The Role of Mouse and Human Lysosomal Sialidase in the Catabolism of Ganglioside G_{M2}.

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ABSTRACT

Tay-Sachs and Sandhoff diseases are autosomal recessive disorders of G_{M2} ganglioside catabolism resulting from deficient activity of lysosomal β-hexosaminidase A (Hex A) or Hex A and Hex B, respectively. This leads to a massive and fatal accumulation of G_{M2} ganglioside in the neurons of affected patients. Mouse models of Tay-Sachs and Sandhoff diseases, created via targeted disruption of the *Hexa* and *Hexb* genes, revealed that while *Hexb* -/- mice suffer a profound, fatal neurodegenerative disease as expected, *Hexa* -/- mice escape disease to about one year. This protection is conferred by an ability to degrade G_{M2} ganglioside by means of a lysosomal sialidase mediated metabolic bypass. To determine if such a bypass could be made to function in humans, a series of experiments were performed to examine and compare the activity of mouse and human lysosomal sialidases in their ability to promote G_{M2} catabolism. The results suggest that human lysosomal sialidase, though sluggish, can indeed degrade G_{M2} when induced sufficiently.

RÉSUMÉ

Les maladies de Tay-Sachs et Sandhoff sont des désordres autosomiques récessifs rares du catabolisme du ganglioside G_{M2} causés par une déficience des enzymes lysosomiales hexosaminidase A (Hex A) ou Hex A et Hex B, respectivement. Les patients souffrant d'une déficience de l'une de ces enzymes génèrent une accumulation massive de G_{M2} menant à une dégénérescence neuronale. Nous avons généré les modèles "knock-out" de souris pour les maladies de Tay-Sachs et Sandhoff. Comme prévu, les souris *Hex b -/-* souffrent de neurodégénérescence; par contre, les souris *Hex A -/-* sont asymptomatiques pendant la première année. Cette différence entre la souris et l'être humain est due à la capacité de l'enzyme lysosomiale sialidase à dégrader le G_{M2} via une voie métabolique alternative. Pour déterminer si cette voie alternative pourrait être exploitée chez l'être humain, nous avons effectué une séries d'expériences comparant la sialidase murine à la sialidase humaine dans leur capacité à cataboliser la dégradation du G_{M2}. Les résultats suggèrent que, malgré une faible efficacité, la sialidase lysosomale humaine peut dégrader le G_{M2}.

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ABBREVIATIONS

Cer:	ceramide
Ci:	Curie
CNS:	central nervous system
cpm:	counts per minute
EM:	electron microscopy
Gal:	galactose
GalNAc:	N-acetylgalactosamine
Glc:	glucose
Hex:	hexosaminidase
kDa:	kilodaltons
Lac-Cer:	lactosylceramide
MCB:	membranous cytoplasmic body
MEM:	modified Eagle's medium
4-MUG:	4-methylymbelliferyl-ß-N-acetylglucosamine
Mu-Gal:	4-methylymbelliferyl-ß-galactoside
4-MUGS:	4-methylymbelliferyl-ß-N-acetylglucosamine-6-sulfate
Mu-Nana:	4-methylymbelliferyl-N-acetylneuraminc acid

NeuNAc or NeuAc: N-acetylneuraminc acid

NG:	neuroglia
PBS:	phosphate buffered saline
TLC:	thin layer chromatography
TSD:	Tay-Sachs disease

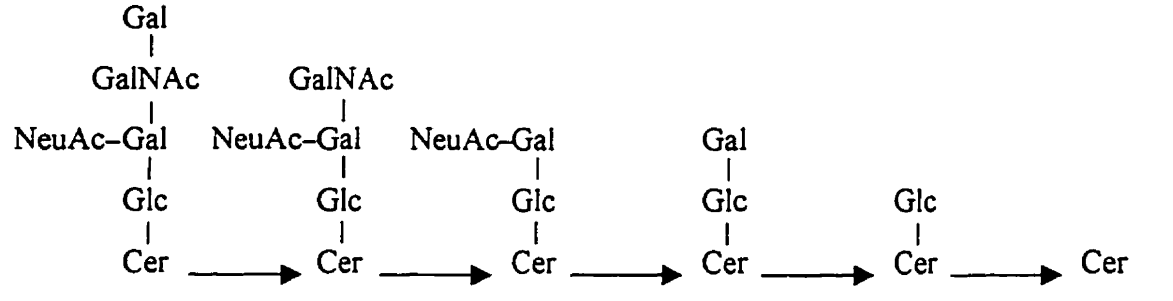
CHAPTER ONE

GENERAL INTRODUCTION

INTRODUCTION

Warren Tay, a British ophthalmologist, was the first to describe Tay-Sachs disease in 1881 when he observed a cherry-red spot in the retina of a 1 year old infant with mental and physical retardation. Later the American neuropathologist Bernard Sachs noted the distended cytoplasm of neurons in patients with this disorder. Tay-Sachs is an autosomal recessive lysosomal storage disorder caused by a deficiency of the lysosomal exohydrolase β-hexosaminidase A (Gravel et al. 1995.) This results in massive intralysosomal accumulation of ganglioside G_{M2} in neuronal cells (Sandhoff et al. 1979.) As such, Tay-Sachs disease is classified as a G_{M2} gangliosidosis and is highly related to two other G_{M2} gangliosidosis disorders. These are Sandhoff disease, caused by the combined deficiencies of *B*-hexosaminidase A (Hex A) and *B*-hexosaminidase B (Hex B) enzymes (Warner et al. 1983) and the AB variant gangliosidosis caused by a deficiency of the G_{M2} activator protein (Conzelmann et al. 1978a, reviewed in Gravel et al. 1995.)

G_{M2} ganglioside is an intermediate in the catabolism of G_{M1} ganglioside, the monosialyl form of a large diversity of gangliosides found in neurons (Echten et al. 1993.) The catabolism of G_{M1} normally proceeds in humans via the sequential hydrolysis of terminal sugars as illustrated in the sequence below (Furst et al. 1992.)



Gal=Galactose, GalNAc=N-acetylgalactosamine, NeuAc=N-acetylneuraminic acid, Glc=Glucose

The conversion of G_{M2} to G_{M3} by removal of the terminal N-acetylgalactosamine residue is catalyzed by Hex A. When Hex A activity is deficient, as in Tay-Sachs disease, G_{M2} can not be degraded and instead accumulates intralysosomally. This differs from the mouse in which G_{M2} apparently is degraded via a different pathway involving removal of the terminal N-acetylneuraminic acid residue by lysosomal sialidase to form the asialo derivative G_{A2} (figure 1) (Phaneuf et al. 1996, Proia et al. 1995.)

This thesis describes an attempt to delineate some of the differences between mouse and human in their abilities to degrade G_{M2}. Specifically, it is an attempt to determine whether the human lysosomal sialidase, if sufficiently induced, can degrade G_{M2} in a manner similar to that of the mouse. Ultimately, this could lead to the use of pharmacologically induced lysosomal sialidase activity as a therapy for Tay-Sachs disease.

GANGLIOSIDE STRUCTURE AND FUNCTION

Gangliosides are glycosphingolipids composed of a hydrophobic ceramide core (N-acylsphingosine) and a hydrophilic oligosaccharide chain that bears one or more sialic acid residues (figure 2.) This sialic acid residue (N-acetylneuraminic acid) confers an anionic charge on gangliosides which is not present in other glycosphingolipids. Typically, gangliosides and other glycosphingolipids are components of the outer leaflet of animal cell plasma membranes and in particular are concentrated in neuronal membranes. With over 100 species identified, gangliosides form a very diverse class of compounds and show a highly variable pattern and content of distribution in the CNS.

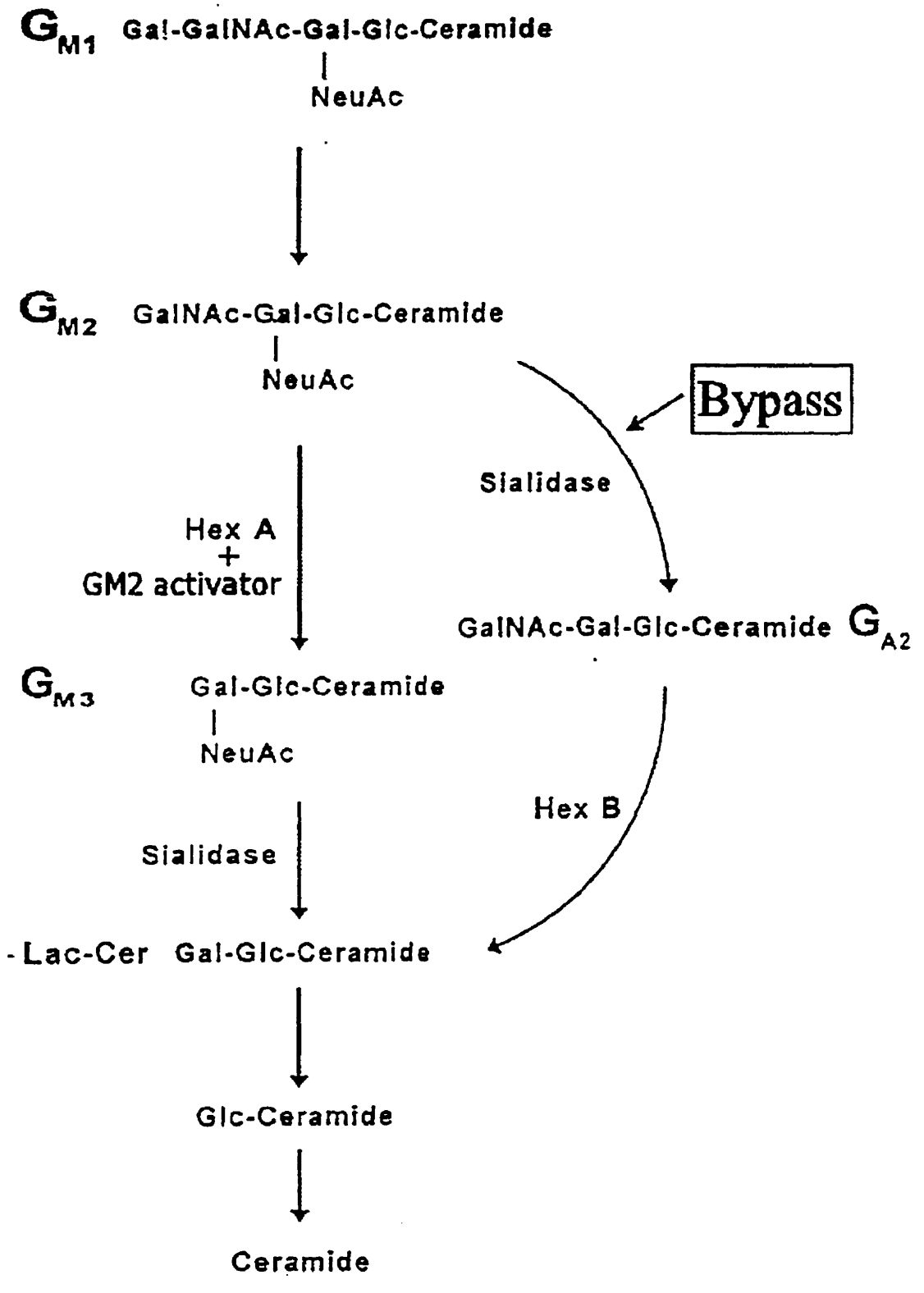


Figure 1. Schematic showing the proposed sialidase mediated bypass for catabolism of G_{M2}. The bypass requires both functional lysosomal sialidase and Hex B.

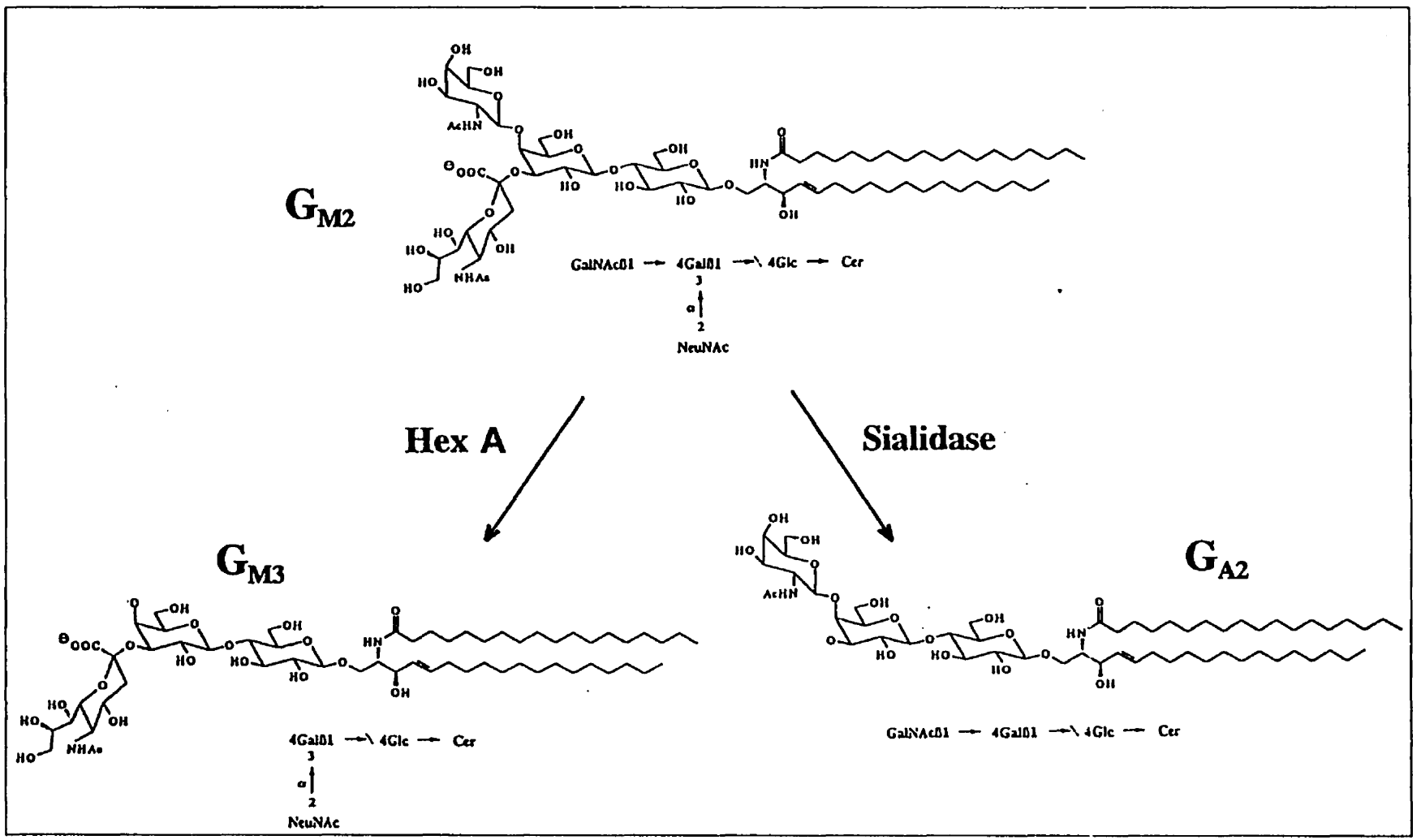


Figure 2. The structure of G_{M2} and two of its derivatives. G_{M3} is derived by removal of the terminal N-acetylgalactosamine residue (GalNac) by Hex A and G_{A2} is derived by removal of the terminal N-acetylneuraminic acid residue (NeuNAc) by sialidase

Anchored into the lipid bilayer via their hydrophobic ceramide core, the oligosaccharide portion of gangliosides extend into the extracellular space where they form cell-type specific patterns believed to act as cell surface markers and to participate in cell-cell interactions (reviewed in Zubay et al. 1993, Echten et al. 1993, Sandhoff et al. 1996, Jeffrey et al. 1998, Furst et al. 1992.) Because of their ubiquitous and complex nature, it has long been assumed gangliosides play a crucial role in cell survival. A recent paper by Takamiya et al. (1996) however has brought this dogma into scrutiny. They show that when the enzyme G_{M2}/G_{D2} synthase is knocked out in mice, thereby preventing the synthesis of gangliosides more complex than G_{M3}, the mice show virtually no neurologic abnormalities. Instead, further examination revealed that the males were sterile and aspermatogenic. These results suggest that complex gangliosides are essential for the transport of testosterone *in vivo* (Takamiya et al. 1998.) Therefore, it is not yet clear what these complex gangliosides have in cell function and survival in the CNS. It is clear however that interference with ganglioside catabolism, as in Tay-Sachs disease, Sandhoff disease, G_{M1} gangliosidosis and sialidosis, is detrimental to cell survival.

G_{M2} GANGLIOSIDOSIS

Hex A is composed of two non-identical subunits, an α-subunit encoded for by the HEXA gene and a ß-subunit encoded for by the HEXB gene. Hex B on the other hand is a homodimer of ß-subunits. (Reviewed in Gravel et al. 1995, Mahuran et al. 1985.) There are three genetically determined metabolic defects that result in G_{M2} gangliosidosis (Gravel et al. 1995.) Tay-Sachs disease is due to a mutation in the α subunit of hexosaminidase, resulting in deficient activity only of the Hex A isozyme. Sandhoff disease is due to a

mutation in the β subunit of hexosaminidase, resulting in deficient activity of both Hex A and Hex B isozymes. Significantly, although Hex A and Hex B share many of the same substrates, Hex B does not act on G_{M2}. Therefore, the presence of Hex B activity in Tay-Sachs patients does not ameliorate the devastating accumulation G_{M2} in this disease. The third form of G_{M2} gangliosidosis is very rare and is due to G_{M2} activator deficiency. It does not affect Hex activities directly but results in G_{M2} accumulation due to an inability to form the G_{M2} ganglioside/G_{M2} activator complex necessary for G_{M2} hydrolysis (Conzelmann et al. 1978a, Hechtman et al. 1982.)

Pathologically all three diseases are characterized primarily by massive intralysosomal accumulations of G_{M2}. Consequently, they have similar clinical phenotypes which can be typified by a description of Tay-Sachs disease. The clinical phenotypes associated with Hex deficiency vary widely and are directly correlated with the severity of the Hex impairment. Null mutants in Hex A for example result in the most severe form of Tay-Sachs whereas mutations that give residual Hex A activity lead to more indolent forms of the disease. The variants of Tay-Sachs disease have been subclassified clinically according to the degree of encephalopathy into infantile, subacute and chronic forms.

Infantile acute Tay-Sachs disease presents stereotypic characteristics that are representative of the prototypic disease once found predominantly amongst Ashkenazi Jewish infants. Affected children appear completely normal at birth. The earliest symptoms, often appreciated only in retrospect by the parents, are mild motor weakness at around 3-5 months of age. Progressive weakness, hypotonia (a decrease in tonic muscle tension), poor head control and decreasing attentiveness are the symptoms that usually draw

parental concern around 6-10 months of age. Visual symptoms such as unusual eye movements or staring episodes also often alert parents of an abnormality. Ophthalmoscopic examination invariably reveals macular pallor with contrasted prominence of the macular fovea centralis (the cherry-red spot.) After 10 months of age the infant becomes continually less responsive to its surroundings, vision deteriorates rapidly and upper and lower motor neuron disease becomes increasingly evident. Further deterioration leads to decerebrate posturing (rigid leg and arm extension), dyscoordinate swallowing and eventually a vegetative state. Death is due to intercurrent infection, usually pneumonia.

The milder forms of Tay-Sachs and Sandhoff diseases show later onset and a less severe clinical course. Chronic forms of these diseases, caused by the least severe mutations of enzyme function, are compatible with life into adulthood. They have some residual Hex A activity resulting in partial G_{M2} catabolism and alleviation of the block.

A characteristic histopathological change in G_{M2} gangliosidoses, is the pronounced swelling of neurons with storage materials (primarily G_{M2} ganglioside) throughout the brain stem, cerebellum and spinal cord. When viewed by EM, the storage material consists of numerous concentrically arranged lamellar structures referred to as membranous cytoplasmic bodies (MCB's). Despite the fact that neurons are engorged with MCB's, mitochondria and other cellular organelles appear normal.

The reason for selective G_{M2} accumulation and subsequent pathology in the CNS is not specifically known. Virtually all cells of the body possess gangliosides and all cells are deficient of Hex A activity in Tay-Sachs patients. Yet most cells do not accumulate gangliosides. This is likely because the rate of biosynthesis of gangliosides vary from cell type to cell type. Ganglioside accumulation in cells would be proportional to the difference

between the rate of substrate biosynthesis and degradation. Therefore, since gangliosides are synthesized predominantly in cells of the CNS, it is expected that these accumulate them most rapidly. It is also possible that cells outside of the CNS, but not within it, express an enzyme that is redundantly protective of Hex A deficiency (eg. lysosomal sialidase). Finally, cells of the CNS do not divide throughout life as do other cells of the body and therefore preclude the simple possibility of reducing accumulated G_{M2} by expansion of cell number. (Reviewed in Gravel et al. 1995, Kandel et al. 1991, Echten et al. 1991, Rowland et al. 1992, Suzuki et al. 1995, Conzelmann et al. 1996, Guazzi et al. 1990, Vanier et al. 1992.)

G_{M2} METABOLISM IN THE HUMAN AND MOUSE

Ganglioside G_{M2} is hydrolyzed by Hex A in the presence of a substrate specific co-factor, the G_{M2} activator protein. Recently, it has been demonstrated by Li et al that this requirement for the activator protein is due to the specific rigid conformation of the G_{M2} oligosaccharide. Although there are two major isozymes of Hex, Hex A and Hex B, only Hex A can cleave the terminal GalNAc residue of G_{M2}. This is because Hex A is capable of cleaving negatively charged substrates such as gangliosides. In addition, Hex A but not Hex B can interact with the G_{M2} activator protein which is a prerequisite for G_{M2} degradation in vivo. (Reviewed in Echten et al. 1991, Sandhoff et al. 1998, Furst et al. 1992, Jeffrey et al. 1985, Gravel et al. 1995.)

Tay-Sachs disease (α-subunit mutation) and Sandhoff disease (ß-subunit mutation) mouse models have been generated by targeted gene disruption. (Phaneuf et al. 1996, Proia et al. 1995.) The *Hexa -/-* mouse completely lacks Hex A enzyme activity and the *Hexb -/-* mouse lacks both Hex A and Hex B activity as expected. The *Hexb -/-* mouse develops a

rapidly progressive neurodegenerative disease similar to that of Sandhoff disease in humans. Surprisingly however, the *Hexa -/-* mouse appears phenotypically normal (until, as we now know, much later in life.) We and Proia et al showed that this is likely due to a metabolic difference between the mouse and human. The mouse can apparently degrade G_{M2} ganglioside by use of a metabolic bypass, which utilizes lysosomal sialidase to remove the terminal sialic acid from G_{M2} to form G_{A2}. Hex B can then act on G_{A2}, converting it to lactosylceramide and hence continue the degradative pathway (figure 1.) In this way, G_{M2} ganglioside does not accumulate to toxic levels and *Hexa -/-* mice evade disease. *Hexb -/-* mice can not make use of this metabolic bypass because they lack a functional Hex B enzyme. It appears that humans do not use this metabolic bypass despite the fact that Tay-Sachs patients have both a functional lysosomal sialidase and Hex B.

LYSOSOMAL SIALIDASE

Sialidases are hydrolytic enzymes that cleave terminal 2 -> 3 and 2 -> 6 sialyl linkages of several oligosaccharides, glycoproteins and glycolipids. Sialidase has been well studied in viruses and bacteria where it destroys the sialic acid-containing receptors at the surface of host cells and mobilizes bacterial nutrients (Galen et al. 1992, Corfield 1992.) In mammals, sialidases have been associated with numerous important biological functions such as antigenic expression, recognition of cell surface receptors, inflammation, infection, cancer and rheumatism (Saito and Yu 1995, Reuter and Gabius 1996.) Traditionally, mammalian sialidases have been characterized based on their subcellular distribution into cytosolic, plasma membrane and lysosomal types (Miyagi et al. 1990.) All three have been cloned in mammals and are present in mouse and human (Miyagi et al. 1993, Pshezhetsky et

al. 1997, Bonten et al. 1996, Campbell et al. 1997, Igdoura et al. 1998, Carrillo et al. 1997, Miyagi et al. 1999.) Though both the cytosolic and plasma membrane sialidases are known to degrade gangliosides generally, they do not degrade G_{M2} to any appreciable extent (Miyagi et al. 1990, Miyagi et al. 1999.) It is the lysosomal sialidase that plays a potential role in the degradation of ganglioside G_{M2} (Fingerhut et al. 1992.) It has been shown that both partially purified mouse and human lysosomal sialidase extracts have catalytic activity towards the sialic acid residue on G_{M2} ganglioside (Tallman et al. 1972, Kolodny et al. 1969, Nagai and Yamada 1988.) The partially purified nature of these extracts however, leaves some ambiguity as to the specific role of lysosomal sialidase in these experiments.

Lysosomal sialidase is part of a multienzyme complex that also contains ß-galactosidase and cathepsin A (protective protein) (Pshezhetsky and Potier 1996.) Sialidase is active only in association with this complex. Van der Spoel et al. (1998) demonstrated by subcellular fractionation of transfected cells that sialidase segregates to mature lysosomes only when accompanied by wild-type cathepsin A. The human lysosomal sialidase cDNA has been cloned and characterized (Pshezhetsky et al. 1997, Bonten et al. 1996, Campbell et al. 1997) and more recently the mouse lysosomal sialidase has also been cloned (Igdoura et al. 1998, Carrillo et al 1997, Rottier et al 1998.) The mouse sialidase cDNA shows a high degree of homology to its human counterpart (80%). Restoration of enzyme activity in human sialidosis cells by expression of the mouse lysosomal sialidase cDNA underlines the structural similarity between mouse and human lysosomal sialidases and suggests a conserved mechanism for complex association and function.

EVIDENCE FOR SIALIDASE MEDIATED G_{M2} CATABOLISM

Evidence suggesting the existence of a sialidase mediated mechanism for G_{M2} catabolism comes from the study of Hex A and Hex B knockout mice as discussed above. Obviously, if mice without a functional Hex A enzyme do not accumulate G_{M2}, it must be degraded by another enzyme. Direct evidence for a G_{A2} route of G_{M2} catabolism was initially suggested by Riboni et al. who treated mouse neuroblastoma cells with ^{3}H-G_{M2} and showed that the principal metabolites were G_{A2} and Lac-Cer rather then G_{M3} and Lac-Cer (Riboni et al. 1995.) A similar incubation with ^{3}H-G_{M1} also resulted in catabolism through G_{A2}. Proia and colleagues went on to examine ^{3}H-G_{M1} catabolism in embryonic fibroblasts cultured from *Hexa -/-* and *Hexb -/-* mice and demonstrated the dominance of the G_{A2} pathway, even in normal cells (figure 3) (Proia et al. 1995.) This contrasts sharply to similar studies performed in human fibroblasts where it was shown that metabolism of G_{M2} proceeds exclusively through the G_{M3} pathway (Srinivasa et al. 1985.) Finally, our study of the pattern of G_{M2} accumulation in the brain and spinal cord of *Hexa -/-* mice correlates with the relative activity of the sialidase bypass to catabolize gangliosides.

There is indirect evidence to suggest that such a bypass could function in humans. Examination of the brains of Sandhoff and Tay-Sachs patients at autopsy revealed a several-fold higher level of G_{A2} accumulation in the former, suggesting that G_{A2} is indeed synthesized from G_{M2} in humans (Sandhoff et al. 1971.) Lack of G_{A2} in the Tay-Sachs brain supports the hypothesis that once formed G_{A2} is degraded by Hex B when present. Therefore, it appears that G_{M2} may be degraded by lysosomal sialidase in humans, but at a level insufficient to be protective. Indeed, northern blot analysis of human brain tissue showed very low levels of lysosomal sialidase expression. The findings in mice and the

insufficiently potent activity of sialidase in humans opens the possibility that elevation of the level of lysosomal sialidase in humans may be all that is needed to confer protection from the devastating affects of Hex A deficiency.

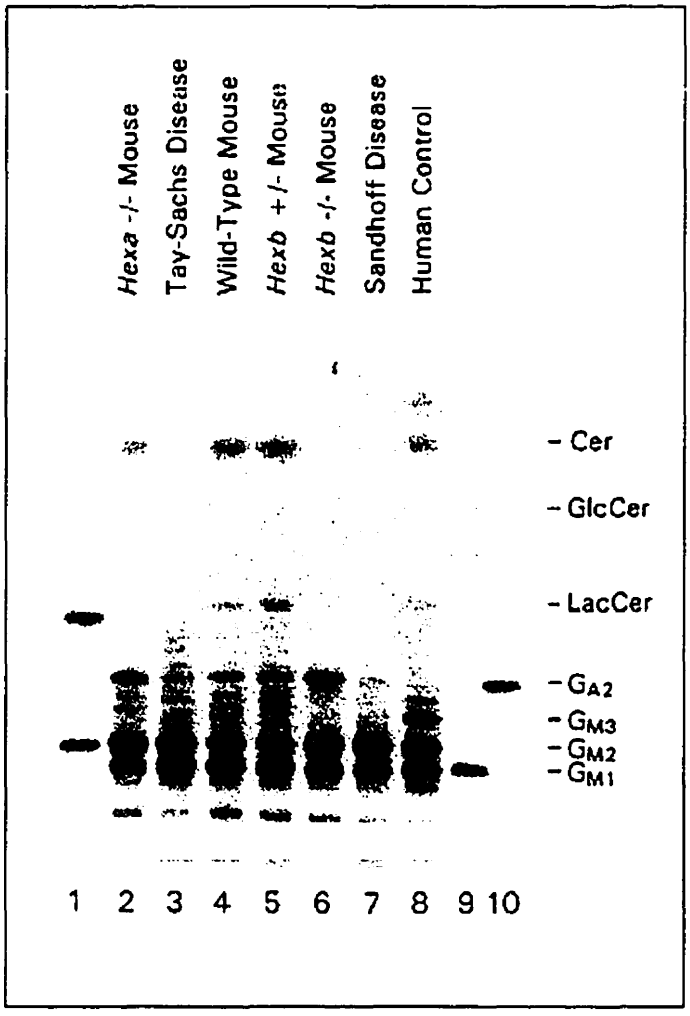


Figure 3. Ganglioside metabolism in mouse and human fibroblasts. ^{3}H-G_{M1} ganglioside was added to fibroblast cultures for 120 hours. The glycolipids were extracted, analyzed by thin-layer chromatography and visualized on a phosphoimager. Lane 1, G_{M2} and lactosylceramide standards; Lane 2, *Hexa -/-* embryonic fibroblasts; Lane 3, Tay-Sachs disease fibroblasts; Lane 4, wild-type mouse embryonic fibroblasts; Lane 5, *Hexb +/-* embryonic fibroblasts; Lane 6, *Hexb -/-* embryonic fibroblasts; Lane 7, Sandhoff disease fibroblasts; Lane 8, normal human fibroblasts; Lane 9, G_{M1} standard; Lane 10, G_{A2} standard (Proia et al. 1995.)

THESIS OUTLINE

The research in this thesis is aimed at determining why the metabolic bypass taken advantage of in *Hexa -/-* mice is not utilized in humans. Is catabolism through the G_{A2} pathway a species-specific feature of mice? Or is it simply that the mouse brain expresses lysosomal sialidase at levels sufficient to activate sialidase mediated G_{M2}

catabolism. If there is a substrate specificity difference, is the difference subtle enough such that it can be overcome with increased sialidase expression in the human brain? That is to say, will induced sialidase expression in the human brain activate the sialidase bypass? Finally, it is possible that the differences between mouse and man in G_{M2} metabolism are completely unrelated to the activities of sialidase. Instead, the metabolic differences may reflect the dissimilarities of other proteins involved in G_{M2} degradation, such as the G_{M2} activator protein or the sulfatide activator. In any case, it is clear that the variation in G_{M2} degradative capacity need only be very subtle to result in dramatic phenotypic differences. Studies of hexosaminidase mutations have revealed that as little as 10% residual Hex A activity can prevent the appearance of disease in humans. This suggests that a low but significant threshold of G_{M2} degradation exists, below which ganglioside accumulation and disease result. An ultimate aim of these studies therefore, is to determine the therapeutic feasibility of inducing sialidase activity above this threshold in human Tay-Sachs patients such that the sialidase mediated bypass becomes available and accumulated G_{M2} can be cleared.

Two techniques were used in an attempt to assess the activity of human lysosomal sialidase against G_{M2}. In the first, mouse and human lysosomal sialidases were purified using classical chromatography techniques and then compared directly for their ability to degrade ^{3}H-G_{M2} *in vitro*. The second technique involved the creation of stably transfected Tay-Sachs neuroglia cell lines expressing mouse and human components of the sialidase bypass. These cell lines were then fed ^{3}H-G_{M2} and the products analyzed to observe the degradation of ^{3}H-G_{M2} *in vivo*.

Chapter 2 contains the paper describing the cloning of the mouse lysosomal sialidase cDNA, a necessary prerequisite for the creation of cell lines expressing this enzyme.

Chapter 3 describes the biochemical purification of mouse and human lysosomal sialidases and their comparison of activity against ^{3}H-G_{M2} *in vitro*.

Chapter 4 describes a series of pulse-chase experiments with ^{3}H-G_{M2} in Tay-Sachs neuroglia cell lines stably expressing mouse and human lysosomal sialidase with cathepsin A.

Chapter 5 contains a summary of thesis work and proposals for future experiments.

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CHAPTER TWO

CLONING OF THE MOUSE LYSOSOMAL SIALIDASE

CLAIM OF ORIGINALITY

This paper is included as a minor component of my thesis. It is published in Human Molecular Genetics 7(1), pages 115-121, 1998. I have included it because the cloning of the mouse lysosomal sialidase cDNA was a vital and necessary prerequisite for the remainder of my research.

My participation was limited to the expression of the cloned cDNA with and without cathepsin A by transient expression into human sialidosis and mouse SM/J fibroblasts and subsequent measurement of sialidase and ß-galactosidase activities.

Carmen Mertineit performed the intracellular localization of the expressed sialidase in sialidosis and SM/J fibroblasts using double immunolabelling of sialidase and LAMP2.

Farzad Saberi evaluated the tissue distribution of sialidase mRNA in different mouse tissues by Northern blot using the mouse sialidase cDNA as a probe.

Suleiman Igdoura performed the majority of the work including the cloning of the sialidase cDNA and gene, determination of the structure of the mouse lysosomal sialidase gene, statistical analysis and preparation of the manuscript.

Roy A. Gravel, Alex V. Pshezhetsky, Michel Potier, and Jacquetta M. Trasler were supervisors of the project.

CLONING OF THE CDNA AND GENE ENCODING MOUSE LYSOSOMAL SIALIDASE AND CORRECTION OF SIALIDASE DEFICIENCY IN HUMAN SIALIDOSIS AND MOUSE SM/J FIBROBLASTS.

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Abstract

Lysosomal sialidase occurs in a multienzyme complex that also contains ß-galactosidase and cathepsin A. We previously cloned the human lysosomal sialidase cDNA and characterized mutations in human sialidosis patients. Here, we report the cloning and expression of the mouse lysosomal sialidase cDNA and gene. The 1.77 kb cDNA encodes an open reading frame of 408 amino acids which shows high homology to the human lysosomal sialidase (80%), the rat cytosolic sialidase (65%) and viral and bacterial sialidases (50-55%). The sialidase gene is approximately 4 kb long and contains 6 exons. The 5 introns range in size from 96 bp to 1200 bp. Northern blot analysis revealed a high expression of multiple sialidase transcripts in kidney and epididymis, moderate levels in brain and spinal cord, and low levels in adrenal, heart, liver, lung and spleen. Transient expression of the cDNA clone in sialidase-deficient SM/J mouse fibroblasts and human sialidosis fibroblasts restored normal levels of sialidase activities in both cell types. Immunocytochemically, expressed sialidase co-localized with a lysosomal marker, LAMP2 confirming its lysosomal nature. Since sialidase activity requires its association with ß-galactosidase and cathepsin A, the expression of mouse sialidase within human sialidosis cells underlines the structural similarity between mouse and human enzymes and suggests that the mechanism for complex formation and function is highly conserved.

INTRODUCTION

Sialidases cleave terminal α2→3 and α2→6 sialyl linkages of oligosaccharides and glycoproteins, a process associated with numerous important biological reactions such as antigenic expression and recognition of cell surface receptors (1,2). Generally, sialidases have been classified based on their subcellular distribution and substrate specificity into 3 types: cytosolic, lysosomal and plasma membrane (3,4). Although the rat and chinese hamster cytosolic sialidases had been cloned previously (5,6), it is only recently that the human lysosomal sialidase cDNA has been successfully identified (7,8,9,10). The identity of the cDNA was confirmed through mutation analysis of sialidosis patients and expression of sialidase activity in deficient human fibroblasts.

The human lysosomal sialidase is a glycoprotein which exists in two isoforms of 44 and 48 kDa (8) and is only active as a part of a high molecular weight lysosomal multienzyme complex that also contains ß-galactosidase and cathepsin A (11). The human lysosomal sialidase gene was mapped to chromosome 6p21 within the human Major Histocompatibility Complex (MHC) (10). Mapping of the mouse sialidase gene was possible because the SM/J strain mouse carries a defective sialidase allele and as a result has a tissue-specific deficiency in sialidase activity (12). The mouse sialidase gene was mapped near the H-2D end of the mouse MHC on chromosome 17, a region which is syntenic to the human MHC region on chromosome 6 (13), suggesting that these may be analogous genes.

Here we report the identification and characterization of the mouse lysosomal sialidase cDNA and gene. Transient expression of the cDNA in sialidase-deficient SM/J

mouse fibroblasts and in human sialidosis fibroblasts restored sialidase activity. Expression in normal cells was dependent on co-transfection with cathepsin A cDNA.

RESULTS

Cloning of sialidase cDNA:

The complete cDNA is 1.77 kb with an open reading frame of 1227 bp encoding a 409 amino acids polypeptide (Fig. 1a). The protein contains a putative 40 amino acids signal peptide, a "FRIP" sequence, four "aspartic boxes" and four potential glycosylation sites. The predicted molecular weight of the protein is 44.6 kDa. With the exception of the signal peptide, the mouse sequence shows high homology with the human lysosomal sialidase sequence (>85%) (Fig. 1b), with the rat cytosolic sialidase (65%) and with viral and bacterial sialidases (50-55%).

Structure of the mouse lysosomal sialidase gene:

Four genomic DNA fragments containing 5 introns were amplified by PCR from mouse (C57BL/6) genomic DNA (Fig. 1c). The mouse sialidase gene is approximately 4 kb long from the translation initiation codon (ATG) to the termination signal (in exon 6) and is structurally similar to the human gene. There is no distinguishable polyadenylation signal. The introns range in size from 96 bp to 1.2 kb (Table 1). All the exon-intron boundary sequences were consistent with the established splice consensus sequences (14).

Intracellular localization:

In order to determine if the expressed sialidase is targeted to the lysosomes, SM/J

lung fibroblasts (Fig. 4a-c) and sialidosis fibroblasts (Fig. 4d-f) transfected with a pCMV-sialidase vector were double immunolabeled for sialidase and for LAMP2 (lysosomal marker)(15). In sialidosis cells, only two successfully transfected cells in Figure 6e show reactivity to the anti sialidase antibody confirming the specificity of the antibody. In SM/J and sialidosis cells transfected with sialidase cDNA, anti sialidase immunofluorescence was observed in perinuclear punctate structures that colocalized with anti LAMP2 immunofluorescence, confirming a lysosomal location for the expressed mouse sialidase (Fig. 4a-f).

Table 1: Intron/exon boundaries in the mouse lysosomal sialidase gene

Primer Pairs	Intron	Exon 5'-splice donor	size (bp)	Exon 3'-splice acceptor
5'-GGCCAGGGCAGAGGATGACTTCAGC	I	AGCCTGgtgagcctt	420	tctgcgcagGTGCAG
5'-GTCGTCCTTACTCCAAACAACATGG	II	ACCAGGgtaacaagc	430	ttcttctagGTAGCA
5'-CCGGAATCTCTCTGTGGATATTGG 5'-CAAAGGGAATGCCGCTCACTCC	III	ATTCAGgtttcaccc	1200	tcttaacagAAACAG
5'-CCCAAACACGATCACGATTTCAACC	IV	TGCCAGgtcaggagt	96	cccacgcagCCCTAC
5'-GGTTCAGGCCTTTCTCGTACAG	V	AGTTCCgtgagtgcc	101	gctctctagGAGTGA

Tissue distribution of sialidase mRNA:

To evaluate the levels of sialidase expression in different mouse tissues, total RNA was analyzed by Northern blot using the mouse sialidase cDNA as a probe. The highest

levels of expression were found in the kidney and epididymis, with moderate levels in the brain and spinal cord, and low levels in adrenal, liver, lung, spleen and heart (Fig. 2). Transcript size varies from 1.8 to 2.6 kb with only the epididymis showing predominantly faster migrating mRNA transcripts. Reprobing the same blot with an 18S oligonucleotide probe confirmed that loading was similar in all lanes, with the exception of the kidney and epididymis samples where only 7.5 μg/lane were used.

Transient Expression of cDNA:

Transfection with mouse sialidase cDNA restored sialidase enzymatic activities in SM/J deficient fibroblasts and in human sialidosis fibroblasts to normal levels (Fig. 3a). Co-transfection of cathepsin A cDNA with the sialidase cDNA in deficient cells increased sialidase activity further to 10-fold in SM/J mouse cells and 40-fold in human sialidosis cells. However, expressing mouse sialidase cDNA in normal human and wild type mouse fibroblasts did not alter levels of sialidase activity. Only when human cathepsin A cDNA was co-transfected with sialidase cDNA did sialidase activity increase by 2-fold in both cell types. In all transfections, levels of a control lysosomal enzyme, ß-galactosidase, remained unchanged (Fig. 3b). The pH curve for expressed sialidase revealed optimum activity at pH 4.5 which is characteristic of lysosomal enzymes.

DISCUSSION

In this study, we report the cloning, sequencing and expression of the mouse lysosomal sialidase cDNA. The alignment of the amino acid sequences demonstrated that

the mouse cDNA sequence shares significant homology with the human lysosomal, rodent cytosolic and bacterial sialidases. This homology includes the highly conserved FRIP domain and aspartic boxes (16). In addition, the residues which bind the carboxylate group of the sialic acid substrate are conserved between bacterial sialidase (Arg 37, 246 and 309) and human (Arg 78, 280 and 347) and mouse (Arg 72, 274 and 341) sialidases (10,17). The three dimensional structure of the mouse lysosomal sialidase appears therefore to resemble that of bacterial sialidases which consist of six four-stranded antiparallel ß-sheets arranged as a propeller (17). The highly conserved sequences shared among the various sialidases support a common phylogenetic origin for mammalian and bacterial sialidases.

The structure of the mouse lysosomal sialidase gene is similar to the human gene with exon-intron junctions at identical sites. However, intron sizes varied considerably between the mouse and human genes, in particular intron III which is 1.2 kb in mouse and 0.425 kb in human. Furthermore, while Northern blot analysis of human tissues revealed a single transcript of 1.9 kb in all tissues (8), mouse tissues show substantial heterogeneity in sialidase mRNA transcripts pointing to the possibility of alternative splicing.

The low endogenous levels of sialidase activity in liver of SM/J mice has been known for many years (12). In this report, we have established a cell line of SM/J lung fibroblasts with a sialidase deficient phenotype. The very low endogenous levels of lysosomal sialidase in these SM/J cells made them suitable for expression of the mouse sialidase clone and for the subsequent detection of elevated sialidase enzymatic activity and sialidase immunolocalization. The increased sialidase activity obtained after co-transfection

of human cathepsin A cDNA with mouse sialidase cDNA confirms that the mouse lysosomal sialidase can form a catabolically active complex with human cathepsin A and mouse ß-galactosidase proteins. Similarly, the expression of the mouse sialidase clone in human sialidosis cells confirms that the mouse lysosomal sialidase can form a catabolically active complex with human cathepsin A and ß-galactosidase proteins. These interspecies interactions among the various components demonstrate a conserved mechanism for complex assembly and activation. Furthermore, the elevation of sialidase activity in normal human fibroblasts by co-transfection of mouse sialidase and human cathepsin A cDNAs together, but not by sialidase alone, illustrates the role of cathepsin A in the activation of sialidase. This is consistent with previous findings that the presence of cathepsin A is essential for the expression of sialidase activity (18).

The lysosomal sialidase has been shown biochemically to cleave sialic acid from oligosaccharides and glycoproteins (1) and from G_{M3} gangliosides (19). We (20) and others (21,22) have described mouse models for Tay-Sachs (*Hexa* -/-) and Sandhoff (*Hexb* -/-) diseases which implicated lysosomal sialidase in the degradation of G_{M2} to G_{A2}, allowing the *Hexa* -/- mice to escape disease symptoms. This sialidase-mediated sparing effect does not occur in human Tay-Sachs patients, although the human brain expresses lysosomal sialidase. This may implicate a substrate specificity difference between mouse and human lysosomal sialidases or it may reflect a difference in the level of the enzyme in the CNS. Further studies will be required to differentiate between these alternatives.

Overall, the molecular characterization of the mouse lysosomal sialidase has

strengthened our understanding of the conserved nature of mammalian sialidases and may shed light on other roles for sialidase revealed through disease.

MATERIALS AND METHODS:

Cell lines:

Normal and sialidosis human fibroblasts were obtained from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital, Montreal (sialidosis cell strain code WG0544). Primary cultures of SM/J and C57BL/6 lung fibroblast were established as follows. Lung tissue was excised from male SM/J or C57BL/6 mice that had been previously perfused through the heart with saline. The tissue was rinsed a few times with cold sterile saline then placed into a glass Petri dish where it was minced thoroughly using sterile blades. Minced tissue was collected into Hanks' Balanced Salt Solution (HBSS) and gently vortexed. The cell masses were then allowed to settle for 10 min on ice. The supernatant was removed and the masses were resuspended in fresh HBSS. An equal volume of HBSS solution containing 0.125% Trypsin and 0.002% DNAse was added and the cells were incubated on a shaker at 37°C for 20 minutes. An equal volume of MEM medium containing 10% FCS was then added to the trypsinized cells and centrifuged at 120xg for 3 minutes. The supernatant contained most of the lung fibroblasts which were retrieved by centrifugation at 3000xg for 5 minutes. All cells types were maintained in Modified Eagle's Medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies:

Mouse and rat monoclonal antibodies against human and mouse LAMP2 respectively were obtained from the Developmental Studies Hybridoma Bank in Baltimore, MD (15). TRITC-conjugated goat anti rabbit IgG, FITC-conjugated goat anti mouse IgG and FITC-conjugated goat anti rat IgG were purchased from Sigma (St.Louis). Rabbit polyclonal antibodies against recombinant human sialidase were prepared as follows. A human sialidase cDNA fragment of 755 bp was obtained by BstEII/TaqI restriction. After treatment with the Klenow fragment DNA polymerase the fragment was inserted into a pGEX-2T vector (Pharmacia) and expressed in E. coli to produce a SIAL-glutathione transferase (GST) fusion protein. The fusion protein was purified from the bacteria homogenate by affinity chromatography on glutathione-Sepharose (Pharmacia) followed by FPLC anion-exchange chromatography on a Mono Q column (Pharmacia). Purified fusion protein, homogeneous by SDS-PAGE analysis, was emulsified with Freunds complete adjuvant and used to immunize a rabbit by subcutaneous injections. The rabbit was boosted ten and twenty days after the first immunization with another 5 μg of the same preparation in incomplete Freunds adjuvant. IgG fraction from the obtained antiserum was further purified using a recombinant GST coupled to CNBr-Sepharose to absorb the anti-GST-specific antibodies. The specificity of the antibody was tested by Western blot analysis (at dilution of 1:2000, data not shown) and by immunofluorescent microscopy (at 1:200, Figure 4e).

Cloning of mouse sialidase cDNA and gene:

Homology searches in the dbEST database (National Center for Biotechnology Information) were performed using the Online BLAST program in order to identify clones with high homology to the human sialidase sequence. The complete mouse lysosomal sialidase cDNA was found in I.M.A.G.E Consortium cDNA clone AA107584 and was obtained from Genome Systems,Inc. (St.Louis).

DNA was amplified from 1 μg of genomic DNA using polymerase chain reaction (PCR) procedures as described elsewhere (23). Based on previously published human sialidase gene structure (9), primers were designed to amplify the introns of the mouse sialidase gene by PCR. The primer pairs used to amplify the introns are given in Table 1. PCR amplified genomic fragments were separated on agarose gels and extracted from the gel using a GeneClean kit (Bio/Can Scientific, La Jolla, CA). PCR products were subcloned into a pCR2.1 vector using the TA-cloning Kit (Invitrogen) and sequenced manually or at the DNA Core facility of the Canadian Genetic Disease Network, Ottawa, Ontario.

Subcloning into expression vectors:

The mouse sialidase cDNA (1.77 kb) flanked with XhoI and Sal I restriction sites was subcloned into the Sal I site of a pCMV-Sport2 vector (GIBCO). The human cathepsin A cDNA was a gift from Y.Suzuki (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The cDNA was excised from a pGEM vector using EcoRI and subcloned into the EcoRI site of the pCMV-Sport2 vector. The orientation of the cDNA was confirmed in both vectors with NcoI restriction analysis.

Northern Blot Analysis:

To evaluate the level of sialidase mRNA in mouse tissues, total RNA from adrenal, spleen, Brain cortex, brain cerebellum, brain stem, spinal cord, heart, kidney, liver, lung and epididymis was analyzed by Northern blot analysis. Total RNA was isolated from different tissues using the acid guanidinium thiocyanate-phenol-chloroform method (24). RNA concentration was measured spectrophotometrically at 260 nm using a Perkin Elmer UV spectrometer and 15 μg of RNA per lane was loaded for all tissues except kidney and epididymis for which 7.5 μg of RNA per lane was loaded. The RNA was denatured and subjected to electrophoresis in 1.5% agarose formaldehyde gels and transferred to Zetabind nylon membranes. Hybridization and washing conditions were as described previously (23). An end-labelled synthetic oligonucleotide recognizing 18S ribosomal RNA was used to assess equivalence of loading between lanes.

Expression in mammalian cells:

For transient expression, cells (normal human fibroblasts, human sialidosis fibroblasts, wild type mouse C57BL/6 fibroblasts and mouse SM/J fibroblasts) were transfected with the mouse sialidase cDNA with or without the human cathepsin A cDNA. Transfection was done using lipofectamine (5 μg vector(s) and 20 μl lipofectamine solution in 2 ml of optimem solution for 75 mm flask of cells) as described by the manufacturer (GIBCO). For control, a pCMV-sport2 vector containing no insert was used as a mock vector. After transfection, cells were incubated for 24 hrs in MEM medium containing 15% FCS with no antibiotics. On the second day, the medium was replaced with medium

containing 15% FCS and antibiotics. Transfection efficiency was variable among cell types and therefore statistical comparisons between Cell types were not considered. After 72 hrs of transfection, the cells were washed once with PBS and then scraped in cold PBS. Cells were pelleted and then sonicated for 5 sec in 100 µl distilled water. Cell homogenates were assayed for lysosomal sialidase and ß-galactosidase enzymes activity using sodium 4-methylumbelliferyl-D-N-acetylneuraminate (Sigma) and 4-methylumbelliferyl-ß-D-galactoside (ICN) substrates respectively (25). The data collected represent three separate experiments. Analysis of pH effect on sialidase activity was performed after expression of the mouse sialidase in sialidosis human fibroblast for 72 hrs. Sialidase activity was measured at the following pHs: 1.5, 2.5, 3.7, 4.2, 5.1, 6.3 and 6.9.

Immunocytochemical localization of lysosomal sialidase:

Transfected cells expressing mouse sialidase cDNA, grown on chambered slides, were washed in cold PBS and fixed for 30 minutes in 3.8% paraformaldehyde-PBS. Cells were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes. After washing twice in cold PBS, cells were blocked with 10% goat serum in PBS for one hour. For double labelling, primary antibodies (diluted 1:200) were incubated with the cells at 4°C overnight. On the second day, cells were washed three times for five minutes each in PBS containing 0.1% Tween-20. Cells were then incubated with secondary antibodies for one hour at room temperature and again washed in PBS containing 0.1% Tween-20. A final wash was performed in distilled water to remove residual salts. Cells were mounted on slides with 50% glycerol in PBS containing 1,4-diazabicyclo-[2,2,2]-octane (DABCO, sigma)

antifading agents. Double antibody labelling experiments were analyzed on a Zeiss LSM 410 inverted confocal microscope (Carl Zeiss Inc. Thornwood, NY) as described previously (26). The fluorescein signal was imaged by exciting the sample with a 488-nm line from an argon or an argon/krypton laser and the resulting fluorescence was collected on a photomultiplier after passage through FT510, FT560 and BP515-540 filter sets. Likewise, the same field was excited with a helium/neon (543-nm line) laser and the Rhodamine signal was imaged on a second photomultiplier after passage through FT510, FT560 and LP590 filter sets. The green and red images were overlaid and pseudo-coloured using built-in LSM software. Images were obtained with 25X/0.8 or 63X/1.4 plan-APOCHROMAT (Zeiss) oil objectives and printed on a Kodak XLS8300 colour printer.

Statistical Analysis:

Data were analyzed using Bartlett's test for homogeneity. One-way analysis of variance was used to test for differences within each cell type (27). For comparisons between treatments of each cell type, a modified least significant difference test was used and significance was set at $P<0.01$ (27).

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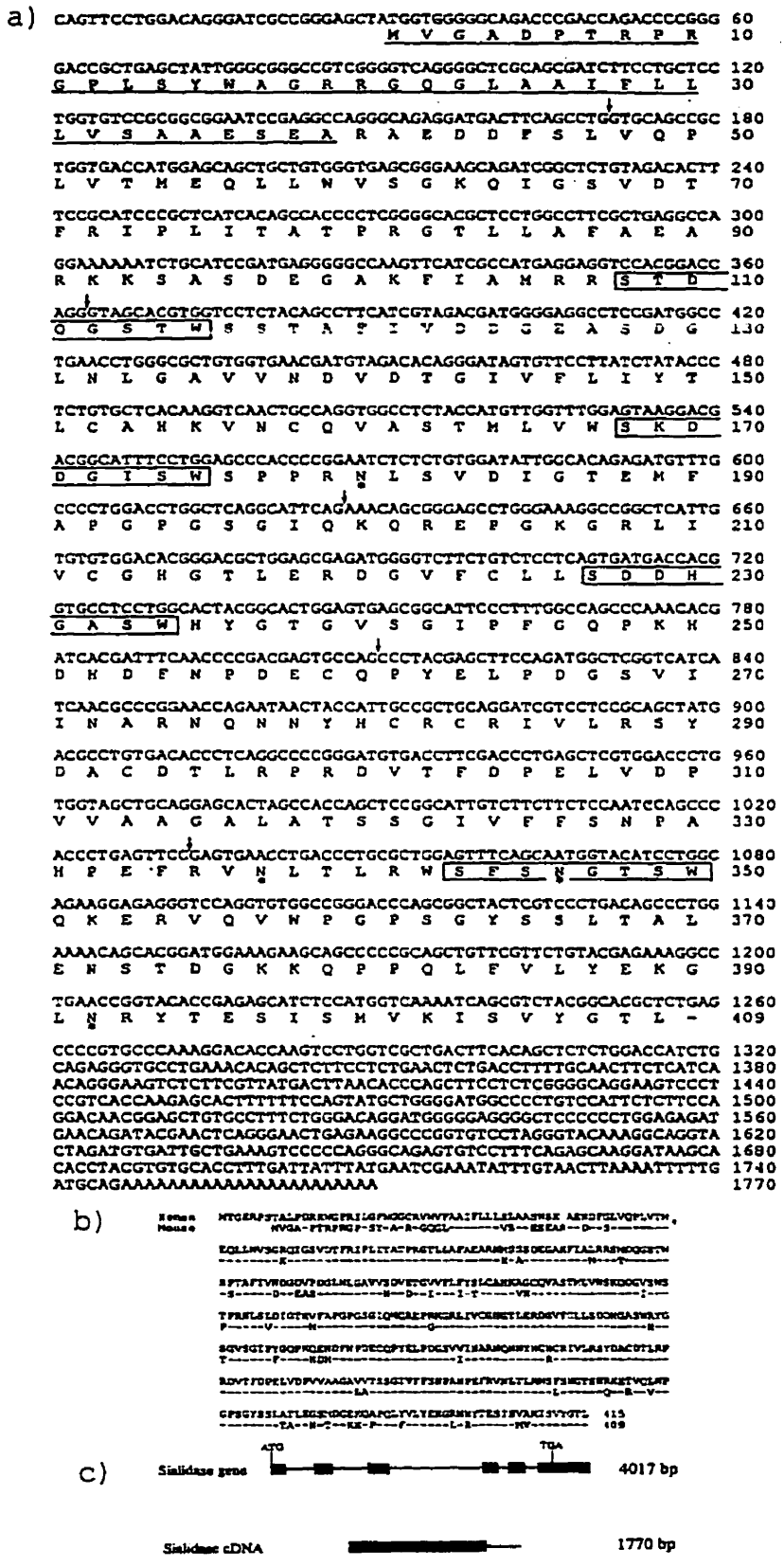


Figure 1

(a) Nucleotide sequence and deduced amino acid sequence of the mouse lysosomal sialidase. The putative signal peptide is underlined. The four potential glycosylation sites are marked with asterisks and the four conserved aspartic boxes are boxed. Arrows indicate positions of intron/exon boundaries. (b) Amino acid sequence alignment of the mouse and human sialidases. While the putative signal peptides show little similarity, the rest of the sequences share extensive homology including identical locations for the aspartic boxes and three of the four potential glycosylation sites. Note that the fourth glycosylation site is substituted by a glycine in the human sialidase sequence. (c) Schematic showing the genomic organization of the mouse lysosomal sialidase gene. The introns vary in size from 96 bp to 1200 bp. The intron/exon junctions are conserved between mouse and human sialidase genes.

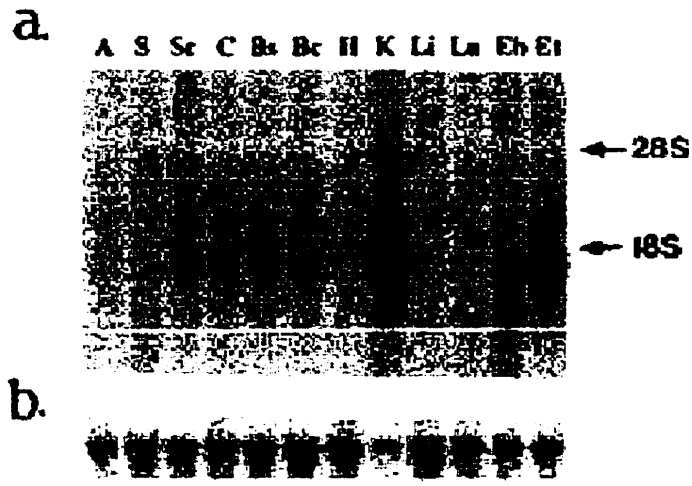


Figure 2
Northern blot analysis of lysosomal sialidase mRNA in various mouse tissues. The multiple transcripts reflect heterogeneity possibly due to alternative splicing. Total RNA from various tissues (10 μg/lane) was electrophoresed though a 1.5% formaldehyde/agarose gel and transferred to a nylon membrane. The membrane was hybridized with ^{32}P-labelled mouse sialidase cDNA. A,adrenal; S, Spleen; Sc,spinal cord; C, cerebellum; Bs, brain stem; Bc, brain cortex; H,heart; K,kidney; Li,liver; Lu,lung; Eh, epididymal head; Et, epididymal tail.

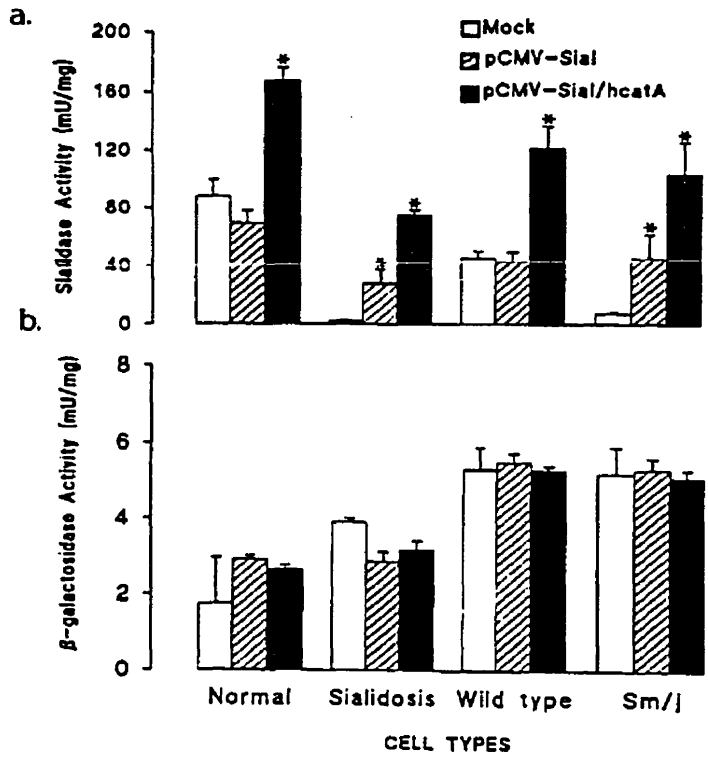


Figure 3

Sialidase (a) or ß-galactosidase (b) activities in transiently transfected normal human fibroblasts (Norm), human sialidosis fibroblasts (sialidosis), mouse C57BL/6 lung fibroblasts (wild type) and mouse SM/J lung fibroblasts (SM/J). Cells were either transfected with mock vector, mouse sialidase cDNA or a combination of mouse sialidase cDNA and human cathepsin A (hCatA) cDNA. * denotes significant differences ($P<0.01$) from mock treated sialidase activity of cells. Error bars represent standard deviation. Note the synergistic effects of cathepsin A and sialidase cDNAs on elevating levels of sialidase expression.

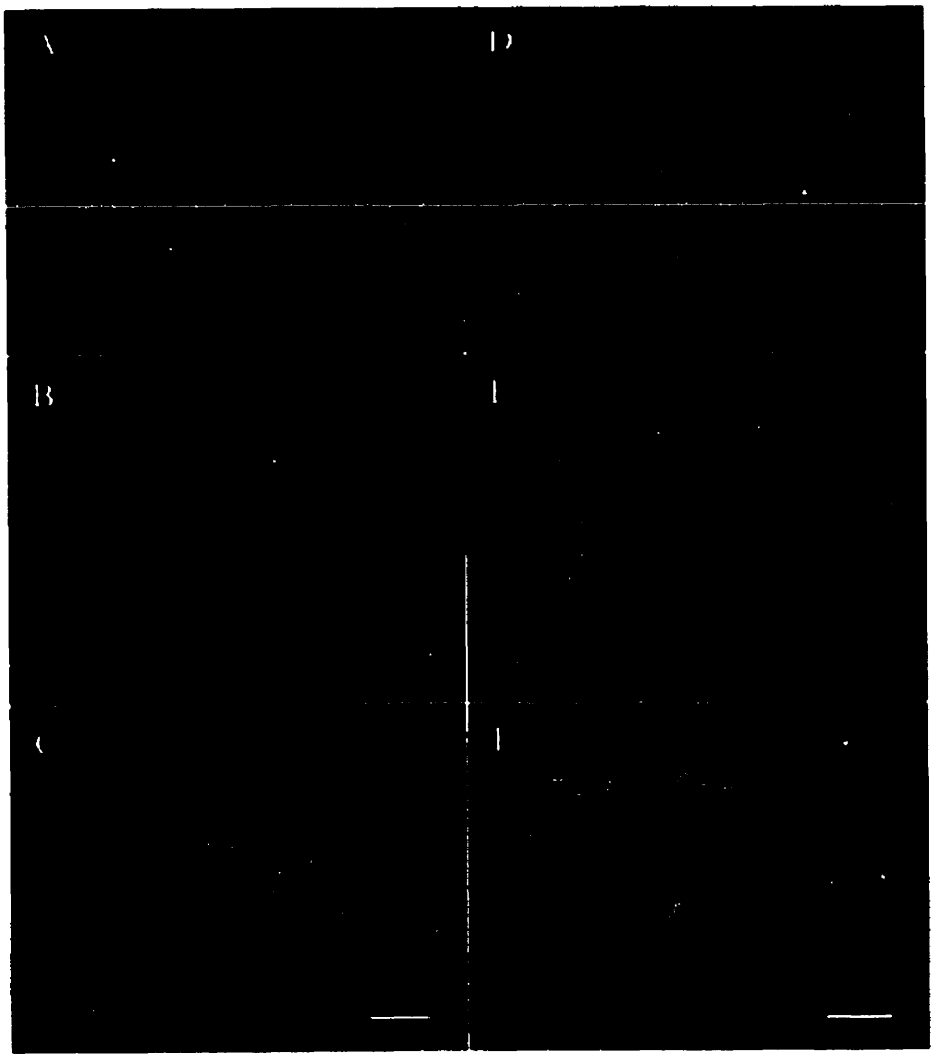


Figure 4
Expressed sialidase colocalizes with LAMP2 within distinct lysosomal compartments. SM/J mouse cells (a-c) and sialidosis human fibroblasts (d-f) transfected with mouse sialidase cDNA were incubated for 72 hrs then fixed and immunolabled for sialidase (b,e) and LAMP2 (a,d) proteins. Note that the lysosomal marker LAMP2 colocalizes with expressed sialidase (c,f) confirming the lysosomal nature of the expressed sialidase enzyme. (Bars in c=6μm and f=13μm).

CHAPTER THREE

PURIFICATION OF MOUSE AND HUMAN LYSOSOMAL SIALIDASE AND COMPARISON OF THEIR ABILITIES TO DEGRADE G_{M2}.

INTRODUCTION

A primary objective of this thesis was to determine whether or not human lysosomal sialidase could cleave G_{M2} to form G_{A2}. Therefore, it was of interest to purify lysosomal sialidase and test this directly *in vitro*. Since it has been suggested by pulse-chase experiments in mouse cells that G_{M2} is cleaved by sialidase (Riboni et al. 1995, Proia et al. 1995), we believed the mouse lysosomal sialidase could serve as a positive control against which the human enzyme could be compared.

To obtain active lysosomal sialidase, it must be stably associated with cathepsin A and β-galactosidase in a multienzyme complex (d'Azzo et al. 1995, Suzuki et al. 1995.) The purification scheme best suited for this purpose was based on a previously published protocol that employed affinity chromatography to purify the entire human lysosomal sialidase complex (Pshezhetsky and Potier 1996.) (Attempts to reconstitute the complex after expression of the individual components in yeast or E. coli have thus far been unsuccessful.) The high degree of homology between mouse and human lysosomal sialidases, both in terms of sequence and functionality (Igdoura et al. 1998) led us to believe that we could adapt this purification scheme for the mouse enzyme. There were however two major differences between the mouse and human purification schemes. One was the source material. For human sialidase, the requirement for large amounts of tissue essentially limited the choice to placenta. In the mouse, tissue assays of sialidase activity revealed the liver and kidneys to be the best source material for lysosomal sialidase (figure 1). The second difference concerns the type of affinity chromatography step used and will be discussed later in the results section.

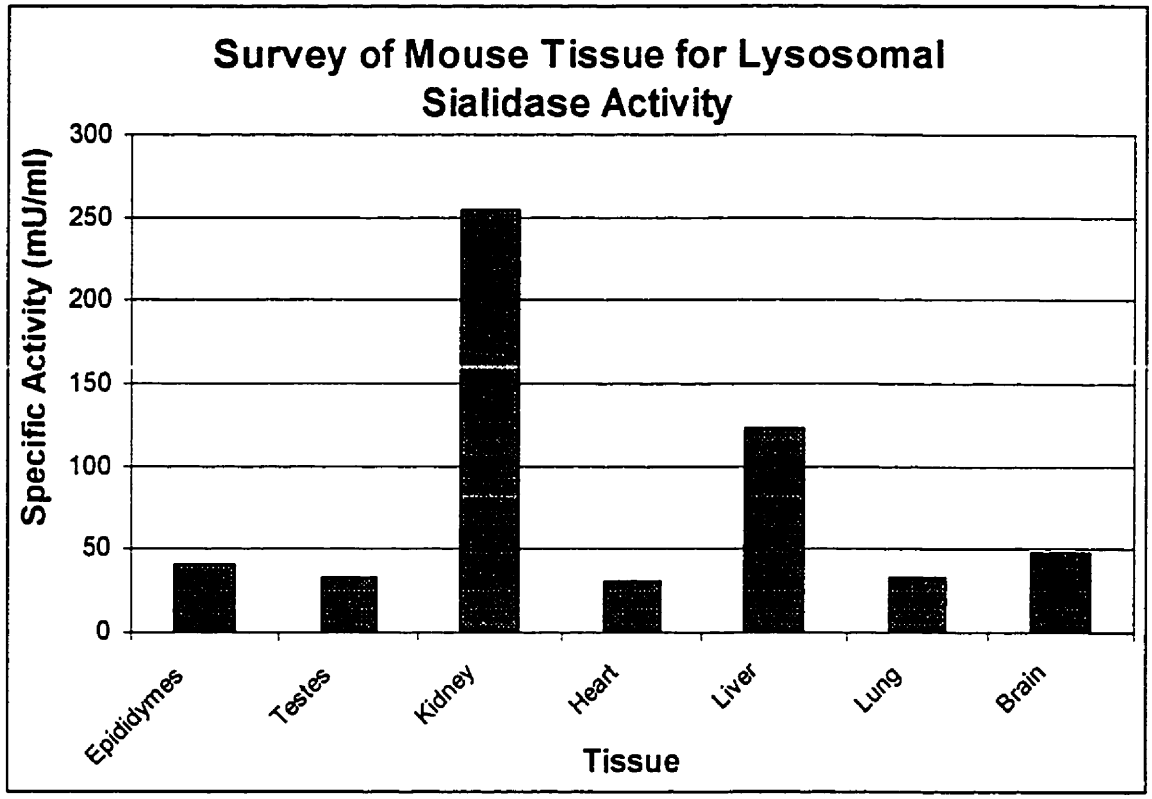


Figure 1. Specific activity of lysosomal sialidase in various mouse tissues. Assays were performed with Mu-Nana as described in the materials and methods section. These data are the average of three mice.

G_{M2} hydrolysis by hexosaminidase requires the G_{M2} activator protein as a cofactor in the reaction. In the absence of such a cofactor, the GalNAc and NeuAc residues in G_{M2} are refractory to hexosaminidase and sialidase, respectively. The resistance of G_{M2} to enzymatic hydrolysis appears to be due to the specific rigid conformation of the G_{M2} oligosaccharide (Li et al. 1999, Li et al. 1981.) Therefore, it was necessary to express and purify a protein cofactor for the *in vitro* hydrolysis of G_{M2}. It is possible to use detergents in lieu of the G_{M2} activator, however, they rapidly inactivate lysosomal sialidase (Fingerhut et al. 1992.) It is not known if there is a correct natural cofactor for degradation of G_{M2} by sialidase, but both saposin B and the G_{M2} activator are potential

candidates. We chose to purify and use mouse and human G_{M2} activator proteins for reasons of availability.

Much consideration was also given to the choice of substrate for our assays. Factors such as cost, safety and available monitoring methods affected our decision. We chose to use ^{3}H-G_{M2} labeled in the ceramide moiety. Labeling in the ceramide moiety allowed us to follow all the products of G_{M2} degradation, rather than just removal of the terminal sialic acid. Though significantly more expensive than tritiated G_{M1}, ^{3}H-G_{M2} offered the advantage of being a more direct substrate with fewer complications than the equivalently labeled ^{3}H-G_{M1}. Since the lysosomal sialidase complex contains β-galactosidase, it can actively cleave G_{M1} to G_{M2}, thereby providing a substrate pool for sialidase indirectly. However the rate of hydrolysis of G_{M1} may differ between mouse and human. This would lead to nonequivalent formation of G_{M2} pools and further complicate a direct comparison between mouse and human lysosomal sialidases.

This chapter reports the purification of mouse and human lysosomal sialidase, purification of mouse and human G_{M2} activator proteins and the results of *in vitro* assays performed using combinations of these components with the ^{3}H-G_{M2} substrate.

MATERIALS AND METHODS

Materials

All materials and chemicals were purchased from Sigma unless otherwise indicated. High performance silica gel-60 TLC plates were purchased from Merck (Darmstadt, Germany) and channeled silica gel-60 TLC plates with a pre-adsorbent zone were purchased from Analtech (Newark, DE.) The CD1 mice were purchased from the

Jax laboratory. Fresh human placenta was kindly donated by patients of St. Justine's Children Hospital (Montreal, Canada.) All chromatography columns, pumps, detectors and recorders were purchased from Pharmacia.

Purification of the Human Lysosomal Sialidase Complex

The human lysosomal sialidase complex was purified following the procedure of Pshezhetsky and Potier (1996.) In brief, about 1 kg of fresh human placenta was perfused with 1 bag of saline, frozen at –80 °C for 2 days and then partially thawed at 4°C overnight. The placenta was cut into pieces with a sharp knife, ground in a meat grinder and finally homogenized in a Waring blender with 3 volumes of 20mM sodium acetate, pH 5.2 (Buffer H) for 6 minutes at 4°C. After centrifugation for 1 hour at 28 000xg the supernatant was filtered through Whatman #4 filter paper and applied overnight at 4°C to a 115 ml concanavalin A - sepharose column equilibrated with 2 volumes Buffer H + 1mM $MgCl_2$, 1mM $CaCl_2$ + 1mM MnCl. The column was washed with 1L of buffer H + 0.15 M NaCl overnight at 4°C and the glycoproteins were eluted at room temperature with buffer H + 50mM EDTA + 0.75 M α-methylmannoside + 0.75 M α-methylglucoside over a period of 4 hours. The eluate was concentrated in an Amicon stirred cell with an PM10 membrane at 4°C for about 48 hours to a final protein concentration of about 50mg/ml (~6ml) and incubated at 37°C for 90 minutes to activate the sialidase complex. This was the concentrated glycoprotein fraction (CGF).

A 1ml aliquot of CGF was applied at room temperature to a FPLC superose-6 gel filtration column and eluted with Buffer H + 50mM EDTA. Fractions containing lysosomal sialidase activity were pooled and concentrated to 200µl as above. This preparation of sialidase was termed the gel filtration purified complex.

A 4ml aliquot of CGF was applied at 4°C to a 10 ml PATGAL-agarose affinity column equilibrated with 10 volumes of Buffer H and washed with Buffer H + 0.15M NaCl until no more protein was eluted. The bound protein was eluted with Buffer H + 50mM EDTA + 0.15M NaCl + 0.5M β-galactono-γ-lactone (~50ml). Fractions containing sialidase activity were pooled and concentrated to 300µl. This preparation of sialidase was termed the affinity purified complex.

A 100µl aliquot of the affinity purified complex was further purified by application onto a FPLC superose-6 column as described above. This was the most highly purified preparation of the sialidase complex since it was subjected to both affinity purification and gel filtration. For this reason it was termed the homogeneously purified complex. (In fact, it is not homogeneous as it contains contaminating enzymes such as hexosaminidase and glucosylceramidase. The term is merely for convenience.)

Finally, all purified complexes, including the remaining aliquot of CGF, were dialyzed overnight at 4°C against Buffer H.

Purification of the Mouse Lysosomal Sialidase Complex

Purification of the mouse sialidase complex was performed using the same method as that for the human with some modifications. The source tissue was 197g liver plus 70g kidney obtained from approximately 103 male CD1 mice. After being frozen for about six months at -80°C, the tissue was thawed 15 minutes at 4°C and homogenized in a Waring blender. After the initial centrifugation, the pellet was resuspended and rehomogenized in 1 volume Buffer H. This second homogenate was centrifuged as before and the two supernatants were combined and filtered through Whatman #4 paper. From this point, the procedure was identical to that of the human until the affinity purification step. Following elution and concentration of the glycoproteins from the Con A column, the CGF was

applied to a Phe-Leu-agarose affinity chromatography column pre-equilibrated with 10 volumes of Buffer H + 0.15M NaCl (Pshezhetsky and Potier 1994.) The column was washed with the same buffer and bound proteins were eluted with ~50ml 5mM Phe-Leu in 0.1M Tris-HCl pH 7.5. The remaining purification steps were identical to the human procedure (figure 2.)

Preparation of the ^{3}H-G_{M2} Substrate

5mg of G_{M2} were purchased from Sigma and forwarded to Moravek Biochemicals (San Francisco, CA) where a reductive tritiation was performed to produce ^{3}H-G_{M2} labeled in the ceramide moiety. The yield was 5 mCi with a specific activity of 10Ci/mol.

Purification of mouse and human G_{M2} activator proteins

E. coli clones expressing high levels of recombinant mouse and human G_{M2} activator-His_6 proteins were kindly donated by Don Mahuran (Hospital for Sick Children, Toronto, Ontario.) Activator proteins were purified as described previously (Smiljanic-Georgivec et al. 1997) using Ni-NTA metal affinity chromatography.

Enzymatic Hydrolysis

For the artificial substrates Mu-Nana, Mu-Gal, 4-MUG and 4-MUGS, each reaction mixture contained 25µl of substrate, 25µl of 20mM sodium acetate, pH 5.2 and 10µl of sample enzyme. Incubations were carried out at 37°C for 10 minutes and stopped by addition of 1ml of 0.4M glycine (the sodium salt), pH 10.4. The fluorescence was then measured in a Perkin-Elmer fluorimeter. For hydrolysis of the ^{3}H-G_{M2} substrate a method similar to that of Jeffrey et al. was used. The specified amount of sample enzyme was incubated with 2.5 or 5µCi of ^{3}H-G_{M2}, with or without 2µg of G_{M2} activator in a total reaction volume of 100µl containing 1mg/ml of BSA and 20mM sodium acetate, pH 5.2. Incubations were carried out at 37°C for 4, 16 or 24 hours as indicated.

Purification of Mouse Lysosomal Sialidase Complex

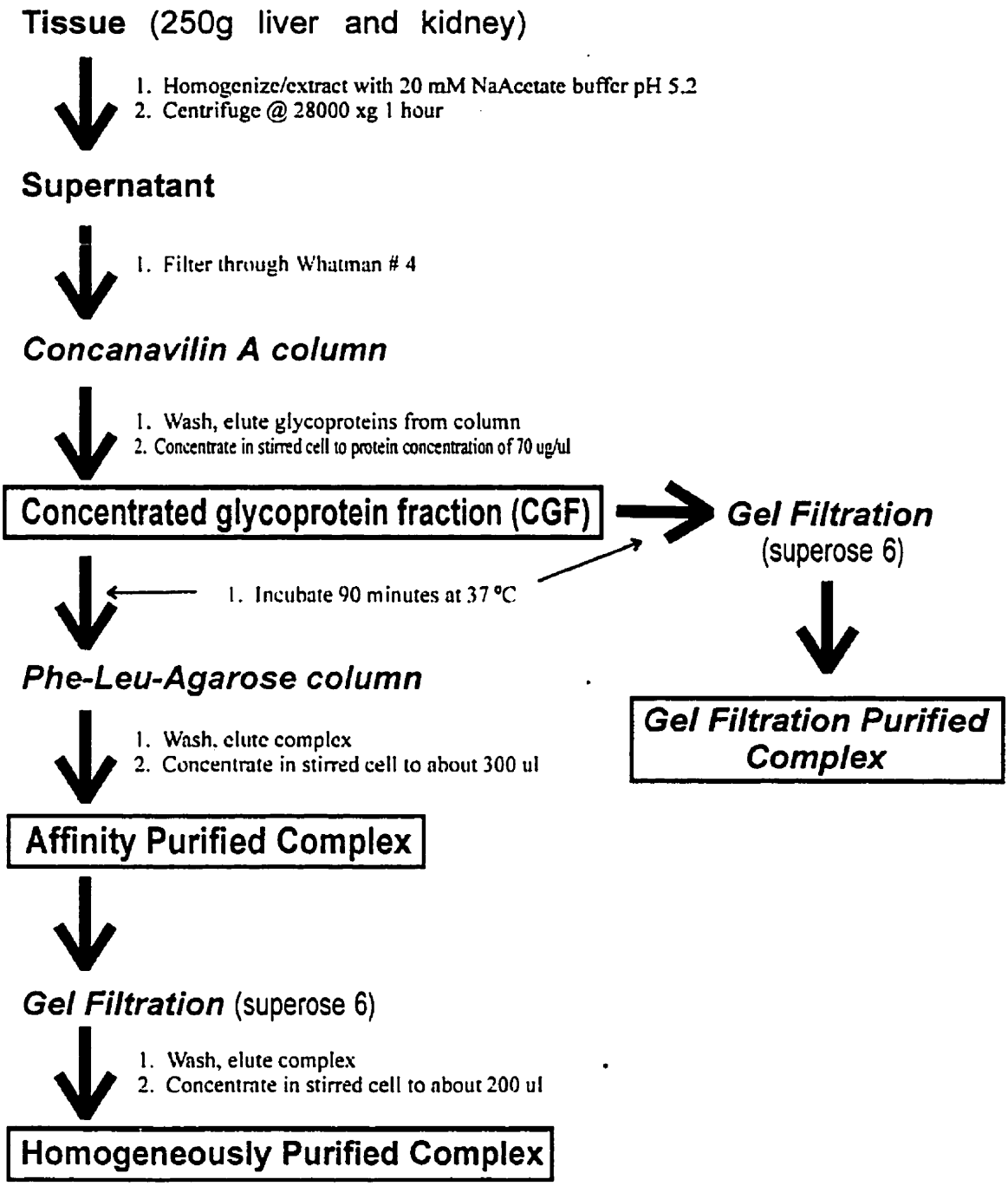


Figure 2. Purification scheme for the mouse lysosomal sialidase complex. The four differently purified preparations of sialidase used for *in vitro* experiments are boxed.

TLC Analysis

After completion of the ^{3}H-G_{M2} assays, reaction mixtures were evaporated to dryness in a speedvac, resuspended in 100µl of 1:1 chloroform/methanol and analyzed by silica gel-60 thin layer chromatography (Jeffrey et al. 1998.) The solvent system used for developing the glycolipids was 60:35:8 chloroform/methanol/water. Quantitative results of the % hydrolysis of ^{3}H-G_{M2} were obtained by scanning the TLC plates with a Bioscan AR2000 radioisotope plate scanner. The radioactive products on the TLC plates were also visualized using a phosphoimager with a tritium sensitive imaging plate.

RESULTS

Purification of the Mouse Lysosomal Sialidase Complex

Since the sialidase complex has never before been purified from mouse tissue it was first necessary to determine the best source tissue. It was found using the artificial substrate Mu-Nana that the kidney and liver of mouse contained the highest specific activity of lysosomal sialidase (figure 1.) From this data it was estimated that approximately 100 mice should be sufficient for a purification of lysosomal sialidase.

For the first purification attempt, the published protocol for the human complex was followed exactly. Unexpectedly however, it failed due to the inability of mouse β-galactosidase to bind PATGAL-agarose, in contrast to the human enzyme (Verheijen et al. 1985.) This was surprising considering the high degree of homology between the mouse and human enzymes, both in terms of sequence and function (Nanba et al. 1990.) and it points to a specificity difference in the binding site of the two enzymes. Based on an alternative protocol (Pshezhetsky and Potier, 1994) the purification was repeated with a Phe-Leu-agarose affinity step in place of the PATGAL-agarose affinity step. Although

the Phe-Leu-agarose affinity purification was successful, the efficiency was extremely poor. Unlike the PATGAL-agarose column, which gives a 30% yield of sialidase activity in the human case, the Phe-Leu-agarose column resulted in only a 2% yield of sialidase activity for the mouse. This was unanticipated because the binding efficiencies of the two columns appeared to be very similar (data not shown.) Possibly this is a reflection of a less stable mouse complex, which may dissociate rapidly when diluted during washing or elution steps. The purification that included both an affinity step and gel filtration step, to give the homogeneously purified mouse complex, is summarized in Table 1. The SDS-PAGE and western analysis of this highly purified sialidase complex is shown in figure 3.

As described in the materials and methods section, the mouse sialidase complex was purified to varying extents using different combinations of the above techniques. These partially purified sialidase preparations were the concentrated glycoprotein fraction, obtained by Con A-sepharose chromatography (the CGF), the CGF plus a gel filtration step and the CGF plus an affinity purification step (boxes in figure 2.) An enzyme profile of each of these partially purified preparations as well as the homogeneously purified sialidase is given in Table 2.

A major problem in all purifications was the dialysis step. It was noticed that during dialysis, a precipitate would form in the dialysis bag. This was accompanied with a reduction in the amount of sialidase activity. Apparently the removal of EDTA during this dialysis step reduced the solubility of proteins in the sample leading to their precipitation. Removal of EDTA is necessary because it interferes with the G_{M2} hydrolysis reaction (data not shown.) It should be possible to minimize protein precipitation by reducing the concentration of proteins before the dialysis step (ie. by increasing the volume of solution.)

ANALYSIS OF PURIFIED COMPLEXES

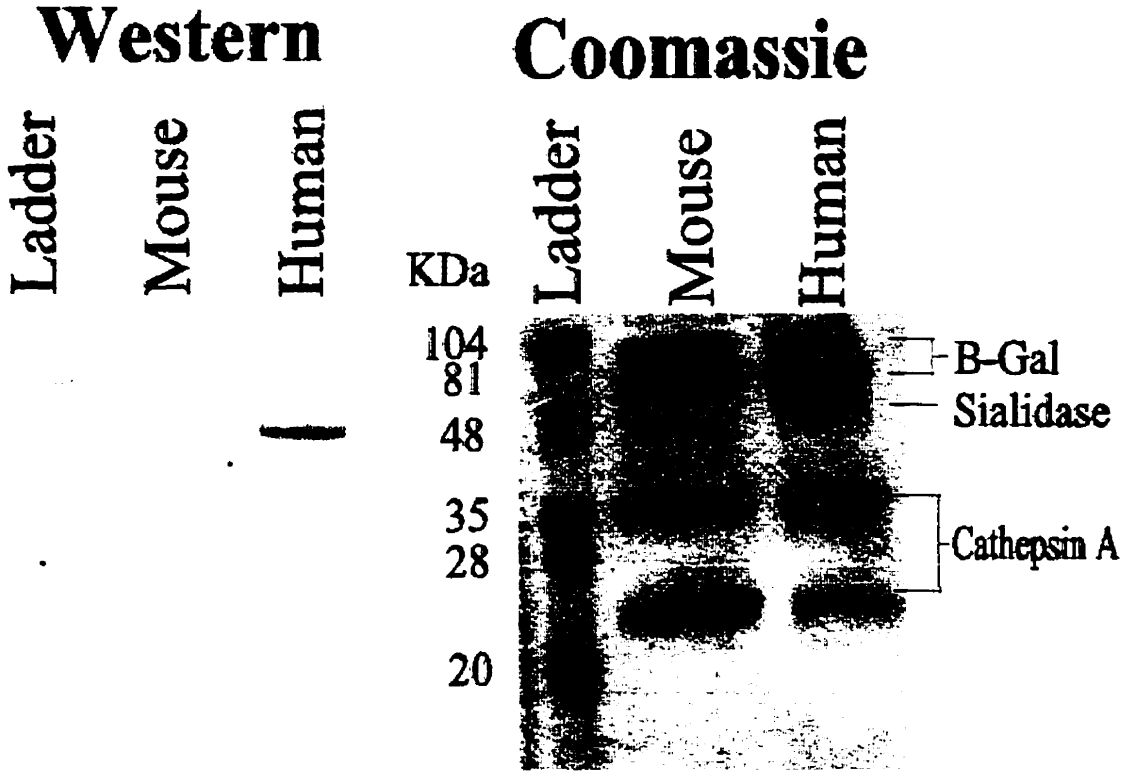


Figure 3. SDS-PAGE analysis of the homogeneously purified mouse and human sialidase complexes. The western was performed using anti-human sialidase antibodies. The antibody shows only slight cross reactivity with the mouse sialidase. Both cathepsin A and ß-galactosidase (B-Gal) are composed of two subunits.

Table 1. Purification results for the mouse lysosomal sialidase complex

	Specific Activity (mU/mg)	Total Activity (mU)	Fold Purification (x)	Yield (%)
Homogenate	0.03	1091		
Supernatant	0.02	567	0.67	52
Con A Sepharose (CGF)	4.3	1513	143	139
Phe-Leu Agarose	45.8	50	1577	4.6
Gel Filtration	96.0	16	3311	1.5

Purification of mouse and human G_{M2} activator proteins

The results for the purification of the mouse and human G_{M2} activator proteins were similar to those described previously (Smiljanic-Georgivec et al. 1997.) For both purifications, the proteins appeared homogenous when visualized by SDS-PAGE (data not shown.) Approximately 1 mg of each activator was purified by the Biorad assay. The activators were concentrated in Centricon-10 spin concentrators to approximately 1ug/ul. Both activators showed activity when tested with hexosaminidase against G_{M2} (as described in materials and methods.)

Table 2. Enzyme profile for partially purified preparations of mouse sialidase

	Con A	Con A + Superose 6	Con A + Phe-Leu-agarose	Con A + Phe-Leu-agarose + superose 6
Total Enzyme Activity in mU	CGF	Gel Filtration Purified	Affinity Purified	Homogeneous Complex
Sialidase	**15**	**8**	**322**	**16**
β-Galactosidase	266	5495	2768	3996
Hexosaminidase A	19	355	942	N/A
Hexosaminidase A+B	73	2943	5650	16

**Note: Data is the average of three assays.*

Purification of the Human Lysosomal Sialidase Complex

Purification of the human enzyme was essentially identical to that of the mouse with the aforementioned changes. The efficiency of each step was similar to that reported previously by Pshezhetsky and Potier (1996) (Data not shown.) An enzyme profile for each preparation of the human lysosomal sialidase complex is given in Table 3.

Table 3. Enzyme profile for partially purified preparations of human sialidase

	Con A	Con A + Superose 6	Con A + Phe-Leu-agarose	Con A + Phe-Leu-agarose + superose 6
Total Enzyme Activity in mU	CGF	Gel Filtration Purified	Affinity Purified	Homogeneous Complex
Sialidase	**202**	**28**	**100**	**60**
β-Galactosidase	14898	2993	28409	9120
Hexosaminidase A	19677	150	3920	N/A
Hexosaminidase A+B	101781	1402	31075	4440

**Note: Data is the average of three assays.*

In Vitro Enzymatic Hydrolysis

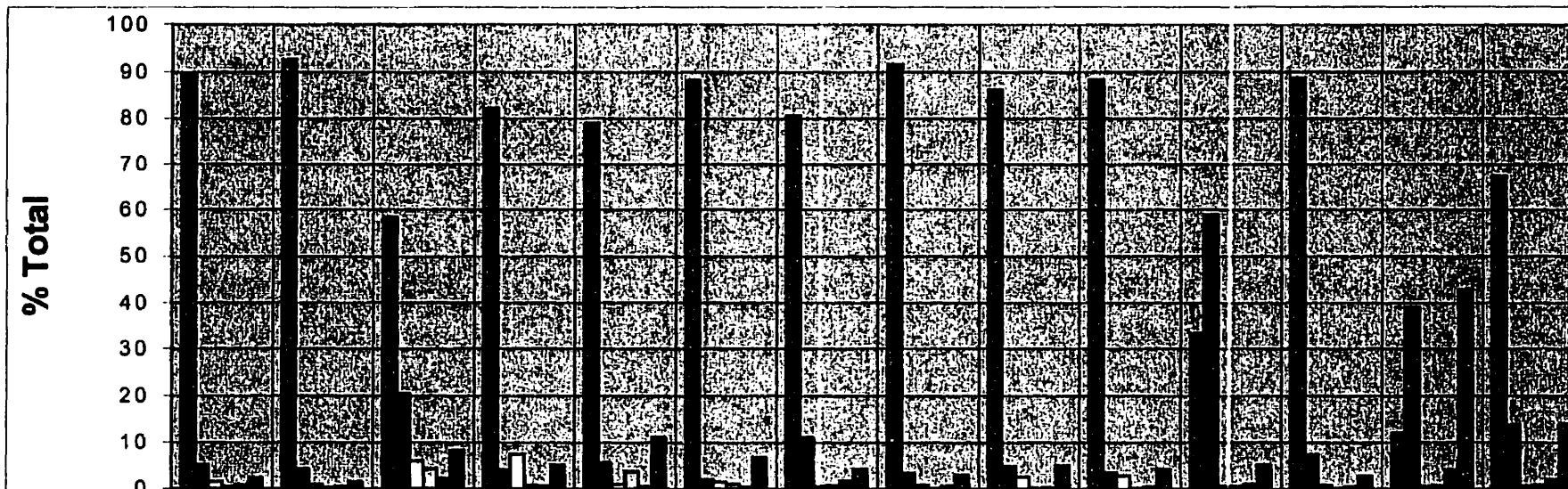
Enzymatic hydrolysis of G_{M2} by differently purified preparations of mouse and human lysosomal sialidases was examined. The results are shown in Figures 4 and 5 along with information pertaining to the incubation conditions and contents of each reaction mixture. The glycolipid products of each reaction are expressed as a percentage of the total radioactivity in the lane. In general, results obtained by the radioisotope scanner were more reproducible than those obtained by imaging with the phosphoimager. Therefore, data was obtained from duplicate scans of TLC plates with the scanner. Pictures obtained from the phosphoimager however were still used for band orientation and identification. A sample image of such a plate is given in figure 6, and a sample tracing of data obtained from radioisotope scanning one lane on this plate is given in figure 7. Data for all reactions were compiled and graphed using Microsoft Excel.

Figure 4 shows the results of hydrolysis of G_{M2} by mouse and human lysosomal sialidase, with mouse G_{M2} activator, in the presence or absence of N-acetylgalactosamine (an inhibitor of hexosaminidase.) The essential findings are that only mouse CGF and the human gel filtration purified complex show appreciable G_{A2} yield (yellow bars in

lanes 3,4,9 and10.) It is evident from these data that the production of G_{A2} is inefficient by either mouse or human sialidase. The poor yield of G_{A2} is further confounded by the fact that any G_{A2} produced is rapidly degraded by contaminating hexosaminidases. This idea is supported by the observation that there was less G_{A2} present in many reactions containing purified sialidase preparations (lanes 7,8 and 11-14) than there was in negative controls (lanes 1 and 2.) Further, inhibition of hexosaminidase activity with N-acetylgalactosamine resulted in slightly higher levels of G_{A2} accumulation (compare lane 3 to lane 4 for example.) In all cases where N-acetylgalactosamine was present, hexosaminidase activity was dramatically reduced. This was reflected by a significant drop in G_{M3} accumulation as compared to when N-acetylgalactosamine was absent (red bars in lanes 3-14.)

In general, figure 4 reveals that most of the purified sialidase preparations contained highly active contaminating enzymes such as hexosaminidase (as discussed above) and glucosylceramidase. Abundant hexosaminidase activity is indicated by the accumulation of G_{M3} in lanes 3, 7 and 11-14 (red bars) and glucosylceramidase activity is indicated by the accumulation of ceramide in lanes 3,5,13 and 14 (orange bars.) Contaminating activities were expected for the CGF since Con A is known to bind most lysosomal enzymes, but they were unanticipated for affinity purified preparations, which should have been fairly pure. Such contamination hints at the possibility of some type of association between the sialidase complex and these other exohydrolases such that any attempt to purify sialidase also results in enrichment of hexosaminidase and glucosylceramidase.

^{3}H-G_{M2} Assays With or Without N-acetylgalactosamine (Hex A inhibitor)



	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gm2	89.48	92.41	58.41	82.02	78.79	88.16	80.38	91.56	86.12	88.34	33.36	88.64	12.10	67.47
Gm3	4.97	4.13	20.44	4.02	5.47	1.95	10.98	3.30	4.73	3.38	58.76	7.34	38.99	13.76
GA2	1.45	0.85	5.82	7.35	0.89	1.47	0.53	0.67	2.54	2.76	0.58	0.74	0.48	0.61
Lac-cer	0.41	0.20	4.30	0.74	3.70	0.89	0.70	0.07	0.12	0.08	0.50	0.11	0.77	0.65
Glu-cer	0.79	0.39	2.14	0.48	0.45	0.31	1.63	0.54	0.23	0.36	0.90	0.39	4.00	2.05
Ceramide	2.27	1.50	8.21	5.02	11.02	6.64	4.20	2.93	4.93	4.11	4.99	2.68	42.78	13.96

Type of purification			CGF				Gel Filtration Purified				Affinity Purified			
Enzyme	Blank		Mouse		Human		Mouse		Human		Mouse		Human	
Inhibitor	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Vol. (ul)	—	—	12.5	12.5	40	40	40	40	40	40	40	40	10	10
Sial (mU)	—	—	4.5	4.5	4.5	4.5	3.4	3.4	2	2	0.6	0.6	1.9	1.9
HexA(mU)	—	—	13	13	440	440	4.2	4.2	11	11	26	26	72	72

Figure 4. Assays were performed using 2.5ul ^{3}H-G_{M2} substrate and 2ug mouse G_{M2} activator in the presence or absence of 100mM GalNAc as indicated by the + or -. Numbers (1-14) immediately below graph correspond to lanes on TLC plates. Incubations were 24 hours at 37°C. Due to time and material limitations these experiments were performed only once.

Figure 5 compares the results of hydrolysis of G_{M2} by mouse and human lysosomal sialidase in the presence of either mouse or human G_{M2} activator protein. It is immediately apparent that the two activators are not equivalent. Overall, the human G_{M2} activator is better for promoting the complete degradation of G_{M2} to ceramide. In every case, more ceramide is formed when the human activator is used than when is the mouse activator (lanes 2-11, orange bars.)

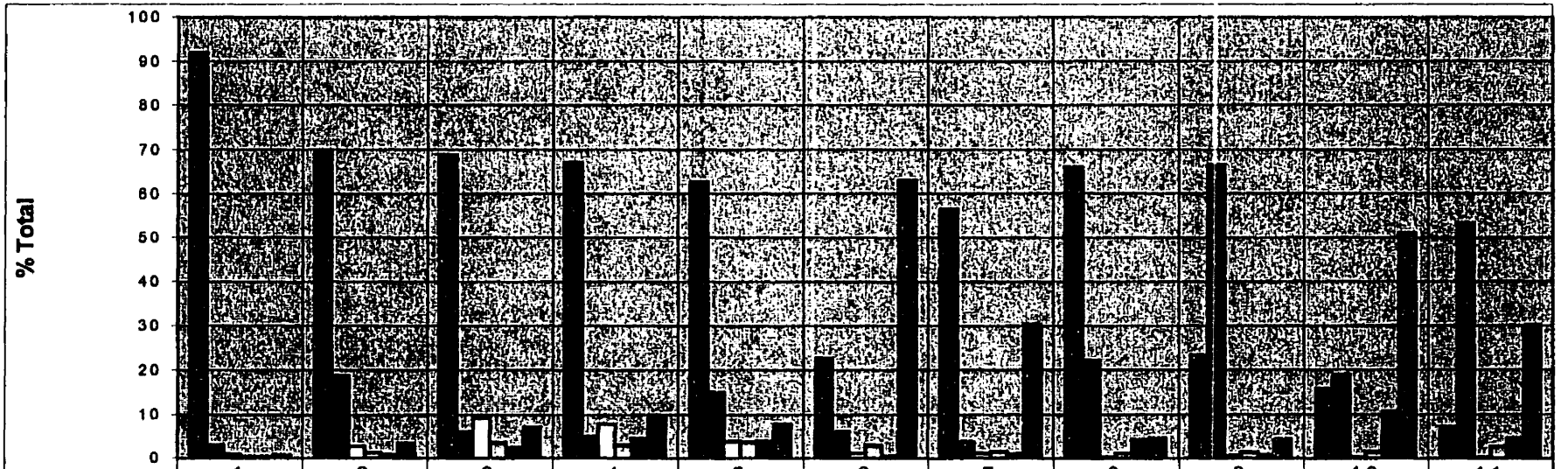
As expected from figure 4, only mouse CGF showed appreciable G_{A2} yield in figure 5 (yellow bars in lanes 2-5.) Accumulation of G_{A2} was significantly more pronounced when mouse CGF was used in conjunction with the human G_{M2} activator (yellow bar in lanes 3 and 4) than it was with no activator (lane 2) or with the mouse activator (lane 5.) In contrast, when mouse activator is added to mouse CGF, G_{M3} accumulates to a much greater extent than when human activator is added to mouse CGF (red bars in lanes 4 and 5.) It seems that for mouse CGF, using the "wrong" G_{M2} activator (ie. the human activator) promotes degradation of G_{M2} via sialidase rather than hexosaminidase.

In general, G_{M2} degradation occurred to a greater extent when the same species activator and enzyme were used together. For example, human CGF with human G_{M2} activator results in much more G_{M2} degradation than human CGF with mouse G_{M2} activator (blue bars in lanes 6 and 7.) Likewise, mouse affinity purified sialidase degrades G_{M2} much more effectively when used with mouse G_{M2} activator than with human activator (blue bars in lanes 8 and 9.) The only exception to this appears to be the human affinity purified sialidase (lanes 10 and 11.) Note however that G_{M2} was almost completely degraded with this preparation irrespective of the type of activator used.

Another observation can be made if the data from figures 4 and 5 are compared with each other. Lane 7 from figure 5 is almost identical to lane 5 from figure 4 (human CGF with mouse G_{M2} activator.) The only significant difference was that 40µl of CGF was used in figure 4, whereas only 20ul of CGF was used in figure 5. A dramatic increase in G_{M2} degradation was observed when less CGF was used (79% G_{M2} versus 59% G_{M2}.) This suggests that the activity of hydrolytic enzymes present in the CGF are inhibited when its concentration is too high.

Data from the homogeneously purified sialidase complexes, obtained utilizing both affinity purification and gel filtration, are not shown because they exhibited virtually no hydrolytic activity against G_{M2}.

^{3}H-G_{M2} Assays Comparing Mouse and Human G_{M2} Activator Proteins



	1	2	3	4	5	6	7	8	9	10	11
Gm2	91.94	69.89	68.72	67.08	62.71	22.67	56.50	65.96	23.36	15.72	7.16
Gm3	3.07	18.54	6.09	4.87	15.02	6.10	3.61	22.15	66.46	18.81	53.32
GA2	0.87	2.75	9.22	7.79	3.77	1.14	1.16	0.15	0.61	0.23	0.21
Lac-cer	0.39	1.23	3.45	3.02	3.57	2.84	1.48	1.17	1.28	1.31	2.40
Glu-cer	0.33	0.81	2.54	4.46	3.69	0.56	0.95	4.11	0.85	10.58	4.48
Ceramide	0.70	3.42	7.00	9.29	7.79	62.99	30.24	4.52	4.28	50.97	30.12

		CGF						Affinity Purified			
Enzyme	Blank	mouse		Mouse		human		Mouse		human	
GM2 activator	---	---	human	Human	mouse	human	mouse	Human	mouse	human	mouse
Vol. (ul)	---	17.0	17.0	8.5	8.5	20.0	20.0	20.0	20.0	2.5	2.5
Sial (mU)	---	6.1	6.1	3.0	3.0	2.3	2.3	0.3	0.3	0.5	0.5
HexA(mU)	---	17.6	17.6	8.8	8.8	220.0	220.0	13.0	13.0	18.0	18.0

Figure 5. 2.5ul (CGF) or 5ul (affinity purified) ^{3}H-G_{M2} and 2ug specified G_{M2} activator were used in each assay. Numbers (1-11) immediately below graph correspond to lanes on TLC plates. Incubations were 16 hours at 37°C. Due to time and material limitation, these experiments were performed only once.

DISCUSSION

To date there has been no evidence to unequivocally demonstrate that human lysosomal sialidase can cleave G_{M2}. Many attempts have been made to delineate the substrate specificity of the lysosomal and other human sialidases, but precise definition remains elusive (Schneider-Jakob et al. 1991, Miyagi et al. 1999, Kopitz et al. 1997.) Despite this, recent advances concerning the activation and stabilization of lysosomal sialidase and cloning and easy purification of activator proteins necessary for ganglioside hydrolysis, have made it easier to test the various mammalian sialidases against ganglioside substrates (Ben-Yoseph et al. 1991, Hiraiwa et al. 1993, Fingerhut et al. 1992.) Evidence to show that lysosomal sialidase can cleave G_{M2} has been more forthcoming in the mouse, but by no means definitive (Miyagi et al. 1986, Saito et al. 1995, Miyagi et al. 1990.) These experiments were inconclusive because they either employed a lysosomal sialidase that was only partially purified or because an unsuitable substrate was used. For example, ^{3}H-G_{M2} labeled in the sialic acid residue is a poor substrate because it does not allow intermediate degradation products to be monitored.

The results presented in this chapter revealed that the purified preparations of lysosomal sialidase contained highly active contaminating enzymes. This resulted in the profound generation of ceramide from G_{M2}, presumably via G_{M3}. Human affinity purified sialidase with human G_{M2} activator and mouse affinity purified sialidase with mouse G_{M2} activator both showed this pattern of G_{M2} degradation. This illustrates two points. One, it shows that the reaction conditions were suitable for the hydrolysis of G_{M2} and that the G_{M2} activator proteins were functioning. Second, it alerted us to the fact that detection of G_{A2} might be difficult because of its rapid degradation by these

contaminating enzymes. Therefore, the absence of G_{A2} does not necessarily mean that sialidase did not catabolize G_{M2}. The use of N-acetylgalactosamine as an inhibitor of hexosaminidase was meant to alleviate this ambiguity, but unfortunately, it was not 100% efficacious and hexosaminidase activity could not be completely eliminated.

One set of reaction conditions that did result in significant catabolism of G_{M2} by sialidase contained mouse CGF and the human G_{M2} activator protein (figure 5, lanes 3 and 4.) Interestingly, addition of the human G_{M2} activator protein also resulted in decreased yield of G_{M3} generated by hexosaminidase and an increase in the amount of downstream products such as lactosylceramide, glucosylceramide and ceramide. (figure 5, lanes 2 and 3.) On the other hand, addition of mouse G_{M2} activator protein to mouse CFG resulted only in a slight increase of downstream products (lane 5.) It appears that human activator in the context of mouse CGF promotes catabolism of G_{M2} via the sialidase pathway, whereas the mouse activator has no such effect or promotes degradation via the hexosaminidase pathway. Why should this be? It is not likely that the human G_{M2} activator simply promotes the G_{A2} pathway, otherwise this would be expected as the normal degradative pathway in humans. The simplest way to explain these results is that the human G_{M2} activator and mouse hexosaminidase are not fully compatible. In other words, the human activator may interfere with and effectively inhibit mouse hexosaminidase, leading to increased sialidase-mediated degradation of G_{M2} by default. In addition, the human G_{M2} activator appears to promote the general degradation of gangliosides more efficiently than the mouse activator. (Hence the greater accumulation of downstream products when the human G_{M2} activator is present.)

The hypothesis that mouse hexosaminidase and human G_{M2} activator interfere with each other is further supported by the data obtained with affinity purified sialidase. Contaminating hexosaminidase in affinity purified mouse sialidase preparations is more efficient at degrading G_{M2} to G_{M3} with mouse activator than with human activator (figure 5, lanes 8 and 9.)

Human CGF does not show significant G_{A2} formation with either activator but shows dramatic degradation of G_{M2} via G_{M3} with both (figure 5, lanes 6 and 7.) This may indicate that human sialidase is not as effective against G_{M2} as mouse sialidase. However, this point cannot be made conclusively because it is also possible that the human sialidase can not compete with the high levels of hexosaminidase present in human CGF. Note that there is almost 30 times the Hex A activity in human CGF compared to mouse CGF, a direct consequence of the source material used for the purification.

The only other conditions that showed a modest amount of G_{A2} formation were those containing human CGF plus a gel filtration step combined with mouse G_{M2} activator (figure 4, lanes 9 and 10.) An increase of 3 to 4 times that of background G_{A2} levels was observed. Unexpectedly however, the equivalent mouse preparation (CGF plus gel filtration) showed no G_{A2} formation despite the fact that the mouse preparation contained higher levels of sialidase and lower levels of contaminating hexosaminidase. The mouse preparation preferred G_{M3} formation (figure 4, lanes 7 and 8.) It is difficult to explain this when mouse CGF showed significant sialidase activity towards G_{M2}. One obvious possibility is that mouse CGF contained a cofactor necessary for sialidase mediated G_{M2} catabolism that is removed with the gel filtration step. Perhaps saposin B

is required. If so, why should the human CGF plus gel filtration preparation suddenly show sialidase activity against G_{M2} when there was none in human CGF? Part of the reason could be the fact that the gel filtration step removes a large proportion of the contaminating and interfering hexosaminidase activity. Perhaps that in combination with the use of mouse activator (inhibitory for human hexosaminidase?) is enough to uncover sialidase activity towards G_{M2}. But then what of the missing cofactor used to explain the mouse situation? Is it possible that the mouse and human lysosomal sialidase use different cofactors for degradation of G_{M2}? This is unlikely given the large degree of homology between the mouse and human enzymes, but possible nonetheless.

It was also surprising that the affinity purified preparations of sialidase did not result in any sialidase mediated G_{M2} degradation. Instead, degradation of G_{M2} via G_{M3} was extremely efficient (figure 4, lanes 11-14 and figure 5, lanes 8-11.) If affinity purified complexes are compared to those purified by gel filtration (figure 4, lanes 7-10), it is evident from the limited catabolism of G_{M2} that hexosaminidase is only fractionally active in the gel filtration prepared samples. Perhaps this is why some sialidase activity against G_{M2} could be detected in the human gel filtration samples. Again, it is possible to postulate that hexosaminidase activity interferes with sialidase activity against G_{M2}. Sialidase activity against G_{M3} however is very active as evidenced by the large amounts of glucosylceramide and ceramide generated in affinity purified preparations. Why was hexosaminidase activity only partially active against G_{M2} in gel filtration preparations? Perhaps it is because a cofactor was missing or an inhibitor was activated.

A limitation of this study is that the yield of highly purified lysosomal sialidase from either mouse or human was too low to allow detection of significant activity against

G_{M2} *in vitro*. Conversion of G_{M2} to G_{A2} is likely a slow reaction and probably can only be detected *in vitro* with relatively large amounts of active sialidase. This problem was not overcome by the use of less highly purified preparations of lysosomal sialidase that contained elevated levels of activity. Impurities in these preparations (such as hexosaminidase) might have interfered with sialidase activity against G_{M2}. In addition, the rate at which contaminating hexosaminidase degraded G_{A2} might have been faster than the rate at which G_{A2} was produced by sialidase. Therefore, G_{A2} would not accumulate and could not be detected in these assays. As mentioned previously, this notion is supported by the fact that inhibition of hexosaminidase with N-acetylgalactosamine slightly increased the amount of detectable G_{A2} in many cases (figure 4.)

It is apparent that interpretation of these data is very complicated and it is difficult to make any definitive conclusions. At best, some inferences can be deduced and some explanations hypothesized. One such hypothesis is that hexosaminidase and sialidase compete with each other for binding sites on G_{M2} and that in the presence of both enzymes, hexosaminidase wins. Only when hexosaminidase is inhibited, such as by association with an incompatible G_{M2} activator or by some other factor (ie. N-acetylgalactosamine), can sialidase effectively compete. This of course is not the only prerequisite for sialidase mediated G_{M2} degradation. There must be sufficiently high levels of sialidase activity and there is likely a requirement for some kind of G_{M2} solubilizing factor. This factor may be something other than the G_{M2} activator used in these experiments, such as saposin B. Ultimately, to test and answer these questions, large amounts of highly purified lysosomal sialidase must be prepared. So far, plausible

techniques for this have not been worked out. Possibilities for circumventing the problems encountered in this chapter are discussed in chapter 5.

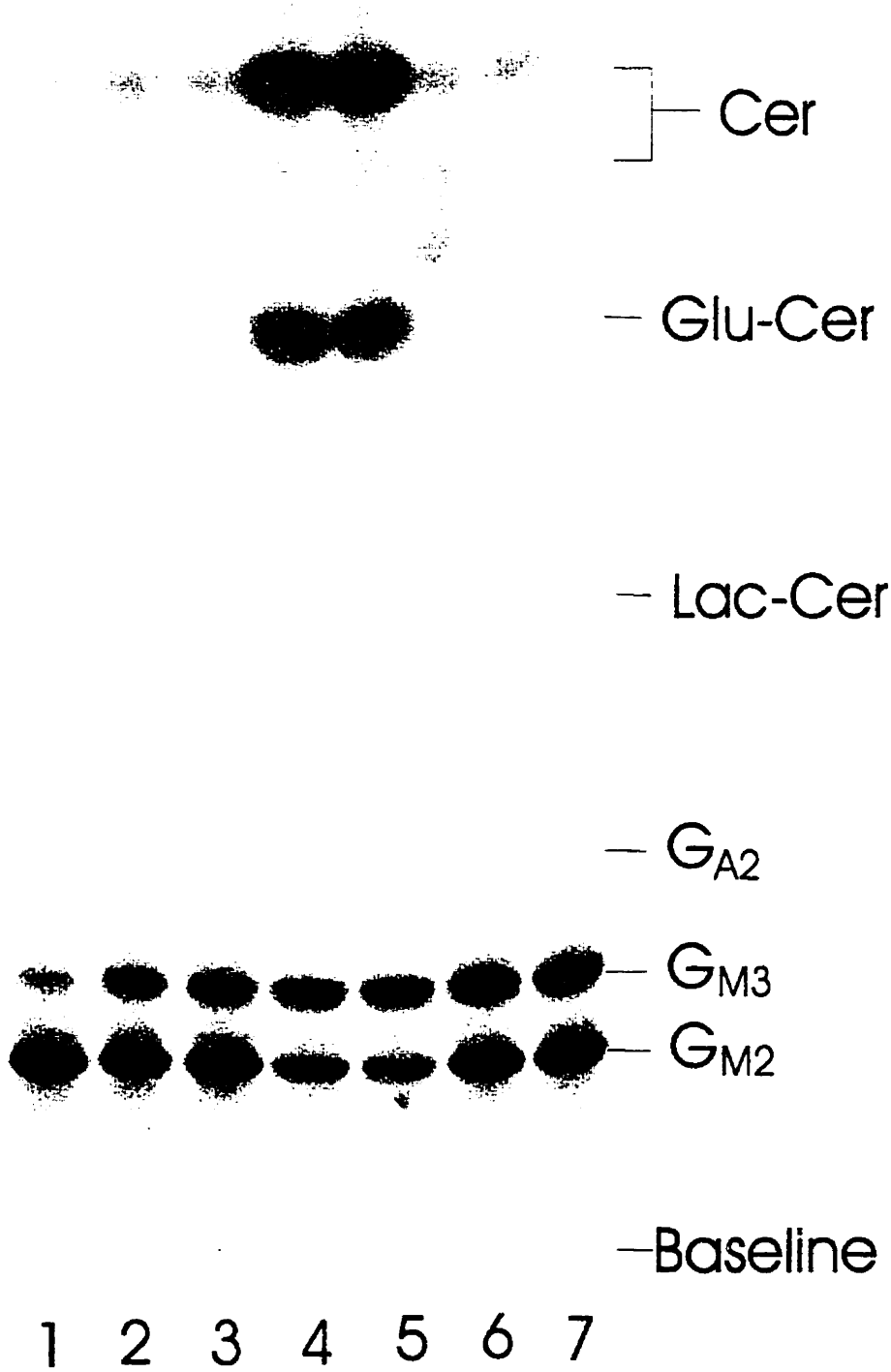


Figure 6. A sample image of a channeled silica gel-60 TLC plate exposed to a tritium sensitive imaging plate for the phosphoimager. Data was obtained from such a plate by scanning each lane with a radioisotope plate scanner.

Sample No.:
Acquired: 17 Apr 1999 17:03 by SU

Sample Name:
Evaluated: 17 Apr 1999 20:46 by SU

Electronic Res: Norm | Bkgnd: None | Norm: None | Base Correct: On

Run Time: 5:42 | Total Counts: 15035 | Total CPM: 2637.7

Total count region: -5 mm - 200 mm

Name	Start (mm)	End (mm)	Max (mm)	RF	Region Counts	Region CPM	% of Total	% of ROIs
GM1	16.4	35.5	20.8	0.104	104	18.2	0.72	0.72
GM2	35.5	49.4	44.2	0.221	2683	470.8	18.57	18.57
GM3	49.4	85.7	54.6	0.273	1680	294.8	11.63	11.63
GA2	62.4	85.7	67.6	0.338	99	17.4	0.69	0.69
LAC-CER	97.9	113.5	105.7	0.528	64	11.2	0.44	0.44
GLU-CER	132.5	161.1	145.5	0.727	2425	425.4	16.78	16.78
CERAMIDE	161.1	198.3	181.0	0.905	7397	1297.8	51.19	51.19
7 Peaks					14452	2535.5	100.02	100.00

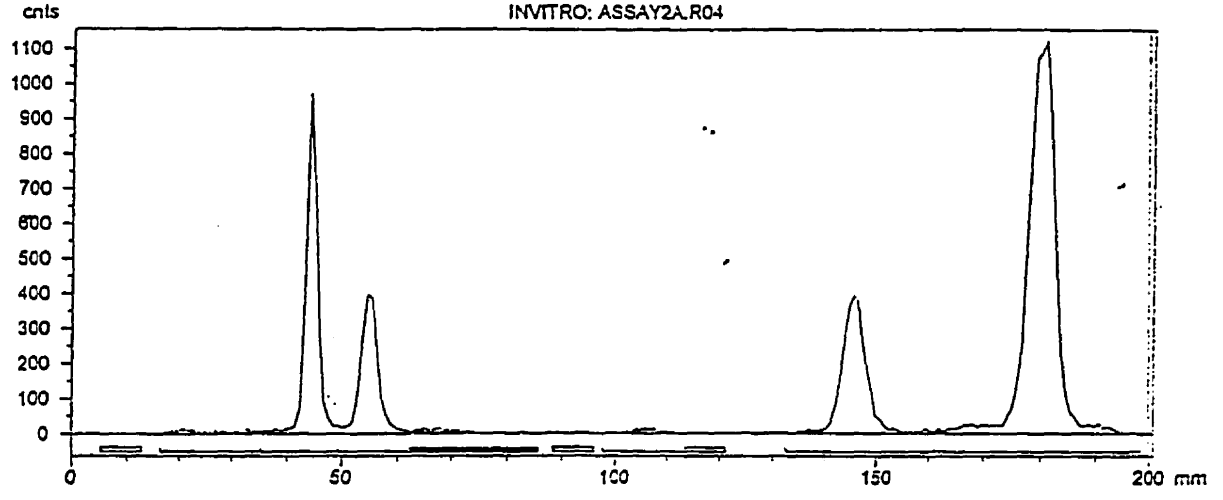


Figure 7. A sample tracing of data obtained by scanning lane 4 of the plate shown in figure 6. The baseline of the TLC plate is represented by 0 mm on the x-axis. Values used for tabulation of data were from %ROIs (regions of interest.) Peaks were identified by their position on the plate and by using phosphoimager data.

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CHAPTER FOUR

ANALYSIS OF G_{M2} GANGLIOSIDE DEGRADATION BY LYSOSOMAL SIALIDASE AS DETERMINED BY PULSE-CHASE EXPERIMENTS

INTRODUCTION

This chapter describes an approach in which the catabolism of ^{3}H-G_{M2} is compared in Tay-Sachs neuroglia cells stably transfected with different human or mouse components of the sialidase bypass. These experiments are similar to those performed by Riboni et al. and Proia et al. who utilized pulse-chase experiments with ^{3}H-G_{M1} to demonstrate that mouse cells preferred catabolism of G_{M2} via G_{A2} rather than G_{M3}. Experiments by Igdoura et al. (1999) provided evidence by immunostaining that high level expression of lysosomal sialidase can clear accumulated G_{M2} from human Tay-Sachs cells. Our objective here was to determine biochemically, by means of pulse-chase experiments, whether flow through the G_{A2} pathway can be induced in human cells if the level of lysosomal sialidase is sufficiently high. Our aim was to correlate increased levels of lysosomal sialidase activity with increased production of G_{A2} in cultured human neuroglia cells.

This approach allowed us to avoid many of the difficulties encountered with the purification-based strategy discussed in chapter three. Any cofactors necessary for sialidase mediated G_{M2} catabolism should have been present as part of the natural lysosomal milieu and thus did not need to be accounted for. In addition, any inhibitors of sialidase mediated G_{M2} degradation would have been maintained at their physiological location and concentration. Therefore, any effects detected in metabolism of ^{3}H-G_{M2}, in theory, must have been due solely to an increased activity of lysosomal sialidase. This allowed for an easier comparison of mouse and human sialidases. Since all else was equal, any differences in ^{3}H-G_{M2} metabolism could be attributed to species-specific differences in the expressed sialidase.

We chose to use the SV-40 transformed Tay-Sachs neuroglia cell line (NG125) derived from the brain of an affected fetus, because of its lack of Hex A activity and because it is an immortal cell line. The lack of Hex A means that ^3H-G_{M2} accumulates in NG125 cells and cannot be degraded. In addition, the lack of Hex A means no interference with sialidase for binding to and degradation of G_{M2}. NG125 cells have endogenous lysosomal sialidase and therefore we expected some background sialidase mediated G_{M2} degradation, however this rate was extremely low under normal circumstances. The transformed nature of NG125 cells allowed us to perform stable transfections instead of transient transfections. We preferred this to the use of primary cells because there was no limit to the length of time we could perform pulse-chase experiments. Assuming that conversion of G_{M2} to G_{A2} is a slow reaction, we anticipated that relatively long periods of time with high-level sialidase expression would be necessary to detect accumulation of G_{A2}. In addition, attempts to transfect the equivalent non-transformed neuroglia cell line (NG18) either transiently or stably, were too inefficient to be useful in pulse-chase experiments. Finally, we chose a neuroglia cell line because we felt it would more closely approximate the situation in the brain than a fibroblast cell line which, does not generate endogenous G_{M2} as do neuroglia cells.

In order to achieve high levels of sialidase activity by transfection into cultured cells, it is necessary to co-transfect sialidase with cathepsin A (Pshezhetsky et al. 1997, Bonten et al. 1996, Campbell et al. 1997.) Van der Spoel et al. (1998) have proposed that this is because sialidase is not transported to the lysosome in the absence of cathepsin A and instead precipitates as an inactive form in the ER. Therefore we used two episomal vectors (figure 1), pREP4 (10.2 kb) and pREP9 (10.5 kb), each with a different drug resistance, to

allow for stable co-transfection of cell lines. We chose episomal vectors in order to eliminate the random and unpredictable effects of integration. In addition, the use of episomal vectors allowed us to eliminate clonal selection of individual colonies since all transfected cells are identical, with the copy number of each vector controlled within a narrow range that is dependent upon drug concentration. This greatly reduced the time required to establish stably transfected cell lines.

Though a large number of vectors containing various components of the sialidase bypass were constructed (Table 1), only a subset of these were used to create stably transfected cell lines (figure 3) due to time constraints. Ultimately, pulse-chase experiments were performed only on cell lines having the highest levels of sialidase expression and on negative and positive control cell lines. This chapter reports the results and conclusions of these pulse-chase experiments and provides evidence that human lysosomal sialidase can degrade G_{M2} ganglioside.

Table 1. Vectors Constructed Containing Components of the Sialidase Bypass*

cDNA	Vector	Name of Construct
Human Sialidase	pREP4	PREPhmneu
Mouse Sialidase	pREP4	PREPmsneu
Human Hex ß	pREP9	PREPhmhexB
Mouse Hex ß	pREP9	PREPmshexB
Human G_{M2}-Activator	pREP4	PREPhmact
Mouse G_{M2}-Activator	pREP4	pREPmsact
Human Cathepsin A	pREP9	pREPhppca
Human Hex α (control)	pREP4	pREPhmHexA

*These vectors are used to create stably transfected cell lines containing various components of the sialidase bypass.

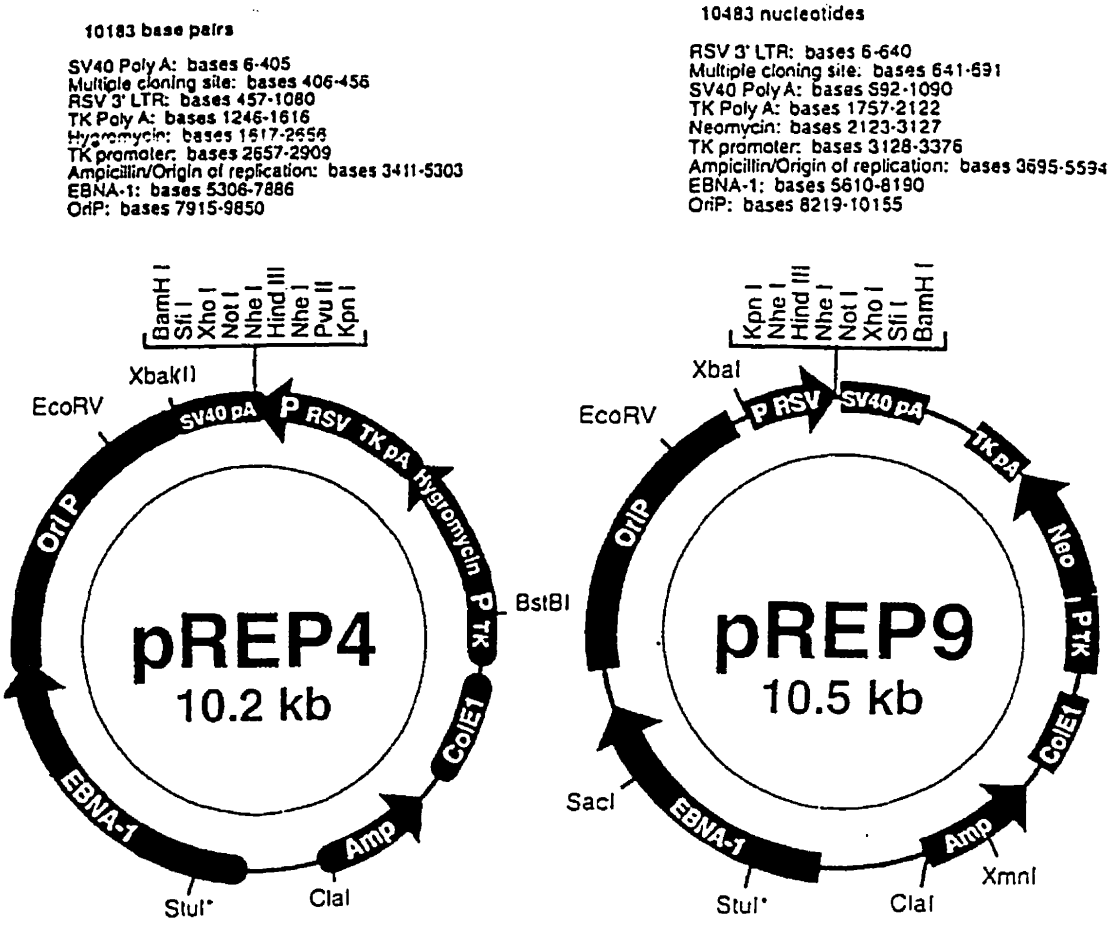


Figure 1. Illustration of the episomal vectors used for stable transfections. pREP4 contains hygromycin resistance and pREP9 contains neomycin (geneticin) resistance.

MATERIALS AND METHODS

Cell Lines

Normal (NG141) and Tay-Sachs (NG125) neuroglia cells and sialidosis fibroblasts (WG544) were obtained from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital, Montreal, Quebec. All cell types were maintained in modified Eagle's medium (α-MEM) supplemented with 10% fetal calf serum (FCS), 10% iron enriched bovine serum and antibiotics.

Subcloning Into Expression Vectors

The mouse sialidase cDNA (1.77kb) was cloned previously in our lab (Igdoura et al. 1998) and subcloned into the pSPORT2 vector (Gibco.) The cDNA was excised from pSPORT2 with *KpnI* and *XhoI* and subcloned directionally into the same sites of pREP4 (Invitrogen.) The human sialidase cDNA (1.92 kb) was obtained royalty free from the IMAGE Consortium (IMAGE clone 26525), Huntsville, Alabama. The cDNA was excised with *NotI* and *HindIII* and subcloned directionally into the same sites of pREP4. The human cathepsin A cDNA (2.03 kb) was a gift from Y. Suzuki (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan.) Initially subcloned into the *SalI* and *XhoI* sites of the pCMV-Sport vector (Gibco), the cDNA was re-excised with *KpnI* and *NheI* and subcloned directionally into the same sites of pREP9. Human hexosaminidase A cDNA in pREP4 was prepared as described previously (Fernandes et al. 1997.) The human and mouse G_{M2} activator protein cDNA's were gifts from Don Mahuran (Hospital for Sick Children, Toronto, Ontario.) The human G_{M2} activator cDNA (coding region only-680 bp) was excised from the pCR2.1 vector (Invitrogen) (Xie et al. 1991) by digestion with *BamHI* and subcloned into the same site of pREP4. The mouse G_{M2} activator (2.05 kb) was excised

from pBluescript (Stratagene) (Yamanaka et al. 1994) using *XhoI* and *NotI* and subcloned into the same sites of pREP4. The human hexosaminidase B cDNA was previously cloned by our lab (Neote et al. 1990) into pBluescript. The cDNA was excised with *XbaI* and *XhoI* and subcloned directionally into the *NheI* and *XhoI* sites of pREP9. The mouse hexosaminidase B cDNA was previously cloned by our lab (Bapat et al. 1988) into pBluescript. The cDNA was excised by *NotI* and *SalI* and subcloned directionally into the *NotI* and *XhoI* sites of PREP9. All constructs were restriction mapped and sequenced manually (Gibco dideoxy sequencing kit) at the vector-insert junctions to verify insert orientation and integrity.

Purification of Plasmid DNA

Small amounts of plasmid DNA for restriction analysis and sequencing were purified using the Wizard miniprep system. Large amounts of plasmid DNA for transfection of cell lines were purified using the Qiagen maxiprep kit.

Transient Expression in Sialidosis Cells

To verify functionality of the constructs, sialidosis cells (deficient in lysosomal sialidase) were transiently transfected with mouse and human sialidase cDNA, with or without human cathepsin A cDNA and with cathepsin A cDNA alone. In addition pREP4 vector with no insert was used as a negative control. Sialidosis fibroblasts were trypsinized, resuspended in α-MEM supplemented with 10% FCS, washed once in phosphate buffered saline (PBS) and washed once in Optimem (Gibco.) Plasmid DNA (21ug) was then electroporated into 7.5 x 10^6 cells, suspended in 500ul Optimem, in a 0.4 cm electroporation cuvette using a Bio-Rad Gene Pulser II set at 240 mV and 950 μF (time constant 35-37.) The cells were seeded in T75 flasks with α-MEM supplemented with 10% FCS for 24 hours

at which time the medium was changed to α-MEM supplemented with 10% FCS and antibiotics. The cells were harvested at 72 hours post-transfection and assayed for sialidase activity and protein concentration.

Establishment of Stably Transfected Cell Lines

Tay-Sachs neuroglia cells (NG125) were stably transfected with pREP4 containing no insert (negative control), mouse and human sialidase with and without cathepsin A, cathepsin A alone and Hex α (positive control.) Transfections were done in T75 flasks using Lipofectamine Plus according to the manufacturer (Gibco) with 4μg DNA, 30μl Lipofectamine, 20μl Plus reagent and 750μl Optimem per transfection. Complexes were incubated with cells 3 hours in 5ml Optimem. After transfection, cells were incubated 24 hours in α-MEM with 15% FCS. On day two, the medium was replaced with α-MEM containing 15% FCS and antibiotics. On day three (72 hours) selective antibiotics were added to a concentration of 400μg/ml. Hygromycin was added to cell lines containing pREP4, geneticin to cell lines containing pREP9 and both to cell lines containing both vectors. Cell lines containing no vector (mock transfection) were also treated with selective antibiotics to ensure efficacy of the drugs at killing non-transformed cells. Selection was maintained at these levels for 10 days at which time the dosage of the drug was reduced to 200μg/ml. This antibiotic concentration was maintained for the remainder of the experiment. An aliquot of cells (one T75) were harvested after 2 months of selection and assayed for sialidase activity or hexosaminidase activity. In addition, one T75 flask of cells was harvested for PCR confirmation of the insert or inserts carried in each cell line. The Wizard miniprep kit was used to purify plasmid DNA from stably transfected cells and the Expand High Fidelity PCR System (Boehringer Mannheim) was used in conjunction with

the primer pair pREP Forward ($^{5'}$-GCCTAGCTCGATACAATAAACGC) and EBV Reverse ($^{5'}$-GTGGTTTGTCCAAA CTCATC) to PCR vector inserts. Products were then analyzed on a 1% agarose gel.

Enzyme Assays

Cells from one T75 were trypsinized, pelleted by centrifugation, resuspended in 200µl 20mM sodium acetate pH 5.2 and sonicated for 5s at 60 Hz. Assays contained 10µl of cell homogenate, 25µl 20mM sodium acetate pH 5.2, and 25µl of substrate. The substrates were 4-methylumbelliferyl-D-N-acetylneuraminic acid for sialidase, 4-methylumbelliferyl-β-galactoside for β-galactosidase and 4-methylumbelliferyl-β-N-acetylglucosamine for total hexosaminidase activity. Assays were incubated at 37°C for 10 or 45 minutes as indicated and stopped with the addition of 1 ml of 0.4M glycine pH 10.4. Fluorescence was measured in a Perkin-Elmer fluorimeter.

Treatment of Cells with ^{3}H-G_{M2}

Confluent T175 flasks of stably transfected cells were split into six T75 flasks and allowed to grow 16 hours to about 70% confluency. Cells were washed in PBS and incubated with 5ml of Optimem containing 1µCi/ml ^{3}H-G_{M2} (pulse). After the indicated amount of time, the medium was removed and replaced with α-MEM containing 10% FCS and 10% iron enriched bovine serum (Chase). After the specified amount of time cells were washed with PBS, trypsinized, pelleted by centrifugation and frozen at -80°C until lipid extraction could be performed.

Lipid Extraction, Separation and Analysis

The extraction and separation method is based on that of Svennerholm et al. (1991). In brief, a pellet from one T75 flask was resuspended in 200µl water, to which 640µl

methanol and 320µl of chloroform were added. After vortexing and incubation in a bath sonicator for 15 minutes, the extract was centrifuged 5 min at 11 000 rpm in a microfuge and the supernatant was collected. The pellet was re-extracted with 1ml of 4:8:3 chloroform /methanol/water and centrifuged as before. The supernatants were pooled and evaporated to dryness in a speedvac. The residue was dissolved in 1.5ml 10:10:1 chloroform/methanol/ water, mixed with 5ml 1:1 methanol/water and applied to a pre-equilibrated Sep-Pak cartridge. (Equilibration of Sep-Pak cartridges was done by sequential washing with 10ml methanol, 10ml 1:1 chloroform/methanol (x3), 10ml methanol and 10ml 0.1M ammonium acetate in 1:1 methanol/water (x2).) The sample was washed with 10ml water (x3) and the lipids eluted with 30ml 1:1 chloroform/methanol. After evaporation to dryness in a vacuum evaporator (Buchi), the lipids were dissolved in 4ml of 9:1 chloroform/methanol and applied to a 1g silica gel 60 column (230-400 mesh, Merck A.G.) packed in chloroform. The column was washed with 8ml 65:25:4 chloroform/methanol/water and the ganglioside fraction was eluted with 10ml 3:6:2 chloroform/methanol/water. The sample was then dried under vacuum and dissolved in 100µl of 1:1 chloroform/methanol. A 5µl aliquot was used for scintillation counting to determine the number of counts in each sample such that an equal amount of radioactivity could be spotted in each lane on the TLC plate. Plates were developed with 60:35:8 chloroform/methanol/0.2% $CaCl_2$ and then either scanned with the radioisotope scanner or exposed to a tritium sensitive imaging plate for the phosphoimager. All data were compiled and analyzed using Microsoft Excel.

RESULTS

Transient Expression of Sialidase

Table 1 shows the constructs created that contain various mouse and human components of the sialidase bypass. To ensure these constructs encoded functioning enzymes before they were used for creation of stably transfected cell lines, some were tested for activity by transient expression (figure 2). That these studies were vital was revealed by the demonstration that one of the original constructs encoding human sialidase was not functional. It was later revealed that this clone contained a T323C mutation that greatly reduced its activity (data not shown). Therefore a second sialidase cDNA was subcloned into pREP4 (pREPhmneu) and this construct showed excellent activity when tested. These data show that the human and mouse sialidase constructs are active, that the human cathepsin A construct is active (co-transfection of mouse or human sialidase with cathepsin A dramatically enhances activity) and that the human Hex α construct is active.

Establishment of Stably Transfected Cell Lines

Once the functionality of the constructs was verified, they were used to stably transfect the transformed Tay-Sachs neuroglia cell line NG125. Transfection efficiency, as determined by β-galactosidase staining (data not shown), was approximately 20% with Lipofectamine. Initial selection with hygromycin or geneticin resulted in the death of nontransfected cells in about 7 days (2 cell divisions). After about 2 months of continuous drug selection, cell lines were assayed for sialidase or hexosaminidase activity (figure 3). Cell lines containing no vector (TSD-NG) or containing the pREP4 vector with no insert (TSD-NG + pREP4) show a modest amount of endogenous sialidase activity. Interestingly the cell line transfected with cathepsin A alone (TSD-NG + h-CA) showed a two-fold

increase in sialidase activity. This suggests that cells normally produce an excess of lysosomal sialidase, some of which remains unused and inactive, and that cathepsin A is the limiting factor. Cells transfected with either mouse or human sialidase alone showed about a four fold increase in sialidase activity and cells with both a sialidase and cathepsin A cDNA showed a nine fold increase above endogenous sialidase levels. The cell line transfected with Hex α showed a massive increase in hexosaminidase activity as compared to the undetectable levels in TSD-NG or TSD-NG + pREP4 (negative controls.) The vector carried in each cell line was confirmed by PCR. All cell lines contained the vector or vectors expected (Data not shown).

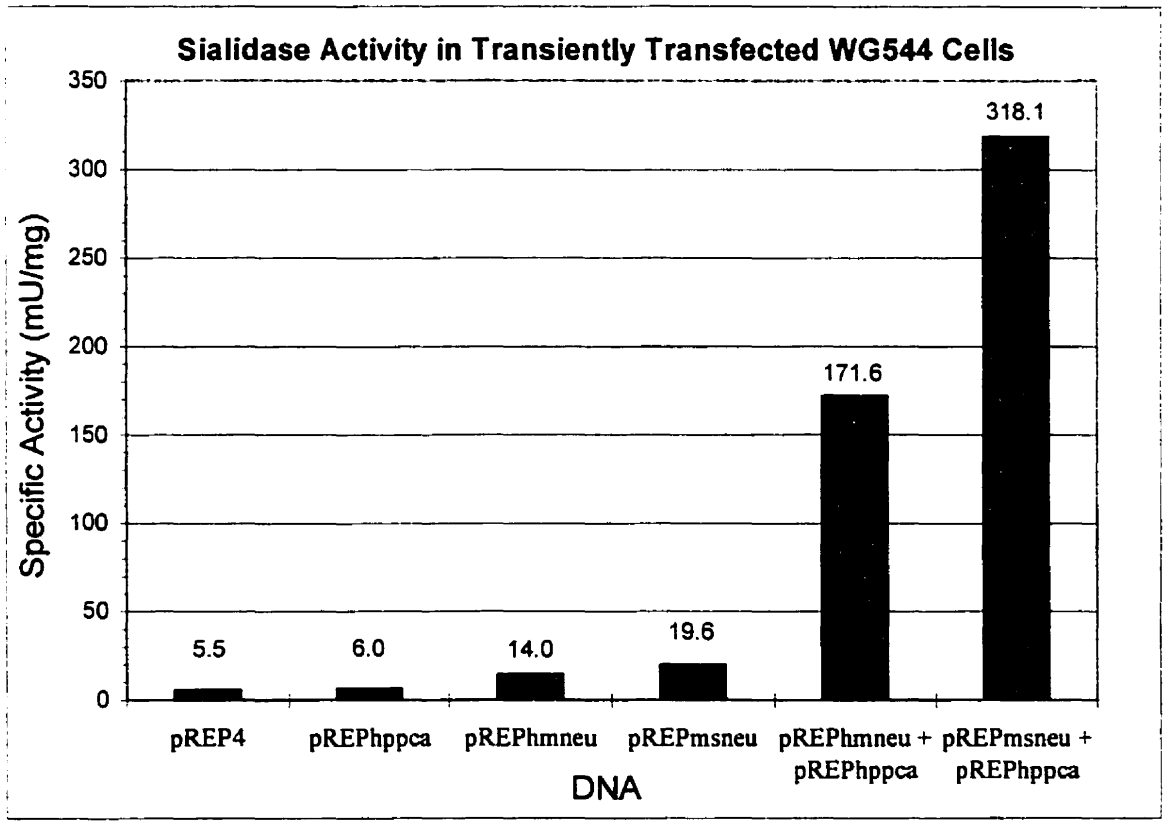


Figure 2. Specific activity of lysosomal sialidase in transiently transfected sialidosis cells. Assays contained 10µl of cell homogenate, 25µl Mu-Nana and 25µl of NaAcetate pH 5.2. Incubations were 45 minutes at 37°C. Data represent an average of three experiments.

ACTIVITIES IN STABLY TRANSFECTED CELL LINES

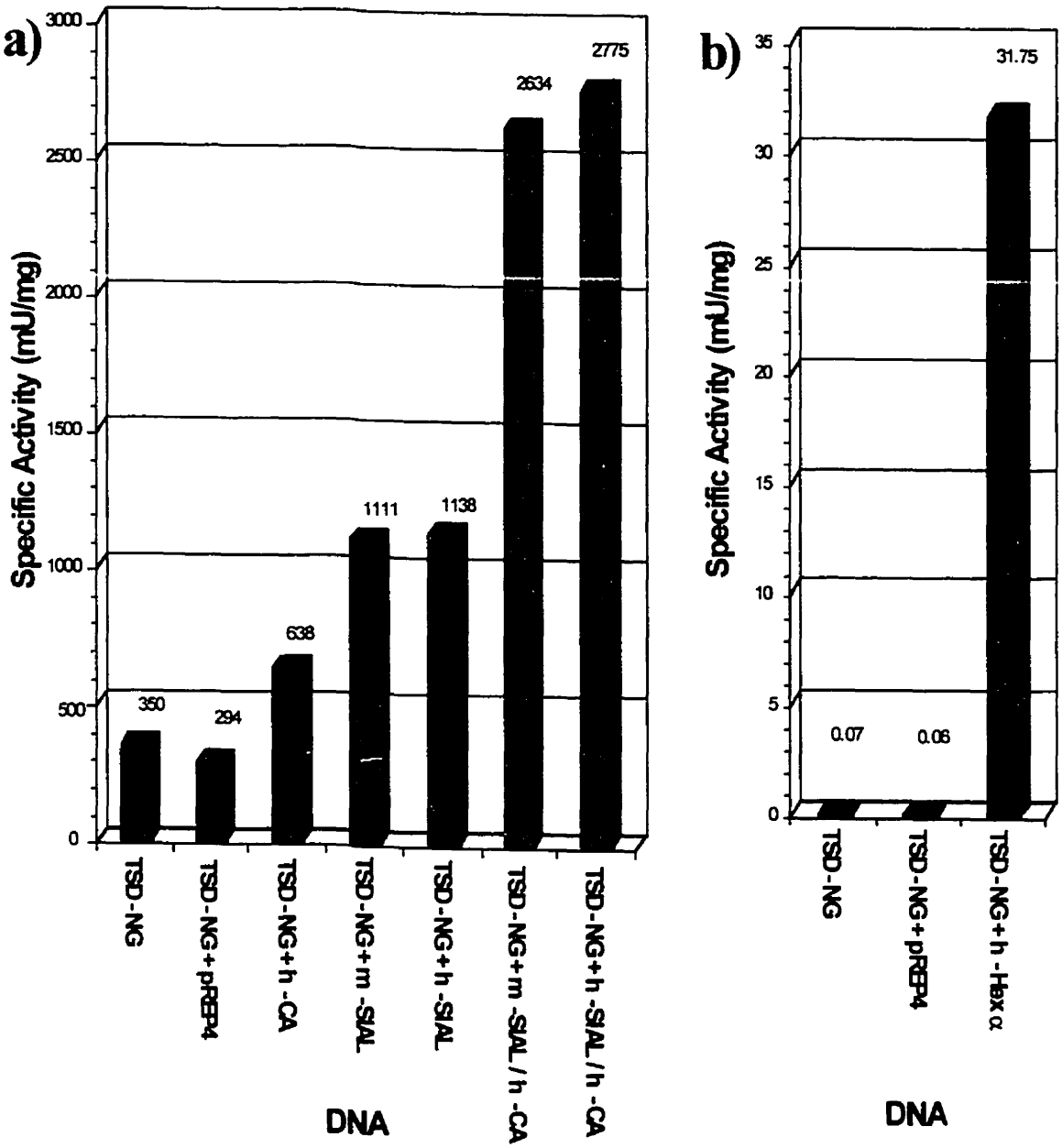


Figure 3. A) Sialidase activity in stably transfected Tay-Sachs neuroglia (TSD-NG) cells. B) Hexosaminidase activity in stably transfected Tay-Sachs neuroglia cells. m=mouse, h=human, CA=cathepsin A, SIAL=sialidase. Activities determined using the artificial substrates Mu-Nana and 4-MUG. Assays contained 10µl of cell homogenate, 25µl substrate and 25µl of NaAcetate pH 5.2. Incubations were 45 minutes at 37°C. Data represent an average of three experiments.

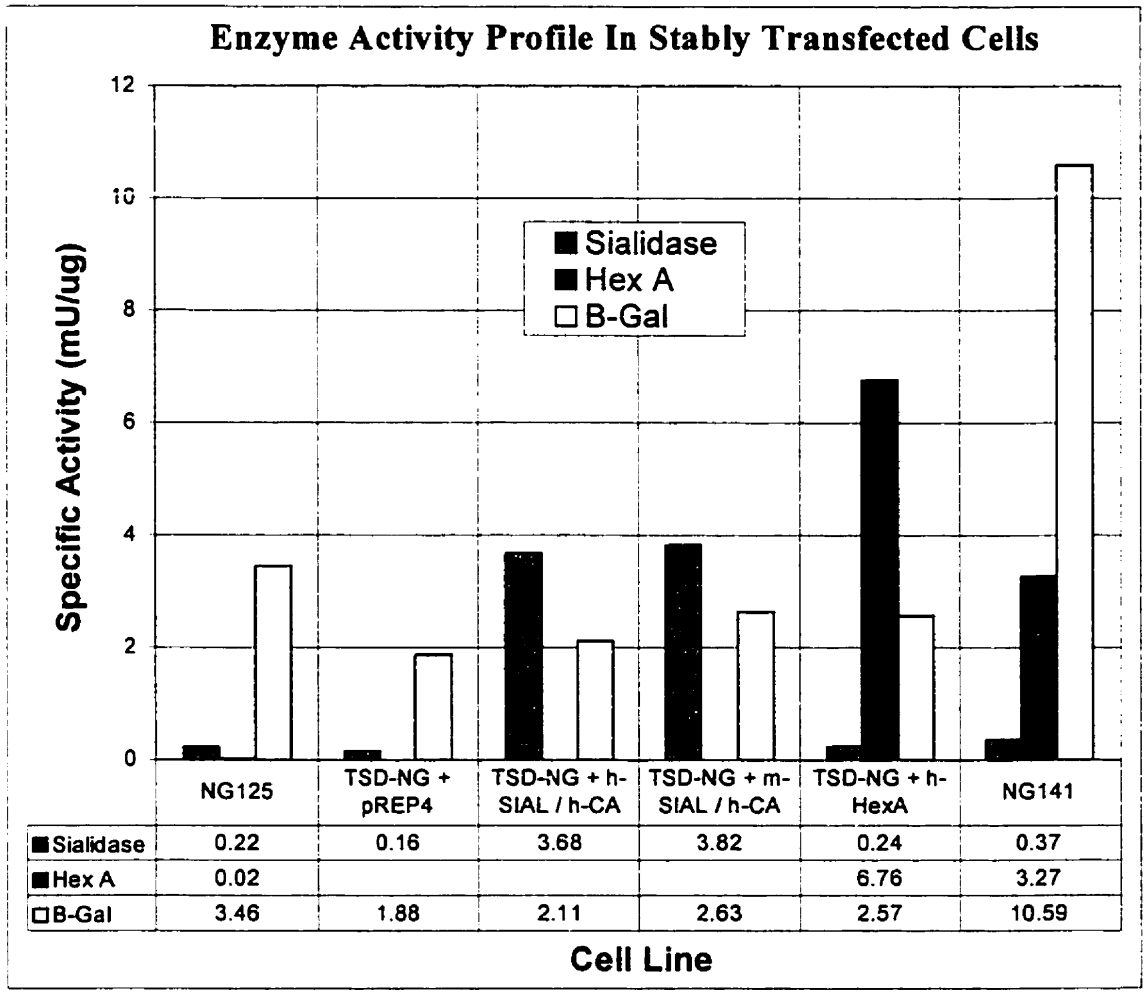


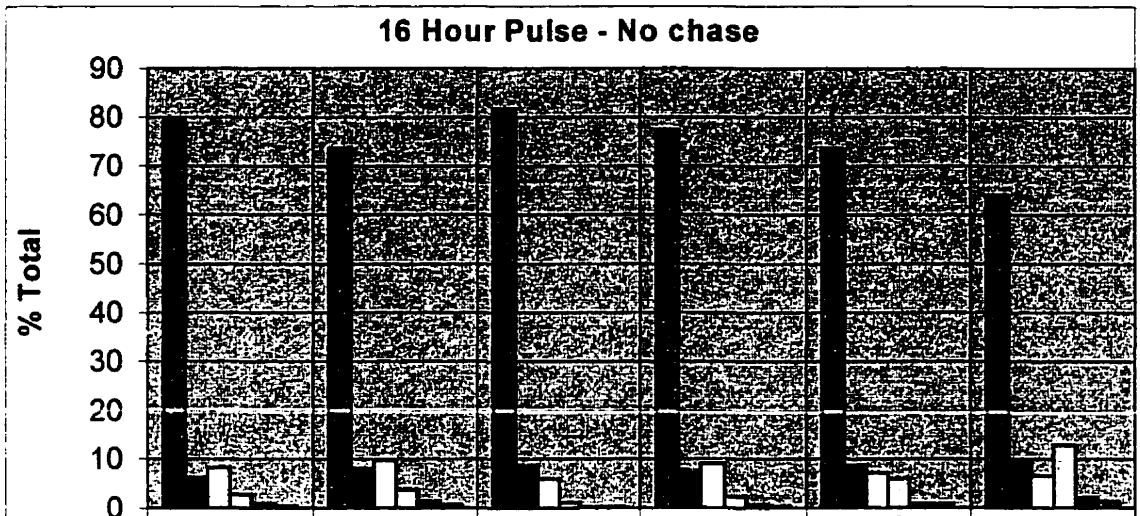
Figure 4. Profile of enzyme activities for the six different cell lines used in pulse-chase experiments. Activities determined using the artificial substrates Mu-Nana and 4-MUG. Assays contained 10µl of cell homogenate, 25µl substrate and 25µl of NaAcetate pH 5.2. Incubations were 10 minutes at 37°C. Abbreviations are as in figure 3. NG141 is the wildtype neuroglia cell line. HexA=hexα, NG125=TSD-NG. Data represent an average of two experiments.

Pulse-Chase Experiments

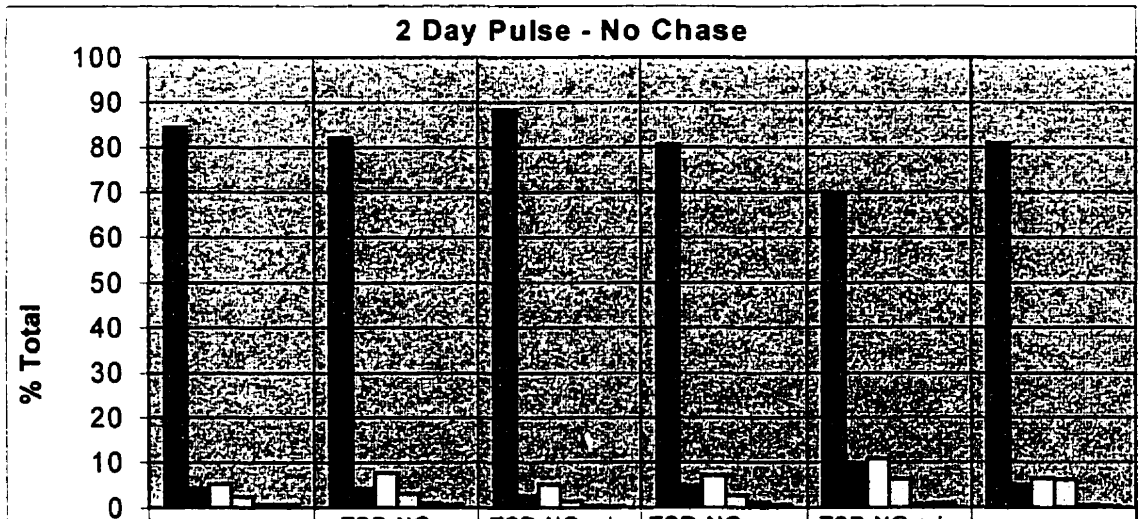
Six cell lines were chosen for pulse-chase experiments. NG125 and TSD-NG + pREP4 were chosen as negative controls and TSD-NG + h-Hexα and the wildtype transformed neuroglia cell line NG141 were chosen as positive controls. The stably transfected cell lines expressing the highest levels of sialidase, TSD-NG + m-SIAL/h-CA (mouse sialidase + cathepsin A) and TSD-NG + h-SIAL/h-CA (human sialidase + cathepsin A) were chosen to test the ability of sialidase to degrade G_{M2}. An enzyme profile for each of these cell lines, showing activity levels for sialidase, hexosaminidase and β-galactosidase, is given in figure 4. Interestingly, the cell lines with sialidase and cathepsin A show approximately a 20-fold increase in sialidase activity above endogenous levels. This is likely because these assays were only 10 minutes long in contrast to 45 minutes for the assays shown in figure 3. Because sialidase is inactivated very rapidly in cell homogenates at 37°C it is likely that the reaction is not linear for much more than 10 minutes (based on sialidase inactivation data; not shown.) Therefore, figure 4 is a more accurate representation of activity levels. Also unexpected was the much higher levels of β-galactosidase activity in NG141 cells. This is likely an artifact of the original SV40 induced transformation process and can be ignored because β-galactosidase should not affect ^{3}H-G_{M2} metabolism.

Four pulse-chase conditions were used. A 16 hour pulse with no chase and 48 hour chase and a 2 day pulse with no chase and 7 day chase. The amount of radioactive incorporation was higher for longer pulse times and when no chase was employed as expected. The most incorporation, for example, was seen with the 2 day pulse-no chase experiment and the least with the 16 hour pulse-48 hour chase experiment. The

quantitative analysis of gangliosides in the cell lines for each experiment is shown in figures 5 and 6. Two things are immediately apparent. First, although it is known that the lipid extraction and separation scheme used results in a ganglioside fraction that contains G_{M1}, G_{M2}, G_{M3} and G_{A2} (Fujita et al. 1996), it is not known where smaller glycolipids such as lactosylceramide, glucosylceramide or ceramide partition. Indeed it appears that while lactosylceramide partitions to the ganglioside fraction at least to some extent, glucosylceramide and ceramide partition mainly to the non-ganglioside fraction as evidenced by their low levels. Second, it is obvious from Figure 6 that the positive controls functioned well as more than 65% of ^3H-G_{M2} is degraded in the cell line expressing Hexα and more than 70% in the NG141 cell line. Closer examination of the data also reveals that there were modest but consistent increases of G_{A2} content in the cell line expressing high levels of human lysosomal sialidase when the chase time was long enough (figure 6.) In other words, the human sialidase appears to degrade G_{M2} at a slow but significant rate. Most surprisingly however is the fact that the mouse lysosomal sialidase did not affect G_{M2} degradation at all. Conveniently, the cell line expressing mouse lysosomal sialidase acted as an excellent negative control. Everything is identical between the cell lines expressing mouse sialidase and human sialidase except the sialidase itself. In both experiments with a chase period, G_{A2} levels increased 2 fold in the cell line expressing human sialidase compared to that expressing mouse sialidase. This increase was not seen when the cells were pulsed but not chased.

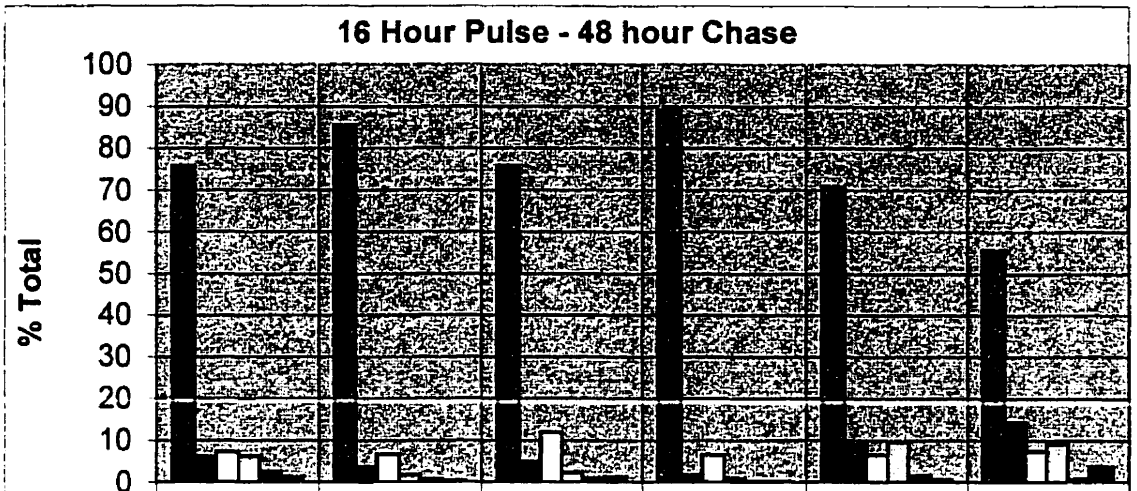


	NG125	TSD-NG + pREP4	TSD-NG + h-SIAL / h-CA	TSD-NG + m-SIAL / h-CA	TSD-NG + h-HexA	NG141
■Gm2	79.16	73.6	81.53	77.32	73.5	63.89
■Gm3	6.08	7.94	8.58	7.57	8.55	9.51
□GA2	8.32	9.65	5.92	9.03	7.15	6.48
□Lac-Cer	2.72	3.86	1	2.34	6.03	12.82
■Glu-cer	0.94	1.22	0.14	0.6	0.68	2.1
■Ceramide	0.39	0.57	0.18	0.36	0.73	1.28

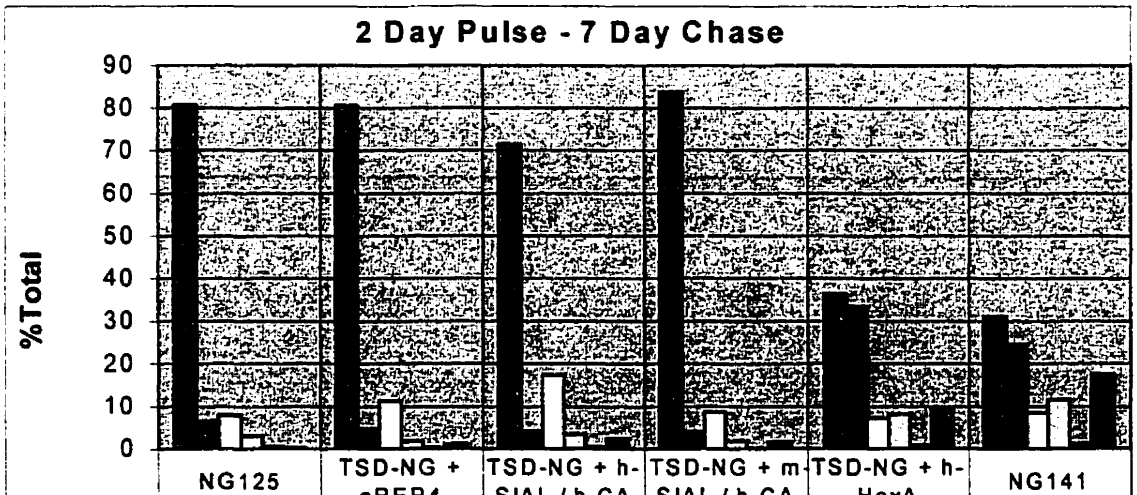


	NG125	TSD-NG + pREP4	TSD-NG + h-SIAL / h-CA	TSD-NG + m-SIAL / h-CA	TSD-NG + h-HexA	NG141
■Gm2	84.25	82.02	88.28	80.6	69.98	80.88
■Gm3	4.2	4.19	2.51	4.77	9.32	4.98
□GA2	5.23	7.57	5.07	7.1	10.71	6.4
□Lac-Cer	2.43	3.13	1.38	2.78	6.24	6.14
■Glu-cer	0.77	0.91	0.41	1.11	0.69	0.56
■Ceramide	0.67	0.7	0.3	0.55	0.95	0.25

Figure 5. Results of experiments with a pulse but no chase. Abbreviations are as in figures 3 and 4. Values refer % total radioactivity in the lane as determined by the Bioscan radioisotope plate scanner. Due to time and material limitations, these experiments were performed only once.



	NG125	TSD-NG + pREP4	TSD-NG + h-SIAL / h-CA	TSD-NG + m-SIAL / h-CA	TSD-NG + h-HexA	NG141
■Gm2	75.73	85.36	75.75	89.15	70.84	55.57
■Gm3	6.08	3.61	4.73	1.7	8.99	14.45
□GA2	7.28	6.72	12.01	6.45	6.47	7.49
□Lac-Cer	6.22	1.73	2.43	0.99	9.58	9.12
■Glu-cer	2.54	0.71	1.02	0.26	1.6	0.91
■Ceramide	1.21	0.34	1.21	0.2	0.79	3.76



	NG125	TSD-NG + pREP4	TSD-NG + h-SIAL / h-CA	TSD-NG + m-SIAL / h-CA	TSD-NG + h-HexA	NG141
■Gm2	80.5	80.59	71.43	83.7	36.41	30.97
■Gm3	6.42	4.62	3.95	3.91	33.4	24.32
□GA2	7.87	11.01	17.24	8.54	7.18	8.43
□Lac-Cer	2.93	1.68	3.44	1.79	8.01	11.48
■Glu-cer	0.48	0.32	0.44	0.09	0.69	1.21
■Ceramide	0.23	1.11	2.31	1.49	9.3	17.35

Figure 6. Results of experiments with a pulse and a chase. Abbreviations are as in figures 3 and 4. Values refer % total radioactivity in the lane as determined by the Bioscan radioisotope plate scanner. Due to time and material limitations, these experiments were performed only once.

DISCUSSION

The data presented here give some compelling evidence that the human sialidase can indeed catabolize G_{M2} to G_{A2} at a slow rate. Despite the fact that the effects were small, they were repeatable in two separate experiments. The data is even more convincing in light of the fact that the mouse lysosomal sialidase showed no effect under identical conditions. This means any increases in the amount of G_{A2} can not be attributed to such things as the presence of cathepsin A alone, or the fact that cell lines expressing a sialidase and cathepsin A were continuously grown in media that contained the drugs hygromycin and geneticin. It is conceivable that such antibiotics could affect the metabolism of a cell. Transfected cell lines grown and treated under selective conditions containing hygromycin and geneticin, exhibited very poor uptake of ^{3}H-G_{M2} compared to the cell lines NG125 and NG141 which were grown without selective antibiotics. However, any effects on the metabolism of gangliosides were not apparent and were probably very minor.

The primary criticism in drawing a definitive conclusion from these data is the minuteness of the effect. More than a 20 fold increase in the activity of lysosomal sialidase resulted in only a 2 fold increase in the amount G_{A2} produced. This effect becomes even smaller when random fluctuation and noise from experimental procedure are considered. In the 16 hour pulse-no chase experiment, levels of G_{A2} range from about 6% to 9%. Such a range is satisfactory and these levels of G_{A2} can be considered background. In the 2 day pulse-no chase experiment, G_{A2} levels vary from 5% to 7.5%, also satisfactory, with the exception of TSD-NG + hHexα which showed 10.7% G_{A2} content. This is likely an artifact as it does not repeat and levels of G_{A2} for TSD-NG +

hHexα fall within the average range for all other experiments. A similar observation is made in the 2 day pulse-7day chase experiment in which negative controls showed a range of 7% to 8.5% for G_{A2} content with the exception of TSD-NG + pREP4 which had 11% G_{A2}. Again, this is likely an artifact, as it never repeats. Finally, for the 16 hour pulse-48 hour chase experiment, all cell lines (except of TSD-NG + hSIAL/h-CA) show a range of 6.5% to 7.5% for G_{A2} content. Therefore, despite some fluctuation in background G_{A2} levels, the cell line expressing high levels of human lysosomal sialidase repeatedly shows the largest increase in the amount of G_{A2} content.

Although the largest detected increase in G_{A2} content was only two fold, it is possible that the rate of G_{M2} conversion to G_{A2} is higher than this. If, as discussed in the previous chapter, G_{A2} is rapidly degraded by Hex B to Lac-Cer, then G_{A2} would not accumulate to high levels. This could explain why a large boost in sialidase activity is accompanied by a modest increase in G_{A2} content. In addition, evidence for increased catabolism of G_{M2} via G_{A2} would not be exhibited by a proportional increase in downstream products such as Glc-Cer or Cer because of their incomplete extraction into the ganglioside fraction.

Finally, it is curious that the mouse sialidase did not effect an increase in G_{A2} production as would be expected based on the literature presented in the introduction of this chapter (Igdoura et al. 1999, Proia et al. 1995.) It can be easily explained however. Perhaps the mouse sialidase cDNA contained a mutation that did not affect its activity against the artificial substrate Mu-Nana, but destroyed its ability to act on G_{M2}. More likely, the lack of activity against G_{M2} probably reflects an incompatibility between the mouse sialidase and the human system used. As suggested in the previous chapter,

perhaps a necessary cofactor for sialidase mediated degradation of G_{M2} could not interact with the mouse sialidase. Such a cofactor could be the G_{M2} activator protein, saposin B or perhaps some other unidentified protein. This incompatibility between mouse sialidase and human cofactors may have increased the threshold for detection of sialidase mediated G_{M2} catabolism beyond the limits of the experiments presented here. The evidence produced by Igdoura et al. (1999) was obtained using immunocytochemistry, a technique which is potentially much more sensitive because of its ability to detect extremely high levels of lysosomal sialidase in individual cells.

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CHAPTER FIVE

FUTURE PROSPECTS

DISCUSSION

The data presented in this thesis provide evidence that both the human and mouse lysosomal sialidase enzymes, under certain conditions, can degrade G_{M2}. In chapter three, the mouse CGF shows significant sialidase activity against G_{M2} (figure 4) as does the human CGF plus gel filtration. In addition, the human sialidase also shows such activity in chapter four as determined by pulse-chase experiments (figure 6.) These data, however, are not conclusive due to complicated interpretations and apparent inconsistencies. The major reason for these problems is that sialidase mediated catabolism of G_{M2} is a slow reaction, making it difficult to detect. In addition, our current understanding of the biology of mammalian sialidases is very limited. Many questions remain concerning the number, type and role of sialidases present in mammalian cells. Lysosomal sialidase is further complicated by the fact that it is part of a multi-enzyme complex. The exact composition and structure of this complex has yet to be delineated. Despite attempts to do so, it is still unclear for example, how sialidase is activated within this complex. Lack of this information makes it more difficult to interpret results concerning how this complex interacts with other proteins, such as the G_{M2} activator or Saposin B and with substrates such as G_{M2}. Recent advances, including the cloning of the mouse (Igdoura et al. 1998, Carrillo et al 1997, Rottier et al 1998) and human (Pshezhetsky et al. 1997, Bonten et al. 1996, Campbell et al. 1997) lysosomal sialidases, cloning of cathepsin A (Galjart et al. 1988), cloning and easy purification of the G_{M2} activators (Xie et al. 1991, Yamanaka et al. 1994) and cloning of the hexosaminidases (Neote et al. 1990, Bapat et al. 1988, Beccari et al. 1992, Korneluk et al. 1986) have all dramatically enhanced our ability to study the lysosomal sialidase complex and the

metabolism of G_{M2} in general. Armed with these tools, the potential for future experiments concerning the sialidase bypass is enormous.

As discussed in chapter three, a means for obtaining high quantities of homogeneously purified lysosomal sialidase must be devised. The current method, based on affinity chromatography (Pshezhetsky and Potier, 1996) is inefficient, especially for the mouse. A feasible alternative approach is to make use of the multitude of new purification techniques based on fusion proteins. For example, it should be possible to reconstitute an active sialidase complex by overexpression and mixing of the individual components. This approach has been attempted without success using a HIS-tag expression system in yeast (personal communication.) It is not clear why this approach failed since all known components of the complex, including sialidase, cathepsin A and ß-galactosidase were produced and purified successfully. Again, this points to our incomplete knowledge of the biology of the lysosomal sialidase. Despite this setback, expression of recombinant proteins holds great promise ultimately for determining and understanding the substrate specificity of lysosomal sialidase and other components involved in the sialidase bypass.

In addition to a purified sialidase, other proteins and factors must be considered for their involvement in sialidase mediated G_{M2} degradation. For example, many of the experiments presented in chapter three could be repeated with mouse and human saposin B in place of the G_{M2} activators. Perhaps the other components of the complex, such as cathepsin A or ß-galactosidase somehow affect the substrate specificity and efficacy of sialidase. Such things would be easily tested if an active complex could be reconstituted from its components.

Similarly, it would be interesting to repeat the experiments presented in chapter four with different cell lines. For example, instead of human Tay-Sachs neuroglia cells, human Sandhoff cells could be used instead. This would offer the advantage of a complete block in G_{M2} metabolism. Any G_{A2} produced in this cell line by sialidase mediated degradation of G_{M2} would remain as G_{A2}. Detection of G_{A2} would be facilitated since there would be no Hex B activity to convert G_{A2} to lactosylceramide. These experiments could also be repeated using mouse cell lines derived from Hex A and Hex B knockout mice. One would expect that increased expression of sialidase would not affect G_{M2} metabolism in mouse cells since it appears to prefer metabolism via sialidase already (Riboni et al. 1995, Proia et al. 1995.) However, it is possible that expression of an incompatible human sialidase could actually prevent sialidase mediated degradation of G_{M2} in these cells.

The ultimate aim of these studies was to evaluate the feasibility of using induced lysosomal sialidase levels as a therapeutic treatment for Tay-Sachs patients. As such, it is essential to determine if the approximate two-fold increase in sialidase mediated G_{M2} degradation that is shown in chapter four is sufficiently high to halt, and in fact reverse G_{M2} accumulation in these cells. It has been demonstrated that very small changes in the accumulation rate of G_{M2} can dramatically affect the overall accumulation of G_{M2} in the long run (Platt et al. 1997.) If such levels are indeed sufficient, then pharmacological induction of lysosomal sialidase activity (either directly or indirectly by induction of factors linked to sialidase activity such as cathepsin A) may be feasible as a therapeutic treatment for Tay-Sachs disease.

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