sialidase of T cells is required for both early production of IL-4 and the IL-4 priming processing of conventional T cells to become active IL-4 producers (Chen et al., 1997; Chen et al., 2000). During the activation sialidase is expressed on the surface of T cells where it participates in desialylation of surface antigen-presenting molecules such as MHC class I, required to render T cells responsive to antigen presenting cells (Landolfi and Cook, 1986) and G_{M3}-ganglioside which, in its turn, modules the Ca^{2+} immobilization and regulates IL-4 production in CD4(+) T cells (Chen et al., 2000). In addition, sialidase of T lymphocytes converts, so called, group specific component or Gc protein into a factor necessary for the inflammation-primed activation of macrophages (Yamamoto and

Kumashiro, 1993; Naraparaju and Yamamoto, 1994; Yamamoto and Naraparaju, 1996). T-cells derived from SM/J or B10.SM strands of mice, deficient in *neu-1* sialidase due to the point mutation (Carillo et al., 1997; Rottier et al., 1998) failed to convert Gc and synthesise IL-4, whereas B cells of these mice were not able to produce IgG_1 and IgE after the immunization with pertussis toxin (Yamamoto and Kumashiro, 1993; Landolfi et al., 1985; Chen et al., 1997).

Our studies of the promoter of human sialidase gene showed that its expression is indeed potently induced by the proinflammatory factors and is inhibited by curcumin and N-acetylcysteine, that has been shown to inhibit inflammatory responses in different tissues (Seyrantepe et al, in preparation). However it is still unclear how de-novo synthesized sialidase can be retained on the surface of activated T cells. One of the possibilities is that the interaction of the sialidase with AP2 adaptor and its inclusion into clatrin-coated pits can be blocked by the phosphorylation of the essential tyrosine in internalization signal. Such mechanism has been recently described for cytotoxic T lymphocyte-associated antigen (CTLA-4), which transmits costimulatory signals essential for optimal activation of T cells (Schwartz, 1992). Cytoplasmic tail of CTLA-4 contains the internalization signal ^{165}YVKM, which provides its binding to AP2 and internalization (Leung et al., 1995). In activated T cells the phosphorylation of the essential ^{165}Tyr residue of CTLA-4 by the T-lymphocyte associated tyrosine Src family (Fyn, Lyn, Lck) and Jak2 kinases prevents its interaction with μ2 subunit of AP2 and results in its expression on the cell surface where it binds CD80 and CD86 molecules (Bradshaw et al., 1997; Miyatake et al., 1998; Chikuma et al., 2000). The recognition pattern for Src family kinases (pTyr-hydrophylic-hydrophylic-[Leu/Ile/Pro] (Songyang et

al., 1993) overlaps the sialidase internalization signal allowing to speculate that similar mechanism may be implicated in the control of the expression of this enzyme on the T cell surface. If proven, this hypothesis will explain the mechanism of sialidase involvement in the immune response.

MATERIALS AND METHODS

Purification of lysosomal membranes from human liver

Samples were obtained from materials discarded from normal donor liver discharged during the reduced liver transplantation were homogenized in osmomolar (0.25 M) sucrose buffered with 10 mM HEPES-NaOH (pH 7.4) and 1 mM EDTA (homogenization buffer) using Teflon and glass Potter-Elvehjem homogenizer. Nucleus, cell debris and heavy mitochondria were removed from the homogenate by 10 min centrifugation at 3,000 g. The pellet was re-homogenized in the same amount of homogenization buffer and centrifugation was repeated. "Light mitochondrial fraction" containing the lysosomes, mitochondria, peroxisomes and partially microsomes was obtained by the centrifugation of combined supernatants at 16,000 g for 18 minutes.

Lysosomes were purified from light mitochondrial fraction by the ultracentrifugation (180,000 g for 3 hours in a Beckman VTi 65 vertical rotor) in a self-forming density gradient of metrizamide (OptiPrep, Nycomed) as described by Wattiaux et al. (1983). Immediately after centrifugation each tube was divided to 15 fractions, using a Beckman Tube slicer kit. Each fraction was essayed for activities of lysosomal membrane and matrix enzymes, marker enzymes for mitochondria, plasma membranes, peroxisomes and Golgi as well as for the presence of lysosomal membrane maker protein LAMP-2 by Western blot as described below. The gradient fractions containing the "light" pool of lysosomes were pulled together, diluted with three volumes of homogenization buffer and centrifuged for 20 min at 35,000 g to obtain the lysosomal pellet. For the purification of lysosomal membranes, the pellet was resuspended in ice-cold water containing the protease inhibitor cocktail (Boehringer Mannheim) and

sonicated (two times for 5 seconds at 50 W). Membranes were precipitated by 1 h centrifugation at 100,000 g and washed several times with ice-cold water containing protease inhibitor cocktail (Boehringer Mannheim). The lysosomal membranes were purified 150-200-fold compared to homogenate as determined by the increase of specific activity of the marker enzyme, β-glucocerebrosidase and were not cross-contaminated by microsomal (marker enzyme, UDP-galactosyl transferase) or plasma (marker enzyme, alkaline phosphatase) membranes.

Construction of sialidase mutants

Sialidase substitution mutants (SIAL-Y412A, SIAL-G413A and SIAL-L415A) were generated by site-directed mutagenesis of the wild-type sialidase cDNA (Pshezhetsky et al. 1997) using three-step overlap-extension PCR as described by Ling and Robinson (1997). Three polymerase chain reactions and four primers were used to introduce each mutation (Table 1). First, using the pCMV-SIAL expression vector as template, we obtained PCR fragment AB with sense primer (A) and mutagenic antisense primer (B) as well as PCR fragment CD with overlapping mutagenic sense primer (C) and antisense primer (D). Second, fragments AB and CD were linked by overlap PCR amplification using primers A and D to obtain the final product containing the desired mutation. In particular, TAT codon of Tyr412 was changed to GCT, GGG codon of Gly413 to GCG and CTC codon of Leu415 to GCC. DNA fragments containing the introduced mutations were obtained by double-digestion with *KpnI/Eco*RV and subcloned into the pCMV-SIAL expression plasmid previously used by us for the expression of sialidase (Lukong et al., 2000). The final constructs were verified by sequencing.

Transgenic expression in COS-7 cells and enzyme assays

COS-7 cells seeded in T-25 tissue culture flasks were transfected with pCMV-SIAL and pCMV-CathA expression vectors using Lipofectamine Plus reagent (Life Technologies, Gaithersburg, MD) as described (Lukong et al., 2000). Sialidase, N-acetyl β-hexosaminidase, β-galactosidase and β-glucosidase activities were assayed in cellular homogenates using the corresponding fluorogenic 4-methylumbelliferyl (muf)-glycoside substrates as described (Potier et al., 1979; Rome et al., 1979; Okada and O'Brien, 1968; Seymour and Peters, 1977). Cathepsin A activity was determined with CBZ-Phe-Leu (Pshezhetsky et al., 1995). Cathepsin B activity was assayed with Z-Arg-Arg-βNA (Gingras et al., 1999). Alkaline phosphatase activity was measured with the assay kit (Sigma, St Louis, MO) using the manufacturer's protocol. One unit of enzyme activity (U) is defined as the conversion of 1 μmol of substrate/min. Proteins were assayed according to Bradford (1976) with bovine serum albumin (Sigma, St Louis, MO) as a standard.

Immunofluorescence based internalization assay

48 h after transfection with wild-type or mutant sialidase, COS-7 cells were treated for 40 min with LysoTracker Red DND-99 (Molecular Probes, Eugene, OR) dye, washed twice with ice-cold PBS and fixed with 3% paraformaldehyde in PBS for 40 min. Cells were permeabilized by incubating with 0.3% Triton X-100, washed twice with PBS and stained with rabbit polyclonal anti-sialidase antibodies and FITC-conjugated monoclonal antibodies against rabbit IgG. Alternatively cells were double stained with rabbit

polyclonal anti-sialidase antibodies and monoclonal antibodies against lysosomal membrane marker LAMP-2 (Washington Biotechnology Inc., Baltimore). Slides were studied on a Zeiss LSM410 inverted confocal microscope (Carl Zeiss Inc., Thornwood).

Sialidase activity based internalization assay

For the purification of lysosomal membranes, 48 h after transfection, COS-7 cells from one T-25 flask were washed with Hank's balanced salt solution and then scraped in the presence of ice-cold homogenization buffer (0.25 M sucrose buffered with 10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA). Cells were homogenized by 20 strokes in glass and glass Potter-Elvehjem homogenizer and then the procedure for the purification of lysosomal membranes followed the protocol described above for the liver tissues. The only modification to that protocol was the reduced speed (1000 versus 3,000 g) during the first centrifugation step, which separated nuclei and cell debris from the rest of the homogenate.

Plasma membranes were purified as described (Kalish et al., 1978) by binding COS-7 cells to polylysine-coated beads (Cytodex 1 beads, Amersham Pharmacia Biotech, Baie d'Urfé, Canada). The preparation was enriched ~80-fold following the specific activity of alkaline phosphatase and did not contain lysosomal marker enzymes (β-hexosaminidase, β-galactosidase or β-glucocerebrosidase). To measure the sialidase activity on the outer cell surface, the cells seeded in 6-well culture dishes were washed several times with Hank's balanced salt solution and overlaid with 500 μl of 20 mM acetate buffer pH 5.2, 0.25 M sucrose and 0.4 mM fluorescent sialidase substrate,

MufNANA. After 30 min of incubation 200 μl aliquots of medium were added to 1.8 ml of 0.4 M glycine buffer pH 10.4 and the concentration of the fluorescent product was measured as described (Potier et al., 1979). To measure the total amount of the enzyme the assay was performed in the presence of 0.03% Triton X-100.

Western blotting

SDS-polyacrylamide gel electrophoresis of proteins was performed under reducing conditions according to the method of Laemmli (1970). After electrophoresis the proteins were electrotransferred to NITRO ME nitrocellulose membrane (Micron Separations Inc, Westboro, MA). The sialidase and cathepsin A detection on Western blots was performed with rabbit antibodies as described (Pshezhetsky and Potier, 1994; Vinogradova et al., 1998) and LAMP-2 detection, with monoclonal anti-human LAMP-2 antibodies (Washington Biotechnology Inc., Baltimore) using the BM Chemiluminiscence kit (Boehringer Mannheim) in accordance with the manufacturer's protocol.

ACKNOWLEDGEMENT

The authors thank Dr. Mila Ashmarina for critical reading of the manuscript and stimulating discussions. This work was supported by the operating grants from Canadian Institutes of Health Research (MT-15079), Fonds de La Recherche en Santé du Québec and Vaincre les Maladies Lysosomales Foundation (France) as well as by the equipment grant from Canadian Foundation for Innovation to A.V.P.

REFERENCES

Akasaki,K., Michihara,A., Fukuzawa,M., Kinoshita,H. and Tsuji,H. (1994) Cycling of an 85-kDa lysosomal membrane glycoprotein between the cell surface and lysosomes in cultured rat hepatocytes. *J. Biochem.,* **116**, 670-676.

Bansal,A. and Gierasch,L.M. (1991) The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. *Cell,* **67(6)**,1195-1201.

Blagoveshchenskaya,A.D., Hewitt,E.W. and Cutler,D.F. (1998) A balance of opposing signals within the cytoplasmic tail controls the lysosomal targeting of P-selectin. *J. Biol. Chem.,* **273(43)**, 27896-27903.

Bonten,E.J., van der Spoel,A., Fornerod,M., Grosveld,G. and d'Azzo,A. (1996) Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes and Devel.*, **10**, 3156-3168.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72**, 248-254.

Bradshaw,J.D., Lu,P., Leytze,G., Rodgers,J., Schieven,G.L., Bennett,K.L., Linsley,P.S., Kurtz,S.E. (1997) Interaction of the cytoplasmic tail of CTLA-4 (CD152) with a clathrin-associated protein is negatively regulated by tyrosine phosphorylation. *Biochemistry,* **36(50),** 15975-15982

Burnside,J. and Schneider,D.L. (1982) Characrerisation of the membrane proteins of rat liver lysosomes. *J. Biol. Chem.,* **204**, 525-534.

Callahan,J.W. (1999) Molecular basis of G_{M1}-gangliosidosis and Morquio disease, type B. Structure-function studies of lysosomal β-galactosidase and the non-lysosomal β-galactosidase-like protein. *Biochim. Biophys. Acta,* **1455(2-3)**, 85-103.

Canfield,W.M., Johnson,K.F., Ye,R.D., Gregory,W. and Kornfeld,S. (1991) Localization of the signal for rapid internalization of the bovine cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor to amino acids 24-29 of the cytoplasmic tail. *J. Biol. Chem.,* **266(9)**, 5682-5688.

Carrillo,M.B., Milner,C.N., Ball,S.T., Snock,M. and Campbell,R.D. (1997) Cloning and characterization of sialidase from the murine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele. *Glycobiology,* **7**, 975-986.

Chen,W.J., Goldstein,J.L. and Brown,M.S. (1990) NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.,* **265(6)**, 3116-3123.

Chen,X.P., Ding,X. and Daynes,R.A. (2000) Ganglioside control over IL-4 priming and cytokine production in activated T cells. *Cytokine,* **12(7)**, 972-985.

Chen,X.P., Enioutina,E.Y. and Daynes,R.A. (1997) The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J. Immunol.,* **158(7)**, 3070-3080.

Chikuma,S., Murakami,M., Tanaka,K., Uede,T. (2000) Janus kinase 2 is associated with a box 1-like motif and phosphorylates a critical tyrosine residue in the

cytoplasmic region of cytotoxic T lymphocyte associated molecule-4. *J. Cell. Biochem.*, **78(2)**, 241-250.

Collawn,J.F., Kuhn,L.A., Liu,L.F., Tainer,J.A. and Trowbridge,I.S. (1991) Transplanted LDL and mannose 6-phosphate receptor internalization signals promote high-efficiency endocytosis of the transferrin receptor. *EMBO J.*, **10(11)**:3247-3253.

Collawn,J.F., Lai,A., Domingo,D., Fitch,M., Hatton,S. and Trowbridge,I.S. (1993) YTRF is the conserved internalization signal of the transferrin receptor, and a second YTRF signal at position 31-34 enhances endocytosis. *J. Biol. Chem.*, **268(29)**, 21686-21692.

Collawn,J.F., Stangel,M., Kuhn,L.A., Esekogwu,V., Jing,S.Q., Trowbridge,I.S. and Tainer,J.A. (1990) Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell,* **63(5)**, 1061-1072.

d'Azzo,A., Andria,G., Strisciuglio,P. and Galijaard,H. (1995) Galactosialidosis. In Scriver,C.R., Beaudet,A.L, Sly,W.S. and Valle D., eds. *Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, pp. 2835-2837.

d'Azzo,A., Hoogeveen,A., Reuser,A.J., Robinson,D. and Galjaard,H. (1982) Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 4535-4539.

Dobrota,M. and Hinton,R. (1980) Large-scale preparation of highly-purified lysosomes from normal rat liver. *Anal. Biochem.*, **102**, 97-102.

Gingras,R., Richard,C., El-Alfy,M., Morales,C.R., Potier,M. and Pshezhetsky,A.V. (1999) Purification, cDNA cloning, and expression of a new human blood plasma glutamate carboxypeptidase homologous to N-acetyl-aspartyl-α-glutamate carboxypeptidase/prostate-specific membrane antigen. *J. Biol. Chem.,* **274(17)**, 11742-11750.

Gottschalk,S., Waheed,A., Schmidt,B., Laidler,P. and von Figura,K. (1989) Sequential processing of lysosomal acid phosphatase by a cytoplasmic thiol proteinase and a lysosomal aspartyl proteinase. *EMBO J.,* **8(11)**:3215-3219.

Guarnieri,F.G., Arterburn,L.M., Penno,M.B., Cha,Y. and August,J.T. (1993) The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein 1. *J. Biol. Chem.,* **268**, 1941-1946.

Hinek,A. (1996) Biological roles of the non-integrin elastin/laminin receptor *Biol. Chem.,* **377(7-8)**, 471-480.

Hirst,J. and Robinson,M.S. (1998) Clathrin and adaptors. *Biochim. Biophys. Acta,* **1404(1-2)**, 173-193.

Hoogeveen,A.T., Verheijen,F.W. and Galjaard,H. (1983) The relation between human lysosomal ß-galactosidase and its protective protein. *J. Biol. Chem.,* **258**, 12143-12146.

Igdoura,S.A., Gafuik,C., Mertineit,C., Saberi,F., Pshezhetsky,A.V., Potier,M., Trasier,J.M. and Gravel,R.A. (1998) Cloning of the cDNA and the gene encoding mouse lysosomal sialidase and correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts. *Hum. Mol. Genet.,* **7**, 115-121.

Jadot,M., Canfield,W.M., Gregory,W. and Kornfeld,S. (1992) Characterization of the signal for rapid internalization of the bovine mannose 6-phosphate/insulin-like growth factor-II receptor. *J. Biol. Chem.*, **267**, 11069-11077.

Jarousee,N. and Kelly,R.B. (2000) Selective inhibition of adaptor complex-mediated vesiculation. *Traffic*, **1**, 378-384.

Kalish,D.I., Cohen,C.M., Jacobson,B.S. and Branton,D. (1978) Membrane isolation on polylysine-coated glass beads. Asymmetry of bound membrane. *Biochim. Biophys. Acta,* **506(1)**, 97-110.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227**, 680-685.

Landolfi,N.F. and Cook,R.G. (1986) Activated T lymphocytes express class I molecules which are hyposialylated compared to other lymphocyte populations. *Mol. Immunol.,* **23(3)**, 297-309.

Landolfi,N.F., Leone,J., Womack,J.E. and Cook,R.G. (1985) Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase. *Immunogenetics,* **22(2)**, 159-167.

Lehmann,L.E., Eberle,W., Krull,S., Prill,V., Schmidt,B., Sander,C., von Figura,K. and Peters,C. (1992) The internalization signal in the cytoplasmic tail of lysosomal acid phosphatase consists of the hexapeptide PGYRHV. *EMBO J.,* **11(12)**, 4391-4399.

Leung,H.T., Bradshaw,J., Cleaveland,J.S., Linsley,P.S. **(1995)** Cytotoxic T lymphocyte-associated molecule-4, a high-avidity receptor for CD80 and CD86, contains an

intracellular localization motif in its cytoplasmic tail. *J. Biol. Chem.,* **270(42)**, 25107-25114

Ling,M.M. and Robinson,B.H. (1997) Approaches to DNA mutagenesis: an overview. Anal. Biochem., **254(2),** 157-178.

Lukong,K.E., Elsliger,M.A., Chang,Y., Richard,C., Thomas,G., Carey,W., Tylki-Szymanska,A., Czartoryska,B., Buchholz,T., Criado,G.R., Palmeri,S. and Pshezhetsky,A.V. (2000) Characterization of the sialidase molecular defects in sialidosis patients suggests the structural organization of the lysosomal multienzyme complex. *Hum. Mol. Genet.,* **9(7)**, 1075-1085.

Miyagi,T, Hata,K., Konno,K. and Tsuiki,S. (1992) Multiple forms of mammalian sialidase: altered expression in carcinogenesis. *Tohoku J. Exp. Med.,* **168(2)**, 223-229.

Miyagi,T., Hata,K., Hasegawa,A. and Aoyagi,T. (1993) Differential effect of various inhibitors on four types of rat sialidase. *Glycoconj. J.,* **10(1)**, 45-49.

Miyagi,T., Sagawa,J., Konno,K. and Tsuiki,S. (1990) Immunological discrimination of intralysosomal, cytosolic, and two membrane sialidases present in rat tissues. *J. Biochem.,* **107**, 794-798.

Naraparaju,V.R. and Yamamoto,N. (1994) Roles of β-galactosidase of B lymphocytes and sialidase of T lymphocytes in inflammation-primed activation of macrophages *Immunol. Lett.,* **43(3)**, 143-148.

Okada,S. and O'Brien,J.S. (1968) Generalized gangliosidosis: ß-galactosidase deficiency. *Science,* **160**, 1002-1004.

Okamoto,C.T., Shia,S.P., Bird,C. and Mostov,K.E. (1992) Roth MG The cytoplasmic domain of the polymeric immunoglobulin receptor contains two internalization signals that are distinct from its basolateral sorting signal. *J. Biol. Chem.,* **267(14)**, 9925-9932.

Okamura-Oho,Y., Zhang,S.Q. and Callahan,J.W. (1994) The biochemistry and clinical features of galactosialidosis. *Biochem. Biophys. Acta.,* **1225**, 244-254.

Ozaki,K., Itoh,N. and Kawasaki,T. (1993) Role of tyrosine-5 in the cytoplasmic tail of the macrophage asialoglycoprotein receptor in the rapid internalization of ligands. *J. Biochem. (Tokyo),* **113(3)**, 271-276.

Pearse,B.M., Smith,C.J. and Owen,D.J. (2000) Clathrin coat construction in endocytosis. *Curr. Opin. Struct. Biol.,* **10(2)**, 220-228.

Peters,C. and von Figura,K. (1994) Biogenesis of lysosomal membranes. *FEBS Lett.,* **346(1)**, 108-114.

Potier,M., Mameli,L., Belisle,M., Dallaire,L. and Melançon,S.B. (1979) Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-N-acetylneuraminate) substrate. *Anal. Biochem.*, **94**, 287-296.

Prince,L.S., Peter,K., Hatton,S.R., Zaliauskiene,L., Cotlin,L.F., Clancy,J.P., Marchase,R.B., Collawn,J.F. (1999) Efficient endocytosis of the cystic fibrosis transmembrane conductance regulator requires a tyrosine-based signal. *J. Biol. Chem.,* **274(6)**, 3602-3609.

Pshezhetsky,A.V. and Potier,M. (1994) Direct affinity purification and supramolecular organization of human lysosomal cathepsin A. *Arch. Biochem. and Biophys.,* **313**, 64-70.

Pshezhetsky,A.V. and Potier,M. (1996) Association of N-acetyl-galactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of ß-galactosidase, cathepsin A and neuraminidase: possible implication for intralysosomal catabolism of keratan sulfate. *J. Biol. Chem.,* **271**, 28359-28365.

Pshezhetsky,A.V., Richard,C., Michaud,L., Igdoura,S., Wang,S., Elsliger, M.A., Qu,J., Leclerc,D., Gravel,R., Dallaire,L. and Potier,M. (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nat. Genet.*, **15**, 316-320.

Pshezhetsky,A.V., Vinogradova,M.V., Elsliger,M-A., El-Zein,F., Svedas,V.K. and Potier,M. (1995) Continuous Spectrophotometric Assay of Human Lysosomal Cathepsin A/Protective Protein in Normal and Galactosialidosis Cells. *Anal. Biochem.,* **230,** 303-307.

Rome,L.H., Garvin,A.J., Allietta,M.M. and Neufeld,E.F. (1979) Two species of lysosomal organelles in cultured human fibroblasts. *Cell,* **17**,143-153.

Rottier,R.J. Bonten,E. and d'Azzo,A. (1998) A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. *Hum. Mol. Genetics,* **7**, 313-321.

Schwartz,R.H. (1992) Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell,* **71(7)**, 1065-1068.

Seymour,C.A. and Peters,T.J. (1977) Enzyme activities in human liver biopsies: assay methods and activities of some lysosomal and membrane-bound enzymes in control tissue and serum. *Clin. Sci. Mol. Med.,* **52(3)**, 229-239.

Songyang,Z., Shoelson,S.E., Chaudhuri,M., Gish,G., Pawson,T., Haser,W.G., King,F., Roberts,T., Ratnofsky,S., Lechleider,R.J., et al. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell,* **72 (5)**, 767-778.

Thomas,G.H. and Beaudet,A.L. (1995). Disorders of Glycoprotein Degradation and structure: α-mannosidosis, β-mannosidosis, fucosidosis, sialidosis, aspartylglucosaminuria, and carbohydrate-deficient glycoprotein syndrome. In Scriver,C.R., Beaudet,A.L., Sly,W.S., Valle,D., eds, *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, N.Y., pp 2529-2561.

van der Spoel,A., Bonten,E. and Azzo,A. (1998) Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. *EMBO J.*, **17**, 1588-1597.

Verheijen,F.W., Janse,H.C., van Diggelen,O.P., Bakker,H.D., Loonen,M.C., Durand,P. and Galjaard,H. (1983) Two genetically different mu-nana neuraminidases in human leucocytes. *Biochem. Biophys. Res. Commun.,* **117**, 470-478.

Vinogradova,M.V., Michaud,L., Mezentsev,A.V., Lukong,K.E., El-Alfy,M., Morales,C.R., Potier,M. and Pshezhetsky,A.V. (1998) Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation. *Biochem. J.,* **330 (Pt 2)**, 641-650.

Waheed,A., Gottschalk,S., Hille,A., Krentler,C., Pohlmann,R., Braulke,T., Hauser,H., Geuze,H. and von Figura,K. (1988) Human lysosomal acid phosphatase is transported as a transmembrane protein to lysosomes in transfected baby hamster kidney cells. *EMBO J.,* **7(8)**, 2351-2358.

Wattiaux,R. and Wattiaux-De Conninck,S. (1983) In Iodinated Density Gradient Media – a practical approach (ed. Rickwood, D.) Oxford Univ. Press. pp 119-137.

Yamamoto,N. and Kumashiro,R. (1993) Conversion of vitamin D3-binding protein (group-specific component) to a macrophage activating factor by the stepwise action of β-galactosidase of B cells and sialidase of T cells. *J. Immunol.,* **151(5)**, 2794-2802.

Yamamoto,N. and Naraparaju,V.R. (1996) Role of vitamin D3-binding protein in activation of mouse macrophages. *J. Immunol.,* **157(4)**, 1744-1749.

Legends to Figures

Figure 1. Distribution of marker enzymes and proteins in fractions obtained during the centrifugation of "light mitochondrial fraction" from mouse liver in a self-forming density gradient of metrizamide.

A. Distribution of marker enzymes for lysosomes (β-hexosaminidase), mitochondria (γ-glutamate dehydrogenase), peroxisomes (peroxidase) and microsomes (UDP-galactosyl transferase).

B. Distribution of lysosomal proteins.

Top: Activities of soluble (β-galactosidase, cathepsin B and β-hexosaminidase) and membrane (β-glucocerebrosidase, acid phosphatase) lysosomal enzymes and sialidase.

Bottom: Western blot showing the distribution of LAMP-2 in the same fractions.

The centrifugation, enzyme assays and western blot were performed as described in Materials and methods. The results represent mean values of three independent experiments.

Figure 2. Amino acid sequence alignment of C-terminus peptide of human lysosomal sialidase with those of membrane lysosomal proteins and endocytosed receptors. Essential conserved tyrosine and hydrophobic amino acid residues of the internalization signal are shown in bold. Above the alignment the secondary structural elements predicted in sialidase sequence from its structural alignment with crystallized bacterial sialidases (Lukong et al., 2000) or using the empirical algorithms are indicated.

α-Helixes are shown by "H", β-sheets, by "E" and turns, by "T". SIAL, human lysosomal sialidase (residues 396-415); MPR300, bovine cation-independent mannose 6-phosphate receptor (residues 2348-2367); TF-R, human transferrin receptor (residues 7-25). LAP, human lysosomal phosphatase (residues 399-418); LAMP-1, human lysosome-associated membrane glycoprotein 1 (residues 397-416); LAMP-2, human lysosome-associated membrane glycoprotein 2 (residues 391-410); CD63, human CD63 antigen (LAMP-3, residues 218-237).

Figure 3. Expression of mutant sialidase in COS-7 cells.

A. Specific sialidase activity (nmol/min mg of total protein) in cellular lysates of COS-7 cells measured 48 h after co-transfection with cathepsin A and mutant sialidase cDNA. Cells were transfected and enzyme activities assayed as described in Materials and methods. **Control** - control COS-7 cells; **WT** – cells transfected with the wild-type sialidase cDNA, **WT + CathA** – cells co-transfected with the wild-type sialidase and cathepsin A cDNA. Enzyme activities in the cell lysates were determined 48 h after transfection. Values represent means ± S.D. of five independent experiments. The cathepsin A activity was similar in all transfected cells suggesting the same transfection efficiency.

B. Detection of sialidase in the cellular lysates of COS-7 cells co-transfected with cathepsinA and mutant sialidase cDNA by Western blot. Lysate aliquotes (20 μg of protein) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and stained with rabbit anti-sialidase antibodies as described. Sample indication is the same as on the upper panel.

Figure 4. Immunohistochemical localization of sialidase mutants expressed in COS-7 cells.

COS-7 cells were probed with 75 mM of lysosomal marker, LysoTracker Red DND-99 for 30 min at 370 C, 48 h post-transfection with cathepsin A and wild-type or mutant sialidase cDNAs, fixed and stained with rabbit polyclonal anti-sialidase antibodies. Slides were studied on a Zeiss LSM410 inverted confocal microscope. Upper panel: anti-sialidase antibodies (green); middle panel: LysoTracker (red); lower panel: co-localization of anti-sialidase antibodies and LysoTracker. Bar =10 μm.

Figure 5. Sialidase activity of the plasma (A) and lysosomal (B) membranes of COS-7 cells transfected with sialidase mutants.

Plasma and lysosomal membranes were purified 48 h after co-transfection of COS-7 cells with cathepsin A and mutant sialidase cDNA as described in Materials and methods. Specific sialidase (black bars) and control endogenous alkaline phosphatase (open bars, **A**) and β-glucosidase (open bars, **B**) activities are shown as a fraction of those measured in the membranes of non-transfected COS-7 cells (**Control**). **WT + CathA** – cells co-transfected with the wild-type sialidase and cathepsin A cDNA. Values represent means ± S.D. of triplicate experiments.

Figure 6. Sialidase activity measured on the cell surface of COS-7 cells transfected with sialidase mutants.

48 h after co-transfection of COS-7 cells with cathepsin A and mutant sialidase cDNA cells were incubated in the osmomolar buffer solution containing sialidase substrate in the absence (black bars) or in the presence (open bars) of 0.03% (v/v) Triton X-100 as described in Material and methods. For each series specific sialidase activity is shown as fraction of that of non-transfected COS-7 cells (**Control**). **WT + CathA** – cells co-transfected with the wild-type sialidase and cathepsin A cDNA. Values represent means ± S.D. of triplicate experiments. The activity of the control endogenous lysosomal enzyme N-acetyl β-hexosaminidase (not shown) demonstrated the same latency of 90-95% for all the cells studied.

Mutation	**Mutagenic oligonucleotide**
Tyr412Ala	B 5'-CAA AAT CAG TGT CGC TGG GAC ACT CTG AGC-3' C 5'-GCT CAG AGT GTC CCA GCG ACA CTG ATT TTG-3'
Gly413Ala	B 5'-CAG TGT CTA TGC GAC ACT CTG AGC-3' C 5'-AGA GTG TCG CAT AGA CAC TGA TTT-3'
Leu415Ala	B 5'-CAG TGT CTA TGG GAC AGC CTG AGC AGC TGT-3' C 5'-GGC ACA GCT CAG GCT GTC CCA TAG ACA CTG-3'
Sense primer	A 5'-CCG AGT GAA CCT GAC CCT GCG ATG G-3'
Antisense primer	D 5'-GGA TCC CCG CGG CCG CCA GTG TG-3'

Table 1. Primers used in overlap-extension PCR. B, mutagenic sense primer; C, mutagenic antisense primer; A, sense primer; D, antisense primer. Mismatched nucleotides are underlined.

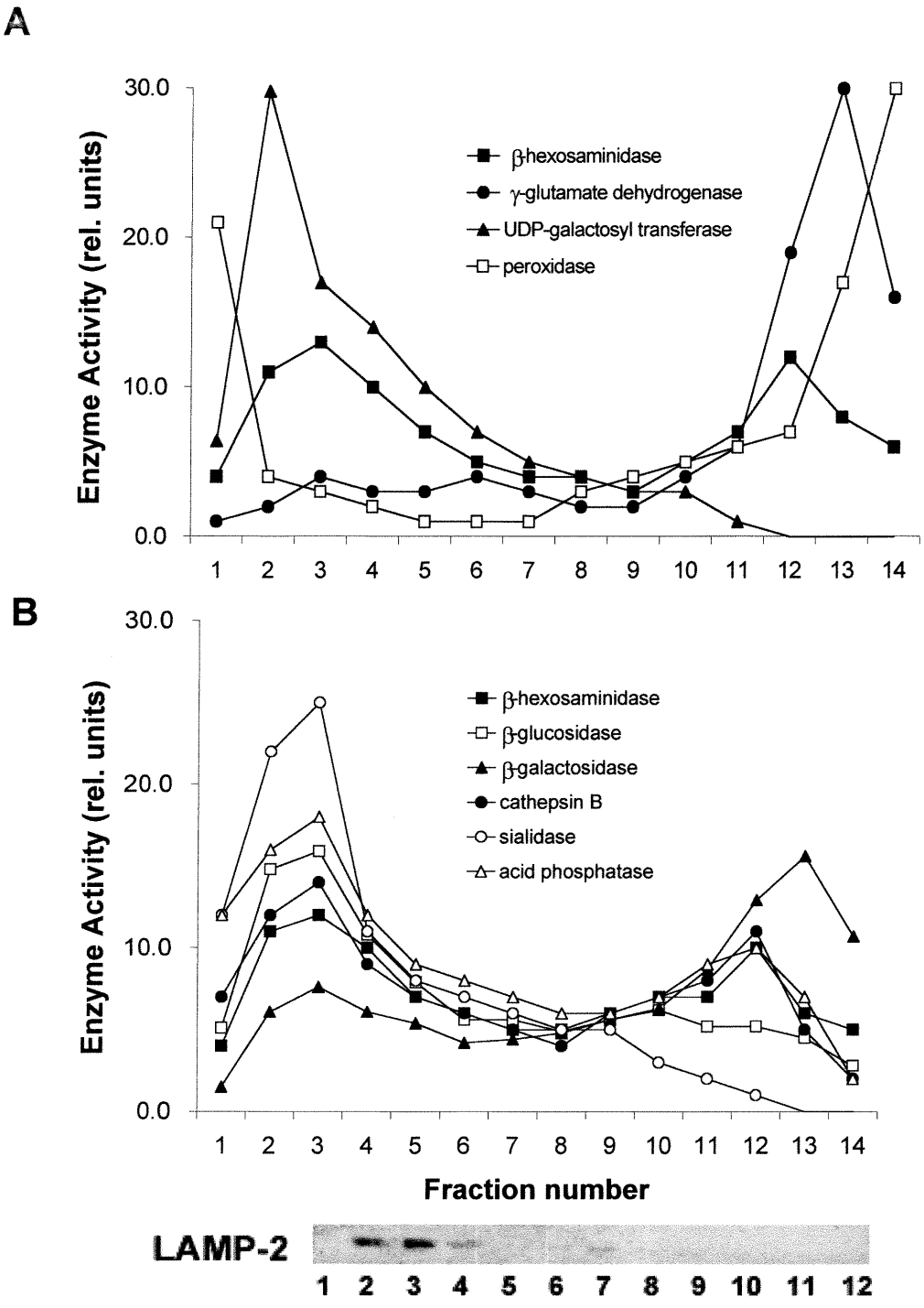
A
30.0
20.0
10.0
0.0
Enzyme Activity (rel. units)
β-hexosaminidase
γ-glutamate dehydrogenase
UDP-galactosyl transferase
peroxidase
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
30.0
20.0
10.0
0.0
Enzyme Activity (rel. units)
β-hexosaminidase
β-glucosidase
β-galactosidase
cathepsin B
sialidase
acid phosphatase
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Fraction number
LAMP-2
1 2 3 4 5 6 7 8 9 10 11 12

FIGURE 1


TTEEEEEEHHHHHHHHTTTT
SIAL (396)GRNHYTESISVAKISVYGTL(415)
MPR300 (2348)LTNCCRRSANVSYKYSKVNK(2367)
TF-R (7)SAFSNLFGGEPLSYTRFSL(25)
LAP (399)LLTVLFRMQAQPPGYRHVAD(418)
LAMP-1 (397)IVLIAYLVGRKRSHAGYQTI(416)
LAMP-2 (391)LVLLAYFIGLKHHHAGYEQF(410)
CD63 (218)LGIVFACCLVKSIRSGYEVM(237)

FIGURE 2

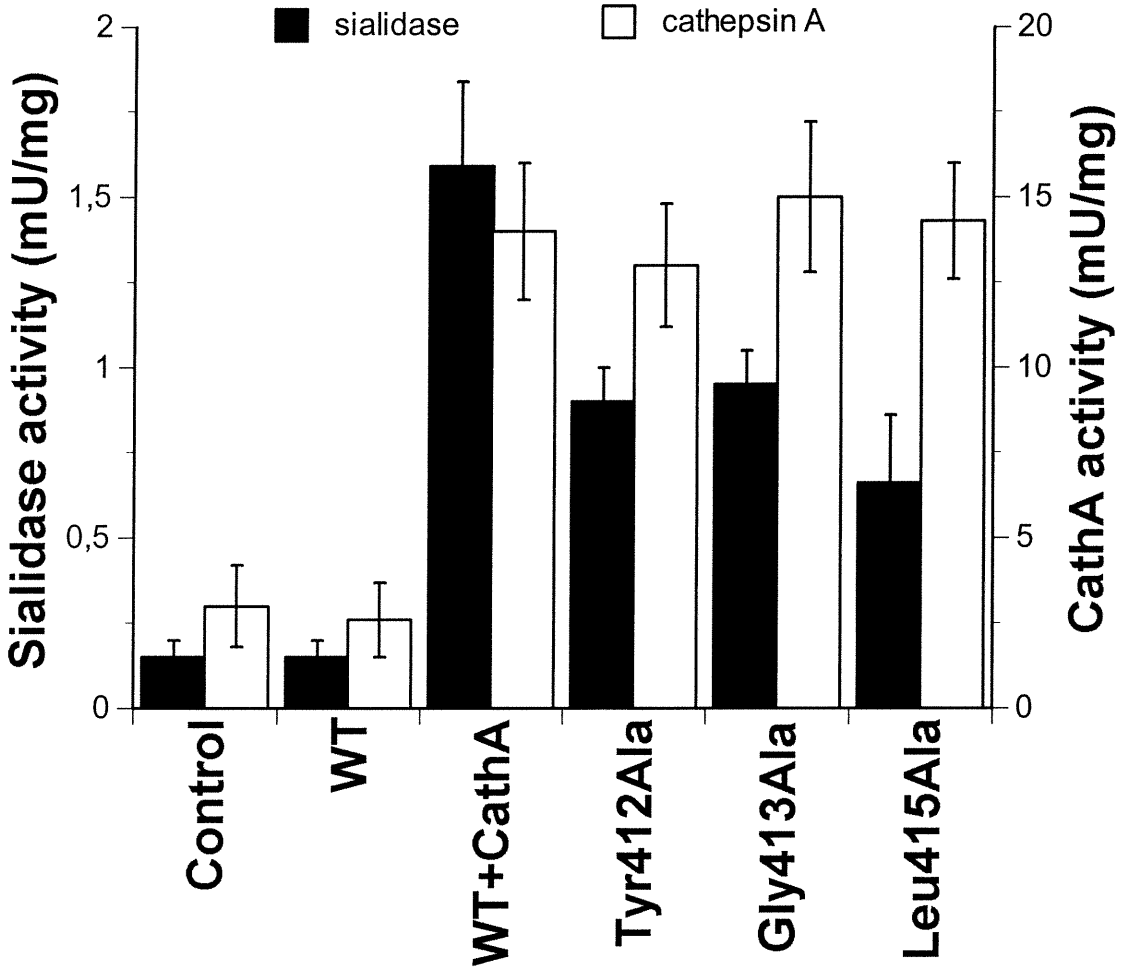
sialidase
cathepsin A
Sialidase activity (mU/mg)
CathA activity (mU/mg)
2
1,5
1
0,5
0
20
15
10
5
0
Control
WT
WT+CathA
Tyr412Ala
Gly413Ala
Leu415Ala

FIGURE 3a

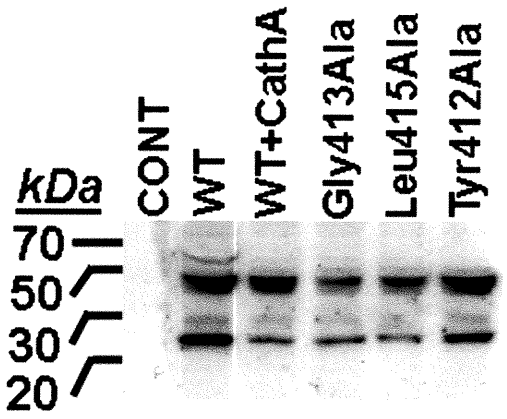
kDa
70
50
30
20
CONT
WT
WT+CathA
Gly413Ala
Leu415Ala
Tyr412Ala

FIGURE 3b

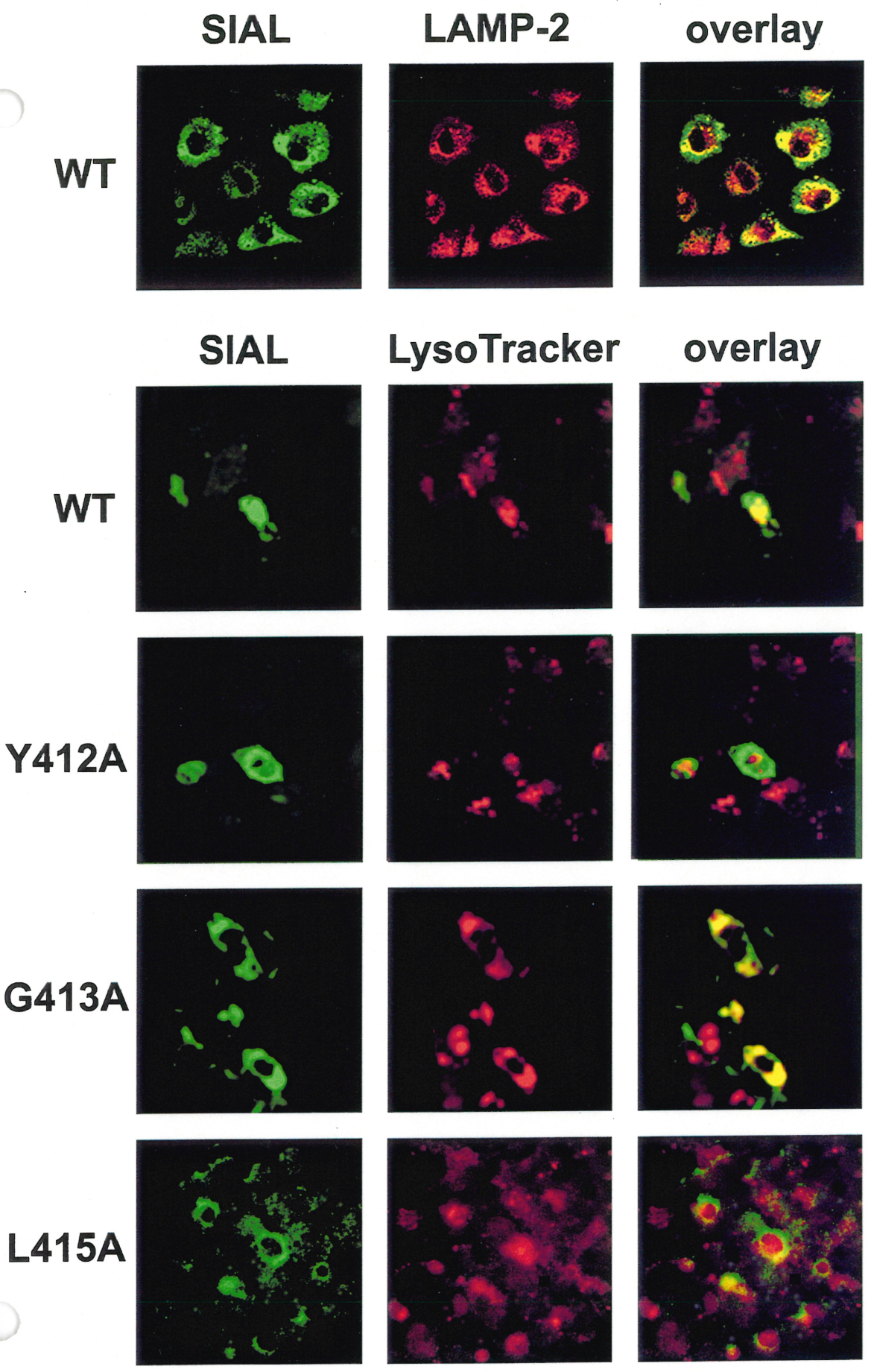
SIAL
LAMP-2
overlay
WT
SIAL
LysoTracker
overlay
WT
Y412A
G413A
L415A

FIGURE 4

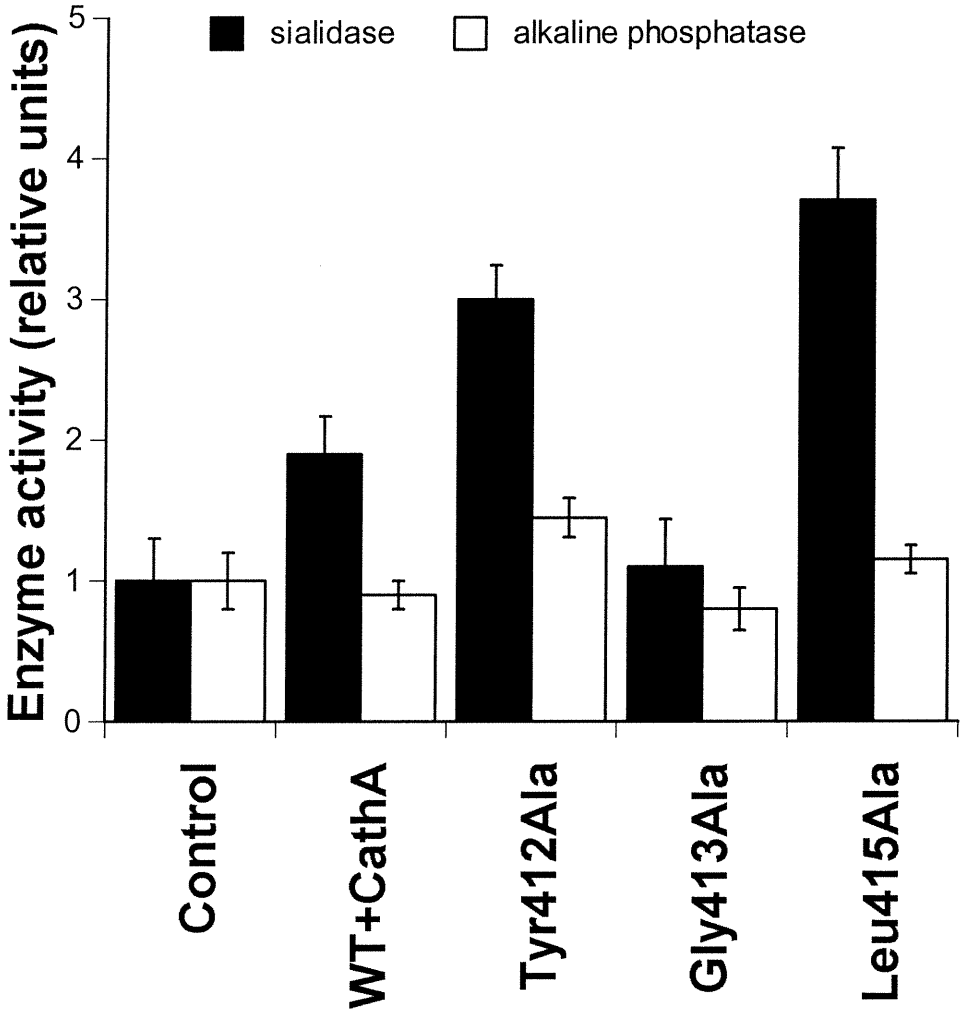
sialidase
alkaline phosphatase
Enzyme activity (relative units)
0
1
2
3
4
5
Control
WT+CathA
Tyr412Ala
Gly413Ala
Leu415Ala

FIGURE 5a

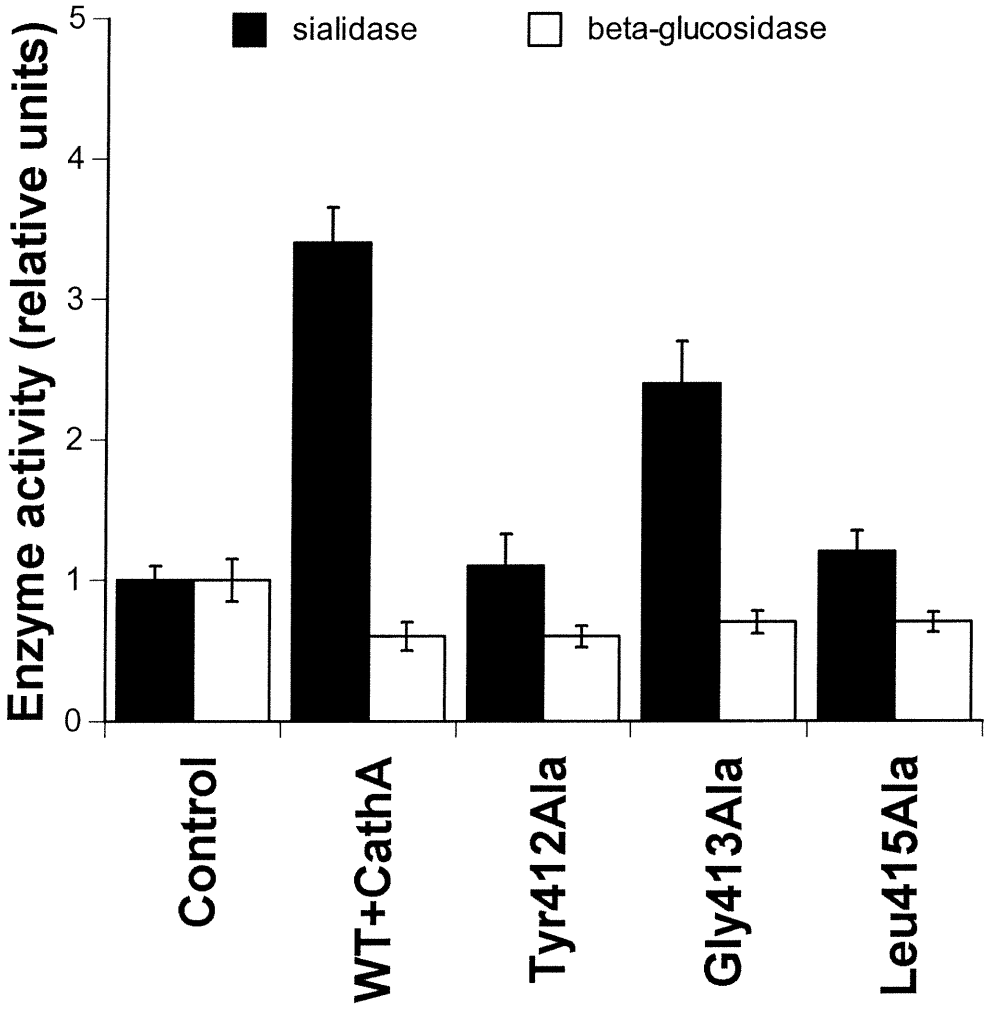
sialidase
beta-glucosidase
Enzyme activity (relative units)
0
1
2
3
4
5
Control
WT+CathA
Tyr412Ala
Gly413Ala
Leu415Ala

FIGURE 5b

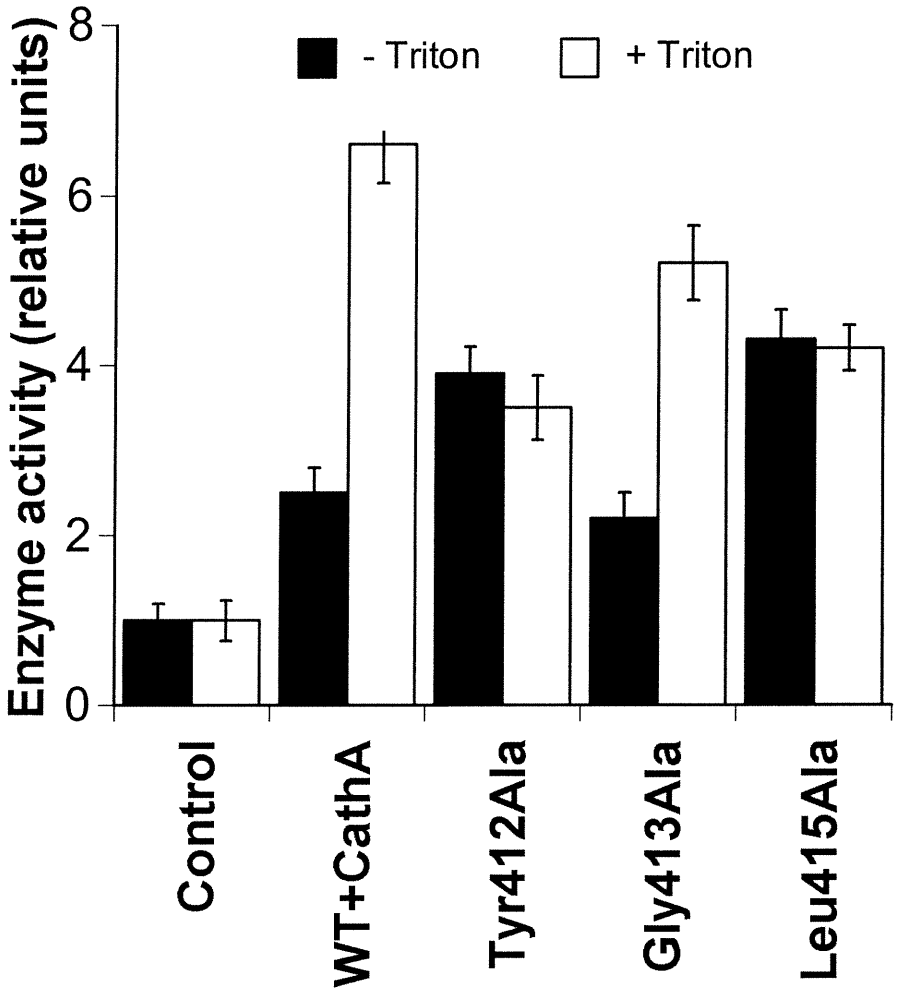
Enzyme activity (relative units)
8
6
4
2
0
- Triton
+ Triton
Control
WT+CathA
Tyr412Ala
Gly413Ala
Leu415Ala

FIGURE 6

CHAPTER 5

General discussion and conclusions

General discussion

The main goal of my project was to study the biochemical properties and biogenesis of SIAL and to understand the molecular mechanisms of its deficiency in two inherited diseases of children, galactosialidosis and sialidosis.

First we studied the mechanism of targeting of SIAL precursor to the lysosome. We demonstrated that although SIAL is N-glycosylated, it does not use the mannose-6-phosphate pathway to reach the lysosome. Furthermore, SIAL does not require the association with CathA for its targeting since it is still sorted to the lysosomes in galactosialidosis cells that lack CathA (Article 1). Instead, SIAL is targeted to the lysosomes via a mechanism involving the interaction of a C-terminal motif (Tyr-X-X-Φ) with an adaptor complex. By measuring the internalization of a series of SIAL substitution mutants we showed that Y412 and L415 residues of SIAL are essential for its rapid endocytosis (Article 6). Our results suggest that lysosomal sialidase is an integral membrane protein containing a single transmembrane domain and a short cytoplasmic tail carying the internalization signal.

The suggested mechanism of SIAL sorting optimally fits its dual physiological role: intralysosomal catabolism of sialylated glycoconjugates and cellular signaling during the immune response (Yamamoto et al., 1993; Landolfi et al., 1985; Chen et al., 1997). SIAL in the multienzyme complex may play a role of an anchor that attaches the complex to the endosomal or plasma membrane. This could be of particular importance for a plasma membrane complex containing SIAL, CathA precursor and an alternatively spliced form of GAL,

elastin-binding protein, essential for cell binding to the extracellular elastic fibres (Hinek, 1996). Our results therefore provide a completely new insight into the physiological role of SIAL.

For years, purification and complete biochemical characterization of SIAL had been hampered by low tissue content and instability of the enzyme (Van der Horst et al., 1989, 1993; Hiraiwa et al., 1988). We purified the multienzyme complex containing SIAL from human placenta by affinity chromatography and separated the components of the complex by FPLC and SDS electrophoresis.

We have raised the antibody against the recombinant SIAL polypeptide that allowed us to demonstrate that SIAL is a 48.3 kDa component of the complex, representing about 10% of its protein. The molecular mass of this protein (43 kDa after the removal of the oligosaccharide chains with endoglycosidase F) corresponded to the predicted mass of the enzyme after cleavage of 47-amino acid signal peptide and N-glycosylation of the three potential glycosylation sites. These data were confirmed by N-terminal sequencing of the mature protein (Article 1).

SIAL is unique amongst the sialidase superfamily in that it requires another protein, CathA, to be catalytically active. Various molecular mechanisms of SIAL activation have been suggested. Verheijen et al. (1985) reported that SIAL can be activated by 90-120 min incubation of concentrated crude glycoprotein fraction at 37°C and concluded that this activation is the result of the SIAL-CathA complex formation. However, according to other authors, activation of SIAL in vitro involved its proteolytic processing (D'Agrosa et al., 1988; Hiraiwa

et al., 1993). We verified both hypotheses and provided a clear picture of the molecular mechanisms involved in SIAL activation. First, SIAL acquires its catalytic active conformation only in association with CathA in the 1.27 MDa complex, which also increases the enzyme's affinity for its substrate. CathA protects SIAL against rapid intralysosomal degradation. In the cells obtained from patients affected with galactosialidosis, an inherited disorder characterized by the combined deficiency of SIAL and GAL due to the mutations in CathA, the 48.3 kDa mature active form of SIAL is rapidly degraded into 38.7 and 24 kDa catalytically inactive forms. This process markedly shortens the half-life of SIAL from 2.7 h in normal fibroblasts to 30 min in galactosialidosis fibroblasts (Article 1). These studies demonstrated the molecular mechanism of SIAL deficiency in galactosialidosis.

Sialidosis is another autosomal recessive lysosomal storage disease characterized by SIAL deficiency, which in this case results directly from mutations in the SIAL gene (Thomas and Beaudet, 1995).

We elucidated the pathogenesis of sialidosis by performing the analysis of SIAL mutations in sialidosis patients of different ethnic origins. To develop a universal strategy for the identification of molecular defects in sialidosis, we have cloned and sequenced the SIAL gene and designed oligonucleotide primers for amplification of all 6 exons of SIAL. We found that one patient had a frameshift mutation (G623delG deletion), which introduced a premature termination codon, truncating 113 amino acids. A complete clinical analysis (Article 4) indicated that this patient suffered from congenital sialidosis and presented uncommon features

such as excessive tortuosity of the retinal arteries and severely dilated coronary arteries. The other patients all had missense mutations which induced amino acid changes: Gly227Arg, Ala298Val, Gly68Val, Ser182Gly, Leu270Phe and Gly328Ser (Article 1). Two other mutations, Val217Met and Gly243Arg, both compound heterozygotes were identified in two unrelated Japanese patients (Article 3). We introduced the new missense mutations and previously identified mutations 779T A (Phe260Tyr) and 1088T C (Leu363Pro) (Pshezhetsky et al., 1997) in the pCMV-SIAL expression vector and co-expressed the mutant sialidase with CathA in COS-7 cells. All mutants showed 0 to 40 % decrease in enzyme activity. Western blot analysis indicated that all mutants are expressed but Ala298Val, Leu270Phe, Phe260Tyr and Gly328Ser are less stable as they are degraded to 37, 26 and 24kDa fragments. Both Val217Met and Gly243Arg mutants expressed lower amounts of protein.

To predict the structural changes caused by the mutations, we modeled the three-dimensional structure of human lysosomal sialidase. The structural models of lysosomal sialidase were built using the atomic coordinates of homologous sialidases from *Micromonospora Viridifaciens* (EUR), *Salmonella Typhimurium* (SIL) and *Vibrio Cholerae* (KIT) as templates (Article 2, Fig. 5 and 6; Artcle 3, Fig. 4).

The derived models predict that SIAL retains the basic fold of bacterial and viral sialidases. This fold consists of six four-stranded antiparallel β-sheets that resemble a six-bladed propeller, arranged around a pseudo six-fold axis (Varghese et al., 1983; Burmeister et al., 1992; Crennell et al., 1993). The topology of the

catalytic domain and the active site residues in our models exactly match those of bacterial and viral sialidases. Arg78 in human enzyme situated in the RIP/RLP motif may be involved in binding the sialic acid carboxylate group and both Arg280 and Arg341 ensure stability of the carboxylate group. The conserved Asp135 in the human enzyme is in the proper location to bind the N-acetyl-/N-glycosyl group of the substrate and Glu394, Tyr370 or Glu264 may donate a proton in the process of the substrate hydrolysis (Crennell et al., 1993; Gaskell et. al., 1995; Crennell et al., 1994). Asp 103 may serve as a proton donor for the glycosidic bond or a stabilizer of the proton-donating water molecule.

So far 21 amino acid substitutions have been identified in sialidosis patients (Bonten et al., 1996, 2000; Pshezhetsky et al., 1997; Milner et al., 1997; Lukong et al., 2000; Naganawa et al., 2000; Sakuraba, private communication). The localization of the missence mutations on SIAL structural model (Article 5, Fig. 4) suggested that several of them affect active site residues (Tyr370Cys) or may interfere with their correct positions (For example, Leu91Arg with the active site residue R^{78}; Pro80Leu with R^{97}; dup399HisTyr with E^{394}; Pro316Ser with R^{280}; and Pro335Gln with R^{341}).

Gly68Val is part of a short α-helix located in the first β-sheet unit. Even though this mutant produced a severe phenotype, its location does not provide any obvious structural impact. Gly227Arg, Val217Met and Gly243Arg are part of the third β-sheet unit. The substitution of Gly227, by Arg, a large charged amino acid residue can conceivably affect structure of the enzyme. From simulation (Article 3, Fig. 6) we can predict that Gly243Arg can cause drastic structural

change that can affect up to 38 residues. The structural effect of Val217Met is mild and compatible with the presence of residual enzyme activity.

Ser182Gly and Ala298Val are situated in the second and fourth Asp boxes respectively. Ser182Gly is exposed in a long flexible loop and completely solvent accessible, and this explains its mild phenotype. Ala 298Val is in the center of a turn loop in the fourth β-sheet unit and incompatible with folding defect. Previously identified Leu270Phe and Phe260Tyr are also part of the fourth β-sheet unit. Leu270 is conserved in *Micromonospora* and *Vibrio* enzymes and its replacement with a bulky Phe is expected to produce significant structural shift, which is consistent with the low expression and instability of the mutant (Article 2, Fig. 4). Phe260 is located in a surface loop region absence in other sialidase structures and may be involve in protein-protein interaction. Gly328Ser and Leu363Pro, (Pshezhetsky et al., 1997) are within the fifth and sixth β-sheets respectively. Substitution of Leu363 for Pro in the model results in a steric clash with the backbone carboxylate of residue Leu344 located in the adjacent β strand. The structural impact of Gly328Ser is less obvious.

As compared to mutations in CathA gene in galactosialidosis (Zhou et al., 1991, 1996; Shimmoto et al., 1990, 1993; Fukuhara et al., 1992; Richard et al., 1998) and those in GAL gene in ß-galactosidosis (Nishimoto et al., 1991; Yoshida et al., 1991, 1992; Oshima et al., 1991, 1992; Chakraborty et al., 1994), sialidosis shows very distinct spectrum of molecular defects. First, most of sialidosis patients studied so far had point mutations in the SIAL cDNA, leading to amino acid substitutions and not the frameshifts or splicing defects (Table 1).

Second, the localization of the mutations on the SIAL structural model showed that in contrast to mutation in CathA, that mostly affect the enzyme central core and cause unfolding of the protein, SIAL mutations involve residues on the surface of the enzyme, and generally are not predicted to result in significant structural changes. Moreover, the distribution of mutations on the SIAL surface is uneven. Our model shows that a majority of mutations resulting in complete or almost complete inactivation of the enzyme and causing severe sialidosis type II phenotype are clustered in one region of the structure (Article 5, Fig. 4). In particular this region contains mutations Gly227Arg, Phe260Tyr, Leu270Phe and Ala298Val identified by us (Article 2), Arg294Ser, Leu231His, and Gly218Ala identified by Bonten (Bonten et al., 2000) and Trp240Arg and Pro316Ser identified by Sakuraba (Sakuraba, private communication). This observation led us to speculate that this cluster may represent the sialidase binding interface to the lysosomal complex and mutations in the region could potentially destabilize the complex, exposing sialidase to inactivation and/or rapid degradation.

We have expressed 8 SIAL mutants 4 of which contained amino acid substitution in the defined surface patch of the fifth ß-sheet (Gly227Arg, Phe260Tyr, Leu270Phe and Ala298Val) and 4 at the opposite surface of the SIAL molecule (Gly68Val, Ser182Gly and Leu363Pro) in COS-7 cells and studied sorting, activity and stability of the produced protein (Article 5). We found that in two cases (Gly227Arg and Leu363Pro) the mutant protein was not sorted to the lysosomes suggesting that these mutations can cause general folding defects

and retention of the mutants in the pre-lysosomal compartments. All other expressed SIAL mutants were targeted to lysosomes and correctly processed.

Subsequent experiments revealed that the SIAL mutants containing amino acid substitutions in the surface patch of the fifth β-sheet have similar properties. First, they have had very low or absent SIAL activity. Second, stability of SIAL mutants in cellular homogenates or their half-life in the cell estimated by metabolic labeling was significantly lower than that of the wild-type enzyme. In addition, previous analysis of COS-7 cells transfected with Ala298Val, Leu270Phe and Phe260Tyr mutants by Western blot (Article 2) demonstrated the presence of 37 kDa, 26 kDa and 24 kDa fragments of SIAL protein similar to those observed in COS-7 cells in which wild-type SIAL was expressed in the absence of human CathA. Similar pattern of degradation products and dramatically reduced half-life was also observed for wild-type SIAL expressed in the cells of a galactosialidosis patient, which lack functional CathA (Article 1). Together these data suggest that Ala298Val, Leu270Phe and Phe260Tyr mutants are not protected by CathA, although the same high amount of functional human CathA was expressed by COS-7 cells in all cases. Indeed in the extracts of cells transfected with Ala298Val, Leu270Phe and Phe260Tyr mutants we could not detect high molecular weight complex of SIAL with CathA (Article 5). Altogether observed data allowed us to hypothesize that Ala298Val, Leu270Phe and Phe260Tyr mutations in SIAL destabilize its association with CathA in the complex, which inactivates the enzyme even though they do not directly affect

activity or stability of SIAL. One can speculate that the other mutations located in and around the fifth ß-sheet patch may also affect the integrity of the complex.

Altogether our results bring new insight into the biogenesis and function of lysosomes and provide the molecular mechanism of two severe inherited disorders.

Table 1

Mutations in the sialidase gene

Mutations in the sialidase gene

Patient	Clinical Phenotype	Origin	Nucleotide Change	Putative consequence	Reference
1	II	Mexican-American	679G→A	Gly227Arg	Article 2
2	II	Jewish	893C→T	Ala298Val	Article 2
3	I	Chinese	544A→G	Ser182Gly	Article 2
4	II	Turkish	623delG	Frameshift, Stop304	Article 2
5	II	Polish	203G→T	Gly68Val	Article 2
6 and 7	II	Spanish	808C→T	Leu270Phe	Article 2
8	I	Italian	982G→A	Gly328Ser	Article 2
9 and 10	I	Japanese	649G→A 727G→A	Val217Met Gly243Arg	Article 3 Article 3
11	II	Anatolian	679delG	Frameshift, Stop 302	Willenbring et al., 2000
12	NA	Japanese	236C→T	Pro80Leu	Sakuraba Private commun.
13	NA	Japanese	718T→C	Trp240Arg	Sakuraba Private commun.

Patient	Clinical Phenotype	Origin	Nucleotide Change	Putative consequence	Reference
14	NA	Japanese	946C→T	Pro316Ser	Sakuraba Private commun.
15	I	German	1127G→T	Frameshift, Stop 377	Bonten et al., 1996
16	II	Italian	401T→G 1337delG	Leu91Arg Frameshift, + 69 amino acids	Bonten et al., 1996 Bonten et al., 1996
17	II	GM01718A cell line	779T→A	Phe260Tyr	Pshezhetsky et al., 1997
			1088T→C	Leu363Pro	Pshezhetsky et al., 1997
18	II	GM11604 cell line	7insACTG	Frameshift	Pshezhetsky et al., 1997
19	I	African-American	878C→T	Arg294Ser	Bonten et al., 2000
			690T→A	Leu231His	Bonten et al., 2000
20	I	African-American	654G→A	Gly218Ala	Bonten et al., 2000
21 and 22	I	German	159G→A	Val54Met	Bonten et al., 2000
23	I	Dutch	Dpl1196 ACCACT	Dpl399 His/Tyr	Bonten et al., 2000
24	II	Mexican-American	1107A→G	Tyr370Cys	Bonten et al., 2000
25	II	Italian	836C→T 1002C→A	Arg280Stop Pro335Gln	Bonten et al., 2000 Bonten et al., 2000

NA= Not available

Conclusions

1) We have studied the synthesis, processing and activation of SIAL and showed that the enzyme is synthesized as a 45.5 kDa precursor and becomes a 48.3 kDa mature active protein after cleavage of the signal peptide and N-glycosylation. SIAL acquires its catalytically active form and is stable only after associating with CathA. SIAL is sorted both to the lysosome and the plasma membrane in a process mediated by a C-terminal motif.
2) We demonstrated that SIAL is targeted to the plasma membrane and then internalized to the endosomal/lysosomal compartment.
3) We showed that in patients affected with the lysosomal storage disease galactosialidosis, SIAL is unstable and undergoes abnormal processing and rapid degradation.
4) We constructed structural models of SIAL which revealed that SIAL shares the same basic folding-pattern and the active site architecture as bacterial and viral sialidases.
5) To understand the pathogenesis of sialidosis, we analyzed mutations in several sialidosis patients from different ethnic origins. All patients had missense mutations except for a patient diagnosed with congenital sialidosis who had a frameshift mutation. Clinical evaluation indicated that this patient presented uncommon features such as excessive tortuosity of retinal arteries and severely dilated coronary arteries.

6) We evaluated the structural impact of the identified mutations and showed that some of the mutations affect active site residues or may cause misfolding of the protein.

7) We selectively expressed 8 SIAL mutants, 4 of which contained amino acid substitutions clustered in one surface domain of the sialidase molecule, and studied their effect on the association of the complex. Biochemical analysis indicated that three of the mutations localized in the cluster are correctly processed and sorted, but do not associate with the complex and are rapidly degraded in the lysosome, suggesting that the cluster containing these mutations may represent the binding site of SIAL with CathA.

Perspectives

The main goal of medical genetics is to provide all the tools required to perform diagnosis, counseling and therapy of inherited diseases. This process requires complete insight into the clinical and biochemical features and the molecular pathology of the disease, which is possible only after the cloning and characterization of the defective gene.

In this project, we have studied the pathogenesis of sialidosis, a rare lysosomal storage disease caused by deficiency of lysosomal sialidase. These studies paved the way for establishing the molecular diagnosis of this disease. However, more analyses need to be performed in the hope of identifying most frequent mutations that can be instrumental in genetic counselling, carrier detection and prenatal diagnosis, as well as in genotype-phenotype correlations.

We defined the sialidase gene structure, identified and characterized mutations in sialidosis patients. We used a homologous model of sialidase to predict the structural impact of the mutations and their influence on the activity and stability of sialidase, but a more precise evaluation requires the knowledge of the crystal structure of the protein. With an efficient expression system in bacteria, yeast or insect cells, sialidase can be produced and purified in quantities sufficient for crystallization studies.

X-ray analysis of the lysosomal complex comprising cathepsin A, sialidase, β-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase will clarify the organization of the complex and provide mechanisms of activation and stabilization of sialidase and β-galactosidase by cathepsin A.

Correction of sialidosis like other lysosomal storage diseases can be approached in three ways: enzyme replacement therapy (ERT), gene transfer therapy (GTT) or bone marrow transplantation (BMT). Each approach has its strengths and weaknesses. However, ERT has been used successfully in the treatment of Type 1 Gaucher disease patients deficient in ß-glucocerebrosidase. In this case, an analogue of the enzyme is designed to target Gaucher cells in the reticuloendothelial system. Since Type I sialidosis, like Type I Gaucher disease, is not associated with neurological complications, enzyme replacement therapy may be applied eventually in the treatment of affected individuals. The main problem with the ERT is that the administered enzyme cannot cross the blood brain barrier. In principle, this problem can be overcome by BMT alone or in conjunction with GTT. Bone marrow-derived macrophages are able to cross the blood brain barrier and reside as microglial cells in the brain, thus delivering the deficient enzyme. Tissue compatibility is the major problem with this approach. Gene therapy has been successful in murine models of some lysosomal storage diseases. For example, Ziegler et al. (1999) constructed a recombinant adenoviral vectors encoding human α-galactosidase A and injected it intravenously in Fabry knock-out mice, resulting in elevated levels of the human enzyme in all tissues. Also, in an effort to correct murine galactosialidosis, Hahn et al. (1998) transplanted galactosialidosis mice with bone marrow from transgenic mice overexpressing human cathepsin A in macrophages under control of the colony stimulating factor-1 receptor promoter. Both examples above can eventually be applied in the correction of sialidase deficiency.

REFERENCES

Aerts J.M., Heikoop J., van Weely S., Donker-Koopman W.E., Barranger J.A., Tager J.M., Schram A.W. Characterization of glucocerebrosidase in peripheral blood cells and cultured blastoid cells. *Exp Cell Res.* **177(2),** 391-8 (1988)

Aguado B., Campbell R.D. Human lysophosphatidic acid acyltransferase is encoded by a gene located in the major histocompatibility complex. *Biochem Soc Trans.* **25(4),** (1997)

Akita H, T. Miyagi, K. Hata, and M. Kagayama. Immunohistochemical evidence for the existence of rat cytosolic sialidase in rat skeletal muscles. *Histochem. Cell. Biol.* **107(6),** 495-503 (1997).

Andria, G., G. Strisciuglio, W.S. Pontarelli, W.S. Sly, and W.E. Dodson. *In* "Sialidases and Sialidoses" (P. Durand et al., eds,) p.365., Edi. Ermes, Milano, 1981.

Aylsworth, A.S., G.H. Thomas, J.L. Hood, N. Malouf, and J. Libert. A severe infantile sialidosis: clinical, biochemical and microscopic features. *J. Pediatr.* **96,** 662-668 (1980).

Baker, E.; X.-H. Guo, A.M. Orsborn, G.R. Sutherland, D.F. Callen, J.J. Hopwood, and C.P. Morris. The Morquio A syndrome (mucopolysaccharidosis IVA) gene maps to 16q24.3. *Am. J. Hum. Genet.* **52,** 96-98 (1993).

Bame, K. J. and Rome, L. H. : Acetyl coenzyme A:alpha-glucosaminide N-acetyltransferase: evidence for a transmembrane acetylation mechanism. *J. Biol. Chem.* **260,** 11293-11299 (1985).

Barrett A..J., Rawlings N.D., and Woessner J.F. eds. Handbook of Proteolytic Enzymes., Acad. Press, London, UK. PP. 393-398, (1998).

Bao M, Booth JL, Elmendorf BJ, Canfield WM Bovine UDP-N-acetylglucosamine: lysosomal-enzyme N-acetylglucosamine-1-phosphotransferase. I. Purification and subunit structure. *J Biol Chem.* **271(49),** 31437-45 (1996).

Bao M., Elmendorf B.J., Booth J.L., Drake R.R., Canfield W.M. Bovine UDP-N-acetylglucosamine:lysosomal-enzyme N-acetylglucosamine-1-phosphotransferase. II. Enzymatic characterization and identification of the catalytic subunit. *J Biol Chem.* **271(49),** 31446-51 (1996).

Barriocanal J.G., Bonifacino J.S., Yuan L., Sandoval I.V. Biosynthesis, glycosylation, movement through the Golgi system, and transport lysosomes by an N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *J Biol Chem.* **261(35),** 16755-63 (1986).

Basner R, H. Kresse, and K. von Figura. N-Acetylglucosamine-6-sulfate sulfatase from human urine. *J. Biol. Chem.* **254(4)**, 1151-1158 (1979).

Beck, M., S.W. Bender, H.L. Reiter, W. Otto, R. Bassler, H. Dancygier, and J. Gehler. Neuraminidase deficiency presenting as non-immune hydrops fetalis. *Eur. J. Pediatr.,* **143**, 135-139 (1984).

Bonten EJ, Arts WF, Beck M, Covanis A, Donati MA, Parini R, Zammarchi E, d'Azzo A. Novel mutations in lysosomal neuraminidase identify functional domains and determine clinical severity in sialidosis. *Hum Mol Genet.* **9(18):**2715-25 (2000).

Bonten, E.J., A. van der Spoel, M. Fornerod, G. Grosveld, and A. d'Azzo. Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes and Devel.* **10**, 3156-3168 (1996).

Bonten, E.J., N.J. Galjart, R. Willemsen, M. Usmany, J.M. Vlak, and A. d'Azzo. Lysosomal protective protein/cathepsin A. Role of the "linker" domain in catalytic activation. *J. Biol. Chem.* **270**, 26441-26445 (1995).

Le Borgne R. and Hoflack B. Protein transport from the secretory to the endocytic pathway in mammalian cells. *Biochim Biophys Acta.* **1404(1-2),** 195-209 (1998).

Braun M., Waheed A., von Figura K. Lysosomal acid phosphatase is transported to lysosomes via the cell surface. *EMBO J.* **8(12),** 3633-40 (1989).

Burmeister, W.P., R.W.H. Ruigrok, and S. Cusack. The 2.2 A resolution crystall structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.* **11**, 49-56 (1992).

Callahan JW and L.S. Wolfe. Isolation and characterization of keratan sulfates from the liver of a patient with GM1-gangliosidosis type I. *Biochim. Biophys. Acta* **215(3)**, 527-543 (1970).

Callahan JW. Molecular basis of GM1 gangliosidosis and Morquio disease, type B. Structure-function studies of lysosomal beta-galactosidase and the non-lysosomal beta-galactosidase-like protein. *Biochim. Biophys. Acta* **1455(2-3)**, 85-103 (1999).

Cantz, M and B. Ulrich-Bott. Disorders of glycoprotein degradation. *J. Inherit. Metab. Dis.* **13**, 523-537 (1990).

Cantz, M., J. Gehler, and J. Spranger. Mucolipidosis I: increased sialic acid content and deficiency of an alpha-N-acetylneuraminidase in cultured fibroblasts. *Biochem. Biophys. Res. Commun.***74**, 732-738 (1977).

Carrillo, M.B., C.N. Milner, S.T. Ball, M. Snock, and R.D. Campbell. Cloning and characterization of sialidase from the murine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele. *Glycobiology* **7**, 975-986 (1997).

Chakraborty, S.; Rafi, M. A.; Wenger, D. A. : Mutations in the lysosomal beta-galactosidase gene that cause the adult form of GM1 gangliosidosis. *Am. J. Hum. Genet.* **54**, 1004 1013 (1994).

Chen XP, E.Y. Enioutina, and R.A. Daynes The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J. Immunol.* **158(7)**, 3070-3080 (1997).

Chen XP, X. Ding, and R.A. Daynes. Ganglioside control over IL-4 priming and cytokine production in activated t cells. *Cytokine* **12(7)**, 972-985 (2000).

Chikuma, T., K. Matsumoto, A. Furukawa, N. Nakayama, R. Yajima, T. Kato, Y. Ishii, and A. Tanaka. A fluorometric assay for measuring deamidase (lysosomal protective protein) using high-performance liquid chromatography. *Anal. Biochem.* **233**, 36-41 (1996).

Chou MY, Li SC, Li YT. Cloning and expression of sialidase L, a NeuAcalpha2-->3Gal-specific sialidase from the leech, Macrobdella decora. *J Biol Chem.* **271(32),** 19219-24 (1996).

Collawn J.F., Kuhn L.A., Liu L..F, Tainer J.A., Trowbridge I.S. Transplanted LDL and mannose-6-phosphate receptor internalization signals promote high-efficiency endocytosis of the transferrin receptor. *EMBO J.* **11**, 3247-53 (1991).

Colman P.M. Influenza virus neuraminidase: structure, antibodies, and inhibitors. *Protein Sci.* **3(10),** 1687-96 (1994)

Cooper D.N. and Youssoufian, H. The CpG dinucleotide and human genetic disease. *Hum. Genet.* **78**, 151-155. (1988)

Corfield, T. Bacterial sialidases--roles in pathogenicity and nutrition. *Glycobiology* **2**, 509-521 (1992).

Conner G.E. The role of the cathepsin D propeptide in sorting to the lysosome. *J Biol Chem.* **267(30),** 21738-45 (1992).

Crennell, S.J., E.F. Garman, W.G. Laver, E.R. Vimr, and G. Taylor. Crystal structure of a bacterial sialidase (from Salmonela typhimurium LT2) shows the same fold as an influenza virus neuraminidase. *Proc. Natl. Acad. Sci. USA* **90**, 9852-9856 (1993).

Crennell, S.J., E.F. Garman, W.G. Laver, E.R. Vimr, and G. Taylor. Crystal structure of a Vibrio cholerae sialidase reveals dual lectin-like domains in addition to the catalytic domain. *Structure* **2**, 535-544 (1994).

Cretin H. The proton gradient across the vacuo-lysosomal membrane of lutoids from the latex of Hevea brasiliensis. I. Further evidence for a proton-translocating ATPase on the vacuo-lysosomal membrane of intact lutoids. *J Membr Biol.* **65(3),** 175-84(1982).

D'Agrosa RM. and J.W. Callahan. In vitro activation of neuraminidase in the beta-galactosidase-neuraminidase-protective protein complex by cathepsin C. *Biochem. Biophys. Res. Commun.* **157(2),** 770-775 (1988).

D'Agrosa RM, M. Hubbes, S. Zhang, R. Shankaran, and J.W. Callahan. Characteristics of the beta-galactosidase-carboxypeptidase complex in GM1-gangliosidosis and beta-galactosialidosis fibroblasts. *Biochem. J.* **285 (Pt 3),** 833-838 (1992).

Dahms NM, Lobel P, Kornfeld S. Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J Biol Chem.* **264(21),** 12115-8 (1989).

d'Azzo, A., G. Andria, P. Strisciuglio, and H. Galijaard. Galactosialidosis. *In* Metabolic and Molecular Bases of Inherited Disease, (Scriver C.R., Beaudet A.L, Sly W.S. and Valle D., eds.) New York: McGraw-Hill, pp. 2835-2837, 1995.

d'Azzo,A., A. Hoogeveen, A.J. Reuser, D. Robinson, and H. Galjaard. Molecular defect in combined ß-galactosidase and neuraminidase deficiency in man. *Proc. Natl. Acad. Sci. U.S.A.* **79,** 4535-4539 (1982).

Dingle, J.T. Vacuoles, vesicles and lysosomes. *British Med. Bull.* **24,** 141-145 (1968).

Dorland L, Haverkamp J, Viliegenthart JF, Strecker G, Michalski JC, Fournet B, Spik G, Montreuil J. 360-MHz 1H nuclear-magnetic-resonance spectroscopy of sialyl-oligosaccharides from patients with sialidosis (mucolipidosis I and II). *Eur J Biochem.* **87(2),** 323-9 (1978).

Durand, P., R. Gatti, S. Cavalieri, C. Borrone, M. Tondeur, J.C. Michalski, and C. Strecker. Sialidosis (mucolipidosis I). *Helvetica Paediatrica Acta,* **32,** 391-400 (1977).

Elsliger M.A., Potier M. Homologous modeling of the lysosomal protective protein/carboxypeptidase L: structural and functional implications of mutations identified in galactosialidosis patients. *Proteins.* **18(1),** 81-93 (1994).

Erickson A.H., Ginns E.I., Barranger J.A. Biosynthesis of the lysosomal enzyme glucocerebrosidase. *J Biol Chem.* **260(26),** 14319-24 (1985).

Erickson A.H., Conner G.E., Blobel G. Biosynthesis of a lysosomal enzyme. Partial structure of two transient and functionally distinct NH2-terminal sequences in cathepsin D. *J Biol Chem.* **256(21),** 11224-31 (1981).

Federico, A., A. Cecio, G.A. Battini, J.C. Michalski, G. Strecker, and G.C. Guazzi. Macular cherry-red spot and myoclonus syndrome. Juvenile form of sialidosis *J. Neurol. Sci.* **48,** 157-169 (1980).

Federico, A., S. Battistini, G. Ciacci, N. de Stefano, R. Gatti, P. Durand, and G.C. Guazzi. Cherry-red spot myoclonus syndrome (type I sialidosis). *Dev. Neurosci.* **13**, 320-326 (1991).

Fingerhut R, G.T. van der Horst, F.W. Verheijen, and E. Conzelmann. Degradation of gangliosides by the lysosomal sialidase requires an activator protein. *Eur. J. Biochem.* **208(3)**, 623-629 (1992).

Frisch A, Neufeld EF. A rapid and sensitive assay for neuraminidase: application to cultured fibroblasts. *Anal Biochem.* **95(1),** 222-7 (1979)

Fukuhara, Y.; Takano, T.; Shimmoto, M.; Oshima, A.; Takeda, E.; Kuroda, Y.; Sakuraba, H.; Suzuki, Y.: A new point mutation of protective protein gene in two Japanese siblings with juvenile galactosialidosis. *Brain Dysfunction* **5,** 319-325, (1992).

Galen,J.E. *et al.* Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infect. Immun.,* **60**, 406-415 (1992).

Galjaard, H., A.T. Hoogeveen, H.A. de Wit-Verbeek, A.J.J. Reuser, M.W. Ho, and D. Robinson. *Nature* **257**, 60-62 (1975).

Galjart, N.J., H. Morreau, R. Willemsen, N. Gillemans, E.J. Bonten, and A. d'Azzo. Human lysosomal protective protein has cathepsin A-like activity distinct from its protection function. *J. Biol. Chem.* **266**, 14754-14762 (1991).

Galjart, N.J., N. Gillemans, A. Harris, T. Gijsbertus, J. van der Horst, F.W. Verheijen, H. Galjaard, and A. d'Asso. Expression of cDNA encoding the human "protective protein" associated with lysosomal ß-galactosidase and neuraminidase: homology to yeast proteases. *Cell* **54**, 755-764 (1988).

Galjart, N.J., N. Gillemans, D. Meijer, and A. d'Azzo. Mouse "protective protein". cDNA cloning, sequence comparison, and expression. *J. Biol. Chem.* **265**, 4678-4684 (1990).

Gaskell, A., S. Crennell, and G. Taylor. The three domains of a bacterial sialidase: a β-propeller, an immunoglobulin module and a galactose-binding jelly-roll. *Structure* **3**, 1197-1205 (1995).

Glickman JN, Kornfeld S. Mannose 6-phosphate-independent targeting of lysosomal enzymes in I-cell disease B lymphoblasts. *J Cell Biol.* **123(1),** 99-108 (1993)

Glossl J, W. Truppe, and H. Kresse. Purification and properties of N-acetylgalactosamine 6-sulphate sulphatase from human placenta. *Biochem. J.* **181(1)**, 37-46 (1979).

Goldberg, M. F.; Cotlier, E.; Fichenscher, L. G.; Kenyon, K. R.; Enat, R.; Borowsky, S. A. : Macular cherry-red spot, corneal clouding, and beta-galactosidase deficiency: clinical, biochemical, and electron microscopic study of a new autosomal recessive storage disease. *Arch. Intern. Med.* **128,** 387-398 (1971).

Gottschalk,A. and P.E. Lind. Product of interaction between influenza virus enzyme and ovomucin. *Nature* **164**, 232-233 (1949).

Gough N.R., Zweifel M.E., Martinez-Augustin O., Aguilar R.C., Bonifacino J.S., Fambrough D.M. Utilization of the indirect lysosome targeting pathway by lysosome-associated membrane proteins (LAMPs) is influenced largely by the C-terminal residue of their GYXXphi targeting signals. *J Cell Sci.* **112 (Pt 23),** 4257-69 (1999)

Groebe H, M. Krins, H. Schmidberger, K. von Figura, K. Harzer, H. Kresse, E. Paschke, A. Sewell, and K. Ullrich. Morquio syndrome (mucopolysaccharidosis IV B) associated with beta-galactosidase deficiency. Report of two cases. *Am. J. Hum. Genet.* **32(2),** 258-272 (1980).

Guarnieri F.G., Arterburn L.M., Penno M.B., Cha Y., August J.T. The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein 1. *J Biol Chem* **268(3),** 1941-6 (1993)

Hahn CN, del Pilar Martin M, Zhou XY, Mann LW, d'Azzo A. Correction of murine galactosialidosis by bone marrow-derived macrophages overexpressing human protective protein/cathepsin A under control of the colony-stimulating factor-1 receptor promoter. *Proc Natl Acad Sci U S A.* **95(25):**14880-5 (1998).

Hakomori S., Igarashi Y. Gangliosides and glycosphingolipids as modulators of cell growth, adhesion, and transmembrane signaling. *Adv Lipid Res.* **25**, 147-62. (1993)

Hanna, W.L., J.M. Turbov, H.L. Jackman, F. Tan, and C.J. Froelich. Dominant chymotrypsin-like esterase activity in human lymphocyte granules is mediated by the serine carboxypeptidase called cathepsin A-like protective protein. *J. Immunol.* **153**, 4663-4672 (1994).

Siebertl H-C, Tajkhorshid E, von der Liet C-W, Reinhard G., Kleineidam R.G., Kruse S., Schauer R., Kaptein R., Gabius H.., Johannes J, Vliegenthart F. G., Knowledge-based homology modelling and experimental determination of amino acid side chain accessiblity by the laser photo CIDNP (chemically induced dynamic nuclear polarization) approach in solution: lessons from the small sialidase of *Clostridium perfringens J.Mol.Model.* **2**, 446 – 455 (1996).

Her, H.G. (1965) Gastroenterology, 48: 625-633

Hers, H.G. The role of the pathogeny of storage diseases. *Biochimie* **54,** 753-757 (1972)

Hickman S, Neufeld E.F. A hypothesis for I-cell disease: defective hydrolases that do not enter lysosomes. *Biochem Biophys Res Commun.* **49(4),** 992-9 (1972).

Hinek A Biological roles of the non-integrin elastin/laminin receptor *Biol. Chem.* **377(7-8),** 471-480 (1996).

Hiraiwa M, M. Nishizawa, Y. Uda, T. Nakajima, and T. Miyatake. Human placental sialidase: further purification and characterization. *J. Biochem. (Tokyo)* **103(1),** 86-90 (1988).

Hiraiwa M, T. Yamauchi, S. Tsuji, M. Nishizawa, T. Miyatake, K. Oyanagi F. Ikuta, and Y. Uda. Activation of human lysosomal sialidase. *J. Biochem. (Tokyo)* **114(6),** 901-905 (1993).

Hiraiwa M, Y. Uda, M. Nishizawa, and T. Miyatake. Human placental sialidase: partial purification and characterization. *J. Biochem. (Tokyo)* **101(5),** 1273-1279 (1987).

Hiraiwa M, Y. Uda, S. Tsuji, T. Miyatake, B.M. Martin, M. Tayama, J.S. O'Brien, and Y. Kishimoto. Human placental sialidase complex: characterization of the 60 kDa protein that cross-reacts with anti-saposin antibodies. *Biochem. Biophys. Res. Commun.* **177(3),** 1211-1216 (1991).

Hirst J. and M.S. Robinson. Clathrin and adaptors. *Biochim. Biophys. Acta* **1404(1-2),** 173-193 (1998).

Hoflack B. and Kornfeld S. Lysosomal enzyme binding to mouse P388D1 macrophage membranes lacking the 215-kDa mannose 6-phosphate receptor: evidence for the existence of a second mannose 6-phosphate receptor. *Proc Natl Acad Sci U S A* **82(13),** 4428-32 (1985)

Hoogeveen A, A. d'Azzo, R. Brossmer, and H. Galjaard. Correction of combined beta-galactosidase/neuraminidase deficiency in human fibroblasts. *Biochem. Biophys. Res. Commun.* **103(1),** 292-300 (1981).

Hoogeveen AT, F.W. Verheijen, A. d'Azzo, and H. Galjaard. Genetic heterogeneity in human neuraminidase deficiency. *Nature* **285(5765),** 500-502 (1980).

Hoogeveen, A.T., F.W. Verheijen, and H. Galjaard. The relation between human lysosomal ß-galactosidase and its protective protein. *J. Biol. Chem.* **258,** 12143-12146 (1983).

Huang JQ, J.M. Trasler, S. Igdoura, J. Michaud, N. Hanal, and R.A. Gravel. Apoptotic cell death in mouse models of GM2 gangliosidosis and observations on human Tay-Sachs and Sandhoff diseases. *Hum. Mol. Genet.* **6(11),** 1879-1885 (1997).

Hunziker W., Geuze H.J. .Intracellular trafficking of lysosomal membrane proteins. *Bioessays.* **18(5),** 379-89 (1996)

Igdoura SA, C. Mertineit, J.M. Trasler, and R.A. Gravel. Sialidase-mediated depletion of GM2 ganglioside in Tay-Sachs neuroglia cells. *Hum. Mol. Genet.* **8(6)**, 1111-1116 (1999).

Igdoura, S.A., C. Gafuik, C. Mertineit, F. Saberi, A.V. Pshezhetsky, M. Potier, J.M. Trasier, and R.A. Gravel. Cloning of the cDNA and the gene encoding mouse lysosomal sialidase and correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts. *Hum. Mol. Genet.* **7**, 115-121 (1998).

Kaplan A., Fischer D., Achord D, Sly W. Phosphohexosyl recognition is a general characteristic of pinocytosis of lysosomal glycosidases by human fibroblasts. *J Clin Invest.* **60(5),** 1088-93 (1977)

Kelly, T.E. and G. Graetz. Isolated acid neuraminidase deficiency: a distinct lysosomal storage disease. *Am. J. Med. Genet.,* **1**, 31-46 (1977).

Koening, H., *Nature*, **195**, 782-785 (1962).

Kornfeld S. Trafficking of lysosomal enzymes. *FASEB J.* **1(6),** 462-8 (1987)

Kornfeld R., Bao M., Brewer K., Noll C., Canfield W. Molecular cloning and functional expression of two splice forms of human N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase. *J Biol Chem.* **274(46),** 32778-85 (1999)

Kopitz J., Muhl C., Ehemann V., Lehmann C., Cantz M. Effects of cell surface ganglioside sialidase inhibition on growth control and differentiation of human neuroblastoma cells. *Eur J Cell Biol.* **73(1),** 1-9 (1997)

Kopitz J, C. von Reitzenstein, K. Sinz, and M. Cantz. Selective ganglioside desialylation in the plasma membrane of human neuroblastoma cells. *Glycobiology* **6(3)**, 367-376 (1996).

Kopitz J, C. von Reitzenstein, M. Burchert, M. Cantz, and H.J. Gabius. Galectin-1 is a major receptor for ganglioside GM1, a product of the growth-controlling activity of a cell surface ganglioside sialidase, on human neuroblastoma cells in culture. *J. Biol. Chem.* **273(18)**, 11205-11211 (1998).

Koster A., von Figura K., Pohlmann R. Mistargeting of lysosomal enzymes in M(r) 46,000 mannose 6-phosphate receptor-deficient mice is compensated by carbohydrate-specific endocytotic receptors. *Eur J Biochem.* **224(2),** 685-9 (1994)

Landolfi NF, J. Leone, J.E. Womack, and R.G. Cook. Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase. *Immunogenetics* **22(2)**, 159-67 (1985).

Landolfi NF and R.G. Cook. Activated T-lymphocytes express class I molecules which are hyposialylated compared to other lymphocyte populations. *Mol. Immunol.* **23(3)**, 297-309 (1986).

Le Borgne R. and Hoflack B., Protein transport from the secretory to the endocytic pathway in mammalian cells. *Biochimica et Biophysica Acta* **1404,** 195-209 (1998)

Lehmann L.E, Eberle W., Krull S., Prill V., Schmidt B., Sander C., von Figura K., Peters C. The internalization signal in the cytoplasmic tail of lysosomal acid phosphatase consists of the hexapeptide PGYRHV. *EMBO J.* **11(12),** 4391-9 (1992)

Leroy, J. G.; Demars, R. I. Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* **157;** 804-806 (1967).

Lin S.C., Hu F.R., Hou J.W., Yao Y.T., Wang T.R. Corneal opacity and congenital glaucoma associated with massive heparan sulfaturia: report of one case. *Taiwan Erh Ko I Hsueh Hui Tsa Chih.* **40(1);** 46-9 (1999)

Loonen M.C., Lugt L, Franke C.L. Letter: Angiokeratoma corporis diffusum and lysosomal enzyme deficiency. *Lancet.* **2(7883);** 785 (1974)

Lowden, J. A.; O'Brien, J. S. : Sialidosis: a review of human neuraminidase deficiency. *Am. J. Hum. Genet.* **31;** 1-18 (1979).

Lukong K.E, M.A. Elsliger, Chang Y., Richard C., Thomas G., Carey W., Tylki-Szymanska A., Czartoryska, B., Buchholz T., Criado G.R., Palmeri S., and Pshezhetsky A.V. Characterization of the sialidase molecular defects in sialidosis patients suggests the structural organization of the lysosomal multienzyme complex. *Hum. Mol. Genet.* **9(7),** 1075-1085 (2000).

Mancini GM, A.T. Hoogeveen, H. Galjaard, J.E. Mansson, and L. Svennerholm. Ganglioside GM1 metabolism in living human fibroblasts with beta-galactosidase deficiency *Hum. Genet.* **73(1),** 35-38 (1986).

Maroteaux P., Humbel R., Strecker G., Michalski J.C., Mande R.. [A new type of sialidosis with kidney disease: nephrosialidosis. I. Clinical, radiological and nosological study]. *Arch Fr Pediatr.* **35(8),** 819-29. (1978) French.

Matoth, Y., Chazan, S.; Cnaan, A., Gelernter, I., Klibansky, C. Frequency of carriers of chronic (type I) Gaucher disease in Ashkenazi Jews. *Am. J.Med. Genet.* **27,** 561-565 (1987).

Matsuda J, O. Suzuki, A. Oshima, A. Ogura, Y. Noguchi, Y. Yamamoto, T. Asano, K. Takimoto, K. Sukegawa, Y. Suzuki, and M. Naiki. Beta-galactosidase-deficient mouse as an animal model for GM1-gangliosidosis. *Glycoconj. J.* **14(6),** 729-736 (1997).

McIntyre G.F and Erickson A.H. Procathepsins L and D are membrane-bound in acidic microsomal vesicles. *J Biol Chem.* **266(23),** 15438-45 (1991).

Meikle P.J., Hopwood J.J., Clague A.E., Carey W.F. Prevalence of lysosomal storage disorders.

JAMA. **281(3),** 249-54 (1999).

Miller, J.J., D.G. Changaris, and R.S. Levy. Conversion of angiotensin I to angiotensin II by cathepsin A isoenzymes of porcine kidney. *Biochem. & Biophys. Res. Commun.*, **154**, 1122-1129 (1988).

Milner, C.M., S.V. Smith, M.B. Carrillo, G.L. Taylor, M. Hollinshead, and R.D. Campbell. Identification of a sialidase encoded in the human major histocompatibility complex. *J. Biol. Chem.*, **272**, 4549-4558 (1997).

Miyagi T, K. Konno, Y. Emori, H. Kawasaki, K. Suzuki, A. Yasui, and S. Tsuik. Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase. *J. Biol. Chem.* **268(35),** 26435-26440 (1993).

Miyagi,T., J. Sagawa, K. Konno, and S. Tsuiki. Immunological discrimination of intralysosomal, cytosolic, and two membrane sialidases present in rat tissues. *J. Biochem.* **107**, 794-798 (1990).

Miyagi T, K. Hata, K. Konno, and S. Tsuiki. Multiple forms of mammalian sialidase: altered expression in carcinogenesis. *Tohoku J. Exp. Med.* **168(2),** 223-229 (1992).

Miyagi T, K. Hata, A. Hasegawa, and T. Aoyagi. Differential effect of various inhibitors on four types of rat sialidase. *Glycoconj J,* **10(1)**, 45-49 (1993).

Miyagi T and Tsuiki S. Purification and characterization of cytosolic sialidase from rat liver. *J Biol Chem.* **260(11),** 6710-6 (1985).

Miyagi T., Wada T., Iwamatsu A., Hata K., Yoshikawa Y., Tokuyama S., Sawada M. Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. *J Biol Chem.* **274(8),** 5004-11 (1999)

Monti E., Bassi M.T., Papini N., Riboni M., Manzoni M., Venerando B., Croci G., Preti A., Ballabio A., Tettamanti G., Borsani G. Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane. *Biochem J.* **349(Pt 1),** 343-351 (2000).

Monti E, A. Preti, C. Nesti, A. Ballabio, and G. Borsani. Expression of a novel human sialidase encoded by the NEU2 gene. *Glycobiology* **9(12)**,1313-1321 (1999).

Morreau, H., N.J. Galjart, R. Willemsen, N. Gillemans, X.Y. Zhou, and A. d'Azzo. Human lysosomal protective protein. Glycosylation, intracellular transport, and association with ß-galactosidase in the endoplasmic reticulum. *J. Biol. Chem.* **267**, 17949-17956 (1992).

Naganawa Y, K. Itoh, M. Shimmoto, K. Takiguchi, H. Doi, Y. Nishizawa, T. Kobayashi, S. Kamei, K.E. Lukong, A.V. Pshezhetsky, and H. Sakuraba. Molecular and structural studies of Japanese patients with sialidosis type 1. *J. Hum. Genet.* **45(4)**, 241-249 (2000).

Naraparaju VR. and N. Yamamoto. Roles of beta-galactosidase of B lymphocytes and sialidase of T lymphocytes in inflammation-primed activation of macrophages *Immunol. Lett.* **43(3)**, 143-148 (1994).

Natowicz M.R., Chi M.M., Lowry O.H., Sly W.S. Enzymatic identification of mannose 6-phosphate on the recognition marker for receptor-mediated pinocytosis of beta-glucuronidase by human fibroblasts. *Proc Natl Acad Sci U S A.* **76(9),** 4322-6 (1979).

Neufeld E.F. Lysosomal storage diseases. *Annu Rev Biochem.* **60**:257-80 (1991).

Nishiyama Y., Sakamoto S., Katayama Y., Mishina M., Terashi A. [Neuroradiological findings on cerebral blood flow and metabolism of a case of adult onset sialidosis]. Rinsho Shinkeigaku. **37(11),** 982-6 (1997) Japanese.

O'Brien, J.S. The cherry red spot-myoclonus syndrome: a newly recognized inherited lysosomal storage disease due to acid neuraminidase deficiency. *Clin. Genet.,* **14,** 55-60 (1979).

O'Brien JS, M.B. Stern, B.H. Landing, J.K. O'Brien, and G.N. Donnell. Generalized gangliosidosis. Another inborn error of metabolism? *Am. J. Dis. Child.* **109**, 338 (1965).

O'Brien JS, E. Gugler, A. Giedion, U. Wiessmann, N. Herschkowitz, C. Meier, and J. Leroy. Spondyloepiphyseal dysplasia, corneal clouding, normal intelligence and acid beta-galactosidase deficiency. *Clin Genet* **9(5)**, 495-504 (1976).

Olcay L., Gumruk F., Boduroglu K., Coskun T., Tuncbilek E. : Anaemia and thrombocytopenia due to haemophagocytosis in a 7-month-old boy with galactosialidosis. *J. Inherit. Metab. Dis.* **21,** 679-680 (1998).

Okada, S. and J.S. O'Brien. Generalized gangliosidosis: beta-galactosidase deficiency. *Science* **160**, 1002 (1968).

Oohira, T., N. Nagata, I. Akaboshi, I. Matsuda, and S. Naito. The infantile form of sialidosis type II associated with congenital adrenal hyperplasia: possible linkage between HLA and the neuraminidase deficiency gene. *Hum. Genet.* **70**, 341-343 (1985).

Oohira,T., N. Nagata, I. Akaboshi, I. Matsuda, and S. Naito. The infantifle form of sialidosis type II associated with congenital adrenal hyperplasia: possible linkage between HLA and the neuraminidase deficiency gene. *Hum. Genet.* **70**, 341-343 (1985).

Orsel J.G., Sincock P.M., Krise J.P., Pfeffer S.R. Recognition of the 300-kDa mannose 6-phosphate receptor cytoplasmic domain by 47-kDa tail-interacting protein. *Proc Natl Acad Sci U S A.* **97(16),** 9047-51 (2000).

Oshima A., Yoshida K., Shimmoto M., Fukuhara Y., Sakuraba H., Suzuki Y., **49,** 1091-1093, 1991.,

Patel MS, Callahan JW, Zhang S, Chan AK, Unger S, Levin AV, Skomorowski MA, Feigenbaum AS, O'Brien K, Hellmann J, Ryan G, Velsher L, Chitayat D. Early-infantile galactosialidosis: prenatal presentation and postnatal follow-up. *Am J Med Genet.* **85(1),** 38-47 (1999).

Pearse BM, Smith CJ, Owen DJ. Clathrin coat construction in endocytosis. *Curr Opin Struct Biol.* **10(2),** 220-8 (2000)

Peters C. and K. von Figura. Biogenesis of lysosomal membranes. *FEBS Lett* **346(1),** 108-114 (1994).

Petersen, G. M., Rotter, J. I., Cantor, R. M., Field, L. L., Greenwald, S., Lim, J. S. T., Roy, C., Schoenfeld, V., Lowden, J. A., Kaback, M. M. The Tay-Sachs disease gene in North American Jewish populations: geographicvariations and origin. *Am. J. Hum. Genet.* **35,** 1258-1269 (1983)

Pinsky L, Miller J, Shanfield B, Watters G, Wolfe L.S. GM1 gangliosidosis in skin fibroblast culture: enzymatic differences between types 1 and 2 and observations on a third variant. *Am J Hum Genet.* **26(5),** 563-77 (1974).

Poorthuis B.J., Wevers R.A., Kleijer W.J., Groener J.E., de Jong J.G., van Weely S, Niezen-Konin KE, van Diggelen OP. The frequency of lysosomal storage diseases in The Netherlands. *Hum Genet.* **105(1-2),** 151-6 (1999)

Potier M, Michaud L, Tranchemontagne J, Thauvette L. Structure of the lysosomal neuraminidase-β-galactosidase-carboxypeptidase multienzyme complex. *Biochem. J.* **267,**197-202 (1990).

Provenzale J.M., Barboriak D.P., Sims K. Neuroradiologic findings in fucosidosis, a rare lysosomal storage disease. *AJNR Am J Neuroradiol.* **16(4 Suppl),** 809-13 (1995).

Pshezhetsky AV, A.V. Levashov, and G.Y. Wiederschain. Regulation of the GM1-galactosidase supramolecular structure and catalytic activity in vitro. *Biochim. Biophys. Acta* **1122(2),** 154-160 (1992).

Pshezhetsky, A.V. and M. Potier. Association of N-acetyl-galactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of ß-galactosidase, cathepsin A and neuraminidase: possible implication for intralysosomal catabolism of keratan sulfate. *J. Biol. Chem.* **271,** 28359-28365 (1996).

Pshezhetsky, A.V. and M. Potier. Direct affinity purification and supramolecular organization of human lysosomal cathepsin A. *Arch. Biochem. and Biophys.* **313,** 64-70 (1994).

Pshezhetsky, A.V. and M. Potier. Stoichiometry of the human lysosomal carboxypeptidase-ß-galactosidase complex. *Biochem. Biophys. Res. Commun.* **195,** 354-362 (1993).

Pshezhetsky, A.V., M.-A. Elsliger, M.V. Vinogradova, and M. Potier. Human lysosomal ß-galactosidase-cathepsin A complex: Definition of the ß-galactosidase-binding interace on cathepsin A. *Biochem.* **34**, 2431-2440 (1995).

Pshezhetsky, A.V., M.V. Vinogradova, M.-A. Elsliger, F. El-Zein, S.K. Svedas, and M. Potier. Continuous spectrophotometric assay of human lysosomal cathepsin A/protective protein in normal and galactosialidosis cells. *Anal. Biochem.* **230**, 303-307 (1995).

Pshezhetsky, A.V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M. A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L. and Potier, M. Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nat. Genet.*, **15**, 316-20 (1997).

Raas-Rothschild A, Cormier-Daire V, Bao M, Genin E, Salomon R, Brewer K, Zeigler M, Mandel H, Toth S, Roe B, Munnich A, Canfield WM. Molecular basis of variant pseudo-hurler polydystrophy. *J Clin Invest.* **105(5),** 673-81 (2000).

Radons J, Biewusch U, Grassel S, Geuze HJ, Hasilik A. Distinctive inhibition of the lysosomal targeting of lysozyme and cathepsin D by drugs affecting pH gradients and protein kinase C. *Biochem J.* **302 (Pt 2),** 581-6. (1994)

Rapin, I., S. Goldfischer, R. Katzman, J. Engel Jr, and J.S. O'Brien. The cherry-red spot-myoclonus syndrome. *Ann. Neurol.*, **3**, 234-242 (1978).

Remington S.J. Serine carboxypeptidases: a new and versatile family of enzymes. *Curr Opin Biotechnol.* **4(4),** 462-8 (1993)

Richard C, J. Tranchemontagne, M.A. Elsliger, G.A. Mitchell, M. Potier, and A.V. Pshezhetsky. Molecular pathology of galactosialidosis in a patient affected with two new frameshift mutations in the cathepsin A/protective protein gene. *Hum. Mutat.* **11(6),** 461-469 (1998).

Rijnboutt S., Aerts H.M., Geuze H.J., Tager J.M., Strous G.J. Mannose 6-phosphate-independent membrane association of cathepsin D, glucocerebrosidase, and sphingolipid-activating protein in HepG2 cells. *J Biol Chem.* **266(8),** 4862-8 (1991)

Robbins A.R., Myerowitz R., Youle R.., Murray G.J., Neville D.M. Jr. The mannose 6-phosphate receptor of Chinese Hamster ovary cells. Isolation of mutants with altered receptors. *J Biol Chem.* **256(20),** 10618-22 (1981)

Rothe B, Rothe B, Roggentin P, Schauer R. The sialidase gene from Clostridium septicum: cloning, sequencing, expression in Escherichia coli and identification of conserved sequences in sialidases and other proteins. *Mol Gen Genet.* **226(1-2),** 190-7 (1991)

Rottier, R.J. E. Bonten, and A. d'Azzo. A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. *Hum. Mol. Genetics,* **7**, 313-321 (1998).

Rudenko G, E. Bonten, W.G. Hol, and A. d'Azzo. The atomic model of the human protective protein/cathepsin A suggests a structural basis for galactosialidosis. *Proc. Natl Acad. Sci. U S A* **95(2)**, 621-625 (1998).

Rudenko, G., E. Bonten, A. d'Azzo, and W.G.J. Hol. Three-dimensional structure of the human protective protein: structure of the precursor form suggests a complex activation mechanism. *Structure* **3**, 1249-1259 (1995).

Sahagian G.G. The mannose 6-phosphate receptor: function, biosynthesis and translocation. *Biol Cell.* **51(2),** 207-14 (1984)

Sakuraba H, Y. Suzuki, M. Akagi, M. Sakai, and N. Amano. beta-Galactosidase-neuraminidase deficiency (galactosialidosis): clinical, pathological, and enzymatic studies in a postmortem case. *Ann. Neurol.* **13(5)**, 497-503 (1983).

Sargent C.A., Dunham I., Campbell R.D. Identification of multiple HTF-island associated genes in the human major histocompatibility complex class III region. *EMBO J.* **8(8),** 2305-12. (1989)

Sato K and Miyagi T. Genomic organization and the 5'-upstream sequence of the rat cytosolic sialidase gene. *Glycobiology.* **5(5),** 511-6 (1995)

Schauer T., Trends Biochem. Sci. **10,** 357-360 (1985)

Schenkman S, Chaves L.B., Pontes de Carvalho L.C., Eichinger D. A proteolytic fragment of Trypanosoma cruzi trans-sialidase lacking the carboxyl-terminal domain is active, monomeric, and generates antibodies that inhibit enzymatic activity.*J Biol Chem.* **269(11),** 7970-5 (1994)

Schneider-Jakob, H.R. and M. Cantz. Lysosomal and plasma membrane ganglioside GM3 sialidases of cultured human fibroblasts. Differentiation by detergents and inhibitors. *Biol. Chem. Hoppe-Seyler* **372**, 443-450 (1991).

Schweizer A, Stahl P.D. and Rohrer J. A Di-aromatic motif in the cytosolic tail of the mannose receptor mediates endosomal sorting. *J Biol Chem.* **275(38),** 29694-700 (2000)

Shigeki Katoh, Taeko Miyagi, Haruko Taniguchi, Yu-ichi Matsubara, Jun-ichi Kadota, Akira Tominaga, Paul W. Kincade, Shigeru Matsukura, and Shigeru Kohno; Cutting Edge: An Inducible Sialidase Regulates the Hyaluronic Acid Binding Ability of CD44-Bearing Human Monocytes1 *The Journal of Immunology* **162,** 5058-5061 (1999).

Shimmoto, M., Y. Nakahori, I. Matsuhita, T. Shinka, Y. Kuroki, K. Itoh, and H. Sakuraba.

A human protective protein gene partially overlaps the gene encoding phospholipid transfer protein on the complementary strand of DNA. *Biochem. Biphys. Res. Commun.* **220**, 802-806 (1996).

Soggs, R.L., L. Steinman, B. Rathjen, B.R. Tharp, J.S. O'Brien, and K.R. Kenyon. Cherry-rep spot-myoclonus syndrome. *Ophtalmology,* **86**, 1861-1874 (1979).

Stone DL, and Sidransky E. Hydrops fetalis: lysosomal storage disorders in extremis. *Adv Pediatr.* **46,** 409-40 (1999).

Strecker G, Herlant-Peers MC, Fournet B, Montreul J. Structure of seven oligosaccharides excreted in the urine of a patient with Sandhoff's disease (GM2 gangliosidosis-variant O). *Eur J Biochem.* **81(1),** 165-71 (1977)

Suzuki Y, N. Nakamura, K. Fukuoka, Y. Shimada, and M. Uono. beta-Galactosidase deficiency in juvenile and adult patients. Report of six Japanese cases and review of literature. *Hum. Genet.* **36(2),** 219-229 (1977).

Suzuki Y., Nanba E., Tsuji A., Yang R.C., Okamura-Oho Y., Yamanaka T.: Clinical and genetic heterogeneity in galactosialidosis. *Brain Dysfunction* **1,** 285-293 (1988) .

Takano T., Shimmoto M., Fukuara Y., Itoh K., Kase R., Takiyama N., Kobayashi T., Oshima A., Sakuraba H., Suzuki Y. Galactosialidosis: Clinical and molecular analysis of 19 Japanese patients. *Brain Dysfunction* **4,** 271-280.(1991)

Tang H.B., DiMango E., Bryan R., Gambello M., Iglewski B.H., Goldberg J.B., Prince A. Contribution of specific Pseudomonas aeruginosa virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. *Infect Immun.* **64(1),** 37-43 (1996)

Thomas G. H. ,Tipton, R. E., Ch'ien L. T., Reynolds L. W., Miller C. S. : Sialidase (alpha-N-acetyl neuraminidase) deficiency: the enzyme defect in an adult with macular cherry red spots and myoclonus without dementia. *Clin. Genet.* **13,** 369-379 (1978).

Tokuyama S, S. Moriya, S. Taniguchi, A. Yasui, J. Miyazaki, S. Orikasa, and T. Miyagi. Suppression of pulmonary metastasis in murine B16 melanoma cells by transfection of a sialidase cDNA. *Int. J. Cancer* **73(3),** 410-415 (1997).

Tylki-Szymanska A., Lugowska A., Czartoryska B. Neuraminidase deficiency presenting as a nephrosialidosis: the first case detected in Poland. *Acta Paediatr Jpn.* **38(5),** 529-32m (1996).

Ulrich-Bott B, B. Klem, R. Kaiser, J. Spranger, and M. Cantz. Lysosomal sialidase deficiency: increased ganglioside content in autopsy tissues of a sialidosis patient. *Enzyme* **38(1-4)**, 262-266 (1987).

van der Horst GT, N.J. Galjart, A. d'Azzo, H. Galjaard, and F.W. Verheijen. Identification and in vitro reconstitution of lysosomal neuraminidase from human placenta. *J. Biol. Chem.* **264(2)**, 1317-1322 (1989).

van der Spoel A, E. Bonten, and A. d'Azzo. Processing of lysosomal beta-galactosidase. The C-terminal precursor fragment is an essential domain of the mature enzyme. *J. Biol. Chem.* **275(14)**, 10035-10040 (2000).

van der Spoel, A., E. Bonten, and A. Azzo. Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. *EMBO J.*, **17**, 1588-1597 (1998).

van Diggelen OP, A.T. Hoogeveen, P.J. Smith, A.J. Reuser, and H. Galjaard. Enhanced proteolytic degradation of normal beta-galactosidase in the lysosomal storage disease with combined beta-galactosidase and neuraminidase deficiency. *Biochim. Biophys. Acta* **703(1)**, 69-76 (1982).

Van Hoof, F [Lysosomal Diseases], [French], Bruxelles. Medical, **56,** 193-203 (1976)

Van Pelt J., Van Kuik J.A., Kamerling J.P., Vliegenthart J.F., Van Diggelen O.P., Galjaard H. Storage of sialic acid-containing carbohydrates in the placenta of a human galactosialidosis fetus. Isolation and structural characterization of 16 sialyloligosaccharides. *Eur J Biochem.* **177(2),** 327-38 (1988)

Varghese, J.N., W.G. Laver, and P.M. Colman. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. *Nature* **303**, 35-40 (1983).

Verheijen FW, Janse HC, van Diggelen OP, Bakker HD, Loonen MC, Durand P, Galjaard H Two genetically different mu-nana neuraminidases in human leucocytes. *Biochem. Biophys. Res. Commun.*, **117**, 470-478 (1983).

Vinogradova MV, L. Michaud, A.V. Mezentsev, K.E. Lukong, M. El-Alfy, C.R. Morales, M. Potier, and A.V. Pshezhetsky. Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation. *Biochem. J.* **330 (Pt 2)**, 641-650 (1998).

Warner T.G., Chang J., Ferrari J., Harris R., McNerney T., Bennett G., Burnier J., Sliwkowski M.B. Isolation and properties of a soluble sialidase from the culture fluid of Chinese hamster ovary cells. *Glycobiology.* **3(5),** 455-63 (1993).

Wenger D.A., Tarby T.J., Wharton C. Macular cherry-red spots and myoclonus with dementia: coexistent neuraminidase and beta-galactosidase deficiencies. *Biochem Biophys Res Commun.* **82(2),** 589-95 (1978).

Winter, R.M., D.M. Swallow, M. Baraitser, and P. Purkiss. Sialidosis type 2 (acid neuraminidase deficiency): clinical and biochemical features of a futher case. *Clin. Gen.* **18**, 203-210 (1980).

Yamamoto N. and R. Kumashiro. Conversion of vitamin D3 binding protein (group-specific component) to a macrophage activating factor by the stepwise action of beta-galactosidase of B cells and sialidase of T cells. *J. Immunol.* **151(5)**, 2794-2802 (1993).

Yamamoto Y, C.A. Hake, B.M. Martin, K.A. Kretz, A.J. Ahern-Rindell, S.L. Naylor, M. Mudd, and J.S. O'Brien. Isolation, characterization, and mapping of a human acid beta-galactosidase cDNA. *DNA Cell Biol.* **9(2)**, 119-127 (1990).

Yoshida K., Oshima, A., Sakuraba, H., Nakano, T., Yanagisawa, N., Inui, K., Okada, S., Uyama, E., Namba, R., Kondo, K., Iwasaki, S., Takamiya, K., Suzuki, Y. , GM1 Gangliosidosis in adults: clinical and molecular analysis of 16 Japanese patients. *Ann. Neurol.* **31,** 328-332 (1992).

Yoshino H, K. Miyashita, N. Miyatani, T. Ariga, Y. Hashimoto, S. Tsuji, K. Oyanagi, E. Ohama, F. Ikuta, A. Suzuki, et al. Abnormal glycosphingolipid metabolism in the nervous system of galactosialidosis. *J. Neurol. Sci.* **97(1)**, 53-65 (1990).

Yutaka Nishiyama, M.D., Shizuki Sakamoto, M.D., Yasuo Katayama, M.D., Masahiro Mishina, M.D. and Akiro Terashi, M.D. Neuroradiological findings on cerebral blood flow and metabolism of a case of adult onset sialidosis *Clin Neurol*, **37,** 982-986 (1997).

Zeigler M, Sury V, Bach G. The identification of lysosomal ganglioside sialidase in human cells. *Eur J Biochem.* **183(2),** 455-8 (1989).

Zhang S. and Callahan J.W. (1994) The biochemistry and clinical features of galactosialidosis. *Biochim. Biophys Acta* **1225,**244-254. (1996)

Zhou, X.Y., H. Morreau, R. Rottier, D. Davis, E. Bonten, N. Gillemans, D. Wenger, F.G. Grosveld, P. Doherty, K. Suzuki, G. Grosveld, and A. d'Azzo. Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes & Dev.* **9**, 2623-2634 (1995).

Zhou X.Y., van der Spoel A., Rottier R., Hale G., Willemsen R., Berry G.T., Strisciuglio P., Morrone A.Zammarchi E. Andria G., d'Azzo A. Molecular and biochemical analysis of protective protein/cathepsin A mutations: correlation with clinical severity in galactosialidosis. *Hum Mol Genet.* **5(12),** 1977-87 (1996).

Ziegler R., Yew N.S., Li C., Cherry M., Berthelette P., Romanczuk H., Ioannou Y.A., Zeidner K.M., Desnick R.J., Cheng S.H. Correction of enzymatic and lysosomal storage defects in Fabry mice by adenovirus-mediated gene transfer. *Hum Gene Ther.* **10(10),** 1667-82 (1999.