This is to certify that the

dissertation entitled

STUDIES ON THE PURIFICATION AND REGULATION OF CMP-SIALIC ACID:LACTOSYLCERAMIDE ALPHA2-3 SIALYLTRANSFERASE

presented by

Lyla J. Melkerson-Watson

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Biochemistry

Charles C. Sweeley
Major professor

Date 11-28-90

STUDIES ON THE PURIFICATION AND REGULATION OF CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE

By

Lyla Jill Melkerson-Watson

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1991

ABSTRACT

STUDIES ON THE REGULATION OF CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE

By

Lyla Jill Melkerson-Watson

A procedure is described for assays of CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1), using Sep Pak C_{18} cartridges. Complete separation of the more polar CMP-sialic acid and sialic acid from the less polar GM_3 is simple and rapid relative to other methods. Chromatographic recovery of GM_3 is high when phosphatidylcholine is added. The procedure may be applicable for other *in vitro* glycosyltransferase assays.

SAT-1 has been purified 40,000-fold from rat liver by affinity chromatography on lactosylceramide-aldehyde Sepharose 4B. Synthesis of the column is described. Purification was verified by immunoaffinity chromatography on M12GC7-Affi Gel-10. The M12GC7 monoclonal antibody specifically inhibits and immunoprecipitates SAT-1 activity. The apparent molecular weight by SDS-PAGE is about 60Kd. Studies on substrate specificity indicate SAT-1 recognition of the glycolipid, Galß1-4Glcß1-O-Cer and to a lesser extent Galß1-O-Cer or Glcß1-O-Cer. SAT-1 is a glycoprotein. The carbohydrate moieties are detected with specific lectins. Deglycosylation of SAT-1 results in a 43Kd band. The two-dimensional electrophoretogram of SAT-1 indicates a pI range of 5.7 to 6.2 for the 60Kd protein.

Lauryldimethylamine oxide (LDAO) is employed in the purification of SAT-1. This detergent has advantages over Triton detergents in the solubilization and stabilization of this sialyltransferase. The ability of LDAO to activate and stabilize

SAT-1 activity may involve the structural similarity between the hydrophobic moieties and quaternary amino groups of LDAO and phosphatidylcholine.

Co-purification of an endogenous proteolytic activity has been proposed as the cause for the size heterogeneity of rat liver sialyltransferases. Addition of protease inhibitors, sulfhydryl-reducing agents and antimicrobial agents to immunoaffinity-purified SAT-1 dramatically affects its activity. All protease inhibitors examined, with the exception of PMSF, inhibited the purified enzyme. The most inhibitory were the cysteine (thiol) protease inhibitors. Further, the apparent activation of SAT-1 activity in the presence of ß-mercaptoethanol was observed.

Turnover of SAT-1 in butyrate-synchronized KB cells is cell-cycle dependent. Regulation of GM_3 synthesis in KB cells may involve the phosphorylation of SAT-1. Analysis of immunoaffinity-purified KB cell SAT-1, during different time points of expression within the G_1 phase, by SDS-PAGE and its immunodetection on Western blots with monoclonal specific for phosphotyrosine residues, indicates SAT-1 is a phosphotyrosine-containing protein. The expression of this tyrosine-phosphorylated form may regulate SAT-1 activity.

To may parents, for their love and desire to educate their childern

To Greg, for his love and support

To Richard, for a new and youthful perspective of life

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Charles C. Sweeley for all of his advice and guidance. He has an extraordinary zest and love for research to which I aspire.

I would also like to thank the following people:

The members of my advisory committee, Drs. Thomas Deits, Walter Esselman, Estelle McGroarty, and William Smith, for their guidance and assistance during my dissertation project.

Drs. Jim Fairley and Richard Anderson for their special guidance and assistance over the years and for just being there...

Dr. Kimihiro Kanemitsu for his friendship and help in teaching me GC analysis of sialic acid and ozonolysis derivatization of LacCer.

Doug Wiesner and Kiyoshi Ogura for their assistance with the quantitation of GM_3 and its sialidase activity, respectively.

Dr. Elizabeth Cowles for her advice on 2D SDS-PAGE and comradeship.

Ms. Patty Voss for all "the tricks of the trade" on monoclonal antibody production and for "babysitting the guys" on occasion.

Dr. Rawle Hollingsworth for the use his/our HPLC.

Mr. Joe Leykam and Ms. Melanie Corlew for assisting me during the long hours on the microbore HPLC and protein analysis.

Carol Smith for her excellent secretarial assistance.

My friends and fellow Grads in Dr. John Wang's lab, Liz Cowles, Jamie Laing, Neera Agrwal, and Kim Hamann, for being "my lab away from lab."

All my laboratory mates who have supported and challenged me over the years, Barbara Myskwiecz-Sullivan, Frank Wilkinson, Kimihiro Kanemitsu, Mitsu Nakamura, Seigo Usuki, Masahiko Shigematsu, Shu-Chen Liu, Dr. Lyu, Patricia Hoops, Lynne Royer, Kazz, Kamada, Musti Swamy, Rawle Hollingsworth, Rob Dodds, Amanda

Poxon, Kiyoshi and Misa Ogura, Barb Kuhns, Bev Chamberlain, Zhi-Heng Huang, Bao-Jen Shyong, and last but not least, Doug Wiesner. My friends...thank you all!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 6

Chapter 7

CHAPTER 1

LITERATURE REVIEW

Introduction

The regulation of cellular growth and differentiation is of fundamental importance to most biological investigations, especially those mechanisms involved in oncogenic transformation. Research during the past decade has implicated a functional role for glycosphingolipids in these phenomena (1-5). Glycosphingolipids comprise a minor proportion of the complex carbohydrate-containing moieties of the plasma membrane forming the glycocalyx; yet, they are major contributors to the communication network modulating cellular activities. Glycosphingolipids serve as membrane receptors for bacteria (6-8) viruses (9,10), toxins (11-14), acetylcholine (15), interferon (16), and fibronectin (17-19)). Glycosphingolipids also act as mediators of cellular interaction (19,20), and Na^+ transport (21), and interact with the cell substratum (17,22) and as modulators of membrane receptor-mediated signal transduction mechanisms (25-31), indicating a role for glycosphingolipids in the regulation of cell proliferation. Further, shedding of gangliosides by tumors leads to enhanced tumor formation, possibly through suppression of the immune response (32,33; for an early review, 34). With increasing diversity in the functional role of glycosphingolipids, specifically gangliosides, research in the glycoconjugate field has three foci: (a) gangliosides as receptors for ligands and mediators in cell-cell interactions; (b) gangliosides as modulators of immune regulation; and (c) gangliosides as modulators of signal transduction mechanisms and cell growth.

The diversity of these functions reflects the structural diversity among the glycosphingolipids. About 300 glycosphingolipid structures have been elucidated (35). However, the functional studies cited above are but a sampling of the potential modulatory roles played by these glycolipids. The functions performed by the

glycosphingolipids may be as diverse as the structures themselves and the cells which express them. The physiological role of these glycoconjugates is not completely understood. Thus, there is great impetus for further investigation of the functional role played by glycosphingolipids in the modulation of cellular activities and the mechanisms which control their biosynthesis.

Ganglioside Structure

The different classes of glycolipids contain different oligosaccharide structures and are tissue- and species-specific (36). Gangliosides are sialic acid-containing glycosphingolipids. The sialic acid[1] confers a net negative charge on the molecule and contributes to its biological activity (37). Gangliosides are amphipathic molecules composed of the hydrophilic carbohydrate head group and the hydrophobic lipid tail (Figure 1). Their lypophilic residue, ceramide[2], is composed of a fatty acid which is amide-linked to a long-chain sphingoid base[3]. The ceramide participates in the formation of the outer leaflet of the plasma membrane bilayer. The carbohydrate sidechain is positioned out into the extracellular matrix. This strategic orientation defines the cell surface and contributes to the multi-functional role of gangliosides in cellular intercellular communication and cell growth.

Glycosphingolipids are structurally unique relative to the glycoproteins (for review, 38) and proteoglycans (for review, 39), two other major families of cell-surface glycoconjugates. Gangliosides, and, in fact, glycolipids in general are unique glycoconjugates in that they contain only one oligosaccharide chain, whereas glycoproteins and proteoglycans contain several such chains as is the case with

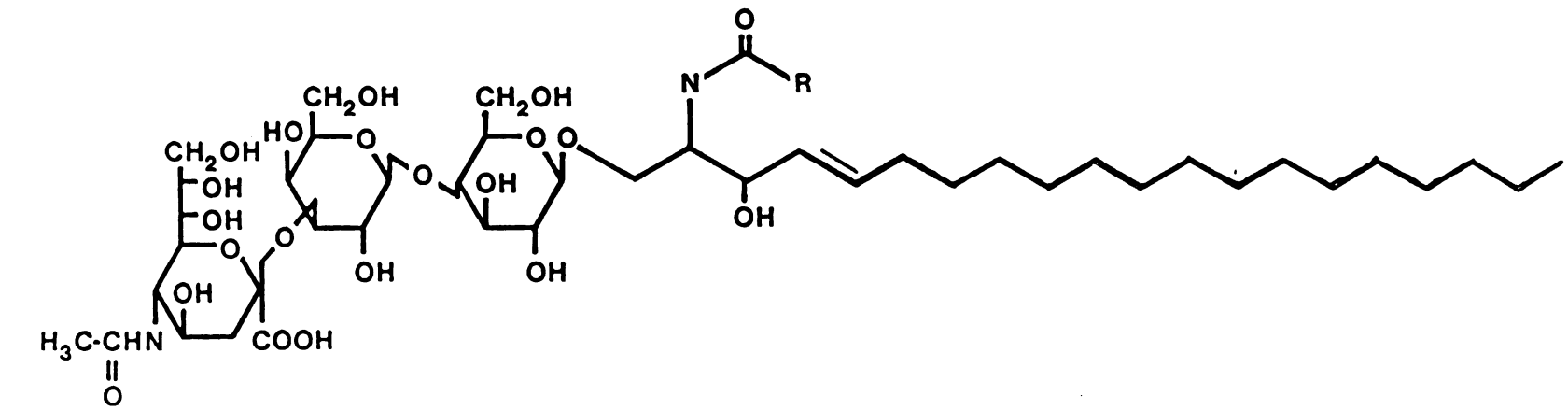
[1]Sialic acid is the generic name for neuramicinic acid.

[2]Ceramide is the trivial name for N-fatty acid acyl sphingosine.

[3]Sphingosine is the name given to D-erythro-1,3-dihydroxy-2-(alkylacetamide)-4,5-*trans*-octadecene or its analogs.

Figure 1. The Chemical Structure of GM_3 Ganglioside.

GM_3 GANGLIOSIDE
(NeuNAc α 2-3 Gal β1-4 Glc β1-1 Cer)



R:$-(CH_2)_n$ or $-CH(OH)-(CH_2)_n$

n = 15 · 25

SIALIC ACID

OLIGOSACCHARIDE

CERAMIDE

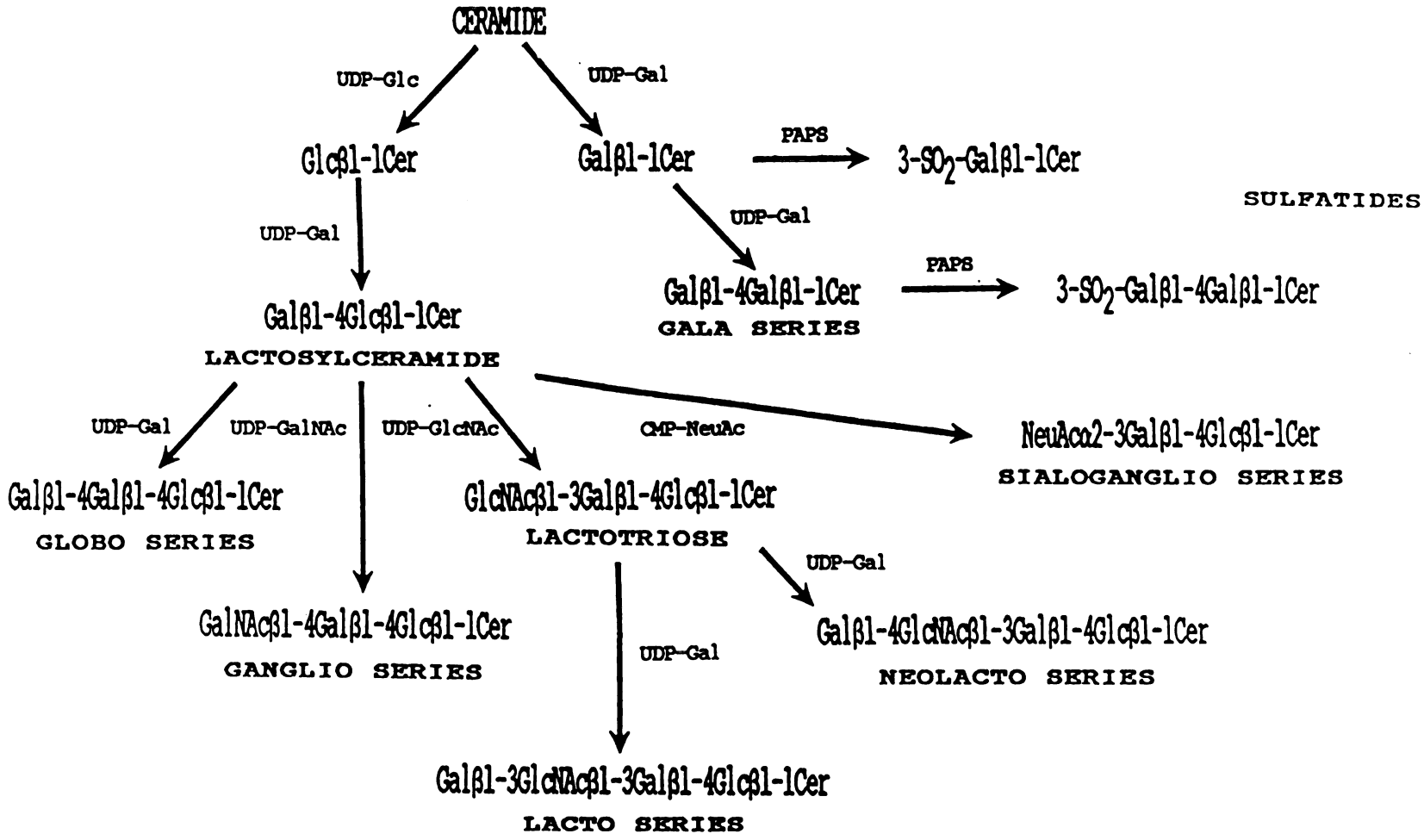
proteoglycans, glycolipids can also be sulfated. The oligosaccharide is O-linked to the ceramide through Glcß (globo, ganglio, sialoganglio, lacto, and neolacto series) and sometimes Galß (series) (Gala series and sulfatides) (See Figure 2). These O-linked saccharides differ from O- and N-linked oligosaccharides of glycoproteins in that the carbohydrate sidechains are typically through a GalNAcα to serine or threonine residues (O-linked) and asparagine (N-linked) in glycoproteins (38). Thus, the lactose (Galß1-4Glcß1-1Cer) linkage is unique to the glycolipid structure. It is important structural differences, such as this that confer the specificity of each particular glycoconjugate to a wide variety of factors or allow them to serve as substrate for a specific glycosyltransferase activity during product-precursor synthesis within the Golgi.

Ganglioside Biosynthesis

Generally, gangliosides are primarily localized on the outer leaflet of the mammalian cell; however, gangliosides have also been observed to be associated with internal membranes (40-43). Subcellular distribution studies with rat liver showed that 76.3% of the total gangliosides (by sialic acid) is localized in the plasma membrane, while lesser amounts reside on internal membrane compartments such as the endoplasmic reticulum (7.4%), Golgi apparatus (1.0%), mitochondria (10.8%) and nuclear membrane (4.8%) (43). The remaining soluble ganglioside were found in supernatant fractions. Individual gangliosides patterns were unique to the membrane examined. Further, Matyas and Morre (43) observed a high concentration of the ganglioside biosynthetic enzymes within the Golgi and minor amounts associated with the endoplasmic reticulum (ER). This was one of the first studies to show that gangliosides are synthesized within the Golgi and then transported, not only to the plasma membrane, but to the ER and other subcompartmental membranes.

Figure 2. Biosynthesis of Glycolipid Oligosaccharide Linkages Specifying the Different Carbohydrate Series.

BIOSYNTHESIS OF GLYCOLIPID CARBOHYDRATE CORE STRUCTURES



Gangliosides[4] (nomenclature used is summarized in Table 1) are synthesized by the stepwise transfer of monosaccharides from sugar nucleotides to growing glycolipid acceptors (44), as shown schematically in Figure 3 for the synthesis of GM_3 ganglioside by CMP-sialic acid:lactosylceramide sialyltransferase (SAT-1). This series of glycosylations occurs largely within the Golgi apparatus where specific glycosyltransferases are localized (45). Little is known about the compartmentalization of the glycosyltransferases though it has been
speculated that their distribution within the Golgi is related to the sequential order of addition of carbohydrates in the ganglioside biosynthetic pathway (for review, 46).

Recently, Ghidoni and coworkers (47-49) have begun detailed investigations on the subcellular biosynthesis and transport of gangliosides in rat liver and the enzymes which catalyze these glycosylations. They have found that the distribution of glycosyltransferases for the monosialo (GM_3->GM_2->GM_1->GD_{1a} catalyzed by GalNAcT-1, GalT-3 and SAT-4, respectively) and disialo (GD_3->GD_2->GD_{1b}->GT_{1b}->GQ_{1b} catalyzed by GD_3-GalNAcT, GD_2-GalT, GD_{1b}-SAT, and GT_{1b}-SAT, respectively) pathways of ganglioside biosynthesis are differentially localized in rat liver Golgi apparatus by the order in which they act and that the glycosyltransferases of monosialo and disialo pathways co-distribute (48). Further, there exists a precursor-product relationship involved in ganglioside metabolism (49) and once formed within the Golgi, a ganglioside is in part made available for transport to the plasma membrane (or other internal membrane), a rapid turnover event, and in part serves as a precursor to subsequent chain elongation forming more complex ganglioside species (47).

The mechanisms through which gangliosides are transported to target membranes within the cell remain to be elucidated. An early proposal for transport of

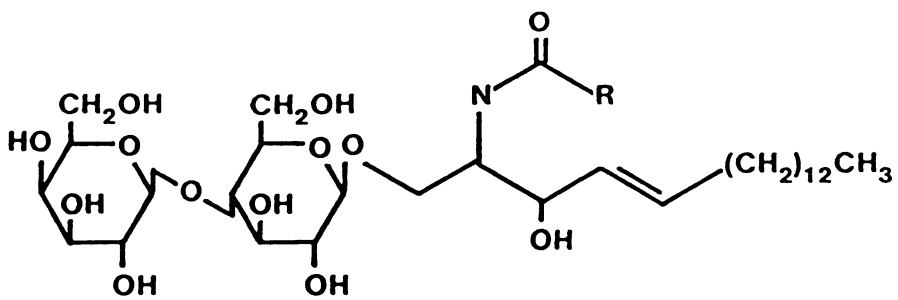
[4]Ganglioside nomenclature is that of Svennerholm (144) as recommended by IUPAC-IBU (145).

Table 1

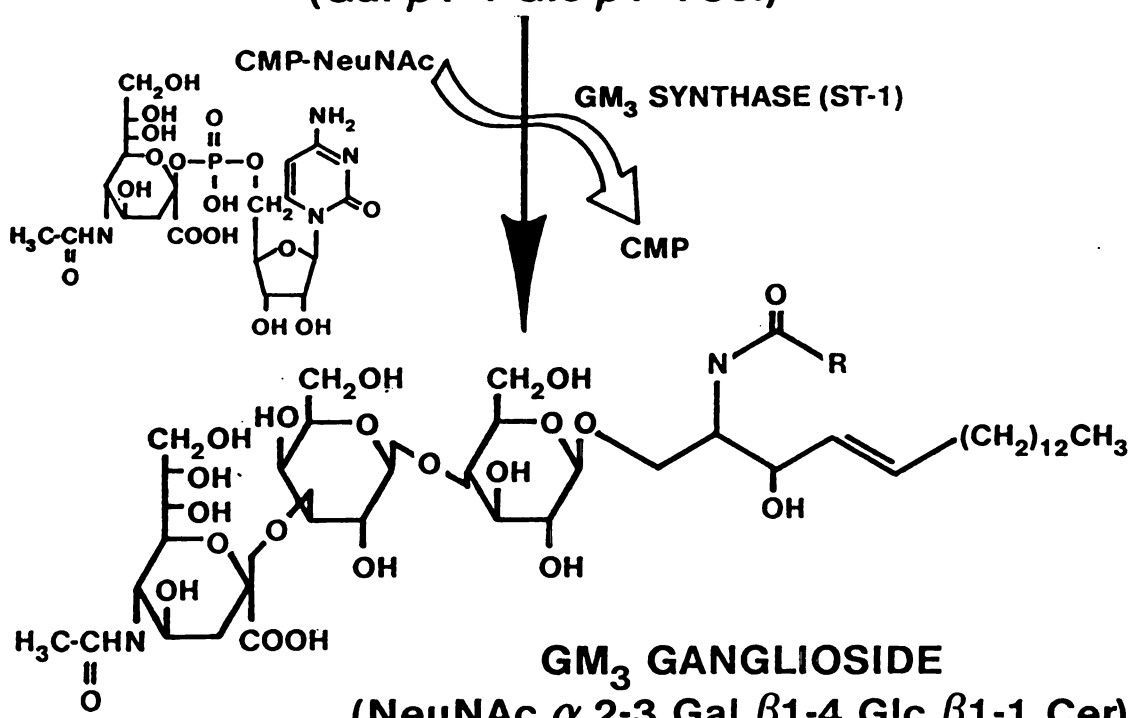
Summary of Ganglioside Nomenclature

Ganglioside	Structure
precursors	
GlcCer	Glcß1-1Cer
LacCer	Galß1-4Glcß1-1Cer
GM_3	NeuAcα2-3Galß1-4Glcß1-1Cer
a-series	
GM_2	GalNAcß1-4(NeuAcα2-3)Galß1-4GlcCer
GM_1	Galß1-3GalNAcß1-4(NeuAcα2-3)Galß1-4GlcCer
GD_{1a}	(NeuAcα2-3)Galß1-3GalNAcß1-4(NeuAcα2-3)Galß1-4GlcCer
GT_{1a}	(NeuAcα2-8NeuAcα2-3)Galß1-3GalNAcß1-4(NeuAcα2-3)Galß1-4GlcCer
b-series	
GD_3	NeuAcα2-8NeuAcα2-3Galß1-4GlcCer
GD_2	GalNAcß1-4(NeuAcα2-8NeuAcα2-3)Galß1-4GlcCer
GD_{1b}	Galß1-4GalNAcß1-4(NeuAcα2-8NeuAcα2-3)Galß1-4GlcCer
GT_{1b}	(NeuAcα2-3)Galß1-4GalNAcß1-4(NeuAcα2-8NeuAcα2-3)Galß1-4GlcCer
GQ_{1b}	(NeuAcα2-8NeuAcα2-3)Galß1-4GalNAcß1-4(NeuAcα2-8NeuAcα2-3)LacCer
c-series	
GT_3	NeuAcα2-8NeuAcα2-8NeuAcα2-3Galß1-4GlcCer
GT_2	GalNAcß1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galß1-4GlcCer
GT_{1c}	Galß1-3GalNAcß1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galß1-4GlcCer
GQ_{1c}	NeuAcα2-3Galß1-3GalNAcß1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)LacCer

Figure 3. Synthesis of GM_3 Ganglioside by CMP-Sialic Acid:Lactosylceramide α2-3 Sialyltransferase



LACTOSYLCERAMIDE
(Gal β1-4 Glc β1-1Cer)



GM_3 GANGLIOSIDE
(NeuNAc α 2-3 Gal β1-4 Glc β1-1 Cer)

R: $-(CH_2)_n$ or $-CH(OH)-(CH_2)_n$

n = 15 - 25

gangliosides to the cell surface was via membrane flow (50,51). However, subsequent studies with monensin, an inhibitor of membrane flow (52), had no effect on either neuroblastoma or glioma cells (53). Therefore, this early model has given way to other eloquent hypotheses. One mechanism suggests regulation of transport through the modulation of temperature (53) or via lipid transfer proteins (54-57). Possibly a mechanism(s) exists, as has been described for glycoprotein movements between the endoplasmic reticulum and Golgi (for review, 58) and within the Golgi cisternae, namely, vesicular transport (e.g., Golgi-coated vesicles) (59). One new reversible drug treatment which may prove useful in addressing glycolipid biosynthesis and transport mechanisms is brefeldin A (BFA), an antifungal metabolite, which blocks intracellular transport and processing of secretory proteins through disassembly of the Golgi apparatus (60).

Another interesting observation involving glycolipid biosynthesis in rat liver Golgi has been the isolation of two asialogangliosides (LacCer->Gg_3->Gg_4->GM_{1b}->GD_{1c}) resulting from the presence of the galactosyl- and N-acetylgalactosylaminyltransferase activities which are typically quiescent in rat liver Golgi (61). Assays *in vitro* of these enzymes gave specific activities and Km values comparable to those observed for the a and b series gangliosides. The presence of these glycosyltransferase activities and the absence of their glycosylation products in normal rat liver leads to interesting speculation as to the mode of regulation of these and other glycosyltransferases forming the glycosphingolipids.

Regulation of Ganglioside Biosynthesis

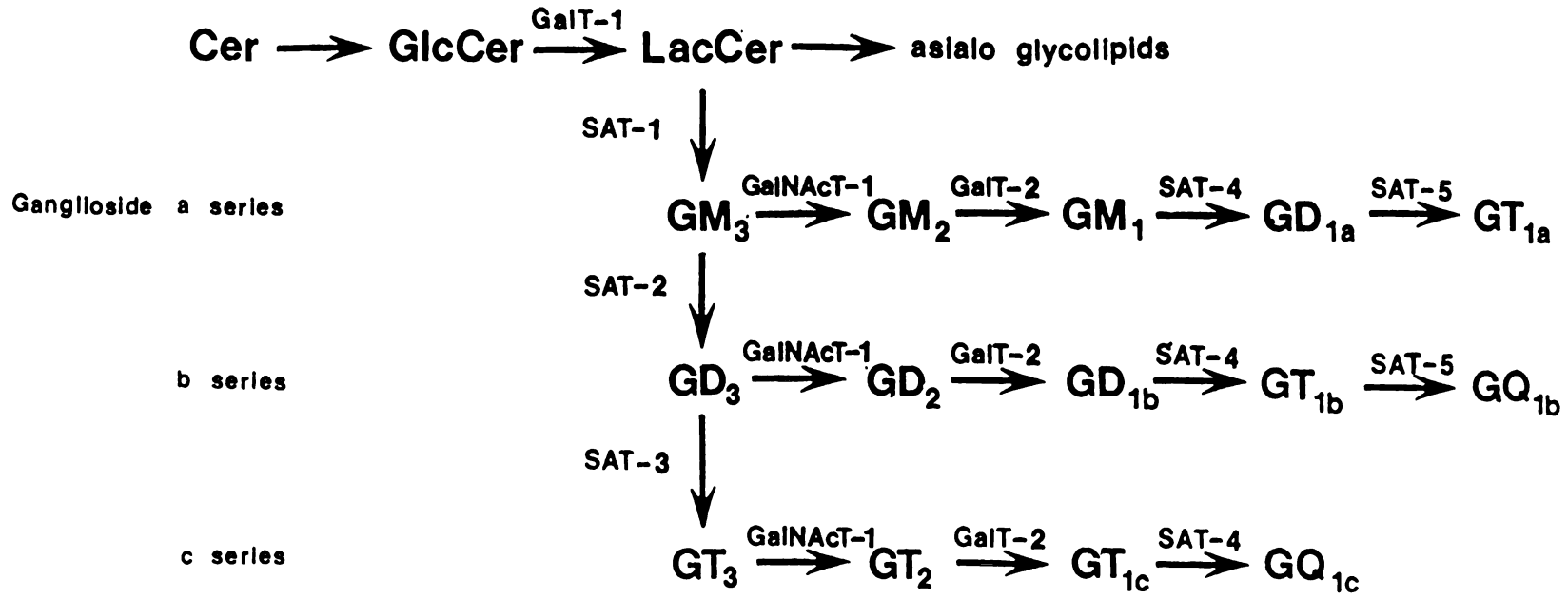
The regulation of all glycosphingolipid biosynthesis may reside within the expression of five glycosyltransferases, specifically, LacCer synthase (62-64), GM_3, GD_3 and GT_3 synthases (64) and GM_2 synthase (63,64). The regulatory model as

proposed by Sandhoff and coworkers (62) for ganglioside biosynthesis is illustrated in Figure 4. As set forth in their model, regulation of the entire ganglioside biosynthetic pathway is dependent upon GalT galactosyltransferase and by SAT-1, SAT-2 and SAT-3 sialyltransferases specificity for the unique carbohydrate-lipid bond, Glcß1-1Cer. Subsequent glycosylations adding GalNAc, Gal and NeuNAc are parallel reactions catalyzed by glycosyltransferases (Table 2) recognizing the terminal carbohydrate structural "backbone." This regulation is likely to be at the transcriptional or post-translational levels, but other factors such as feedback inhibition (65), membrane fluidity (66), availability of sugar nucleotide as well as its translocation into the lumen of the Golgi (67,68), protein matrix effects (69), influx of divalent cations (70) and temperature (71) may also play an important function. However, the molecular details of ganglioside biosynthesis and functions are still speculative due to the lack of information on the mechanisms governing the regulation of the glycosyltransferases involved. This will evolve as these enzymes are purified and their cDNA sequences are determined.

Topography of Glycosyltransferases

Glycosyltransferases are type-2 membrane (single α-helix) enzymes with a lumenal orientation within the Golgi (Figure 5) (for reviews, 68,72). Evidence supporting this type of topography include: complex carbohydrate substrates are known to have a lumenal orientation; Golgi vesicles must be disrupted or permeabilized with detergent for activity; glycosyltransferases are glycoproteins; glycosyltransferases are secretory proteins, being derived from membrane-bound forms through proteolysis. The lumenal orientation of glycosyltransferases requires a mechanism for the transport of sugar nucleotides into the Golgi (for review, 68). The proposed mechanism involves entry of each sugar nucleotide through a specific antiporter (Figure 5). The sugar is

Figure 4. Biosynthetic Pathway of Glycosphingolipids.

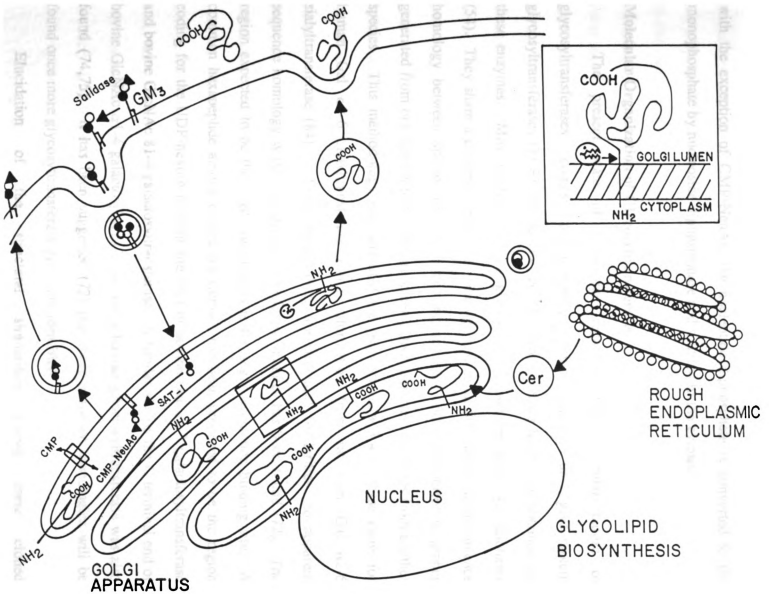


Ganglioside Biosynthetic Pathway

Table 2. Glycolipid Glycosyltransferase Nomenclature

Glycosyltransferase	Designation	Ganglioside Formed
CMP-NeuAc:LacCer α2-3 sialyltransferase	SAT-1	GM_3
UDP-GalNAc:GM_3 ß1-4N-acetylgalactosaminyl transferase	GalNAcT-1	GM_2
UDP-Gal:GM_2 ß1-3 galactosyltransferase	GalT-3	GM_1
CMP-NeuAc:GM_1 α2-3 sialyltransferase	SAT-4	GD_{1a}
CMP-NeuAc:GD_{1a} α2-8 sialyltransferase	SAT-5	G_{T1a}
CMP-NeuAc:GM_3 α2-8 sialyltransferase	SAT-2	GD_3
UDP-GalNAc:GD_3 ß1-4 N-acetylgalactosaminyl transferase	GD_2-GalNAcT-1	GD_2
UDP-Gal:GD_2 ß1-3 galactosyltransferase	GD_2-GalT	GD_{1b}
CMP-NeuAc:GD_{1b} α2-3 sialyltransferase	GD_{1b}-SAT-4	GT1b
CMP-NeuAc:GT_{1b} α2-8 sialyltransferase	GT_{1b}-SAT-5	GQ1b
CMP-NeuAc:GD_3 α2-8 sialyltransferase	GD_3-SAT-3	GT_3
UDP-GalNAc:GT_3 ß1-4 N-acetylgalactosaminyl transferase	GT_3-GalNAcT-1	GT_2
UDP-Gal:GT_2 ß1-3 galactosyltransferase	GT_2-GalT	GT_{1c}
CMP-NeuAc:GT_{1c}α2-3 sialyltransferase	GT_{1c}-SAT-4	GQ_{1c}

Figure 5. Topography and General Structure of Glycosyltransferases within the Golgi Apparatus.


COOH
COOH
Sialidase
GM3
COOH
COOH
GOLGI LUMEN
CYTOPLASM
NH2
NH2
SAT-I
CMP
CMP-NeuAc
NH2
COOH
NH2
NH2
COOH
COOH
NH2
NH2
COOH
NH2
Cer
ROUGH
ENDOPLASMIC
RETICULUM
NUCLEUS
GLYCOLIPID
BIOSYNTHESIS
GOLGI
APPARATUS

transferred to lumenal glycoconjugate acceptors via specific glycosyltransferases and, with the exception of CMP-NeuAc, the nucleotide diphosphate is converted to the monophosphate by nucleoside diphosphatase and shuttled to the cytosol.

Molecular Organization of Glycosyltransferases

The present state of knowledge regarding the molecular nature of glycosyltransferases comes predominantly from studies on glycoprotein glycosyltransferases (73-83; for review see 72). The cDNA's have been obtained for these enzymes. Most mRNA encoding glycosyltransferases are about 4.5 kilobases (54). They share a common structural homology and about 80% amino acid sequence homology between species (72,84) permitting antibody and oligonucleotide probes generated from one species to be used to screen cDNA libraries for clones from another species. This methodology has permitted the recovery of a 3.4 kilobase clone for human Gal α2-6 sialyltransferase using information from rat liver Gal α2-6 sialyltransferase (84). Even though there are structural similarities, no apparent sequence homology within the glycosyltransferase families has been found (72). The region expected to be the most homologous is the sugar nucleotide binding site. A common hexapeptide among cloned transferases has been proposed to be the region coding for the UDP-hexose binding site (82) of bovine Gal α1-3 galactosyltransferase and bovine GlcNAc ß1-4 galactosyltransferase. A similar site on the C-terminal end of bovine GlcNAc α1-4 galactosyltransferase and a human galactosyltransferase was also found (74,75). It has been suggested (72) that more sequence homologies will be found once more glycosyltransferase genes are identified.

Elucidation of the structural similarities among these cloned glycosyltransferases, as determined from deduced amino acid sequences (for review, 72), indicate that they all possess a short 6-41 amino acid cytosolic NH_2 terminus, a 16-20 amino acid transmembrane domain and a lumenal domain containing a stem

region and the catalytic site (Figure 5). It is the stem region which is the least homologous for the cloned glycosyltransferases (74-83; for review, 72), and which is the proposed site of proteolytic degradation of these enzymes (72,73,85). A cathepsin D-like activity has been associated with this endogenous proteolytic processing, releasing a soluble catalytically-active enzyme from the mature membrane-associated form (86). The mechanism and the regulation of this proteolytic activity are yet to be resolved. Further, the stem region seems to play a role in the targeting of the enzyme within the Golgi as has been identified for Gal α2-6 sialyltransferase by the use of altered sequences and fluorescent antibody detection (85). Other glycosyltransferases have also been localized to the Golgi network (85,87-90). Further, these Golgi membrane-associated glycoprotein transferases are glycosylated (76).

Purification of Glycosphingolipid Glycosyltransferases

Little information regarding glycosphingolipid (GSL) glycosyltransferases is known because most current studies in the GSL field are still focussing on the purification of these enzymes from the Golgi. Presumably, it is the nature of the lipid substrate, the membrane environment surrounding the enzymes, instability of the purified GSL glycosyltransferase, and endogenous proteolytic processing which have lead to difficulties in advancing the state of knowledge of glycosyltransferases in the GSL field. Thus, there exists a need to purify the various GSL glycosyltransferases and isolate the genes encoding these enzymes to determine their role and that of their products (glycolipids) in the regulation of cell growth and differentiation, thereby defining the role of gangliosides in the transformation process.

Some progress in this regard has been made. Employing standard protein purification strategies, a few GSL glycosyltransferases have been purified (82,91-97) and of these only one has been successfully cloned and sequenced (94).

Studies on the regulation of purified and cloned Gal:Lc_3 ß1-4 galactosyltransferase (GalT-4) and Fuc:LM1 α1-3 fucosyltransferase (FucT-3) from human colon carcinoma Colo 205 cells and P-1698 mouse lymphoma cells (94) indicate a role for sphingosine in the regulation of GSL glycosyltransferases. Sphingosine inhibits GalT-4 enzyme activity at low concentration while at high concentration FucT-3 is inhibited. Apparently, sphingosine interacts with a specific octameric amino acid sequence within the catalytic domain of the glycosyltransferase.

Recently, through the efforts of Lowe and coworkers (98-103) the gene sequences of three more glycosyltransferases are known. These are α1-3GalT, α1-2FucT, and α1-3/1-4 FucT, the galactosyl- and fucosyltransferases responsible for the synthesis of the blood group B, H and Le antigens, respectively. By employing an expression cloning by gene transfer approach, they have cloned, sequenced and expressed genomic and cDNA sequences of the blood group H antigen (100-102) and the Le determination (103) into mouse LM fibroblasts to define the gene structure and study the complex level of regulation required for the human blood group H α1-2 fucosyltransferase gene and Le antigen fucosyltransferase gene. Multiple functional transcripts have been generated with potential to encode either the transmembrane or catalytic domains. These colinear transcripts differ at the 5' ends suggesting a multiple polypeptides can be generated from a single fucosyltransferase gene (100-103). Presumably other glycosyltransferase genes, perhaps existing as a gene family, are regulated by switching on and off their expression.

Regulation of Cell Proliferation by Glycosphingolipids.

The cell surface has been postulated to play an important role in the regulation of cell growth and differentiation (4,30,104,105). Gangliosides, ubiquitous sialic acid-containing glycosphingolipids of mammalian plasma membranes (106), have been implicated in these phenomena (4). Changes in ganglioside composition and

metabolism have been associated with oncogenic transformation (1); during cellular transformation, there is often an accumulation of precursor (less complex) gangliosides, compared with nontransformed cells, due to incomplete synthesis that results from a block or impairment of the ganglioside biosynthetic pathway. Alternatively, there may be synthesis of novel gangliosides (enhanced synthesis of the neoglycolipids i.e., the Le antigens) due to activation of specific quiescent embryonic or fetal glycosyltransferases (2,4,107).

Several lines of evidence suggest a specific role for glycosphingolipid composition and metabolism in oncogenic transformation. Exogenous addition of gangliosides can either stimulate or inhibit cell growth depending on the cell type. Specifically, GQ_{1b}, a polysialylganglioside, promotes neurite outgrowth (for review, 109) by stimulating cell surface protein phosphorylation via an ecto-protein kinase activity (110). Addition of GD1a, a disialylganglioside, activates Ca^{2+}/calmodulin-dependent kinase activity (111,112) have shown that exogenously added GM_1 monosialylganglioside inhibits cAMP-dependent protein kinase (cAK) of histone IIA and autophosphorylation of cAK, but stimulates cyclic nucleotide phosphodiesterase (PDE). Other studies have shown that a decrease of GM_3 or GM_1 ganglioside in several lines of fibroblasts has been associated with oncogenic transformation of these cells. Addition of exogenous GM_3 or GM_1 restores normal contact-inhibited cell growth (3) by altering the binding affinity of the cells to growth factors such as PDGF, EGF and FGF (24,25). GM_3 functions to inhibit cell proliferation by inhibiting the autophosphorylation of EGF-R tyrosine kinase (24,25). Further, chemical compounds such as retinoic acid (113-115), tumor-promoting phorbol esters (113,116-120) and sodium butyrate (121-126) have been shown to modulate specific glycosyltransferases and cell growth and differentiation. Specifically, retinoic acid, phorbol-12-myristate-13-acetate (PMA) and sodium butyrate stimulate CMP-sialic acid:lactosylceramide sialyltransferase (SAT-1) activity 5- to 10-fold (113,120,124-126).

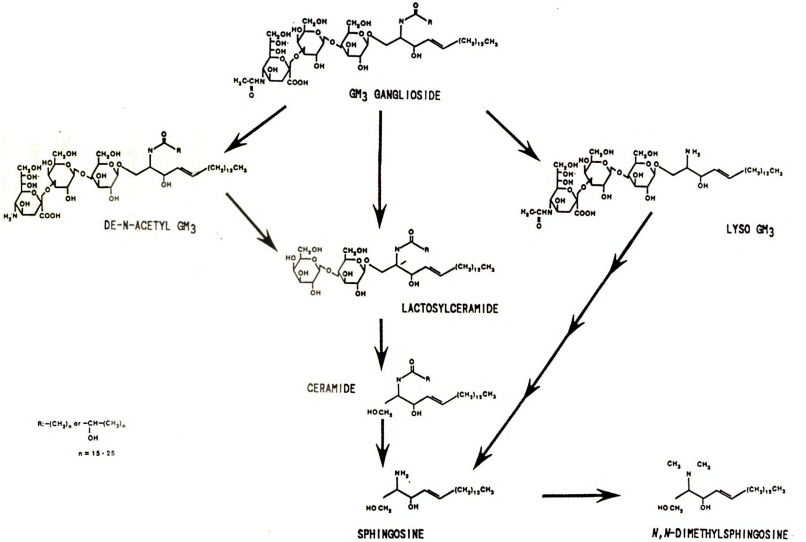
GM_3 in the Regulation of Cell Growth

An "allosteric regulation" model for the interaction of GM_3 with the EGF-receptor has been proposed by Schlessinger in 1983 (80) and supported by Hakomori in 1984 (3), in which the growth factor receptor has multiple loci for growth factor binding, protein kinase activity and gangliosides or other components serve to mediate the effect. Specifically, GM_3 ganglioside inhibits EGF-stimulated phosphorylation of the EGF receptor without affecting the binding. EGF binding to its receptor is a necessary pre-replicative factor controlling commitment of cells for DNA replication. Thus, enrichment of specific gangliosides, like GM_3, in the plasma membrane indirectly or directly affects the receptor functions and possibly receptor-receptor interaction.

GM_3 ganglioside has been demonstrated to interact directly with the EGF-R. Immunoprecipitation of the EGF-R with a specific monoclonal antibody co-precipitated GM_3 along with the receptor (26,27). GM_3 was demonstrated to inhibit the autophosphorylation of EGF/EGF-R complex by interfering with the EGF-dependent dimerization of the receptor (27). Further, these researchers could enhance the effect on GM_3 inhibition of EGF-dependent tyrosine kinase activity in the presence of lyso-PC (27).

The degradation of cell-surface GM_3 ganglioside to LacCer by endogenous sialidase activity has been demonstrated to stimulate cell growth (128,129), presumably through the release of the inhibition of the EGF-dependent dimerization of the EGF-R; autophosphorylation continues and cell growth is stimulated (130). The fate of the LacCer may be further degraded by glycosidases or recycling of LacCer back to the endoplasmic reticulum and Golgi for resynthesis of GM_3 (129-131). The effects of

Figure 6. Molecular Species Formed Metabolically from GM_3 Ganglioside Involved in Cell Growth Regulation Via Modulation of Signal Transduction Mechanism.


GM3 GANGLIOSIDE
DE-N-ACETYL GM3
LYSO GM3
LACTOSYLCERAMIDE
CERAMIDE
SPHINGOSINE
N,N-DIMETHYLSPHINGOSINE
R:-(CH2)n or -CH-(CH2)n
OH
n = 15 - 25

other naturally occurring GM3 catabolites (see Figure 6 for their structures), lyso GM3 (26,27) and de-N-acetyl-GM3 (28), have been investigated. In A431 cells, lyso-GM_3 was found to inhibit cell growth by dramatically inhibiting PK-C and EGF-R signal transduction (26,27). The effect of exogenous addition of de-N-acetyl-GM_3 was opposite to the inhibition of cell growth by GM_3 and lyso-GM_3. De-N-acetyl-GM_3 stimulated EGF-dependent EGF-R tyrosine kinase activity (28).

Complete enzymatic degradation of GM_3 by glycosidases and ceramidase yields sphingosine. The effect of sphingosine on protein kinase C (PK-C) and EGF tyrosine kinase is being investigated, because of a suggested role for sphingosine as a modulator of transmembrane signaling (132-135; for review, 136). Observations including those by Igarashi and Hakomori (137), who have recently shown N,N-dimethyl enzymatic derivatization of erythro-sphingosine to give a strong inhibitor of PK-C, whereas the D-erythro unsubstituted form is not. They have indicated that this effect is different from that observed for the EGF tyrosine kinase. In a related study, the inhibition of PK-C by sphingosine was found to be pH dependent; inhibition of PK-C required a positive charge (138).

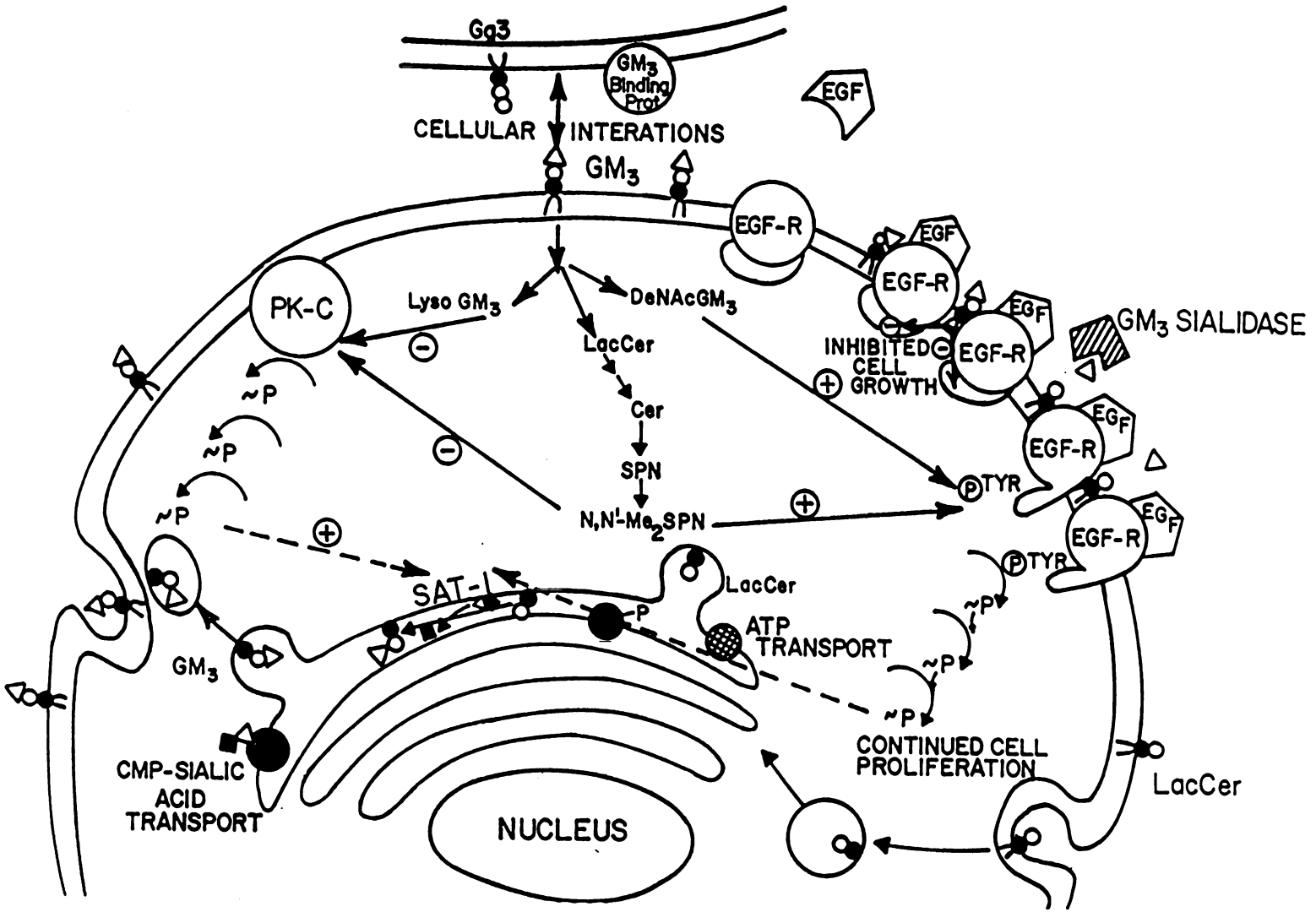
Regulation of GM_3 Synthesis

In consideration of the down-regulation by exogenous GM_3 on the autophosphorylation of tyrosine on EGF-R, a similar role for endogenous GM_3 in regulation of cell growth has been proposed (131). GM_3 is a naturally occurring constituent of most mammalian cell membranes. The amount of cell surface GM_3 and the level of CMP sialic acid:lactosylceramide sialyltransferase activity is cell-cycle dependent and is maximal at late M/early G_1 of the cell cycle (124,126). One proposed mechanism for increased sialyltransferase activity may involve phosphorylation/dephosphorylation (139). PMA, which can bind to and stimulate PK-C activity (140,141), stimulates SAT-1 activity in cultured cells (113, 120,140).

Further, Burczak et al. (139) have shown that SAT-1 is activated following treatment with a cAMP-dependent protein kinase (cAK). In studies on the regulation of GM_3-GalNAcT-1 (GM_2 synthase) by Dawson *et al.* (142), showed the activity of this N-acetylgalactosaminyltransferase was stimulated by cAK-activators. Further, phosphoproteins and an ATP transport mechanism have been identified within the Golgi (143). Accordingly, the regulatory mechanism for the synthesis (see chapter 6) and degradation (128,129,131) of GM_3 becomes paramount to the role of GM_3 ganglioside in the modulation of cell growth and oncogenic transformation. As such, the purification and investigation of the mechanism of regulation of SAT-1 activity was the focus of this dissertation research project. Based on the aforementioned data, recognizing GM_3 as a key modulator of cell growth, a working hypothetical model for the regulation of SAT-1 is presented (Figure 7).

Figure 7. Hypothetical Model of SAT-1 Regulation. This schematic diagram summaries reported data on the regulation of cell growth by GM_3 ganglioside and its catabolites and the potential of phosphorylation as a regulatory mechanisms for this key enzyme of ganglioside biosynthesis.

HYPOTHETICAL MODEL OF SAT-I REGULATION



REFERENCES

1. Hakomori, S. (1981) Ann Rev. Biochem. **50**, 733.
2. Hakomori, S. and Kannagi, R (1983) J. Natl. Cancer Inst. **71**, 231-251.
3. Hakomori, S. (1984) Annu. Rev. Immunol. **2**, 103-126.
4. Hakomori, S. (1985) Cancer Res. **45**, 24-5-2414
5. Ledeen, R.W. (1989) in Neurobiology of Glycoconjugates (Margolis, R.U. and Margolis, R.K., eds.) Plenum Press, NY, p.p.43-83.
6. Kirivan, H.C., Ginsburg, V. and Roberts, D.D. (1988) Arch. Biochem. Biophys. **260**, 493-496.
7. Karlsson, K.-A. (1989), Annu. Rev. Biochem. **58**, 309-350.
8. Deal, C.D. and Krivan, H.C. (1990) J. Biol. Chem. **265**, 12774-12777.
9. Haywood, A.M. (1974) J. Mol. Biol. **83**, 427-436.
10. Markwell, M.K., Svennerholm, L. and Paulson, J.C. (1981) Proc. Natl. Acad. Sci., U.S.A. **78**, 5406-5410.
11. Clowes, A.W., Cherry, R.J. and Chapman, D. (1972) J. Mol. Biol. **67**, 49-57.
12. Critchley, D.R. and Vicker, M.G. (1977) Cell Surface Rev. **3**, 307-370.
13. Van Heyningen, W.E., Carpenter, C.C.J., Pierce, N.F. and Greenough, W.B. (1971) J. Infect. Dis. **124**, 415-418.
14. Fishman, P.H. (1982) J. Membrane Biol. **69**, 85-97.
15. Cuatrecasas, P. (1974) Annu. Rev. Biochem. **43**, 169-214.
16. Besamcon, F. and Ankel, H. (1974) Nature **252**, 478-480.
17. Kleinman, H.K., Martin, G.R. and Fishman, P.H. (1979) Proc. Natl. Acad. Sci., U.S.A. **76**, 3367-3371.
18. Perkins , R.M., Kellie, S., Patel, B. and Critchley, D.R. (1982) Exp. Cell Res. **141**, 231-243.

19. Matyas, G.R., Evers, D.E., Radinsky, R. and Morre, D.J. (1986) Exp. Cell Res. **162**, 296-318.

19. Clausen, H., Levery, S.B., Nudelman, E., Baldwin, M. and Hakomori, S. (1986) Biochem. **25**, 7075-7085.

20. Tiemeyer, M., Swank-Hill, P., and Schnaar, R.L. (1990) J. Biol. Chem. **265**, 11990-11999.

21. Spiegel, S., Handler, J.S. and Fishman, P.H. (1986) J. Biol. Chem. **261**, 15755-15760.

22. Cheresh, D.A., Pierschbacher, M.A., Herzig, M.A. and Mujoo, K. (1986) J. Cell Biol. **102**, 689-696.

24. Bremmer, E. G., Hakomori, S., Bowen-Pope, D.F., Raines, E. and Ross, R. (1984) J. Biol. Chem. **259**, 6818-6825.

25. Bremmer, E.G., Schlessinger, J. and Hakomori, S. (1986) J. Biol. Chem. **261**, 2334-2340.

26. Hanai, N. Nores, G., Torres-Mendez, C.R., and Hakomori, S. (1987) Biochem. Biophys. Res. Commun. **127**, 134.

27. Hanai, N., Nores, G.A., MacLeod, C., Torres-Mendez, C.-R. and Hakomori, S. (1988) J. Biol. Chem. **263**, 10915-10921.

28. Hanai, N., Dohi, T., Nores, G.A. and Hakomori, S. (1988) J. Biol. Chem. **263**, 6296-6301.

29. Igarashi, Y., Kitanura, K., Toyokuni, T., Dean, B., Fenderson, B.A., Ogawa, T., and Hakomori, S. (1990) J. Biol. Chem. **265**, 5385-5389.

30. Hakomori, S. (1990) J. Biol. Chem. **265**, 18713-18716.

31. Weis, F.M.B. and Davis, R.J. (1990) J. Biol. Chem. **265**, 12059-12066

32. Ladisch, S. (1989) in Gangliosides and Cancer (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 217-238.

33. Portoukalian, J. (1989) in Gangliosides and Cancer (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 207-216.

34. Marcus, M.D. (1984) Molec. Immunol. XXX, 1083-1091.

35. Stults, C.L.M., Sweeley, C.C., and Macher, B.A. (1989) Methods Enzymol. **179**, 167-214.

36. Sweeley, C.C. (1985) in *Biochemistry of Lipids and Membranes,* (Vance, D.E. and Vance, J.E., eds.) The Benjamin/Cummings Publishing Co., Inc., p.p. 361-403.

37. Reutter, W., Kottgen, E., Bauer, C. and Gerok, W. (1982) "Biological Significance of Sialic Acids" in Sialic Acids: Chemistry, Metabolism and Function (Schauer, R., ed.) Springer-Verlag Wien, New York.

38. Kornfeld, R. and Kornfeld, S. (1984) Annu. Rev. Biochem. **54**, 631-664.

39. Hassell, J. R., Kimura, J. H., and Hascall, V.C. (1986) Annu. Rev. Biochem. **55**, 539-567.

40. Keenan, T.W., Huang, C.M. and Morre, D.J. (1972) Biochem. Biophys. Res. Commun. **47**, 1277-1283.

41. Keenan, T.W., Morre, D.J. and Huang, C.M. (1972) FEBS Lett. **24**, 204-208.

42. Miller-Prodraza, H., Bradley, R.M. and Fishman, P.H. (1982) Biochemistry **21**, 3260-3265.

43. Matyas, G.R. and Morre, D.J. (1987) Biochim. Biophys. Acta **921**, 599-614.

44. Roseman, S. (1970) Chem. Phys. Lipids **5**, 270-297.

45. Keenan, T.W., Morre, D.J. and Basu, S. (1974) J. Biol. Chem. **249**, 310-315.

46. Kornfeld, R. and Kornfeld, S. (1985) Ann. Rev. Biochem. **54**, 631-664.

47. Trinchera, M. and Ghidoni, R. (1989) J. Biol. Chem. **264**, 15766-15769.

48. Trinchera, M., Pirovano, B. and Ghidoni, R. (1990) J. Biol. Chem. **265**, 18242-18247.

49. Trinchera, M., Vallenzasca, C., Ghidoni, R. and Tettamanti, G. (1990) FEBS Letters **260**, 23-26.

50. Fishman, P.H. and Brady, R.O. (1976) Science **194**, 906-915.

51. Morre, D.J., Kartenbeck, J. and Franke, W.W. (1979) Biochim. Biophys. Acta **559**, 71-152.

52. Morre, D.J., Boss, W.F., Grimes, J. and Mollenhauer, H.H. (1983) Eur. J. Cell Biol. **30**, 25-32.

53. Miller-Podraza, H. and Fishman, P.H. (1984) Biochim. Biophys. Acta **804**, 44-51.

54. Yamada, K. and Saski, T. (1982) J. Biochem. **92**, 457-464.

55. Abe, S., Yamada, K. Sakagami, T. and Sasaki, T. (1984) Biochim. Biophys. Acta **778**, 239-244.

56. Wong, M., Brown, R.E., Barenholz, Y. and Thompson, T.E. (1984) Biochemistry **23**, 6498-6505.

57. Kadowaki, H., Symanski, L.A. and Koff, R.S. (1988) J. Lipid Res. **29**, 52-62.

58. Balch, W.E. (1989) J. Biol. Chem. **264**, 16965-16968.

59. Rothman, J.E. and Orci, L (1990) FASEB J. **4**, 1460-1468.

60. Shite, S., Seguchi, T., Mizoguchi, H., Ono, M., and Kuwano, M. (1990) J. Biol. Chem. **265**, 17385-17388.

61. Pohlentz, G., Klein, D., Schmitz, D., Schwarzmann, G., Peter-Katalinic, J. and Sandhoff, K. (1988) Biol. Chem. Hoppe-Seyler, **369**, 55-63.

62. Pohlentz, G., Klein, D., Schwarzmann, G., Schmitz, D. and Sandhoff, K. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7044-7048.

63. Senn, H.-J., Manke, C., Dieter, P., Tran-Thi, T.-A., Fitzke, E., Gerok, W. and Decker, K. (1989) Arch. Biochem. Biophys., in press.

64. Schuz-Henninger, R., Ullmer, E., Prinz, C. and Decker, K. (1989) Arch. Biochem. Biophys., in press.

65. Yusuf, et al. (1987) Biol. Chem. Hoppe-seyler **368**, 455-462.

66. Sandhoff, K. (1980) Naturwissenschaften **67**, 431-444.

67. Schuz-Henninger, R., Prinz, C. and Decker, K. (1988) Arch. Biochem. Biophys. **262**, 49-58.

68. Hirschberg, C.B. and Snidem, D. (1987) Ann. Rev. Biochem. **56**, 63-87.

69. Shao, M.-C., Krudy, G., Rosevear, P.R. and Wold, F. (1989) Biochem. **28**, 4077-4083.

70. Speigel, S. and Panagiotopoulos, C. (1988) Exp. Cell Res. **177**, 414-427.

71. Mitranic, M., Sturgess, J.M. and Moscarello, M.A. (1976) Biochim. Biophys. Acta **443**, 190-197.

72 Paulson, J.C. and Colley, (1989) J. Biol. Chem. **264**, 17615-17618.

73. Weinstein, J., Lee, E.U., MeEntee, K., Lai, P.-H. and Paulson, J.C. (1987) J. Biol. Chem. **262**, 17735-17743.

74. Narimatsu, H., Sinha, S., Brew, K., Okayama, H. and Qasba, P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4720-4724.

75. Appert, H.E., Rutherford, T.J., Tarr, G.E., Wiest, J.S., Thomford, N.R. and McCroquodale, D.J. (1986) Biochem. Biophys. Res. Commun. **139**, 163-168.

76. Smith, C.A. and Brew, K. (1977) J. Biol. Chem. **252**, 7294-7299.

77. Shaper, N.L., Hollis, G.F., Douglas, J.G., Kirsch, I.R. and Shaper, J.H. (1988) J. Biol. Chem. 263, 1042-10428.

78. Nakazawa, K., Ando, T., Kimurs, T. and Narimatsu, H. (1988) J. Biochem. (Tokyo) **104**, 165-168.

79. Masri, K.A., Appert, H.E. and Fukuda, M.M. (1988) Biochem. Biophys. Res. Commun. **157**, 657-663.

80. D'Agostaro, G., Bendiak, B. and Tropak, M. (1989) Eur. J. Biochem. **183**, 211-217.

81. Shaper, N.L., Shaper, J.H., Meuth, J.L., Fox, J.L., Chang, H., Kirsch, I.R. and Hollis, G.F. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1573-1577.

82. Joziasse, D.H., Shaper, J.H., van den Eijnden, D.H., van Tunen, A.J. and Shaper, N.L. (1989) J. Biol. Chem. **264**, 14290-14297.

83. Larsen, R.D., Rajan, V.P., Ruff, M.M., Kukowska-Latello, J., Cummings, R.D. and Lowe, J.B. (1989) Proc. Natl. Acad. Sci. U.S.A., in press.

84. Lance, P., Lau, K.M. and Lau, J.T.Y. (1989) Biochem. Biophys. Res. Commun. **164**, 225-232.

85. Colley, K.J., Lee, E.U., Adler, B., Browne, J.K. and Paulson, J.C. (1989) J. Biol. Chem. **264**, 17619-17622.

86. Lammers, G. and Jamieson, J.C. (1988) Biochem. J. **256**, 623- 631.

87. Roth, J. (1984) J. Cell Biol. **98**, 399-406.

88. Roth, J. (1987) Biochim. Biophys. Acta **906**, 405-436.

89. Lee, E.U., Roth, J. and Paulson, J.C. (1989) J. Biol. Chem. **264**, 13848-13855.

90. Taatjes, D.J., Roth, J., Weinstein, J. and Paulson, J.C. (1988) J. Biol. Chem. **263**, 6302-6309.

91. Taniguchi, N., Yokosawa, N., Gasa, S. and Makita, A. (1982) J. Biol. Chem. **257**, 10631-10637.

92. Taniguchi, N. and Makita, A. (1985) J. Biol. Chem. **259**, 5637-5642.

93. Taniguchi, N., Yangisawa, K., Makita, A. and Naiki, M. (1985) J. Biol. Chem. **260**, 4908-4913.

94. Basu, M., Weng, S.-A., Das. K.K. and Zang, B.-H. (1989) Joint Meeting of Society for Complex Carbohydrates and Midwest Connective Tissue Workshop at University of Michigan, Ann Arbor, MI, November 8-11, 1989.

95. Gu, X.-B., Gu, T.-J. and Yu, R.K. (1990) Biochem. Biophys. Res. Commun. **166**, 387-393.

96. Clausen H., White, T., Taiko, K., Titani, K. Stroud, M. Holmes, E., Karkov, J. Thim, L. and Hakomori, S. (1990) J. Biol. Chem. **265**, 1139-1145.

97. Melkerson-Watson, L.J. and Sweeley, C.C. (1991) J. Biol. Chem., in press (See Chapter 3).

98. Larsen, R.D., Rajan, V.P., Ruff, M.M., Kukowska-Latallo, J., Cummings, R.D. and Lowe, J.B. (1989) Proc. Natl. Acad. Sci, U.S.A. **86**, 8227-8231.

99. Larsen, R.D., Rivera-Marrero, C.A., Ernst, L.K., Cummings, R.D. and Lowe, J.B. (1990) J. Biol Chem. **265**, 7055-7061.

100. Rajan, V.P., Larsen, R.D., Ajmera, S., Ernst, L.K. and Lowe, J.B. (19989) J. Biol Chem. **264**, 11158-11167.

101. Ernst, L.K., Rajan, V.P., Larsen R.D., Ruff, M.M. and Lowe, J.B. (1989) J. Biol. Chem. **264**, 3436-3447.

102. Larsen, R.D., Ernst, L.K., Nair, R.P. and Lowe, J.B. (1990) Proc. Natl. Acad. Sci., U.S.A., (personal commun.) in press.

103. Kukosska-Latallo, J.F., Larsen, R.D., Nair, R.P. and Lowe, J.B. (1990) Genes and Development, (personal commun.) in press.

104. Spiegel, S. and Fishman, P.H. (1987) Proc. Natl. Acad. Sci., U.S.A. **84**, 141-145.

105. Kreutter, D., Kim, J.Y.H., Goldenring, J.R., Rasmussen, H., Ukomadu, C., de Lorenzo, R.J. and Yu, R.K. (1987) J. Biol. Chem. **262**, 1633-1637.

106. Wiegandt, H. (1982) Adv. Neurochem. **4**, 149.

107. Feizi, T. (1985) Nature **314**, 53-57.

108. Hakomori, S. (1989) in *Gangliosides and Cancer*, 1st ed., VCH Publisher, New York, NY, p.p.57-68.

109. Ledeen, R.W. (1984) J. Neurosci. Res. **12**, 147.

110. Nagai, Y. and Tsuji, S. (1989) in *Gangliosides and Cancer*, 1st ed., (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 33-39.

111. Goldenring, J.R., Otis, L.C., Yu, R.K. and De Lorenzo, R.J. (1985) J. Neurochem. **44**, 1229-1234.

112. Yates, A.J., Walters, J.D., Stock, S.M., McIlroy, B.K. and Johnson, J.D. (1989) in *Gangliosides and Cancer*, 1st ed., (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 41-56

113. Moskal, J.R., Lockney, M.W., Marvel, C.C., Trosko, J.E. and Sweeley, C.C. (1987) Cancer Res. **47**, 787-790.

114. Deutsch, V. and Lotan, R. (1983) Exp. Cell Res. **149**, 237-245.

115. Lotan, R., Lotan, D. and Meromsky, L. (1984) Cancer Res. **44**, 5805-5812.

116. Payatte, R., Biehl, J., Toyama, T. and Holtzer, H. (1980) Cancer Res. **40**, 2465-2474.

117. Koeffler, H.P., Bar-Eli, M. and Territo, M.C. (1981) Cancer, Res. **41**, 919-926.

118. Fukuda, M. (1981) Cancer Res. **41**, 4621-4628.

119. Huberman, E., Heckman, C. and Langenbach, R. (1979) Cancer Res. **39**, 2618-2624.

120. Burczak, J.D., Moskal, J.R., Trosko, J.E., Fairley, J.L. and Sweeley, C.C. (1983) Exp. Cell Res. **147**, 281-286.

121. Leder, A. and Leder, P. (1975) Cell **5**, 319-322.

122. Ginsburg, E., Salomon, D., Sreevalsan, T. and Freese, E. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 2457-2461.

123. Fishman, P.H., Simmons, J.L., Brady, R.O. and Freese, E. (1974) Biochem. Biophys. Res. Commun. **59**, 292-299.

124. Chatterjee, S., Sweeley, C.C. and Velicer, L.F. (1975) J. Biol. Chem. **250**, 61-66.

125. Macher, B.A., Lockney, M., Moskal, J.R., Fung, V.K. and Sweeley, C.C. (1978) Exp. Cell Res. **117**, 95-102.

126 Moskal, J.R., Lockney, M.W., Marvel, C.C., Mason, P.A. and Sweeley, C.C. (1980) in ACS Symposium Series, No. 128, Cell Surface Glycolipids (Sweeley, C.C., ed.) p.p. 241-263.

127. Schlessinger, et al. (1983) Crit. Rev. Biochem. **14**, 93-111.

128. Usuki, S., Lyu, S.-C. and Sweeley, C.C. (1988) J. Biol. Chem. **263**, 6847-6853,

129. Usuki, S., Hoops, P. and Sweeley, C.C. (1988) J. Biol. Chem. **263**, 10595-10599.

130 Usuki, S. and Sweeley, C.C. (1988) Ind. J. Biochem. Biophys. **25**, 102-105.

131. Swamy, M.J. and Sweeley, C.C. (1989) Biochem. Biophys. Res. Commun. **162**, 1188-1193

132. Hampton, R.Y. and Morand, O.H. (1989) Science, **246**, 1050.

133. Hannun, Y.A., Loomis, C.R., Merril, A.H., Jr. and Bell, R.M. (1986) J. Biol. Chem. **261**, 12604-12609.

134. Hannun, Y.A. and Bell, R.M. (1989) Science **243**, 500-507.

135. Faucher, M.F., Girones, N., Hannun, Y.A., Bell, R.M. and Davis, R.J. (1988) J. Biol. Chem. **263**, 5319-5327.

136. Merrill, A.H. and Stevens, V.L. (1989) Biochim. Biophys. Acta **101**, 131-139.

137. Igarashi, Y. and Hakomori, S.-I. (1989) Biochem. Biophys. Res. Commun. **164**, 1411-1416.

138. Bottega, R., Epand, R.M. and Ball, E.H. (1989) Biochem. Biophys. Res. Commun. **164**, 102-107.

139. Burczak, J.D., Soltyziak, R.M. and Sweeley, C.C. (1984) J. Lipid Res. **25**, 1541-1547.

140. Nishizuka, Y. (1984) Nature **308**, 693-698.

141. Mitchell, B. (1983) Trends Biochem. Sci. **8**, 263-265.

142. Dawson, G., McLawhon, R. and Miller, R.J. (1980) J. Biol. Chem. **255**, 129-137.

143. Capasso, J.M., Keenan, T.W., Abeijon, C. and Hirshberg, C.B. (1989) J. Biol. Chem. **264**, 5283-5240.

144. Svennerholm, L. (1964) J. Lipid Res. **5**, 145-155.

145. IUPAC-IUB Recommendations (1977) Lipids **12**, 455-468.

CHAPTER 2

A QUANTITATIVE METHOD FOR SEPARATING REACTION COMPONENTS OF CMP-SIALIC ACID:LACTOSYLCERAMIDE SIALYLTRANSFERASE USING SEP PAK C_{18} CARTRIDGES

L. J. Melkerson-Watson, K. Kanemitsu, and C. C. Sweeley

November 11, 1986

Department of Biochemistry
Michigan State University
East Lansing, MI 48824

Revised March 9, 1987

Glycoconjugate J. (1987) **4**, 7-16

Running Title: Studies on the Purification and Regulation of SAT-1

ABSTRACT

A rapid procedure is described for the separation of CMP-sialic acid:lactosylceramide sialyltransferase reaction components using Sep Pak C_{18} cartridges. The quantitative separation of the more polar nucleotide sugar, CMP-sialic acid, and its free acid from the least polar GM_3 ganglioside is simple and rapid relative to previous described methods. Recovery of GM_3 is optimized by the addition of phosphatidylcholine to the reaction mixture prior to the chromatographic step. Using rat live Golgi membranes as a source of CMP-sialic acid:lactosylceramide sialyltransferase activity (GM_3 synthase, SAT-1), the transfer of [^{14}C]-sialic acid from CMP-[^{14}C]-sialic acid to lactosylceramide can be quantified by this assay. The procedure is reliable and may be applicable to the isolation of ganglioside products in other in vitro glycosyltransferase assays.

INTRODUCTION

The cell surface has been postulated to play an important role in the regulation of cell growth and differentiation. Gangliosides, ubiquitous sialic acid-containing glycosphingolipids of plasma membrane, have been implicated in these phenomena. Changes in ganglioside composition and metabolism have been associated with oncogenic transformation (1); during which there is often an accumulation of less complex gangliosides than in the non-transformed cells, due to a block or impairment of the ganglioside biosynthetic pathway. Alternatively, there may be synthesis of novel gangliosides due to activation of specific quiescent embryonic or fetal glycosyltransferases (2).

Several lines of evidence suggest that synthesis of GM_3 ganglioside, catalyzed by CMP-sialic acid:lactosylceramide sialyltransferase (SAT-1), may be critical in the regulation of ganglioside biosynthesis and cell growth. SAT-1 catalyzes the first committed step in the synthesis of gangliosides of the ganglio type. An especially important finding was the inhibition of cell growth via platelet dependent growth factor and epidermal growth factor mediated mitogenesis by the GM_3 ganglioside incorporated into the plasma membrane (3,4).

The present assay was developed for use in the purification and characterization of SAT-1 to homogeneity from Golgi membranes and for studies of the mechanisms involved in the regulation of GM_3 synthesis by this enzyme. Like other glycosyltransferases, SAT-1 is relatively unstable in solubilized preparations and its specificity for lactosylceramide (LacCer) and the sugar nucleotide is low (5). *In vivo* sialyltransferase activity may be dependent upon the composition of the surrounding membrane, leading to difficulties in assays of its activity in partially purified preparations. Separation and analysis of the labeled GM_3 product from its labeled precursors by most chromatographic methods (6-13) is time consuming. For example, determination of GM_3 synthase activity in our laboratory by the paper chromatographic

method described by Basu and coworkers (10) requires two to three days (14). On the other hand, employing Sep Pak C_{18} cartridges (Waters Associates) for the analysis requires only 2-3 h and gives yields of labeled GM_3 that is completely separated from the labeled sugar nucleotide precursors and free acid.

MATERIALS AND METHODS

Materials

Sep Pak C_{18} cartridges were obtained from Waters Associates (Milford, MA, USA); Leur-lok tip syringes from Becton-Dickinson (Rutherford, NJ, USA); reagent grade solvents from Fisher Scientific (Pittsburgh, PA, USA); cytidine 5'-monophosphate [4-^{14}C]-sialic acid, (NEC-636, 1.8 mCi/mmol) from New England Nuclear (Boston, MA, USA); [4,5,6,7,8,9-^{14}C]-sialic acid (20 mCi/mmol) from ICN Pharmaceuticals (Irvine, CA, USA); phosphatidylcholine type III-L (from bovine liver) and type V-E (from frozen egg yolk); sodium cacodylate, cytidine 5'-monophosphate sialic acid (CMP-sialic acid) and Triton CF-54 from Sigma Chemical Co. (St. Louis, MO, USA); and HPTLC silica gel 60 from EM Science (Cherry Hill, NJ, USA).

Three sources of lactosylceramide were employed: a product from human erythrocytes prepared according to the method of Kundu (13), N-palmitoyl-DL-dihydro-lactosylcerebroside purchased from Sigma Chemical Co., and synthetic lactosylceramide prepared in our laboratory (15). The labeled substrates [^{3}H]-GM_3, [^{3}H]-LacCer, [^{3}H]-GD_{1a}, [^{3}H]-GD_3], [^{3}H]-$FucGM_1$ and [^{3}H]-$GgOse_4Cer$ ([^{3}H]-asialo-GM_1) were prepared according to the potassium [^{3}H]-borohydride reduction method of Schwarzmann (16). The $FucGM_1$ was a gift from Dr. S. Hakomori; asialo-GM_1 was derived by mild acid hydrolysis of GM_1.

Rat Liver Golgi Membranes

CMP-sialic acid:lactosylceramide sialyltransferase (SAT-1) was partially purified from rat liver Golgi membranes. The Golgi microsomal fraction was prepared by a method similar to those previously described by Morre et al. (197), Fleischer and Kervina (18) and Carey and Hirschberg (19). Briefly, livers were removed from 176-200 g male CD rats (Charles River, Wilmington, MA, USA) and homogenized in 5 vol of 25 mM sodium cacodylate buffer (pH 6.5) containing 0.25 M sucrose and 1 mM

phenylmethyl sulfonyl fluoride (PMSF). Golgi microsomes were collected by differential centrifugation followed by sucrose density gradient centrifugation. Protein determinations were by the method of Peterson (20).

CMP-Sialic Acid:Lactosylceramide Sialyltransferase Assay and Sep Pak C_{18} Separation Strategy

Assays of SAT-1 activity were performed as previously described (5,21). The reaction was terminated by addition of 1000 μg phosphatidylcholine in 0.4 ml 100 mM KCl and frozen. this reaction mixture, containing LacCer, [^{14}C]-GM_3, CMP-[^{14}C]-sialic acid and, possibly, free [^{14}C]-sialic acid formed by the hydrolysis of the sugar nucleotide, was transferred to a Sep Pak C_{18} cartridge (pre-conditioned as suggested by the manufacturer). Typically, the cartridges were attached to the end of a 5 ml Luer tip syringe, and washed with 5 ml methanol followed by 10 ml water. The sample was applied repeatedly with slight pressure until the eluate, after loading the sample mixture, was clear. Typically 2-3 times was adequate. However, when analyzing SAT-1 activity in crude preparations, the assay mixture may need to be applied to more than one cartridge or the same cartridge may be reutilized. The cartridges are re-usable after repeating the washing procedure, three times for assays containing crude homogenates and about eight times in assays with Golgi vesicles. The radiolabeled nucleotide sugar, CMP-[^{14}C]-sialic acid, and free [^{14}C]-sialic acid were eluted from the cartridge with 10 ml water. Chloroform/methanol, 2/1 by vol, 0.5 ml, was added to change the solvent system in the cartridge. This step reduced the amount of time required to evaporated the organic solvent. The GM_3 ganglioside was subsequently eluted with 5.0 ml chloroform/methanol, 2/1 by vol, and collected directly into a glass scintillation vial. The organic solvent was evaporated to dryness (traces of chloroform quench the radioactive signal) and the amount of [^{14}C]-GM_3 was determined by liquid

scintillation spectrometry in a Packard Tri-Carb 460C in 5 ml Flo Scint^{TM}II (Radiomatic Instruments and Chemical Co., Tampa, FL, USA).

Quantitative Recovery of SAT-1 Reaction Components from Sep Pak C_{18} Cartridges

Solutions containing 40 nmol CMP-sialic acid (donor substrate), 100 nmol LacCer (acceptor substrate), 5 nmol GM_3 (product) and 5 nmol sialic acid (free acid) in a final volume of 500 μl (see above) were applied to the reverse-phase cartridge. The elution profiles of the various reaction components were ascertained for reaction mixtures containing either CMP-[^{14}C]-NeuAc, [^{14}C]-NeuAc, or [^{3}H]-GM_3. The radiobiologicals were eluted with sequential applications of 0.5 ml aliquots of various aqueous and organic solvents and processed as described above.

Recovery of each reaction component was also examined by GLC for sialic acid content. A modification of the method using trimethylsilyl (TMS) ethers, first developed by Sweeley and coworkers (22, 23) and later amended by Ledeen and Yu (24) was employed. The reaction mixtures for these studies contained only one sialic acid-containing component per assay. All other conditions were as described above. All samples were taken to dryness, TMS derivatized, and fractions analyzed. Methanol was added to aid in evaporation of the solvent.

Qualitative Recovery of the [^{14}C]-GM_3 Product

Identification of the SAT-1 product was verified by TLC. The SAT-1 activity assay was performed and GM_3 product recovered as described above. The assay was repeated ten times and the organic phases pooled. The sample was taken to dryness and the phosphatidylcholine and [^{14}C]-GM_3product were separated according to a published procedure (25). The [^{14}C]-GM_3 was chromatographed on HPTLC silica gel 60 plates in chloroform/methanol/water, 60/30/5 by vol. The TLC plate was scraped

in 0.5 cm segments, transferred to glass scintillation vials containing 1.0 ml water and sonicated. The amount of radioactivity was quantified by liquid scintillation spectrometry in a Packard Tri-Carb 460C in 5 ml Flo Scint^{TM}II.

RESULTS

Separation of SAT-1 Reaction Components

Conditions for optimal recovery of the GM_3 ganglioside from the SAT-1 assay mixture were determined from experiments employing a variety of eluting solvents. Under the conditions of glycosyltransferase assays, in addition to the expected transfer of sialic acid to the LacCer acceptor substrate to form GM_3, hydrolysis of the radiolabeled sugar nucleotide to radiolabeled free sugar and unlabeled nucleotide may occur if phosphodiesterases or phosphatases are present in crude preparations (26). Therefore, it was necessary to compare recovery of CMP-[^{14}C]-sialic acid, [^{14}C]-sialic acid and [^{14}C]-GM_3 from our applied sample.

The separation of the radiolabeled reactants and the GM_3 product of the SAT-1 reaction mixture was performed as described in the Materials and Methods section. The recoveries of CMP-[^{14}C]-sialic acid, [^{14}C]-sialic acid and [^{3}H]-GM_3 were determined in H_20, 100 mM KCl, phosphate-buffered saline (PBS, 137 mM NaCl, 2.68 mM KCL, 1.47 mM KH_2PO_4, 8.06 mM NaH_2PO_4) and 25 mM sodium cacodylate (pH 6.5): methanol, chloroform/methanol, 1/1 by vol; chloroform/methanol, 2/1 by vol; chloroform/methanol/water, 60/30/5 by vol; and in the absence and presence of phosphatidylcholine. Employing the strategy of Williams and McCluer (27), application of GM_3 gangliosides to the cartridge in 100 mM KCl gave recoveries of only 39-53% with the aqueous solvents surveyed and 47-61% with the organic solvents from the SAT-1 reaction mixture. This method has been reported to give 94% recovery of rat brain gangliosides from the theoretical upper phase containing 100 mM KCL (27). Our inability to recover GM_3 completely was attributed to the presence of 0.3% Triton CF-54 in our SAT-1 assay buffer. To enhance the recovery of the [^{3}H]-GM_3 by increasing the size of the lipid-detergent-lipid micelles, phosphatidylcholine (PC) was added. PC enhances SAT-1 activity and

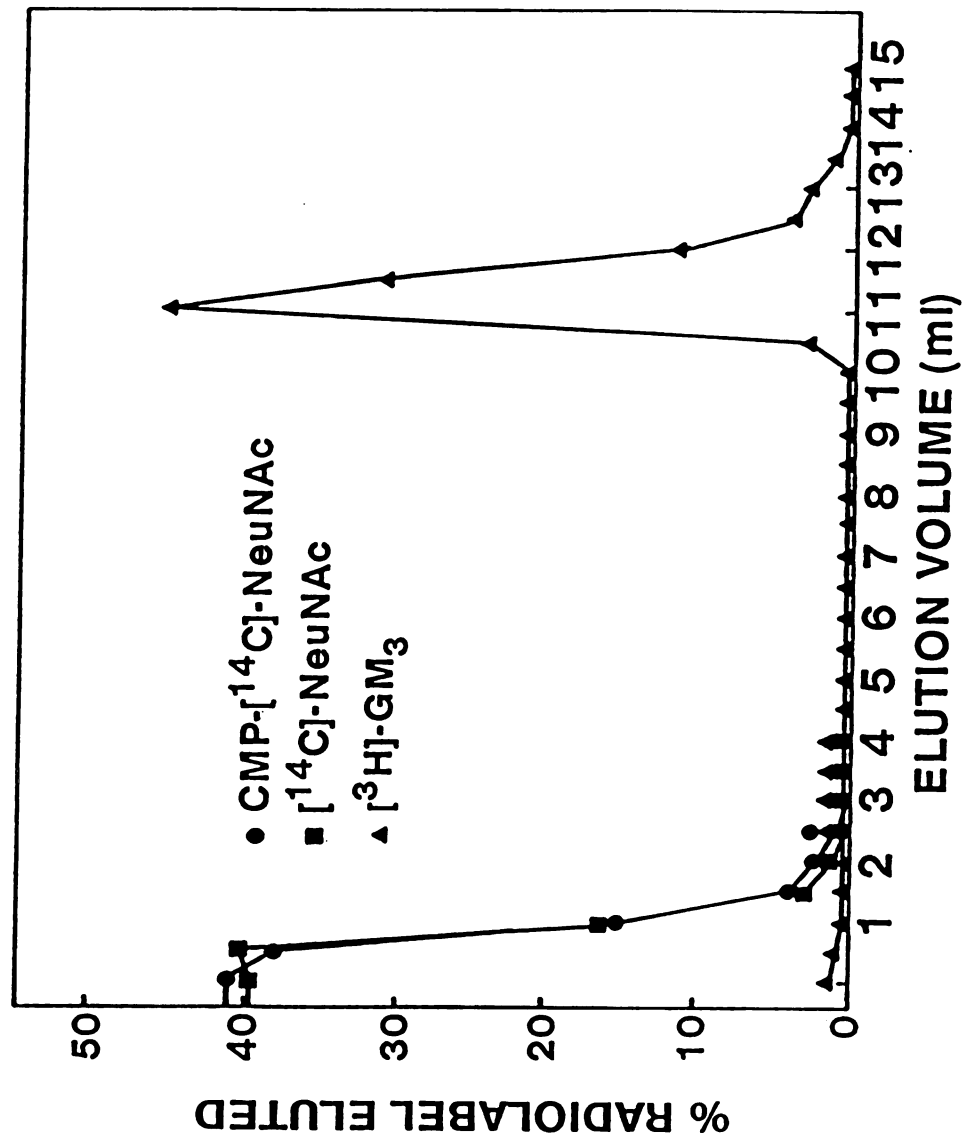
is probably a membrane-associated lipid (14). PC from two different sources (bovine liver and egg yolk) was examined and both gave similar results. Other phospholipids were not assayed. The recovery of GM_3 was dependent upon the amount of PC added (10-1000 μg). Formation of the PC/Triton CF-54/GM_3 micelles gave recoveries of 70-100% of the ganglioside from the SAT-1 reaction mixture in the organic was (Table 1). Complete recovery of GM_3 was accomplished with the addition of 1000 μg PC in 100 mM KCl. Further, the detergent served to reduce the interaction of the nucleotide sugar with thecartridge. Williams and McCluer (27) reported that 0.39% of added CMP-NeuAc was absorbed on Sep Pak C_{18} cartridges and that with extensive washing of the cartridge with water, retention of nucleotide sugars could be reduced. In our experiments, the nucleotide sugar and free sialic acid were recovered in good yield (99-100%) from the cartridge regardless of the aqueous solvent used. The recovery of CMP-sialic acid and sialic acid was completed within 4-5 ml aqueous solvent; however, 10 ml was typically employed. Negligible amounts (0.01 - 0.04%) were recovered in the organic solvent and as shown by GLC analysis for sialic content, the anomeric mixture of sialic acid recovered from the CMP-sialic acid after methanolysis was different from that obtained form GM_3. Thus, critical differentiation can be made between the amount of product formed and any possible contamination from nucleotide sugars. Additionally, the recovery of the ganglioside correlated with the degree of polarity of the solvent employed. With the exception of methanol (92% recovery of GM_3), all the organic solvents examined gave complete recovery of the GM_3 ganglioside. The most reproducible results were obtained when chloroform/methanol, 2/1 by vol, was the solvent. A typical elution profile for the recovery of the GM_3 ganglioside according to our separation scheme, as described in the Materials and Methods section, is shown in Fig. 1.

Table 1
Recovery of [^{3}H]-GM_3 from Sep Pak C_{18} Cartridges. The Effect of Phosphatidylcholine on Recovery.

Sample no.	100mM KCl (ml)	PC[a] (mg)	H_2O (ml)	[^{3}H]-GM_3 cpm Recovered	[^{3}H]-GM_3 Average	% Recovered
1	-	-	-		209490 ±6060	100.0
2	0.4	-	-	122270	129340	61.7
3	0.4	-	-	136400	±7070	
4	-	10	0.4	148960	144080	68.8
5	-	10	0.4	139210	±4880	
6	-	100	0.4	181020	182930	87.3
7	-	100	0.4	184850	±1920	
8	-	1000	0.4	198010	206340	87.3
9	-	1000	0.4	214670	±8330	
10	0.4	10	-	156870	165890	79.2
11	0.4	10	-	174900	±9020	
12	0.4	100	-	175000	180000	85.9
13	0.4	100	-	185000	±5000	
14	0.4	1000	-	210850	210850	101.0
15	0.4	1000	-	228920[b]	(219890 ±9030)	(106.0)[b]
16	-	10	0.4	186400	174810	83.4
17	-	10	0.4	163220	±11590	
18	-	100	0.4	173690	184290	88.0
19	-	100	0.4	194890	±10600	
20	-	1000	0.4	209080	200730	95.8
21	-	1000	0.4	191730	±9000	
22	0.4	10	-	125970	144280	68.9
23	0.4	10	-	162600	±18320	
24	0.4	100	-	184290	181360	86.6
25	0.4	100	-	178430	±2930	
26	0.4	1000	-	201560	207045	98.8
27	0.4	1000	-	212530	±5500	

[a] For samples 2-15, the PC was added to the 0.4 ml additive solution. For samples 16-27, the PC was added to the SAT-1 reaction matrix.
[b] Value outside confidence limits of standard.

Figure 1. Elution Profile of Radiolabeled SAT-1 Reaction Components on Sep Pak C_{18} Cartridges. Chromatography on Sep Pak C_{18} was performed on complete reaction mixtures composed of 100 ml of a protein fraction containing SAT-1, and 400 ml 100 mM KCl containing 1000 μg PC (sonicated before addition to the reaction mixture). The final concentrations of components contained in the SAT-1 reaction mixture were 1 mM LacCer, 0.4 mM CMP-NeuAc, 0.05 mM GM_3, 0.05 mM NeuAc, 112.5 mM sodium cacodylate (pH 6.5), 15 mM $MnCl_2$, 0.125 mM sucrose, 0.05 mM PMSF and 0.3% (w/v) Triton CF-54. CMP-[^{14}C]-NeuAc (o), [^{14}C]-NeuAc (o) or [^{3}H]-GM_3 (o) were individually substituted into the SAT-1 reaction mixture and their recovery monitored by collecting 0.5 ml fractions from cartridges.


% RADIOLABEL ELUTED
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ELUTION VOLUME (ml)
● CMP-[^{14}C]-NeuNAc
■ [^{14}C]-NeuNAc
▲ [^{3}H]-GM$_3$

The recovery of the SAT-1 reaction components, as investigated by GLC analysis of both the polar and non-polar eluants for sialic acid content, gave similar, results for the devised separation scheme of: 1000 mg in 100 mM KCl, water, and $CHCl_3$/MeOH, 2/1 by vol (applied volume, aqueous solvent, and organic solvent, respectively). The sugar nucleotide and free sialic acid eluted with the aqueous solvent and the GM_3 ganglioside was recovered with the organic solvent. Methanolysis of GM_3 gave the methyl ketal of N-acetylneuraminic acid in the ß-anomeric configuration which was recovered in the organic solvent without contamination from other reaction components. The free sialic acid liberated from the other reactions components was primarily in the a configuration and recoverable in the aqueous solvent. Some traces of α-NeuAc (0.05 ± 0.02 μg) were detected in the organic fractions. However, these amounts did not significantly increase the background in blanks for the analysis of the GM_3 by radioactivity.

The capacity of the Sep Pak C_{18} cartridge for GM_3 was examined by analysis of radioactivity and by gas chromatographic analysis. In analysis of radioactivity, application of up to 50 nmol of [^{3}H]-GM_3 was completely recoverable. Gas chromatographic analyses indicated that application of GM_3 equivalent to 25 μg of sialic acid (81 nmol) overloaded the Sep Pak C_{18} cartridge wheres 13.6 μg of the GM_3 sialic acid (44 nmol) was retained by the cartridge. To determine the maximum capacity of the cartridge with optimal recovery, the recovery of 0-15 μg GM_3 sialic acid was examined. For amounts of GM_3 no exceeding 2.5 μg (8 nmol) of sialic acid, 96% of the GM_3 was recovered. This is in contrast to the 5 μmol reported by Williams and McCluer for ganglioside sialic acid recovery from theoretical upper phase (27). When 5-15 μg (16-40 nmol) of GM_3 sialic acid was applied to the cartridge using our SAT-1 system, the recovery was reduced to 75-87%.

Application of SAT-1/SepPak C_{18} Strategy in Determining SAT-1 Activity

The transfer of [^{14}C]-NeuAc from CMP-[^{14}C]-NeuAc to LacCer to form [^{14}C]-GM_3 by SAT-1 was examined using the Sep Pak C_{18} separation strategy described in the Materials and Methods section. The enzymatic synthesis of GM_3 in rat liver Golgi was studies as a functions of time and Golgi microsomal protein (Fig. 2). The synthesis of GM_3 proceeded linearly for at least 180 min and the synthesis of the product was proportional to the amount of protein in the reaction mixture up to 0.25 mg of microsomal protein. The cartridges were saturated by excess protein in the crude enzyme preparation, resulting in non-linearity of the curve beyond 0.3 mg of Golgi fraction.

The acid glycosphingolipid formed form LacCer and CMP-sialic acid was identified as GM_3 ganglioside by TLC. The SAT-1 activity assays were set up with two different formats. Pooled eluates of glycosphingolipid were obtained from 10 assays employing CMP-[^{14}C]-sialic acid and LacCer while separate products were pooled from a second set of 10 assays employing CMP-sialic acid and [^{3}H]-LacCer. The organic eluants from each set were dried under nitrogen and the phosphatidylcholine and salts removed by the procedure described by Ladisch and Gillard (25). The radiolabeled products comigrated with a reference sample of [^{3}H]-GM_3 on HPTLC plates (data not shown).

The applicability of this reverse-phase chromatography strategy on Sep Pal C_{18} cartridges for the recovery of other glycosyltransferase products was briefly investigated. Table 2 shows that while the conditions have been optimized for the recovery of GM_3; other glycosphingolipids such as LacCer, Fuc-GM_1, $GgOse_4Cer$, GD_3 and GD_{1a} can be recovered form the detergent-containing SAT-1 reaction mixture by this procedure. The recovery and analysis of SAT-1 sialyltransferase with slight modifications may be equally effective for assays of other glycosyltransferase activities.

Figure 2. Enzymatic Synthesis of [^{14}C]-GM_3 from CMP-[^{14}C]-NeuAc and LacCer as a Function of Time and Protein. A. Reaction mixtures with 2.28 x 10^{-2} mg Golgi-enriched microsomal protein were incubated for the indicated period of time at 37°C and the [^{14}C]-GM_3 product was recovered as described in the Materials and Methods section. **B.** Reaction mixtures containing the indicated amount of Golgi-enriched microsomal protein were incubated for 155 min at 37°C and processed as described in the Materials and Methods section. These data were collected from two different Golgi membrane preparations from rat liver.

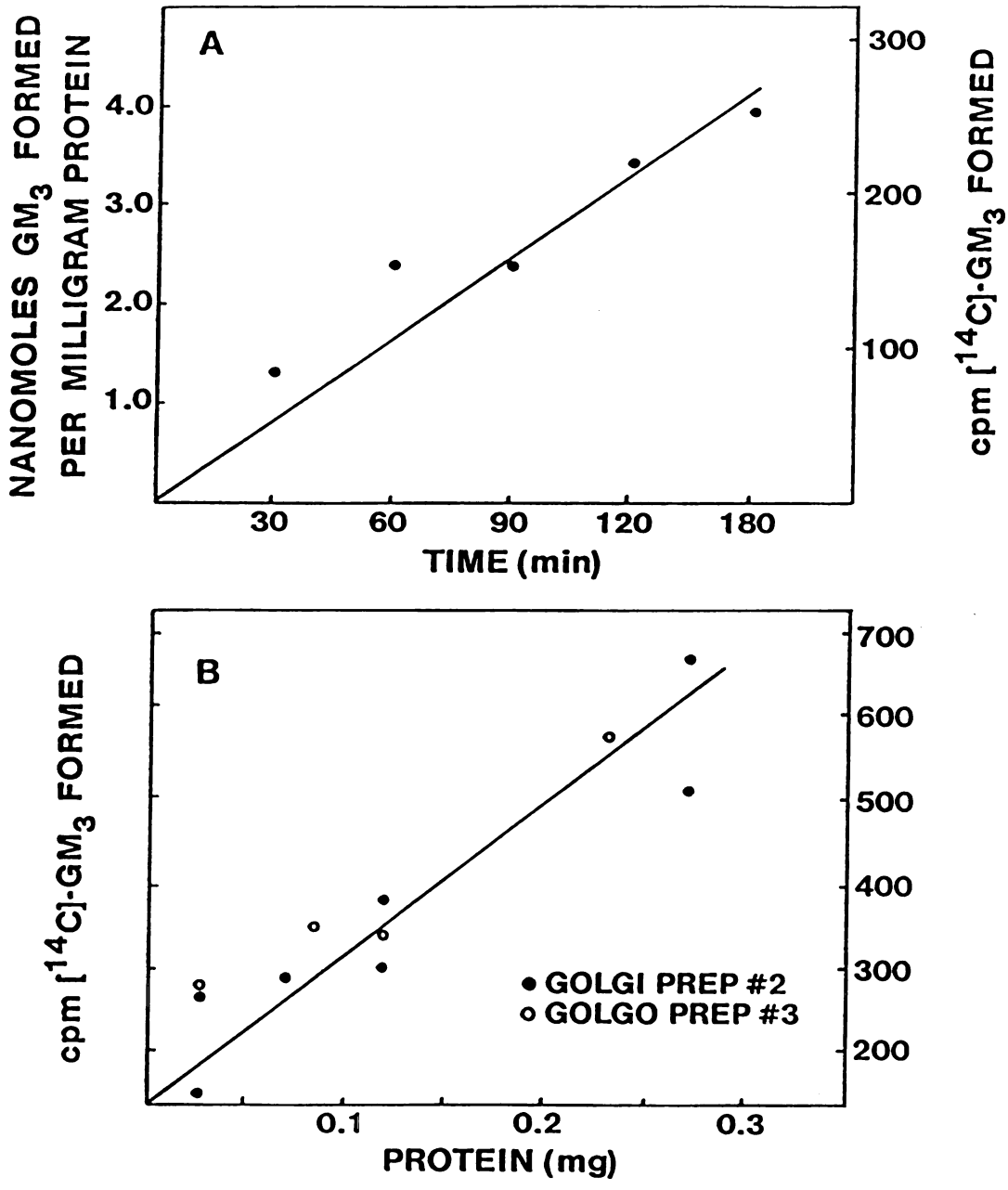

A
NANOMOLES GM3 FORMED PER MILLIGRAM PROTEIN
cpm [14C]-GM3 FORMED
4.0
3.0
2.0
1.0
300
200
100
30
60
90
120
180
TIME (min)
B
cpm [14C]-GM3 FORMED
700
600
500
400
300
200
0.1
0.2
0.3
PROTEIN (mg)
● GOLGI PREP #2
o GOLGO PREP #3

Table 2

Recovery of Other Gangliosides from SAT-1 Reaction Mixtures from Sep Pak C_{18} Cartridges.

Glycosphingolipid	Amount added (cpm)	cpm [^{3}H]-GSL Recovered Above Background	% Recovered
[^{3}H]LacCer	300,000	297460±8680	99.2
[^{3}H]-GM_3	184,000	188580±5250	102.5
[^{3}H]-FucGM_1	1600	1970±110	123.1
[^{3}H]-GgOse_4Cer	20,000	17350±750	86.8
[^{3}H]-GD_3	9800	8380±60	84.9
[^{3}H]-GD_{1a}	5700	4900±610	85.9

DISCUSSION

The major purpose of this investigation was to simplify the analysis of CMP-sialic acid:lactosylceramide sialyltransferase (SAT-1) activity. We have developed a rapid and convenient system for the separation and analysis of GM_3 ganglioside from crude reaction mixtures. Sep Pak C_{18} cartridges (octadecylsilane bonded-phase packings) are useful for the separation of problem sample preparations containing components of differing polarities in complex aqueous solvents. A common problem in studies of ganglioside synthesis and regulation is the need for complete separation of radiolabeled sugar nucleotides from the radiolabeled ganglioside product. Several methods have been developed for removal of non-ganglioside reactants (6-13).

Williams and McCluer (27) have previously reported that Sep Pak C_{18} reverse-phase cartridges can be employed for the recovery of gangliosides from theoretical upper phases of Folch-extracted tissues with the addition of 100 mM KCl to the sample matrix. However, their modification is not applicable for the recovery and analysis of gangliosides synthesized from detergent-solubilized Golgi membrane preparations. With detergent in our SAT-1 assay mixtures, the recovery of GM_3 was 39-62%, which is not acceptable for enzymatic analyses. Since purification and characterization of membrane-bound SAT-1 requires detergent solubilization for *in vitro* analysis of enzyme activity, we found it necessary to modify the Sep Pak C_{18} procedure to accommodate the composition of our reaction mixtures. The Sep Pak C_{18} cartridges provide a rapid, convenient and reliable system for determining SAT-1 activity during its purification and characterization. SAT-1 activity can be quantified accurately up to the formation of 8 nmol of GM_3. Enzyme activity can be determined by the recovery of radiolabeled GM_3 or by GLC analysis of sialic acid recovered from the GM_3 by methanolysis. This method can be applied to quantitative analysis of other glycosyltransferase activities involved in the biosynthesis of gangliosides. The described procedure has been modified in our laboratory for the analysis of GD_3

synthase activity. The only problem we have encountered is that some detergents, e.g., Triton X-100 (>0.5%, by vol), completely solubilize 1000 μg PC in the sample matrix. Therefore, depending on the nature of the detergent employed and the nature of the lipid environment of the glycosyltransferase, a different amount or type of phospholipid may have to be used to obtain optimal recovery of the ganglioside product.

ACKNOWLEDGEMENTS

This study was supported in part by a research grant from the National Institutes of Health (AM12434).

We thank Kimihiro Kanemitsu for sharing his expertise and training on the GC analysis of sialic acid.

REFERENCES

1. Hakomori, S. (1981) Ann. Rev. Biochem. **50**, 733-764.
2. Hakomori, S. and Kannagi, R. (1983) J. Natl. Cancer Inst. **71**, 231-251.
3. Bremmer, E. G., Hakomori, S., Bowen-Pope, D.F., Raines, E., and Ross, R. (1984) J. Biol. Chem. **259**, 6818-6825.
4. Bremmer, E.G., Schlessinger, J. and Hakomori, S. (1986) J. Biol. Chem. **261**, 2434-2440.
5. Burczak, J.D., Fairley, J.L., and Sweeley, C.C. (1984) Biochim. Biophys. Acta **804**, 442-449.
6. Walton, K.M. and Schnaar, R.L. (1986) Anal. Biochem. **152**, 154-159.
7. Byrne, M.C., Sbaschnig-Agler, M., Aquino, D.A., Slcafani, J.R., and Ledeen, R.W. (1985) Anal. Biochem. **148**, 163-173.
8. Yates, H.C., Ueno, K., Chang, N.-C., Glaser, G.H., and Yu, R.K. (1980) J. Neurochem. **34**, 560-568.
9. Carter, T.P. and Kanfer, J. (1973) Lipids **8**, 537-548.
10. Chien, J., Williams, T., and Basu, S. (1973) J. Biol. Chem. **245**, 1778-1785.
11. Kanfer, J. (1969) Methods Enzymol. **3**, 41-90.
12. Chien, J., Williams, T., and Basu, S. (1966) Methods Enzymol. **8**, 365-368.
13. Kundu, S.K. (1981) Methods Enzymol. **62**, 174-185.
14. Burczak, J.D. (1984) Ph.D. Dissertation, Michigan State University, East Lansing, MI, USA.
15. Kanemitsu, K. and Sweeley, C.C. (1986) Glycoconjugate J. **3**, 143-151.
16. Schwarzmann, G. (1978) Biochim. Biophys. Acta **529**, 106-114.
17. Morre, D.J., Merlin, L.M., and Keenan, T.W. (1969) Biochem. Biophys. Res. Commun. **37**, 813-819.
18. Fleischer, S. and Kervina, M. (1974) Methods Enzymol. **31**, 6-41.
19. Carey, D.J. and Hirschberg, C.B. (1980) J. Biol. Chem. **255**, 4348-4354.

20. Peterson, G.L. (1977) Anal. Biochem. **83**, 346-356.

21. Kaufman, B., Basu, S., and Roseman, S. (1966) Methods Enzymol. **8**, 365-368.

22. Sweeley, C.C. and Walker, B. (1964) Anal. Chem. **36**, 1461.

23. Vance D.E. and Sweeley, C.C. (1967) J. Lipid Res. **8**, 621.

24. Ledeen, R.W. and Yu, R.K. (1982) Methods Enzymol. **83**, 179-183.

25. Ladisch, S. and Gillard, B. (1984) Anal. Biochem. **146**, 220-231.

26. Yu, P.H., Barclay, S., Davis, B., and Boulton, A.A. (1981) Biochem. Pharmacol. **30**, 3089-3094.

27. Williams, M.A. and McCluer, R.H. (1980) J. Neurochem. **35**, 266-269.

CHAPTER 3

PURIFICATION TO APPARENT HOMOGENEITY BY IMMUNOAFFINITY CHROMATOGRAPHY AND PARTIAL CHARACTERIZATION OF THE GM_3 GANGLIOSIDE FORMING ENZYME, CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE (SAT-1), FROM RAT LIVER GOLGI*

L. J. Melkerson-Watson and C. C. Sweeley+

July 30, 1990

Submitted August 22, 1990

Revised November 9, 1990

J. Biol. Chem. (1991), in press.

Department of Biochemistry
Michigan State University
East Lansing, MI 48824

Running Title: Studies on the Purification and Regulation of SAT-1

ABSTRACT

CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) has been purified approximately 40,000-fold to apparent homogeneity from rat liver Golgi. The enzyme was solubilized from Golgi vesicles in 5% lauryl dimethylamine oxide (LDAO), "partially" purified by affinity chromatography twice on CMP-hexanolamine and once on lactosylceramide-(LacCer-) aldehyde Sepharose 4B. Final purification was achieved by immunoaffinity chromatography on M12GC7-Affi Gel-10. The M12GC7 monoclonal antibody specifically inhibits and immunoprecipitates SAT-1 activity. Identification of the protein, with an apparent molecular weight by SDS-PAGE of about 60,000 daltons, was confirmed by Western Blot and immunodetection with M12GC7. SAT-1 specifically catalyzes the transfer of N-acetylneuraminic acid (NeuAc, sialic acid) to lactosylceramide (Galß1-4Glcß1-O-Cer), forming GM_3 ganglioside. Studies on substrate specificity indicate that the preferred acceptors have the general structure saccharideß1-O-Cer, a disaccharide being preferred to a monosaccharide. SAT-1 is a glycoprotein. The carbohydrate moieties are detected with specific lectins. Deglycosylation of SAT-1 with N-glycanase results in an increase in a 43,000 dalton band. The two-dimensional electrophoretogram of SAT-1 indicates a pI range of 5.7 to 6.2 for the 60,000 dalton protein.

INTRODUCTION

Glycoconjugates have been implicated in a variety of cell surface interactions, cellular differentiation, and oncogenic transformation (for reviews, 1-3 and references therein). The synthesis of these complex carbohydrates is catalyzed by linkage-specific glycosyltransferases, several of which have been purified and cloned (for review, 4 and references therein). Many of these purified and characterized enzymes are involved in the synthesis of various glycoproteins. Very limited success has been achieved in purifying the glycosyltransferases specifically involved in glycosphingolipid (GSL) biosynthesis. This may reflect the difficulty in solubilization, the relative instability of the enzymes once extracted from their native lipid environment, low substrate specificities (5), laborious activity assays and proteolytic degradation during purification (6, for review 4).

The general approach for this group of glycosyltransferases has been the affinity chromatography employing either an acid modification of the acceptor glycolipid (5,7-10) or Sepharose 4B followed by FPLC MonoS ion exchange and proRPC reverse-phase chromatography (11). While these protocols have proven to be applicable for purifying some GSL glycosyltransferases, they are not optimal for those glycosyltransferases involved in the early stages of the glycosphingolipid cascade. A recent report (12) identifies two classes of GSL glycosyltransferases based on their substrates specificities. The first class recognizes acceptor glycolipid substrates strictly on the basis of carbohydrate-carbohydrate linkages, and the second recognizes carbohydrate-lipid linkages. Thus, a glycolipid-acid Sepharose, or alternative, may not provide the optimal glycolipid structure to successfully purify both classes of glycolipid glycosyltransferases.

We report here the purification to apparent homogeneity of rat hepatic lactosylß-O-ceramide α2-3 sialyltransferase (SAT-1). Purification of this GM_3 ganglioside forming enzyme was accomplished using a glycolipid-aldehyde Sepharose affinity resin

and monoclonal antibody, M12GC7, directed against SAT-1. We describe the synthesis of this affinity ligand, production and characterization of a specific anti-SAT-1 monoclonal antibody, and immunoaffinity chromatography in verification of SAT-1 apparent homogeneity.

EXPERIMENTAL PROCEDURES

Materials - Cytidine 5'-monophosphate sialic acid, (CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid, 286.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and its specific activity adjusted to 22,200 dpm/nmol with unlabeled CMP-sialic acid from Sigma (St. Louis, MO). Lactosylceramide was purified from AB+ human red blood cells obtained from the American Red Cross, Lansing, MI or was synthesized as described (13). The sphinganine-containing form of lactosylceramide was purchased from Sigma and used in activity assays. Ammonyx LO (lauryldimethylamine oxide, LDAO) was obtained from the Stepan Company (Chicago, IL). Sodium cacodylate, CMP-hexanolamine-Sepharose 4B, Hexanolamine-Sepharose 4B, fetuin, asialofetuin, mucin, asialomucin, glycophorin, asialoglycophorin, α_1-acid glycoprotein, lactose, 6-aminohexyl-Sepharose 4B and pristane were purchased from Sigma Chemical Company (St. Louis, MO). RPMI 1640, NCTC-109, penicillin, streptomycin sulfate, HEPES, fetal bovine serum, donor horse serum, hypoxanthine, thymidine and L-glutamine were purchased from GIBCO Laboratories (Gaithersburg, MD). Affi-Gel 10, Goat anti-mouse IgG (H+L), goat anti-mouse IgG-alkaline phosphatase conjugate, SDS-PAGE low molecular weight standards, prestained molecular weight standards, MAPS II Protein A HPLC column, MAPS II buffers and ultrapure electrophoresis reagents were bought from BioRad (Richmond, CA). Mouse Isotyper test kit was from Amersham (Arlington Heights, IL). Rabbit anti-mouse IgG's were from Chemicon (Temecula, CA). Protein A-positive Staph A cells, polyethylene glycol-1500 (PEG-1500), aminopterine, Nutridoma-SP, α2-6 sialyltransferase, PMSF, leupeptin, aprotinin, pepstatin, TPCK, TLCK, E-64, and the glycan differentiation kit were purchased from Boehringer Mannheim (Indianapolis, IN). Genzyme (Boston, MA) was the source of N-glycanase, as well as α2-6 and α2-3 sialyltransferases. Sep Pak C_{18} cartridges were purchased from Waters Associates (Milford, MA). HPTLC plates with fluorescent indicator were from EM Science (Cherry Hill, NJ). Polyisobutylmethylacrylate

(PIBMA) was obtained through the Aldrich Chemical Co. (Milwaukee, WI). Ampholines, Pharmalytes and carbamylated pI standards were from Pharmacia-LKB (Piscataway, NJ). All other reagents were ultrapure or ACS grade from commercial sources.

Animals and Cell Lines - Male CD rats (six to eight weeks old) and female Swiss albino mice (four to six weeks old) were purchased from Charles River (Wilmington, MA). Rats were anesthetized with CO_2 and the liver perfused with ice-cold 25 mM sodium cacodylate (pH 6.5) containing 0.25 M sucrose and 1 mM PMSF before excising the tissue. The mice were anesthetized with ether and cervically dislocated. SP2/O-Ag14 cells (ATCC CRL 1581) were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained between 1 x 10^5 to 1 x 10^6 cells/ml in RPMI 1640 containing 10% fetal bovine serum, 10% horse serum, NCTC- 109, hypoxanthine, thymidine, penicillin, streptomycin and 1% Nutridoma-SP at 7.5% CO_2.

1. ASSAYS.

Protein concentration. Protein was assayed by the procedure of Peterson (14) or spectrophotometrically at A_{280} (15).

SAT-1 Activity Assay. SAT-1 assays, with radiolabeled CMP-sialic acid as the donor substrate and lactosylceramide as the acceptor substrate, were carried out by a modification of a previously described procedure (16). Assay mixtures contained the following components in 100 μl: lactosylceramide, 0.2 μmoles; CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid (22,200 dpm/nmole), 0.04 μmoles; sodium cacodylate buffer (pH 6.5), 10 mmoles (100 mM final concentration); manganese chloride, 1 mmoles (10 mM final concentration); 5.0% (v/v) LDAO and 0.005-0.1 mg of the protein. GM_3 reaction product was recovered by reverse phase chromatography on Sep Pak C_{18} cartridges as previously described (17).

ST2-6 Sialyltransferase Assay. ß-galactosyl α2-6 (ST2-6) sialyltransferase was assayed according to the conditions previously described for this enzymes (18-21) using 50 μg asialo-α_1-acid glycoprotein as acceptor. The α_1-acid glycoprotein was desialylated by acid hydrolysis (22) in 0.05 M H_2SO_4 at 80°C for 60 min. The reaction was neutralized with 0.1 M NaOH and dialyzed versus double-distilled H_2O overnight and lyophilized.

Thiamine Pyrophosphatase Activity. The assay was performed as previously described (24).

Quantitation of Lauryldimethylamine Oxide (LDAO). The amount of LDAO in solution was quantitated as described by the manufacturer (Stepan Chemical Co., Chicago IL).

2. AFFINITY PURIFICATION OF SAT-1.

Preparation of Golgi-enriched Microsomes. Procedures for the preparation of a highly enriched fraction of Golgi vesicles from rat liver have been well-established. Golgi-enriched microsomes were prepared by differential centrifugation similar to methods previously described (23-27). All fractionation steps were carried out at 4°C. Livers (100 g wet weight) were from six- to eight-week old (approximately 175 g) male CD rat. After anesthetizing rats with CO_2 and decapitation, the livers were perfused with 10-20 ml of 25 mM sodium cacodylate (pH 6.5) containing 0.25 M sucrose and 1 mM phenylmethylsulfonyl fluoride (PMSF), removed and immediately homogenized in five volumes of the same buffer using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). After centrifugation at 4°C for 10 min at 5000 x g, the supernatant fraction was centrifuged on 1.2 M sucrose cushions at 26,000 rpm (approximately 100,000 x g) for 30 min in a Beckman SW27 rotor. The microsomes were collected and pelleted at 100,000 x g for 30 min at 4°C. The microsomal pellets were resuspended in 25 mM sodium cacodylate (pH 6.5) containing 0.25 M sucrose and 1 mM PMSF to a final ratio of about 0.7 to 1.0 g liver per ml buffer. Discontinuous

sucrose gradients were routinely prepared by layering 10 ml of the microsomal suspension on top of 5.0 ml of 10%, 20%, 25%, 32%, 35% and 40% (w/v) sucrose, prepared in 25 mM sodium cacodylate (pH 6.5), and centrifuging at 26,000 rpm for 3 h in a Beckman SW27 rotor at $4^{o}C$. Employing this methodology, the Golgi were recovered at the sucrose interfaces between 10-30%, as verified by increased thiamine pyrophosphatase and SAT-1 activity. The Golgi were concentrated by centrifugation at 100,000 x g and resuspended in a minimal volume of 25 mM sodium cacodylate (pH 6.5).

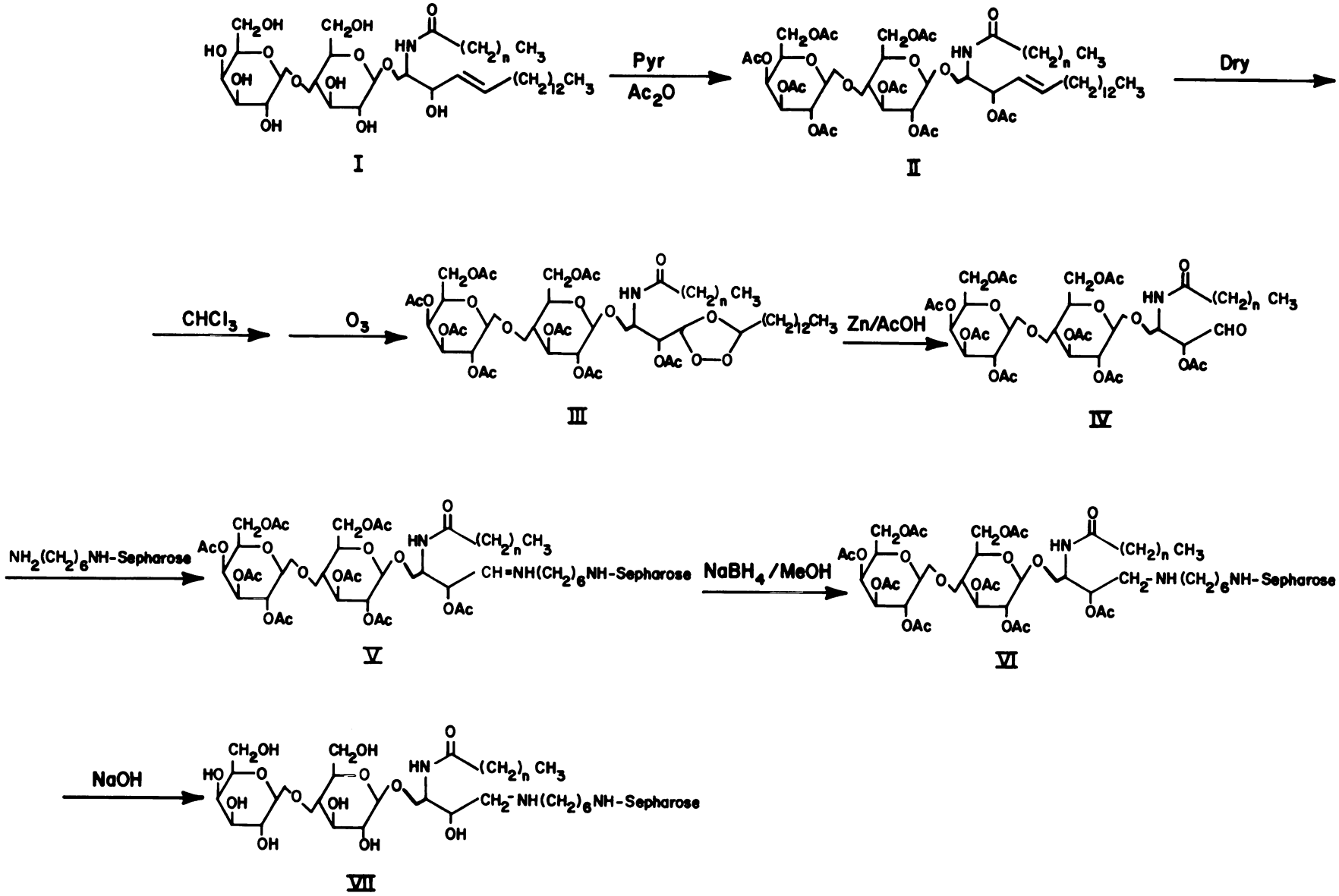
Detergent Extraction. The Golgi-enriched microsomal fraction was brought to a final concentration of 15% (v/v) LDAO in 25 mM sodium cacodylate (pH 6.5) containing 10 mM $MnCl_2$, 1 mM PMSF. The suspension was sonicated for 15-20 min. Following gentle stirring for 12-18 h (overnight) at $4^{o}C$, the detergent-solubilized fraction was recovered by centrifugation at 150,000 x g for 90 min at $4^{o}C$. The supernatant fraction contained the detergent-solubilzed SAT-1 activity. The pellet was re-extracted and the supernatants pooled and the final volume adjusted to give 5% LDAO final concentration. Special considerations in using LDAO for SAT-1 purification are described elsewhere (29).

Affinity Chromatography on CMP-hexanolamine-Sepharose I. The detergent-extracted proteins from Golgi vesicles were applied to a column (1.5 cm x 30 cm) of CMP-hexanolamine Sepharose (2-4 μmoles CMP per ml gel) which had been equilibrated with 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO and 10 mM $MnCl_2$ (affinity chromatography buffer). The column was washed with a minimum of 10 column volumes of the same buffer to remove excess proteins at a flow rate of 40 ml/h. The absorbance at A_{280nm} of the fractions was monitored. Elution of the sialyltransferase activity was carried out with a linear salt gradient established between 500 ml (total volume) of the affinity chromatography buffer and buffer containing 0.5 M NaCl.

Rechromatography on CMP-hexanolamine Sepharose II. The SAT-1 active fractions were pooled from CMP Sepharose I and concentrated on an Amicon PM10 membrane filter. The concentrate was dialyzed against 25 mM sodium cacodylate (pH 6.5) and applied on a second CMP-hexanolamine Sepharose 4B column (1.5 cm x 30 cm) equilibrated with the 25 mM sodium cacodylate (pH 6.5) affinity chromatography buffer containing 5% (v/v) LDAO and 10 mM $MnCl_2$. The flow rate for loading the sample was 2-4 ml/h. The column was washed with a minimum of 10 column volumes of buffer at a flow rate of 40 ml/h until no protein was detected by A_{280nm}. The SAT-1 enzyme activity was eluted with a linear gradient (500 ml total volume) of affinity chromatography buffer and buffer containing 1 mM CMP. SAT-1 active fractions were pooled and concentrated. The CMP was removed during protein concentration with PM10 membrane and chromatography on Sephadex G-25 (fine) (1.5 cm x 25 cm) in the affinity chromatography buffer.

Preparation of LacCer-aldehyde Sepharose. Steps in the synthesis of the lactosylceramide (LacCer)-aldehyde affinity column are shown in Fig. 1. Thirty mg of LacCer (I), purified from AB+ human red blood cells as previously described (28), was solubilized in 1.2 ml pyridine to which 0.9 ml acetic anhydride was slowly added with gentle stirring. The acetylation was allowed to proceed overnight at room temperature. The reaction mixture was evaporated to dryness via addition of toluene. The acetylated LacCer (II), AcLacCer, was resuspended in 5.0 ml chloroform and the allylic double bond of the sphing-4-enine residue was oxidized, in the presence of excess ozone for 40 min at -50^{o}C with 90 volts at 6 psi O_2, to the ozonide derivative (III). The AcLacCer ozonide was converted to the aldehyde (IV) by reduction of the ozonide with excess Zn in acetic acid. This derivative was taken to dryness under a stream of N_2. The AcLacCer-aldehyde (IV) was recovered in the aqueous methanolic phase by aqueous methanol/hexane (1:1, v/v) partition and verified by HPTLC in a dual solvent system, dichloroethane/acetone (1:1, v/v) followed by

Figure 1. Chemical Synthesis of LacCer-aldehyde Sepharose. The synthesis of the lactosylceramide-aldehyde-Sepharose 4B affinity resin is described under Experimental Procedures. The compounds are identified as follows: I.) LacCer; II.) acetylated lactosylceramide (AcLacCer); III.) AcLacCer ozonide; IV.) AcLacCer-aldehyde; V.) Schiff base intermediate; VI.) AcLacCer-aldehyde-Sepahrose 4B; VII.) LacCer-aldehyde-Sepharose 4B.


Pyr
Ac2O
Dry
CHCl3
O3
Zn/AcOH
NH2(CH2)6NH-Sepharose
NaBH4/MeOH
NaOH
I
II
III
IV
V
VI
VII

chloroform/methanol/water (60:30:5, v/v/v). The AcLacCer-aldehyde (IV) was linked to a methanolic suspension of prewashed 6-aminohexyl-Sepharose 4B. The Sepharose was prewashed twice with double distilled H_2O and three times with MeOH. The AcLacCer-aldehyde and gel were allowed to react for 18 h at 4°C. The Schiff base (V) was reduced with a 10-fold excess of $NaBH_4$ in methanol for 1 h, and the acetyl groups (VI) were removed by mild alkali-catalyzed hydrolysis to give the LacCer-aldehyde affinity ligand (VII). The affinity resin was equilibrated with affinity chromatography buffer.

Chromatography on LacCer-aldehyde Sepharose. Active fractions of SAT-1 from the CMP-Sepharose II step were directly subjected to LacCer-Sepharose affinity separation on a 1.0 cm x 10 cm column. The column was previously equilibrated with 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO and 10 mM $MnCl_2$ affinity chromatography buffer at a flow rate of 2.5 ml/h. After washing the column with five volumes of affinity buffer at a flow rate of 10 ml/h, SAT-1 was eluted from the column with affinity buffer containing 1 mM CMP. Active fractions were pooled, concentrated and the nucleotide removed by gel filtration on Sephadex G-25 (fine). The enzyme was stored at -20°C in the 25 mM sodium cacodylate containing 5% (v/v) LDAO for 6-12 months without appreciable loss of activity (29).

3. STRATEGY FOR DEVELOPING A MONOCLONAL ANTIBODY TO SAT-1 AND ITS APPLICATION IN SAT-1 PURIFICATION

Production of Monoclonal Antibodies to SAT-1. Monoclonal antibodies to rat liver SAT-1 were raised to the 40,000-fold CMP/LacCer affinity-purified enzyme. Fifty μg of active affinity-purified SAT-1 was suspended in 2.0 ml of Ribi Probe MPL-TDM Emulsion (Ribi Immunochem Research Co.) by sonication. The fusion protocol used for the production of anti-SAT-1 hybridomas and the Western blot and ELISA screening protocols were performed as described (30). Hybrids were produced by the fusion between SP2/0-Ag14 mouse myeloma cells (2-3 x 10^6 cells/ml) and mouse

spleen cells (2-3 x 10^7 cells/ml) three days post booster. Hybridomas were cultured and maintained in HT-containing RPMI 1640 with 10% (v/v) fetal bovine and 10% (v/v) donor horse sera at a cell density of 1 x 10^5 to 1 x 10^6 cells/ml. Hybridomas were screened by ELISA and immunoprecipitation for anti-SAT-1 activity. The anti-SAT-1 positives were cloned by the method of limiting dilution (30) and the resulting monoclonal hybridomas were grown and the culture media screened as described before for the hybridomas. Anti-SAT-1 producing monoclonal hybridomas were established, expanded and cultured in serum-free RPMI 1640 containing 1% (v/v) Nutridoma-SP, 10% NCTC- 109, hypoxanthine, thymidine, penicillin and streptomycin with 7.5% CO_2. The medium containing the secreted monoclonal antibody (MAb M12GC7) to SAT-1 was collected, clarified by centrifugation at 2000 x g for 10 min at 22°C and concentrated using an Amicon YM100 membrane. Alternatively, M12GC7 anti-SAT-1 antibodies were recovered from ascites fluid.

The isotype of M12GC7 was determined using the Amersham's mouse antibody isotyper kit according to the manufacturer's directions.

Monoclonal antibodies were purified using the BioRad MAPS II Protein A HPLC system according to the manufacturer with some modification. M12GC7 was bound to the MAPS II column in MAPS II binding buffer according to the manufacturer. The column was washed with a 1:10 dilution of MAPS II elution buffer. The monoclonal antibodies were eluted on a linear gradient established between the diluted elution buffer and the MAPS II elution buffer.

Inhibition of SAT-1 Activity by M12GC7. The effect of monoclonal antibody M12GC7 on SAT-1 activity was determined by preincubating 10 μg of affinity-purified sialyltransferase with 1.2, 2.4, 6.0 and 12 μg antibody, in duplicate, for 30 min prior to performing the standard SAT-1 activity assay described above. Controls were run with heat-inactivated enzyme and no enzyme.

Specificity of M12GC7. The specificity of M12GC7 to a variety of Proteins was examined by Western Blot and ELISA of M12GC7 . 1-10 μg of mouse IgG, purified SAT-1, detergent-solubilized Golgi, ST2-3 and ST2-6 (Genzyme), α2-6 sialyltransferase (BMB), human transferrin, human fetuin, human glycophorin, soybean lectin, *and E. coli* ß-galactosidase, and *E. coli* acetate kinase were examined. M12GC7 in PBS, pH 7.2, (for ELISA) or in TBS, pH 7.4, (for Western) containing 1% (w/v) BSA at a final concentration of 5 μg/ml was incubated under the appropriate conditions. Immunodetection, with the appropriate reagents, was accomplished with goat anti-mouse IgG alkaline phosphatase as a secondary antibody.

Immunoaffinity Purification of SAT-1. Anti-SAT-1 monoclonal antibody (3 mg), isolated and characterized as described above, was bound to 6 ml of Affi-Gel 10 (BioRad) according to the manufacturer's instructions. The anti-SAT-1-Affi-Gel 10 affinity column was equilibrated with 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO, 10 mM $MnCl_2$, 1 mM PMSF, and 0.1 M NaCl. Rat liver Golgi vesicles were prepared and detergent-solubilized, as described above except that several cathepsin and thiol protease inhibitors (1 mM PMSF, 0.3 mM aprotinin, 1 mM pepstatin, 1 mM leupeptin, 135 mM TLCK-HCl, 284 mM TPCK, and 2.8 mM E-64) were added. SAT-1, from the detergent extract, was batch-adsorbed to the immunoaffinity ligand overnight with gentle rocking at 4^{o}C. The column was washed with 10-20 column volumes of the same salt-containing buffer until no protein could be detected by A_{280nm}. The SAT-1 sialyltransferase was then eluted with 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO, 10 mM $MnCl_2$ and 1.0 M NaCl.

4. ASSESSMENT OF PURITY AND BIOLOGICAL ACTIVITY

Verification of SAT-1 Homogeneity. SDS polyacrylamide gel electrophoresis (31) was carried out on 5-20 μg of SAT-1 and silver-stained (32). Prior to electrophoresis, the enzyme was removed from its detergent-containing buffer first by precipitation in absolute ethanol (1:9 ratio sample to ethanol) at -20^{o}C for 24-48 h

followed by acetone (HPLC grade) precipitation 12-18 h. With each precipitation step, the sample was centrifuged at 14,000 x g for 30 min, all but 25 μl of supernatant removed so as not to disrupt the protein pellet, and the sample taken to dryness in a Savant speedvac. The standard proteins (BioRad) for SDS-PAGE were lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (42,700), bovine serum albumin (66,200) and phosphorylase B (97,400).

Localization of SAT-1 Activity with Silver-Stained Protein. Duplicate SDS-PAGE gels containing 5 μg SAT-1 were run for detection of CMP/LacCer affinity-purified and M12GC7 immunoaffinity-purified SAT-1. Detection of SAT-1 was made using either of two parallel approaches. First, enzyme activity of the protein was assessed by cutting the gel lane into slices and incubating the samples in SAT-1 assay mixture to localize the enzyme activity with the silver-stained protein. Alternatively, Western blots (described above) of SAT-1 on Immobilon (Millipore Corp.) were detected with anti-SAT-1 monoclonal antibody M12GC7 (5 μg/ml) and Goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibodies (BioRad).

SAT-1 Specificity. The specificity of SAT-1 to various glycoconjugates was examined. The methods are described below.

Method #1: Specificity for Various Glycosphingolipid Substrates. The specificity of SAT-1 toward various glycosphingolipid substrates was examined by direct assay of the sialyltransferase activity on substrates chromatographed on high-performance-thin-layer-plates (HPTLC) as described (33), with some modifications. Briefly, 10 nmol of each glycosphingolipid substrate was chromatographed on Merck HPTLC plates containing fluorescence indicator. (Note: being of a harder composition, this type of plate withstands the washing process better than plates without indicator. Also, lipids can be detected prior to fixing by UV illumination or iodination.) The lipids were chromatographed with chloroform/methanol/0.2% $CaCl_2$ (60:40:9, v/v/v). The HPTLC plate was dried *in vacuo* 18 h and then developed with

iodine to clearly define and mark the glycolipid boundaries. Once the lipid boundaries were marked lightly with a pencil, the iodine was evaporated from the plate by incubating the plate at 55°C for a few min. This treatment to remove the iodine did not appear to affect the glycosphingolipids or their ability to serve as receptor substrate (C. Sranka and R. Laine, Glycomed, Alameda, CA, *personal communication*). The dried plates were immersed in n-hexane for about 30 sec, fixed in 0.1% (w/v) polyisobutylmethylacrylate, PIBMA, (Aldrich Chemical Co., lot #03639KT) in n-hexane for 30 sec, and then dried completely and stored at -20°C until used. The PIBMA fixing solution was prepared from a stock solution of 10% (w/v) PIBMA in chloroform. Prepared HPTLC plates were incubated with 0.1 mg/ml purified SAT-1 enzyme in 25 mM sodium cacodylate (pH 6.5) containing 10 mM $MnCl_2$, 5% (v/v) LDAO and 240 nmol of CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid with a specific activity of 22,200 dpm/nmol (NEN lot #2655-018, CMP-NeuNAc from Sigma lot #86F-7931). A final volume of 1.0 ml was pipetted onto a 2.0 x 4.0 cm plate and incubated for 60 min at 37°C in a 6 cm tissue culture dish. Following incubation, the plate was carefully transferred to a clean dish and was washed five times with 5.0 ml phosphate-buffered saline (PBS) containing 0.05% Tween 20 (ELISA grade). The positions of the glycosphingolipids were detected with orcinol and the radioactivity quantitated by scraping the glycosphingolipids into scintillation vials containing 0.2 ml double distilled H_2O. Following sonication for 10 min, 5 ml Safety-Solve (Research Products International Corp.) was added and the amount of [^{14}C]-sialic acid transferred determined on a Packard Tri-Carb 460C Liquid Scintillation System. Three controls were with no acceptor, with no enzyme, and with boiled enzyme.

Method #2: Specificity for Various Glycoprotein Substrates. SAT-1 specificity to various glycoprotein substrates was determined following incubation of 50 μg of each glycoprotein with 10 μg immunoaffinity-purified SAT-1, 50 nmol of CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid (NEN lot #2655-018, specific activity adjusted to 22,200

dpm/nmol with CMP-sialic acid Sigma lot #86F-7931) in a final volume of 0.1 ml in 25 mM sodium cacodylate (pH 6.5) containing 10 mM $MnCl_2$ and 5% (v/v) LDAO. The reactions were terminated by the addition of 0.9 ml absolute ethanol (final ethanol/H_2O ,90:10). The proteins were precipitated at -20°C for 48 h in 90% ethanol followed by 18 h in acetone, as described. The amount of radiolabeled sialic acid incorporated was determined by counting the protein pellets in scintillant. Additionally, duplicates of the glycoproteins were subjected to SDS-PAGE followed by staining with Coomassie Blue. The radioactivity associated with each glycoprotein was determined by cutting the protein from the gel and counting the amount of [^{14}C]-sialic acid transferred in 5 ml of Safety-Solve (Research Products International), sonicated 5 min and counted on a Packard Tri-Carb 460C Liquid Scintillation System. Controls were run with α2-6 ß-galactosyl sialyltransferase and the same glycoprotein acceptors.

Glycan Detection. Immunoaffinity-purified SAT-1 was electrotransferred onto Immobilon and the Western blot was cut into 0.5 cm strips, each containing approximately 0.7 μg/cm SAT-1. The SAT-1 was incubated with biotinylated lectins obtained from E-Y Labs (San Mateo, CA) and digoxigenin-labeled lectins from Boehringer (Indianapolis, IN) under the conditions suggested by the manufacturer for analysis of the carbohydrate moieties of SAT-1.

Two-Dimensional Gel Electrophoresis. Analysis of rat liver Golgi SAT-1 by 2D SDS-PAGE was performed as described by Dunbar (34). The purified sialyltransferase, 10 μg, was isoelectrofocussed in the first dimension in tube gels according to the O'Farrell method (35) in the second dimension on 10-20% SDS-PAGE according to the Laemmli system (31), as described by Dunbar (34) using the BioRad mini 2D Protean II system. The carrier ampholytes were a mixture of 4 parts Pharmacia Pharmalytes pH 5-8 and 1 part (LKB Ampholines pH 3.5-10/Pharmacia Pharmalytes pH 3-10, 1:1). The 2D SDS-PAGE patterns were analyzed by the Bio

Image Visage 110 computerized digital image analysis system (Millipore, Milford, MA).

RESULTS

Detergent Extraction. As described under "Materials and Methods," Golgi were prepared from rat liver homogenates by differential centrifugation and discontinuous sucrose density gradient centrifugation. Analysis of the preparation for enzyme markers showed that SAT-1 and thiamine pyrophosphatase, a Golgi enzyme marker, were recovered from the same fractions. The rat liver Golgi, prepared by established TEM procedures (36,37), were observed to be pure intact vesicles. Sonication and gentle agitation of the Golgi vesicles in 25 mM sodium cacodylate containing 10 mM $MnCl_2$ and 15% (v/v) lauryl dimethylamine oxide (LDAO) gave the most effective solubilization. The merits of this surfactant for the purification of SAT-1 are described (29).

Preliminary Characterization of SAT-1. For optimal enzyme assay conditions and affinity purification, SAT-1 activity from rat liver Golgi vesicles was characterized with respect to substrate binding, stability, requirements for maximal activity and potential inhibitors. The data correlated well with those reported for SAT-1 in studies using homogenates of embryonic chicken (38) and cultured hamster fibroblasts (16). The apparent K_m values for the CMP-sialic acid and LacCer substrates were 0.26 and 0.11 mM, respectively; the V_{max} values were 26.3 and 18.7 pmoles of GM_3 formed per min per mg protein. SAT-1 was inhibited to the same extent by either 1.0 mM CMP, 0.5 mM CDP or 0.1 mM CTP. The enzyme required 10 mM $MnCl_2$ for optimal activity . SAT-1 was most active at pH 6.5 in sodium cacodylate with detectable activity in the pH range of 6.1 to 7.6.

Affinity Purification of SAT-1 from Rat Liver Golgi. The affinity purification of SAT-1 on CMP- and LacCer-Sepharoses is summarized in Table 1. SAT-1 was purified 43,000-fold over crude homogenate from detergent-extracted rat liver Golgi. Conditions were optimized for affinity purification based on preliminary

Table 1

AFFINITY PURIFICATION OF SAT-1

Fraction	Volume (ml)	Protein (mg)	Units (pmol/min)	Specific Activity (units/mg)	Purification (-fold)
100g rat liver					
Crude Homogenate	325	34100	3750	0.11	1.00
Post Mitochondrial Supernatant	365	17700	4500	0.25	2.28
Detergent Extracted Golgi	57.0	73.0	1050	14.4	130
CMPI(NaCl)	12.5	4.0	350	87.5	795
CMPII(CMP)	2.6	0.5	82	160	1450
LacCer(CMP)	11.0	1.0	4660	4710	42800

characterization of the enzyme activity in rat liver Golgi. Throughout the purification procedure, SAT-1 sialyltransferase activity wasassayed with human RBC LacCer or the commercially available LacCer containing N-palmitoylsphinganine.

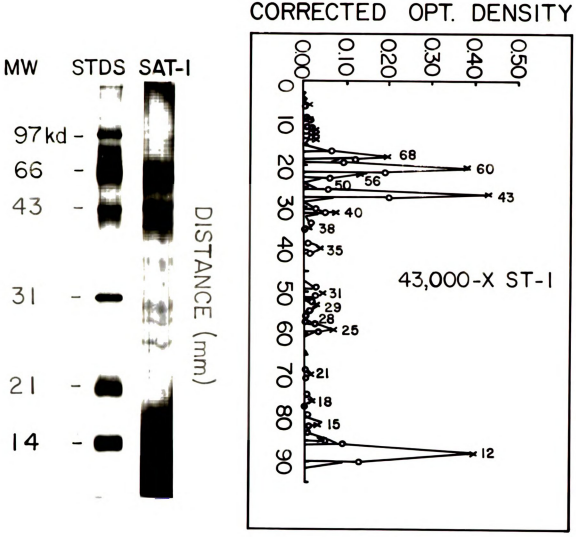
SAT-1 activity was recovered from CMP I with a linear salt gradient between 0.36-0.4 M NaCl. The SAT-1 active fraction was pooled, desalted and rechromatographed onto the CMP II column. SAT-1 was recovered at 0.1-0.15 mM CMP using a linear CMP gradient. Further washing of the CMP II column with buffer containing 5 mM and 10 mM CMP resulted in no further recovery of enzyme. The final sialyltransferase affinity step was LacCer-aldehyde Sepharose. The enzyme was eluted with 1 mM CMP.

Assessment of Affinity-Purified SAT-1 by Gel Electrophoresis. The 43,000-fold purified enzyme gave nine polypeptides on a 12.5% SDS-PAGE (Fig. 2) with five predominant molecular weight species at 68, 60, 43, 25 and 12 Kd. Similar size heterogeneity, attributed to proteolytic degradation (6,21, for review 4), has been reported for other purified sialyltransferases (9,10,18-21). Further, the electrophoretic profile of the 40,000-fold affinity-purified enzyme could be varied with time and temperature of solubilization in SDS-PAGE sample buffer (data not shown). Only four polypeptides were resolved following solubilization of SAT-1 in sample buffer for 2 min at 95^{o}C, while nine polypeptides were present in gels when the SAT-1 preparation had been treated for 30 min at 37^{o}C.

The enzyme activity of each of these polypeptides was examined following electroelution from the gel. As judged by specific activity, 27% of the total activity applied to the gel was recovered. The pooled 68 and 60 Kd polypeptides exhibited the most activity (57% of the total recovered activity). A small amount of activity was associated with the 25 and 12 Kd polypeptides. No activity was associated with the 43 Kd band.

Figure 2. Gel Electrophoresis of Affinity-Purified SAT-1. SDS-PAGE gel electrophoresis was performed on 15 μg of SAT-1, treated as described under Methods to remove the LDAO detergent and then electrophoresed under the conditions of Laemmli (31) on a 12% SDS-PAGE gel. Five predominant molecular weight species were resolved at 68, 60, 43, 25 and 12 Kd. Similar size heterogeneity has been reported for other purified sialyltransferases (9,10,18-21).

AFFINITY PURIFIED SAT-1



Treatment of SAT-1 with cathepsin D gave an increases in the 56 Kd polypeptide. Digestion of both the 60 Kd and 56 Kd species with cathepsin D and N-glycanase gave an increase in the 43 Kd band, suggesting that they were the same activity and that SAT-1 was a glycoprotein. A Cathepsin D-like activity has been found to be associated with the conversion of the "mature" 47 Kd membrane-bound form of the ß-galactoside α2-6 sialyltransferase to its soluble 41 Kd form (39, for review 4).

The ability to manipulate the electrophoretic pattern of the sialyltransferase suggested there may be aggregation of the membrane protein when solubilized at high temperature in SDS. Likely too is enhanced proteolytic degradation at 37°C even in the denaturing conditions of SDS sample buffer. The loss of activity following elution to verify the SAT-1 protein may have been due to loss of protein during electroelution, failure to renature or production of an inactive proteolytic fragment. The 60 Kd polypeptide was suspected to be the mature form of the enzyme and the other peptides were either subunits of the mature form or proteolytic fragments.

Since the possibility of protein contaminants could not be ruled out, monoclonal antibodies were raised to the 40,000-fold affinity-purified SAT-1. The advantages of immunoaffinity purification of SAT-1 were that SAT-1 could be isolated from contaminants and minimal time was required for the purification (4 days total), lessening the amount of time the enzyme would be subjected to conditions allowing proteolytic degradation.

Specificity of M12GC7 Anti-SAT-1 Monoclonal Antibody. With these several possible explanations for the heterogeneity of the 43,000-fold affinity purified SAT-1 (i.e., contaminating protein(s), proteolytic degradation, subunits, etc.), monoclonal antibodies were raised against this "partially-purified" SAT-1 to resolve the issue and purify SAT-1. As described under "Materials and Methods" mouse anti-rat liver Golgi SAT-1 monoclonal antibodies were obtained. One monoclonal (M12GC7),

on the basis of its ability to immunoprecipitate and inactivate SAT-1, was chosen for immunoaffinity purification of SAT-1. M12GC7 markedly inhibited SAT-1 (Fig. 3).

The specificity of M12GC7 was examined by Western Blot analysis. M12GC7 was found to react only with SAT-1 and Goat anti-mouse IgG. No reaction was observed with ST2-6, ST2-3, human transferrin, human albumin, human fetuin, *E. coli* ß-galactosidase and acetate kinase (data not shown). The results were confirmed by ELISA (data not shown).

Purification of SAT-1 to Apparent Homogeneity by Immunoaffinity Chromatography. Anti-SAT-1 monoclonal antibody, M12GC7, coupled to Affi-Gel 10 (BioRad) was used to purify SAT-1 to apparent homogeneity. To reduce the amount of fragmentation possibly caused during the mechanical disruption of the enzyme, sonication of the detergent extract was reduced to 5 min. To limit endogenous proteolysis of SAT-1 during purification, possibly by a cathepsin-like activity similar to that identified for ß-galactoside α2-6ST (40, reviewed 4), several cathepsin and thiol protease inhibitors were kept in mixtures during the purification. All of these protease inhibitors, with the exception of PMSF, inactivated the enzyme (data not shown). However, they allowed recovery of a homogeneous protein, as assessed by SDS-PAGE and verified by Western Blot to Immobilon and immunodetection with M12GC7. The apparent molecular weight of SAT-1 was approximated at 60,000 daltons as judged by SDS-PAGE. This analysis is shown in Fig. 4.

The capacity of the anti-SAT-1 Affi-Gel 10 column for SAT-1 from detergent-extracted Golgi, as tested so far, is about 100-150 μg SAT-1 from 20 g rat liver and purification data from a subsequent preparation with only PMSF and without the cathepsin and thiol protease inhibitors is summarized in Table 2. Purification of SAT-1 by immunoaffinity chromatography to 37,000-fold was found to be comparable to that obtained by affinity chromatography on CMP- and LacCer-aldehyde- Sepharoses. Employing the same immunoaffinity column, SAT-1 has also been isolated from human

Figure 3. Inhibition of SAT-1 activity by M12GC7 monoclonal antibody. A monoclonal antibody was raised to the 40,000-fold affinity-purified sialyltransferase as described under the Methods section. Increasing amounts of M12GC7 were preincubated for 30 min with 10 μg of the "partially-purified" SAT-1 enzyme. The open squares represent SAT-1 activity present in reaction samples without added M12GC7. The inhibition of SAT-1 by the addition of increasing amounts of M12GC7 is shown with closed diamonds. All SAT-1 activity was abolished in samples containing 2 or more μg of M12GC7. Controls were run with heat-inactivated enzyme and no enzyme.

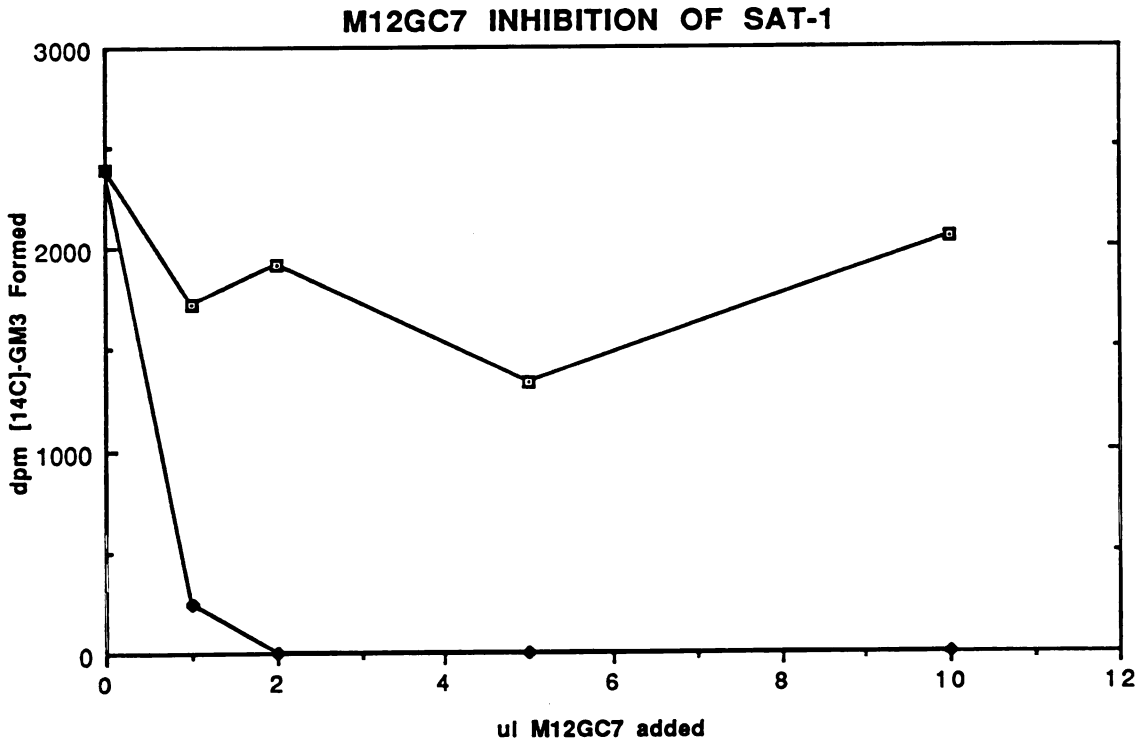

M12GC7 INHIBITION OF SAT-1
dpm [14C]-GM3 Formed
3000
2000
1000
0
0
2
4
6
8
10
12
ul M12GC7 added

Figure 4. Assessment of Immunoaffinity-Purified SAT-1 Homogeneity by SDS-PAGE Gel Electrophoresis. Electrophoresis and Western blotting of 5 μg of the immunoaffinity-purified SAT-1 was described in the methods. Verification of the protein as SAT-1 was by immunodetection with M12GC7 and alkaline phosphatase-conjugated secondary antibody. Immunolocalization of SAT-1 was necessary because addition of protease inhibitors inhibited the enzyme both in standard assay protocols and activity assays preformed on gel slices from a duplicate gel. Following immunodetection, only one band was resolved at 60,000 daltons.

B
2
1

A
1 2

Table 2

IMMUNOAFFINITY PURIFICATION OF SAT-1

Fraction	Volume (ml)	Protein (mg)	Units (pmol/min)	Yield (%)	Specific Activity (units/mg)	Purification (-fold)
20g rat liver						
Crude Homogenate	106	27880	5940	100	0.15	1.00
Post Mitochondrial Supernatant	88	3476	5100	86	0.8	5.33
Detergent Extracted Golgi	17.2	N.D.	1170	31	N.D.	-
αSAT-1	1.2	0.1	440	7.4	5500	36670

KB cells. One note of caution, the proteases associated with these sialyltransferases can also degrade the Affi-Gel bound antibody.

Specificity of SAT-1. The preferred substrate for the purified SAT-1 is LacCer with glucosylceramide (GlcCer), galactosylceramide (GalCer) and asialoGM_1 also serving as substrates, but to a lesser extent (Table 3). SAT-1 appears to recognize the saccharide ß1-O-Cer linkage, with the disaccharide being preferred over the monosaccharide. There was not any significant amount of sialic acid (NeuAc) incorporated into any other glycoconjugate examined. The radioactivity recovered with GM_1 and GD_{1a} was due to a minor asialoGM_1 contaminant in the glycosphingolipid substrate. These data were verified following sialyltransferase activity assays with glycosphingolipid immobilized on polystyrene (Falcon) microtiter plates (40) or by SDS-PAGE for glycoprotein substrates. Control assays with ST2-6 were performed.

SAT-1 Glycan Detection. The carbohydrate moieties of SAT-1, bound to Immobilon, were characterized with biotin- and digoxigenin-labeled lectins. These data are summarized in Table 4. Positive binding of the biotinylated lectins and digoxigenin-conjugated lectins enables the immunological detection of the carbohydrate structures. There may be both N- and O-linked carbohydrate side chains as indicated by the binding of DSA to the SAT-1 glycoprotein. The carbohydrate side chain(s) on SAT-1 contain galactose, indicated by the positive reaction of BPA, GS-I, and PNA. The carbohydrate structures may also contain GlcNAc as indicated by the strong reaction with ConA and WGA. Further, the positive reaction of ConA indicates the carbohydrate structures are branched N-glycans containing mannose. It is unlikely that there are terminally linked mannose residues as the reaction with GNA was negative. The presence of N-linked sugars is also indicated by a positive reaction of SAT-1 with WGA, which recognizes N-acetyl chitobiose units of asparagine-linked oligosaccharides. Further, binding of WGA could result from the presence of NeuAc. Positive reaction with the LPA, SNA and MAA lectins, which recognize sialic acid

Table 3

SAT-1 SPECIFICITY

Acceptor	Structure	NeuAc Incorporated *pmol*	Specific Activity *pmol/min x mg*	Relative Intensity %
Method #1				
LacCer	Galβ1-4Glcβ1-O-Cer	80.5	2680	100.0
GlcCer	Glcβ1-O-Cer	52.7	1760	65.7
GalCer	Galβ1-O-Cer	32.7	1090	40.7
AsialoGM$_1$	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-O-Cer	67.3	2240	83.6
GD$_3$	(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-O-Cer	7.3	243	9.1
GD$_{1b}$	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-O-Cer	0.0	0	0.0
GT$_{1b}$	(NeuAcα2-3)Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcb1-O-Cer	0.0	0	0.0
GM$_3$	(NeuAcα2-3)Galβ1-4Glcβ1-O-Cer	2.5	83	0.3
GM$_2$	GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-O-Cer	5.9	197	7.4
GM$_1$	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-O-Cer	4.5	152	5.7
GD$_{1a}$	(NeuAcα2-3)Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-O-Cer	11.8	394	14.7
Method #2				
Fetuin		1.1	5	0.2
Asialofetuin	(Galβ1-4GlcNAc)$_3$(Man)$_2$(GlcNAc)$_2$-N-Asn	18.4	82	3.1
	(Galβ1-4GlcNAc)$_2$(Man)$_2$(GlcNAc)$_2$-N-Asn			
	Galβ1-3GalNAcα1-O-Thr/Ser			
Mucin		4.0	18	0.7
Asialomucin	GalNAcβ1-3(Fucα1-2)Galβ1-3GalNAcα1-O-Thr/Ser	6.9	31	1.2
	Galβ1-3GalNAcα1-O-Thr/Ser			
α_1-acid glycoprotein		0.5	2	0.0
asialo α_1-acid glycoprotein	(Galβ1-4GlcNAc)$_4$(Man)$_3$(GlcNAc)$_2$-N-Asn	0.0	0	0.0
Glycophorin		0.0	0	0.0
Asialoglycophorin	Galβ1-3GalNAcα1-O-Thr/Ser	0.2	1	0.0

Table 4

GLYCAN DIFFERENTIATION OF SAT-1 CARBOHYDRATE SIDECHAINS

Lectin	Carbohydrate Specificity	Reaction
E-Y Labs - Biotinylated Lectins		
BPA	Gal & GalNAc	+
ConA	α-Man > α-Glc > GlcNAc	+
GS-I	melibiose & α-D-Gal	+/-
LPA	NeuNAc	+
PNA	Galß1-3GalNAc > α-Gal & ß-Gal	+/-
SBA	α- & ß-GalNAc > α- & ß-Gal	+
UEA-I	α-L-Fucose	+/-
WGA	$(GlcNAcß1\text{-}4GlcNAc)_{1\text{-}4}$ > ß-GlcNAc > Neu5Ac	+
Controls		
M12GC7	SAT-1	+
DBA	Me-2-acetamide-2-deoxy-D-Gal	+[1]
BMB - Digoxigenin-labeled Lectins		
GNA	Mannose (terminally linked)	-
SNA	NeuAcα2-6Gal or NeuAcα2-6GalNAc	+
MAA	NeuAcα2-3Gal	+
PNA	Galß(1-3)-GalNAc	-
DSA	Galß(1-4)GlcNAc-(in N-glycans) & GlcNAc-Ser/Thr	+

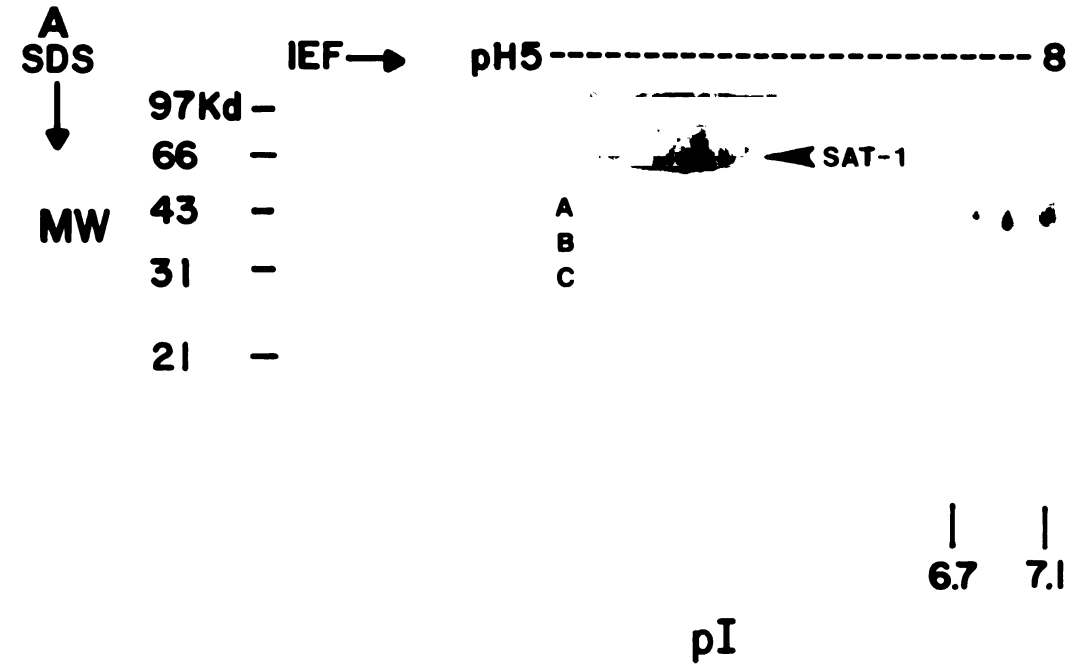
[1] Detection of LDAO by lectin.

residues, substantiate the presence of NeuAc on SAT-1 and suggest both α2-3 and α2-6 NeuAc linkages as defined by MAA and SNA, respectively. Reaction with DBA may have been due to the presence of some LDAO in the SAT-1 preparation.

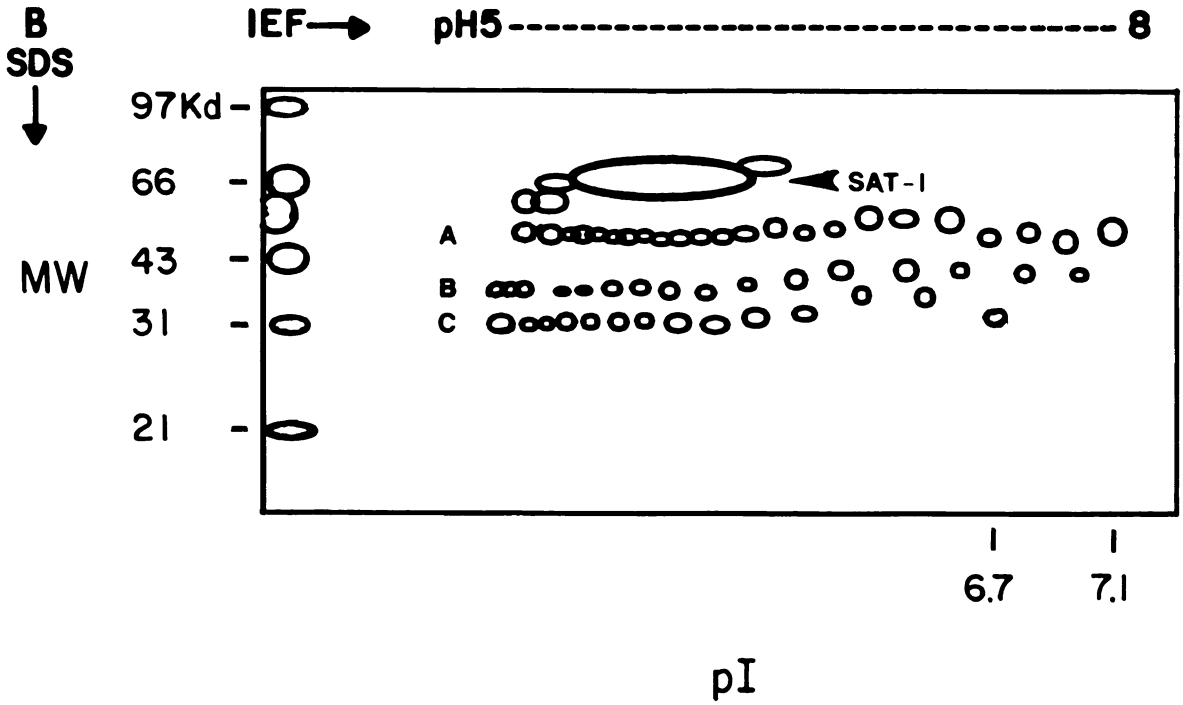
Two-Dimensional Gel Electrophoresis of SAT-1. The two-dimensional protein pattern of rat liver SAT-1 was established by standard 2D SDS-PAGE methods (34,35,31,32). The 60 Kd SAT-1 was estimated to be in the pI range of 5.7 to 6.2 following computerized digital image analysis on the Bio Image Visage 110 using internal molecular weight (BioRad) and carbamylated pI standards (Pharmacia) (Fig. 5A and B). The high degree of glycosylation of SAT-1 is indicated by the lateral spread of the protein due to greater molecular and charge heterogeneity. Establishment of the 2D pattern of SAT-1 is a necessary step for analyzing post-translational covalent modification of SAT-1 by metabolic labeling experiments.

Figure 5. Analysis of SAT-1 by 2D SDS-PAGE. SAT-1, 10 μg, was analyzed by 2D SDS-PAGE (34) following ethanol and acetone precipitation as described in the text. The first dimensional IEF gel was according to the method of O'Farrell (35). The ampholyte mixture was 1 part pH 3-10 and 4 parts pH 5-8. A standard 12% polyacrylamide electrophoretic gel was run according to Laemmli (31) and silver-stained by the method of Merril *et al.* (32). The approximate molecular weight and pI range of SAT-1, 60,000 daltons and 5.7 to 6.2, respectively, were estimated following a computerized digital image analysis on the Bio Image Visage 110 relative to internal molecular weight (BioRad) and carbamylated pI (Pharmacia) marker. Panel A is the SAT-1 protein pattern obtained and Panel B is the computer diagrammatic representation of the detected protein and standards.

2-D SDS-PAGE (pH5-8) SAT-1



A = CPK
B = GADPH
C = CA



DISCUSSION

The cell surface plays an important role in the regulation of mammalian cell growth and differentiation. Gangliosides, ubiquitous sialic acid-containing glycosphingolipids of the plasma membrane, are implicated in these phenomena. Changes in ganglioside composition and metabolism are associated with cell proliferation and oncogenic transformation (1-3). Further, marked variations in ganglioside composition found during differentiation and aging (1), following transformation (2) or treatment of cells with hormones (3) or chemicals (41-52) are largely due to alterations of the glycosyltransferase activities. Purification of the enzymes regulating specific glycosylation steps within the lumen of the endoplasmic reticulum and Golgi apparatus may lead to an understanding of the functional role of gangliosides in cellular proliferation.

It has been postulated that several glycosyltransferases may be involved in the main level of regulation of ganglioside biosynthesis (53-57). Whether at the transcriptional or post-translational level, regulation appears to be primarily dependent on one galactosyltransferase, GalT-1, and three sialyltransferases, SAT-1, SAT-2 and SAT-3 (53-57). These enzymes function at branch points between the asialo, a, b and c series of glycosphingolipids and are involved in the conversion of GlcCer to LacCer, LacCer to GM_3, GM_3 to GD_3, and GD_3 to GT_3, respectively.

There has been limited success in the purification of these glycosphingolipid glycosyltransferases. The acceptor glycolipid acid-Sepharoses first synthesized by Makita and coworkers (5,7-8) have been found to be ineffective in the purification of GalT-1 (S. Chatterjee, *personal communication*) and SAT-1. Gu *et al.* (10) have recently reported a 10,000-fold purification of SAT-2 using a glycolipid-acid column; however, no substrate specificity was reported.

Purification of SAT-1 to apparent homogeneity was achieved by affinity chromatography on CMP-Sepharose and LacCer aldehyde-Sepharose. The size

heterogeneity, as originally observed by SDS-PAGE, may have been due to proteolytic degradation during purification by a cathepsin-like activity, as described by others (21,39, for review 4). Digestion of SAT-1 with cathepsin and N-glycanase indicated this possibility.

Verification that SAT-1 was purified to apparent homogeneity required the production of an anti-SAT-1 specific monoclonal antibody, M12GC7, capable of immunoprecipitating and inactivating SAT-1. Using an M12GC7-Affi Gel 10 immunoaffinity column, in the presence of several thiol and cathepsin protease inhibitors, SAT-1 was resolved as a homogeneous band by SDS-PAGE. The apparent molecular weight of the 37,000-fold purified SAT-1 was 60,000 daltons as judged by SDS-PAGE. Immunoresolution of a minor band at 56,000 daltons suggests some endogenous proteolytic activity may have been associated with the enzyme even after antibody chromatography.

SAT-1 catalyzes the transglycosylation reaction between its sugar nucleotide donor, CMP-NeuAc, and oligosaccharide-lipid acceptor substrate Galß1-4Glcß1-O-Cer. SAT-1 appears to be specific for the saccharide-ß1-O-Cer linkage. LacCer is the preferred substrate although GlcCer (Glcß1-O-Cer), GalCer (Galß1-O-Cer), and asialo GM_1 (Galß1-3GalNAcß1-4Galß1-4Glcß1-O-Cer) serve as substrate to a lesser extent. Sandhoff and coworkers (53-55) have suggested that SAT-1, SAT-2 and SAT-3 are unique regulatory enzymes in the ganglioside biosynthetic cascade in that they recognize the carbohydrate lipid linkage and not the carbohydrate "backbones" (53). In contrast, SAT-4, the α2-3 sialyltransferase purified by Joziasse *et al.* (9), exhibits specificity for the particular carbohydrate sequence, Galß1-3GalNAcß1-4Galß1-4Glc and recognizes this sequence in gangliosides (GM_1 and GD_{1b}) and glycoproteins. Basu *et al.* (12) have categorized several glycosyltransferases into two distinct groups based on their ability to recognize specific acceptor substrate sequences. These recognition sites are either for a specific hydrophobic and carbohydrate sequence or for

a particular carbohydrate acceptor sequence. They termed these two classes of glycosyltransferases HY-CAR and CAR, respectively (12).

The ability of SAT-1 to recognize GalCer is supported by early studies of Yu and Lee (58) which suggest SAT-1 catalyzes the transfer of sialic acid to both GalCer and LacCer, forming GM_4 and GM_3, respectively. In their experiments with a mouse brain microsomal fraction, both substrates served equally well. The activity of our purified SAT-1 is two-fold greater for LacCer than for GalCer (or GlcCer). It is presumed that the carbohydrate/hydrophobic recognition site of SAT-1 prefers the conformation of the disaccharide linked to ceramide over the monosaccharide-ceramide linkage. This does not, however, discount the possibility of tissue specificity of the SAT-1 sialyltransferase for these two acceptor substrates; GM_4 is found almost exclusively in the brain and GM_3 is found in liver and many other tissues. Tissue-specific expression of ß-galactoside α2-6 and α2-3 sialyltransferases mRNA has been observed by Paulson and coworkers (59) and by O'Hanlon *et al.* (60).

The observation that asialo GM_1 is also a substrate for SAT-1 is more difficult to rationalize. While we expect SAT-1 to be involved in the sialylation of terminal Gal moieties, we have not ruled out the possibility that SAT-1 is capable of sialylating either one or both Gal residues of asialo GM_1, forming GM_{1b}, GM_{1a}, or GD_{1a}. We are investigating this possibility through substrate competition analyses, specific neuraminidase digestion of the labeled product and ability of asialo GM_2 to serve as acceptor.

Two modes of regulation of SAT-1 have been proposed, (1) negative feedback inhibition (53-55) and (2) covalent modification of SAT-1 by a phosphorylation/dephosphorylation mechanism (61). SAT-1 activity is cell cycle-dependent, increasing during late M/early G_1 (48). During early G_1, both SAT-1 activity and GM_3 expression are maximal (48,52,62). Apparently, the cyclic accumulation of GM_3 at G_1 (53) may serve to inhibit progression through the cell cycle

since exogenously added GM_3 has been shown to inhibit growth factor-induced mitogenesis via EGF-receptor tyrosine kinase autophosphorylation (for review 3), an event associated with cellular proliferation. Hopefully, the availability of SAT-1 will make it possible to investigate the involvement of SAT-1 activity and GM_3 ganglioside in cellular proliferation and to identify the regulatory mechanism involved.

ACKNOWLEDGEMENTS

We thank Dr. Kimihiro Kanemitsu for assisting with the ozonolysis of LacCer.

We thank Carol Smith for her excellent secretarial support.

FOOTNOTES

* This work was supported in part by a research grant (DK12434) from the National Institutes of Health.

+ To whom correspondence should be addressed: Dept. of Biochemistry, Michigan State University, Rm. 401 Biochem. Bldg., E. Lansing, MI 48824-1319.

ABBREVIATIONS

2D, two-dimensional

BPA, *Bauhinia purpurea* agglutinin

CMP, cytidine 5-monophosphate

Con A, *Canavalia ensiformis* agglutinin

DBA, *Colichos biflorus* agglutinin

DSA, *Datura stramonium* agglutinin

Glycosphingolipid*

GM_3, NeuAcα2-3Galß1-4Glcß1-1Cer

GNA, *Galanthus nivalis* agglutinin

GS-I, *Griffonia simplicifolia* agglutinin

HPLC, high pressure liquid chromatography

HPTLC, high performance thin layer chromatography

IEF, isoelectric focusing

LDAO, lauryl dimethylamine oxide

LacCer, Galß1-4Glcß1-1Cer

LPA, *Limulus polyphemus* agglutinin

MAA, *Maackia amurensis* agglutinin

MAPSII, monoclonal antibody purification system II

NeuAc, neuraminic acid, also known as sialic acid

PAGE, polyacrylamide gel electrophoresis

PBS, Phosphate-buffered saline

PIBMA, polyisobutylmethylacrylate

PMSF, phenylmethane-sulfonyl fluoride

PNA, *Arachis hypogaea* agglutinin

SAT-1, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase, also known as GM_3 synthase

SDS, sodium dodecyl sulfate

SNA, *Sambucus nigra* agglutinin

ST2-3, ß-galactoside α2-3 sialyltransferase

ST2-6, ß-galactoside α2-6 sialyltransferase

TBS, TRIS-buffered saline

TLCK-HCl, (L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl

TPCK, L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone

WGA, wheat germ agglutinin

*All abbreviations for Glycosphingolipids are according to the Svennerholm nomenclature (63) and the IUPAC - IUB recommendations (64).

REFERENCES

1. Hakomori, S. (1984) Ann. Rev. Immunol. **2**, 103-126.
2. Hakomori, S. (1985) Cancer Res. **45**, 2405-2414.
3. Hakomori, S. (1990) J. Biol. Chem. **265**,.18713-18716.
4. Paulson, J. C. and Colley, K. J. (1989) J. Biol. Chem. **264**, 17615-17618.
5. Taniguchi, N., Yokosawa, N., Gasa, S. and Makita, A. (1982) J. Biol. Chem. **257**, 10631-10637.
6. Paulson, J. C. (1987) Trends Biochem. Sci. **14**, 272-276.
7. Taniguchi, N. and Makita, A (1984) J. Biol. Chem. **259**, 5637, 5642.
8. Taniguchi, N. and Makita, A. (1985) J. Biol. Chem. **260**, 4908-4913.
9. Joziasse, D.H., Bergh, M.L.E., ter Hart, H.G.J., Koppen, P.L., Hooghwinkel, G.J.M. and Van den Eijnden, D.H. (1985) J. Biol. Chem. **260**, 4941-4951.
10. Gu, X.-B., Gu, T.-J. and Yu, R.K. (1990) Biochem. Biophys. Res. Commun. **166**, 387-393.
11. Clausen, H., White, T., Takio, K., Titani, K., Stroud, M., Holmes, E., Karkov, J., Thim, L. and Hakomori, S. (1990) J. Biol. Chem. 265, 1139-1145.
12. Basu, S., Ghosh, S., Basu, M., Wang, S., Das, K.K., Hawes, J.W., Li, Z., and Zhang, B. (1990) FASEB J. Vol. **4**, No. 7, Abstract #2584.
13. Kanemitsu, K. and Sweeley, C. C. (1986) Glycoconjugate J. **3**, 143-151.
14. Peterson, G.L. (1977) Anal Biochem. **83**, 346-356.
15. Layne, E. (1957) Methods Enzymol. **3**, 447-454.
16. Burczak, J.D., Fairley, J.L., and Sweeley, C.C. (1984) Biochim. Biophys. Acta **804**, 442-449.
17. Melkerson-Watson, L.J., Kanemitsu, K., and Sweeley, C.C. (1987) Glycoconjugate J. 4, 7-16

18. Paulson, J.C., Beranek, W.E. and Hill, R.L. (1977) J. Biol. Chem. **252**, 2356-2362.

19. Sadler, J.E., Rearick, J.I., Paulson, J.C., and Hill, R.L. (1979) J. Biol. Chem. **254**, 4434-4443.

20. Sadler, J.E., Rearick, J.I., and Hill, R.L. (1979) J. Biol. Chem. **254**, 5934-5941.

21. Weinstein, J., de Souza-e-Silva, U., and Paulson, J.C. (1982) J. Biol. Chem. **257**, 13835-13844.

22. Baubichon-Cortay, H., Broquet, P., George, P. and Louisot, P. (1989) Glycoconjugate J. **6**, 115-127.

23. Morre, D.J., Merlin, L.M., and Keenan, T.W. (1969) Biochem. Biophys. Res. Commun. **37**, 813-819.

24. Morre, D.J. (1971) Methods Enzymol. **22**, 130-148.

25. Fleischer, S., and Kervina, M. (1974) Methods Enzymol. **31**, 6-41.

26. Carey, D.J., and Hirschberg, C.B. (1980) J. Biol. Chem. **255**, 4348-4354.

27. Leelavathi, D.E., Estes, L.W., Feingold, D.S. and Lombardi, B. (1970) Biochim. Biophys. Acta **211**, 124-138.

28. Kundu, S.K. (1981) Methods Enzymol. **72**, 174-204.

29. Melkerson-Watson, L. J. and Sweeley, C. C. (1990) Biochem. Biophys. Res. Commun. **172**, 165-171.

30. Fuller, S.A., Takahashi, M., and Hurrell, J.G.R. (1987) in *Current Protocols in Molecular Biology*, Vol. #2, Ch. 11 (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds.) Greene Publ. Asso. & Wiley-Interscience, NY, NY.

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

32. Merril, C.R., Goldman, D., Van Keuren, M.L. (1984) Methods Enzymol. **104**, 441-447.

33. Gu, X., Gu, T., and Yu, R. K. (1990) Anal. Biochem. **185**, 151-155.

34. Dunbar, B. S. (1987) in Two-Dimensional Electrophoresis and Immunological Techniques, pp. 227-271. Plenum Press, New York.

35. O'Farrell, P.H. (1975) J. Biol. Chem. **250**, 4007-4021.

36. Roth, J., Bendayan, M., and Orci, L. (1978) J. Histochem. **26**, 1074-1081.

37. Roth, J., Taatjes, D.J., Lucocq, J.M., Weinstein, J., and Paulson, J.C. (1985) Cell **43**, 287-295.

38. Kaufman, B., Basu, S., and Roseman, S. (1966) Methods Enzymol. **8**, 365-368.

39. Lammers, G. and Jamiesson, J.C. (1988) Biochem. J. **256**, 623-631.

40. Holmgren, J., Svennerholm, L., Elwing, H., Fredman, P. and Strannegard, O. Proc. Natl. Acad. Sci., U.S.A., (1980) **77**, 1947-1950.

41. Moskal, J.R., Lockney, M.W., Marvel, C.C., Trosko, J.E., and Sweeley, C.C. (1987) Cancer Res. **47**, 787-790.

42. Deutsch, V. and Lotan, R. (1983) Exp. Cell Res. **149**, 237-245.

43. Lotan, R., Lotan, D. and Meromsky, L. (1984) Cancer Res. **44**, 5805-5812.

44. Payatte, R., Biehl, J., Toyama, T. and Holtzer, H. (1980) Cancer Res. **40**, 2465-2474.

45. Koeffler, H.P., Bar-Eli, M. and Territo, M.C. (1981) Cancer, Res. **41**, 919-926.

46. Fukuda, M. (1981) Cancer Res. **41**, 4621-4628.

47. Huberman, E., Heckman, C. and Langenbach, R. (1979) Cancer Res. **39**, 2618-2624.

48. Burczak, J.D., Moskal, J.R., Trosko, J.E., Fairley, J.L. and Sweeley, C.C. (1983) Exp. Cell Res. **147**, 281-286.

49. Leder, A. and Leder, P. (1975) Cell **5**, 319-322.

50. Ginsburg, E., Salomon, D., Sreevalsan, T. and Freese, E. (1973) Proc. Natl. Acad. Sci., U.S.A. **70**, 2457-2461.

51. Fishman, P.H., Simmons, J.L., Brady, R.O. and Freese, E. (1974) Biochem. Biophys. Res. Commun. **59**, 292-299.

52. Macher, B.A., Lockney, M., Moskal, J.R., Fung, V.K. and Sweeley, C.C. (1978) Exp. Cell Res. **117**, 95-102.

53. Pohlentz, G., Klein, D., Schwarzmann, G., Schimtz, D. and Sandhoff, K. (1988) Proc. Natl. Acad. Sci., U.S.A., **85**, 7044-7048.

54. Yusuf, H.K.M., Schwarzmann, G., Pohlentz, G. and Sandhoff, K. (1987) Biol. Chem. Hoppe-Seyeler **368**, 455-462.

55. Yusuf, H.K.M., Schwarzmann, G., Pohlentz, G. and Sandhoff, K. (1987) Biol. Chem. Hoppe-seyler **368**, 455-462.

56. Schuz-Henninger, R., Ullmer, E., Prinz, C. and Decker, K. (1989) Eur. J. Biochem. **185**, 327-330.

57. Schuz-Henninger, R., Prinz, C. and Decker, K. (1988) Arch. Biochem. Biophys. **262**, 49-58.

58. Yu, R. K., and Lee, S.H. (1976) J. Biol. Chem. **251**, 198-203.

59. Paulson, J. C., Weinstein, J., and Schauer, A. (1989) J. Biol. Chem. **264**, 10931-10934.

60. O'Hanlon, T. P., Lau, K. M., Wang, X. C., and Lau, J. T. Y. (1989) J. Biol. Chem. **264** 17389-17394.

61. Burczak, J.D., Soltyziak, R.M. and Sweeley, C.C. (1984) J. Lipid Res. **25**, 1541-1547.

62. Chatterjee, S., Sweeley, C.C. and Velicer, L.F. (1975) J. Biol. Chem. **250**, 61-66.

63. Svennerholm, L. (1964) J. Lipid Res. **5**, 145-155.

64. IUPAC-IUB Recommendations (1977) Lipids **12**, 455-468.

CHAPTER 4

SPECIAL CONSIDERATIONS IN THE PURIFICATION OF THE GM_3 GANGLIOSIDE FORMING ENZYME, CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE (SAT-1): SOLUBILIZATION OF SAT-1 WITH LAURYLDIMETHYLAMINE OXIDE*

L.J. Melkerson-Watson and C.C. Sweeley[1]

Department of Biochemistry
Michigan State University
East Lansing, MI 48824

August 16, 1990

Biochem. Biophys. Res. Commun. (1990) **172**, 165-171.

Running Title: Studies on the Purification and Regulation of SAT-1

SUMMARY

Lauryldimethylamine oxide (LDAO)[2] was employed in the purification of the GM_3 ganglioside forming enzyme, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) (4). This detergent has advantages over the typically employed Triton detergents in the solubilization and stabilization of this sialyltransferase. Crude protein fractions solubilized from rat liver Golgi by several such detergents are very similar in composition as determined by two-dimensional gel electrophoresis. However, LDAO appears to activate and stabilize SAT-1 activity. It is possible that SAT-1 activation involves the structurally similar hydrophobic moieties and quaternary amino groups of LDAO and phosphatidylcholine.

INTRODUCTION

Detergent solubilization is essential for the purification of integral membrane proteins. Detergents serve to replace the native lipids of the membrane bilayer about the hydrophobic domain(s) of the protein so that routine biochemical and chromatographic methods can be employed for purification. Many times the choice of detergents is by trial and error, optimized to stabilize a particular enzyme activity. Although the stability of a protein in detergent in not clearly understood, it is likely related to the artificial conformation imposed on the protein by a particular detergent environment. These properties of detergents have been reviewed (1,2) and recently summarized (3). We report here the application of a nonionic/cationic detergent, lauryldimethylamine oxide (LDAO) in the purification of a sialyltransferase, the GM_3-forming Golgi enzyme, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1).

METHODS

Materials: Lauryldimethylamine oxide (LDAO), also called Ammonyx LO, was obtained from the Stepan Chemical Co, (Northfield, IL). Triton CF-54, ß-octylglucoside and myosin were purchased from Sigma Chemical Co. (St. Louis, MO). Triton X-100, Gelcode molecular weight markers and Extracti-Gel D were purchased from Pierce Chemical Co. (Rockford, IL). CMP [$^{14}C_{4,5,6,7,8,9}$] sialic acid was obtained from New England Nuclear (Boston, MA). Sep Pak C_{18} cartridges were purchased from Waters (Milford, MA). Carbamylated pI standard markers were from Pharmacia (Piscataway, NJ). The reverse-phase RP-300, C_8 HPLC column was obtained from Applied Biosystems (Foster City, CA). All other reagents and chemicals were of reagent grade.

Rat Liver Golgi: Rat liver Golgi was prepared as described previously (4) using well-established procedures (5-7).

CMP-sialic acid:Lactosylceramide α2-3 Sialyltransferase (SAT-1) Activity Assay: SAT-1 activity was assayed as previously described (8,9).

Two-Dimensional Gel Electrophoresis: Rat liver Golgi, 0.3% Triton X-100 extracted Golgi, 1% Triton CF-54 extracted Golgi, and 15% LDAO extracted Golgi proteins were isoelectrofocussed in the first dimension in tube gels according to the O'Farrell method (25) and in the second dimension on 10-20% SDS-PAGE according to the Laemmli system as described by Dunbar (10). The carrier ampholytes were a mixture of 2 parts Pharmacia Pharmalytes pH 5-8 and 1 part (LKB Ampholines pH 3.5-10/Pharmacia Pharmalytes pH 3-10, 1:1). The 2D SDS-PAGE patterns were analyzed by the Bio Image Visage 110 computerized digital image analysis system (Millipore, Milford, MA).

RESULTS AND DISCUSSION

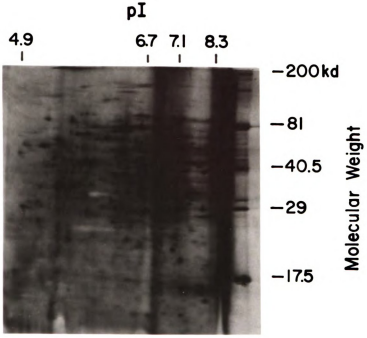
LDAO was employed in the purification to homogeneity of CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) from rat liver Golgi (4). LDAO is a nonionic/cationic detergent which protonates under acidic conditions (pH < 7) conferring a net positive charge to its hydrophilic head group (3). It's use has been primarily for the solubilization of photoreactive centers from chloroplasts (11,12). This is the first report of the use of LDAO for the solubilization of a glycosyltransferase.

Typically, Triton X-100 (13-19) or Triton CF-54 (20) have been the surfactants employed in sialyltransferase solubilizations from Golgi vesicles. Two-dimensional computerized digital image analyses of the proteins solubilized from rat liver Golgi vesicles by either 0.3% Triton X-100, 1% (w/v) Triton CF-54 or 15% (w/v) LDAO gave very similar protein patterns under the conditions employed relative to the reference image obtained with unextracted rat liver Golgi. Each detergent resolved about 300 silver-stained polypeptides within the pI range of 4.7 to 8.3. LDAO resolved 284 polypeptides, Triton CF-54 318, and Triton X-100 295. The reference pattern of rat liver Golgi protein (no detergent digestion) contained 315 proteins. The Bio Image Visage 110 match-paired 156, 175 and 191 (for LDAO, Triton X-100 and Triton CF-54, respectively) of these proteins to the reference image. The two-dimensional map of the LDAO-soluble Golgi proteins is shown in Figure 1.

LDAO offers several advantages over the Triton surfactants. First, LDAO enhances SAT-1 activity from rat liver Golgi 12 to 15-fold higher than either Triton CF-54, Triton X-100, or ß-octylglucoside (Figure 2). This activation of SAT-1 may reflect a stabilization of the solubilized enzyme in a structural motif similar to its native conformation in a phosphatidylcholine (PC) membrane. PC, a major phospholipid of the Golgi, has been shown previously to give a 16-fold activation of SAT-1 (21).

Figure 1. Two-Dimensional SDS-PAGE of Detergent-Solubilized Rat Liver Golgi Proteins. Protein from rat liver Golgi was extracted in the presence of 3.0% (w/v) Triton X-100, 1.0% (w/v) Triton CF-54 or 15% (v/v) lauryldimethylamine oxide. The proteins solubilized from the Golgi membranes under these conditions were analyzed by two-dimensional gel electrophoresis. First-dimension tube gels were performed according to the method of O'Farrell (25) with an ampholyte mixture of 1 part pH 3-10 and 2 parts 5-8 on sixty micrograms of total protein. The second-dimension slab gels were standard 10-20% Laemmli SDS-polyacrylamide gels. The two-dimensional patterns were analyzed following silver staining (26) by computerized digital imaging using the Bio Image Visage 110 (Millipore/Bio Image, Ann Arbor, MI). Bio Image parameter settings were filter width 15, spot threshold 6, minimum spot width 4, minimum filter width volume 15, and minimum spot size 60. A 2D SDS-PAGE reference image was from sixty micrograms of unextracted Golgi membrane. Panel A shows the 2D pattern from 13.1 μg 15% LDAO soluble Golgi proteins. A composite image of the analysis of unextracted rat liver Golgi proteins, LDAO extracted protein, Triton X-100 extracted proteins and Triton CF-54 extracted proteins gave very similar 2D patterns (for discussion see text). Panel B illustrates a typical diagrammatic representation plotted for these samples obtained from the digital image analysis on the Bio Image Visage 110. This pattern is from the LDAO extracted Golgi proteins. The boxed area on the plot indicates the pI and M.W. range of the purified SAT-1, a 60,000 dalton glycoprotein in the pI range of approximately 5.7 to 6.2 (4). The molecular weight markers 200 Kd, myosin; 81 Kd transketolase; 40.5 Kd, creatinine phosphokinase; 29 Kd, phosphoglucomutase; and 17.5 Kd myoglobin. The carbamylation standards for determining pI were creatinine phosphokinase (pI 4.9-7.1); glyceraldehyde-3-Phosphatedehydrogenase (pI 4.8-8.3) and carbonic anhydrase (pI 4.7-6.7).

A


pI
4.9
6.7
7.1
8.3
200kd
81
40.5
29
17.5
Molecular Weight

B

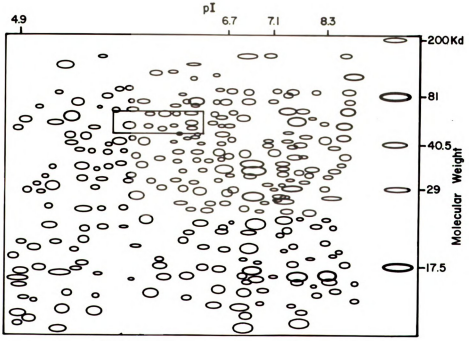
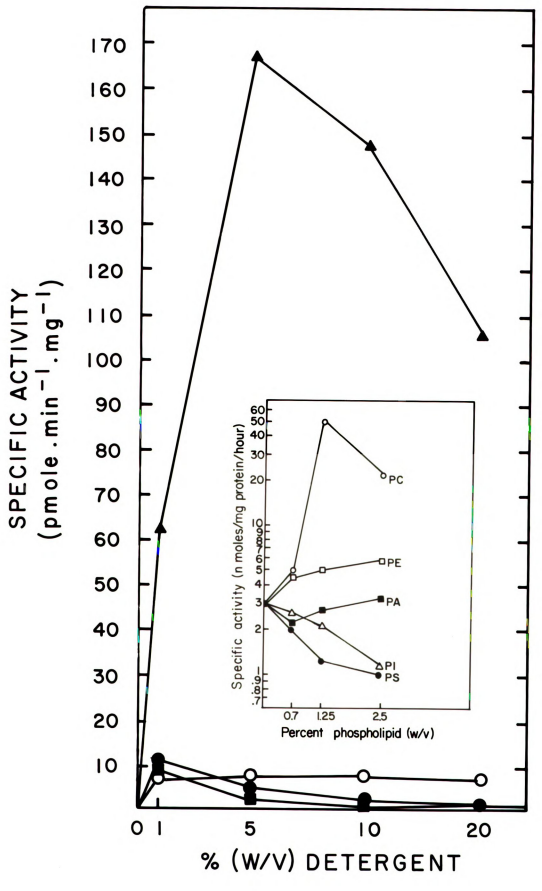

pI
4.9
6.7
7.1
8.3
200Kd
81
40.5
29
17.5
Molecular Weight

Figure 2. Effects of Various Surfactants on SAT-1 Activity in Rat Liver Golgi. Golgi vesicles were prepared from rat liver by well-established procedures (5-7) and is described in detail elsewhere (4). Increasing concentrations of LDAO, Triton CF-54, Triton X-100 and ß-octylglucoside were added to standard reaction mixtures (4,8-9) with Golgi-enriched microsomes as the enzyme source and assayed immediately. The GM_3 product was recovered as described (8) and the SAT-1 activity determined. The inserted panel serves for comparison of SAT-1 activated by LDAO and PC and is reproduced with permission from Journal Lipid Research 25, 1541-1547 and the authors (21).


SPECIFIC ACTIVITY
(pmole.min⁻¹.mg⁻¹)
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0 1
5
10
20
% (W/V) DETERGENT
Specific activity (n moles/mg protein/hour)
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1
.9
.8
.7
PC
PE
PA
PI
PS
0.7
1.25
2.5
Percent phospholipid (w/v)

A second advantage of LDAO is that it does not absorb at A_{280nm}, as do the Tritons, permitting the monitoring of protein elution at A_{280nm} during purification steps involving column chromatography. LDAO exhibits end-group absorption below 215 nm.

The hydrophobic nature and positive charge of LDAO below pH 7.0 confers considerable stability on SAT-1 for purification and storage. SAT-1 has been stored in 25 mM sodium cacodylate (pH 6.5) containing 15% (w/v) LDAO at -80°C for periods of 6-12 months without appreciable loss of activity (Table 1). We attribute this to the hydrophobicity of the detergent and the structural similarity between LDAO and PC. Glew and coworkers (*private communication*) have observed a similar stabilization by charge with glucocerebrosidase, a membrane-bound lysosomal protein, and phosphatidylserine (PS). Acyl CoA, a negatively charged amphipatic molecule, confers the same effect as PS.

LDAO remains tightly associated with the enzyme, a factor which may complicate some protein analyses. Several methods have been explored for the removal of LDAO. LDAO will dialyze, but the process is slow. Extracti-Gel D (Pierce Chemical Co.) is not a viable alternative for SAT-1 purification because both LDAO and the hydrophobic SAT-1 sialyltransferase are adsorbed. TCA precipitation is ineffective. Further, analysis of the glycan residues on SAT-1 (4) indicate SAT-1 is a glycoprotein containing sialic acid. The use of TCA to precipitate sialylated proteins for carbohydrate analysis is not recommended as sialic acid hydrolysis occurs under the acidic conditions. Combined ethanol and acetone precipitations, which serve to precipitate proteins by changing their solvation properties, removes sufficient LDAO from SAT-1 sialyltransferase to allow SDS-PAGE gel electrophoresis. For electrophoretic analysis of SAT-1, 5-20 μg of the sialyltransferase is precipitated in 90% (v/v) ethanol at -20°C for 36-48 hrs followed by centrifugation at 14,000 x g for 30 min. The ethanol is carefully removed and the pellet taken to dryness in a

TABLE 1

STABILITY OF LDAO SOLUBILIZED SAT-1 IN 15%(V/V) LDAO AT -20°C FOR 6 MONTHS

Fraction	Specific Activity t_0	Specific Activity t_6	% Activity Remaining after 6 mos. at -20°C
CH	0.43	0.07	16.5%
PMS	0.74	0.23	31.3%
DE	0.52	0.56	106.3%
CMPI(NaCl)	1036	1170	113%
CMPII(CMP)	5110	4980	97.5%
LacCer(CMP)	15350	15640	102%

Speedvac. The sialyltransferase pellet is resuspended in HPLC-grade acetone to desalt. Precipitation of SAT-1 in acetone is allowed to proceed at -20°C for 18 hrs. Good resolution of SAT-1 by 2D SDS-PAGE is achieved (4). However, the hydrophobic nature of LDAO allows some of it to co-precipitate with SAT-1 even in ethanol/acetone.

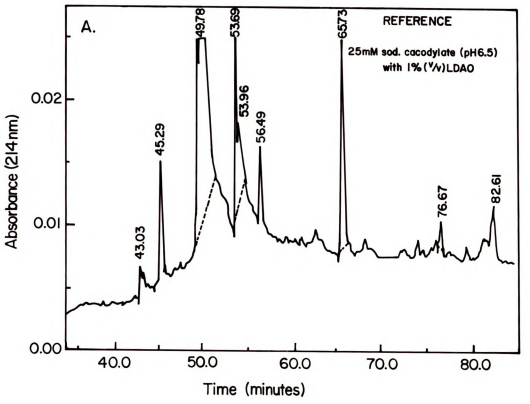
LDAO at high concentration is toxic. The LD_{50} of LDAO is 3.6 g/kg (value supplied by the manufacturer). For immunological work, LDAO can be exchanged with PC at a minimum of a 1:10 ratio of detergent to phospholipid and dialyzed. The exchange of PC for LDAO decreases the toxicity of the immunogen. Further, incorporating SAT-1 antigen into PC liposomes has the advantage that the half-life of the antigen in circulation is increased. This methodology permitted us to raise a monoclonal antibody to rat liver SAT-1 (4).

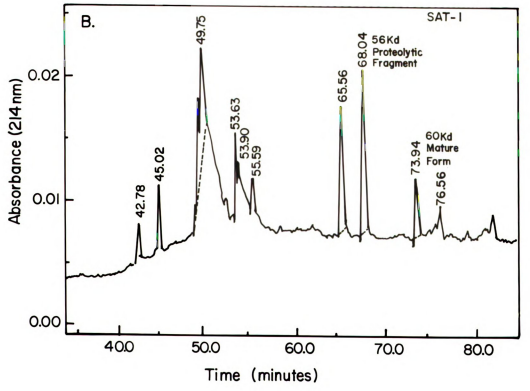
The most effective means of LDAO removal is reverse-phase HPLC. Resolution of purified SAT-1 sialyltransferase from LDAO is achieved on a C_8 microbore HPLC system (Applied BioSystems). SAT-1 in 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO is applied onto an Aquapore C_8, RP300 (250 x 1.0 mm x 7 μm) reverse-phase microbore column and eluted on a 90 min linear gradient established between 0.1% (v/v) trifluoroacetic acid and 90% (v/v) acetonitrile. The differences in hydrophobicity permit the separation of SAT-1 from LDAO (Figures 3a and 3b). SAT-1 sialyltransferase exhibits greater hydrophobicity than LDAO and elutes at 76% and 82 % of the B solvent (i.e., 68.4% and 73.8% acetonitrile). LDAO resolves into several peaks, with the majority of the detergent eluting from 47% to 54% of the B solvent (i.e., 42.3 to 48.6% acetonitrile). The two SAT-1 peaks, 56 Kd and 60 Kd by SDS-PAGE, respectively, were verified to be immunologically reactive on Western Blots with M12GC7, a specific anti-SAT-1 monoclonal antibody (4). The 56 Kd polypeptide may be a proteolytic product of the 60 Kd SAT-1. SAT-1, like other purified sialyltransferases, is subject to degradation by an associated endogenous

proteolytic activity (for review, 22 and references therein). Further, this activity is present even after one week of storage at -80°C following SAT-1 purification. One possible explanation is that a brush border protease associates with SAT-1 via the LDAO detergent, enhancing its stability and activity. Preliminary investigation by limited digestion of the HPLC 56 Kd and 60 Kd, M12GC7 positive, SAT-1 polypeptides with 0.2 units cathepsin D and 2 units N-glycanase for 30 minutes at 37°C prior to SDS gel electrophoresis resulted in formation of 56 Kd proteolytic and 43 Kd deglycosylated polypeptide/products from both protein peaks obtained by HPLC. The endogenous proteolytic activity is suspected to be a cathepsin D-like activity (23) and is believed to be important in the release of the soluble catalytic domain of the glycosyltransferase from the membrane anchor (22).

One other consideration for the use of LDAO is important. Since it is an amphipathic compound, some is recoverable from SAT-1 product assays on Sep Pak C_{18} cartridges. This does not present a problem for optimal recovery of the GM_3 product under the conditions described by Melkerson-Watson *et al.* (9). We recommend, however, that an alternative method of analysis be employed for studies of enzyme specificity as the LDAO and PC, used in the Sep Pak C_{18} recovery, have relative mobilities on HPTLC in the range of some glycolipid substrates. Modifications of the method of Yu *et al.* (24), reported elsewhere (4), were well-suited for analyzing sialyltransferase activity with glycolipids chromatographed on HPTLC plates.

Figure 3a and 3b. Reverse-phase C_8 HPLC of SAT-1 vs. Reference Buffer Containing 5% (v/v) Lauryldimethylamine Oxide (LDAO). Resolution of purified SAT-1 sialyltransferase from LDAO was achieved on a C_8 microbore HPLC system (Applied Biosystems). SAT-1 in 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO was applied onto an Aquapore C_8, RP300 (250 x 1.0 mm x 7 μm) reverse-phase microbore column and eluted on a 90 min linear gradient established between 0.1% (v/v) trifluoroacetic acid and 90% (v/v) acetonitrile. Sample volume injected was 250 μl. Panel A illustrates a typical chromatogram obtained from buffer containing LDAO. B shows the resolution of unique SAT-1 proteins from the same LDAO containing buffer.


A.
REFERENCE
25mM sod. cacodylate (pH6.5)
with 1% (v/v) LDAO
43.03
45.29
49.78
53.69
53.96
56.49
65.73
76.67
82.61
Absorbance (214nm)
0.00
0.01
0.02
40.0
50.0
60.0
70.0
80.0
Time (minutes)


B.
SAT-1
42.78
45.02
49.75
53.63
53.90
55.59
65.56
68.04
56Kd
Proteolytic
Fragment
73.94
76.56
60Kd
Mature
Form
Absorbance (214nm)
0.00
0.01
0.02
40.0
50.0
60.0
70.0
80.0
Time (minutes)

FOOTNOTES

[1]To whom correspondence should be addressed.

*This work was supported in part by a research grant from the National Institutes of Health (DK12434).

[2]The abbreviations used are:

SAT-1, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase, also known as GM_3 synthase;

LDAO, lauryl dimethylamine oxide, also known as Ammonyx LO;

GM_3, NeuAcα2-3Galß1-4Glcß1-1Cer;

PC, phosphatidylcholine;

2D, two-dimensional;

SDS, sodium dodecyl sulfate;

PAGE, polyacrylaminde gel electrophoresis;

LD_{50}, lethal dose with 50% survival;

HPLC, high performance liquid chromatography;

Kd, kilodalton.

ACKNOWLEDGEMENTS

We thank Carol Smith for her excellent secretarial support.

We thank David Hicks, formerly of Integrated Separation Systems (assisting with the 2D SDS-PAGE of the detergent-solubilized proteins from Golgi vesicles (Fig. 2A).

REFERENCES

1. Hjelmeland, L. M., and Chrambach, A. (1984) Methods Enzymol. **104**, 305.
2. Neugebauer, J.M. (1990) Methods Enzymol. **189**, 239-264.
3. Neugebauer, J.M. "Guide to the Properties and Uses of Detergents in Biol. and Biochem.", Calbiochem, San Diego, CA.
4. Melkerson-Watson, L. J., and Sweeley, C. C. (1990) "Purification to Homogeneity by Immunoaffinity Chromatography and Partial Characterization of the GM_3 Ganglioside Forming Enzyme, CMP-Sialic Acid:Lactosylceramide α2-3 Sialyltransferase(SAT-1), From Rat Liver Golgi", submitted for publication.
5. Morre, D. J. (1971) Methods Enzymol. **22**, 130-148.
6. Fleischer and Kervina, M. (1974) Methods Enzymol. **31**, 6-41.
7. Carey, D. J., and Hirschberg, C. B. (1980) J. Biol. Chem. **255**, 4348-4354.
8. Burczak, J. D., Fairley, J. L., and Sweeley, C. C. (1984) Exp. Cell Res. **147**, 281-286.
9. Melkerson-Watson, L. J., Kanemitsu, K., Sweeley, C. C. (1987) Glycoconjugate J. **4**, 7-16.
10. Dunbar, B. S. (1987) in Two-Dimensional Electrophoresis and Immunological Techniques, pp. 227-271. Plenum Press, New York.
11. Kendall-Tobias, M. W., and Seibert, M. (1982) Arch. Biochem. Biophys. **216**, 255-258.
12. Bowes, J. M., Stewart, A. C., Bendall, D. S. (1983) Biochim. Biophys. Acta **725**, 210-219.
13. Sadler, J. E., Paulson, J. C., and Hill, R. L. (1979) J. Biol. Chem. **254**, 2112-2119.
14. Sadler, J. E., Rearick, J. I., Paulson, J. C., and Hill, R. L. (1979) J. Biol. Chem. **254**, 4434-4443.

15. Taniguchi, N., Yokosawa, N., Gasa, S., and Makita, A. (1982) J. Biol. Chem. **257**, 10631-10637.

16. Taniguchi, N. and Makita, A.(1984) J. Biol. Chem. **259**, 5637-5642.

17. Joziasse, D. H., Bergh, M. L. E., ter Hart, H G. J., Koppen, P. L., Hooghwinkel, G. J. M., and Van den Eijnden, D. H. (1985) J. Biol. Chem. **260**, 4941-4951.

18. Clausen, H., White, T., Takio, K., Titani, K., Stroud, M., Holmes, E., Karkov, J., Thim, L., and Hakomori, S. (1990) J. Biol. Chem. **265**, 1139-1145.

19. Gu, X.-B., Gu, T.-J., and Yu, R.K. (1990) Biochem. Biophys. Res. Commun. **166**, 387-393.

20. Weinstein, J., de Souza-e-Silva, U., and Paulson, J. C. (1982) J. Biol. Chem. **257**, 13835-13844.

21. Burczak, J. D., Soltyziak, R. M., and Sweeley, C. C. (1984) J. Lipid Res. **25**, 1541-1547.

22. Paulson, J. C., and Colley, K. J. (1989) J. Biol. Chem. **264**, 17615-17618.

23. Lammers, G. and Jamiesson, J. C. (1988) Biochem. J. **256**, 623-631.

24. Gu, X., Gu, T., Yu, R.K. (1990) Anal. Biochem. **185**, 151-155.

25. O'Farrell, P.H. (1975) J. Biol. Chem. **250**, 4007.

26. Merril, C.R., Goldman, D. and Van Keuren, M.L. (1982) Electrophoresis **3**, 17.

CHAPTER 5

SPECIAL CONSIDERATIONS IN THE PURIFICATION OF THE GM_3 GANGLIOSIDE-FORMING ENZYME, CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE (SAT-1): EFFECTS OF PROTEASE INHIBITORS ON RAT HEPATIC SAT-1 ACTIVITY

L. J. Melkerson-Watson and C. C. Sweeley

Department of Biochemistry
Michigan State University
East Lansing, MI 48824-1319

November 6, 1990

Running Title: Studies on the Purification and Regulation of SAT-1

SUMMARY

Co-purification of an endogenous proteolytic activity has been proposed as the cause for the size heterogeneity of sialyltransferases. Reported herein are results on the effects of various protease inhibitors, sulfhydryl-reducing agents and antimicrobial agents on SAT-1 activity. Addition of protease inhibitors to immunoaffinity-purified rat liver SAT-1 dramatically affects its activity. All protease inhibitors examined, with the exception of PMSF, inhibited the purified enzyme. The most inhibitory were the cysteine (thiol) protease inhibitors. This effect is less spectacular when the effect of these inhibitors was studied on SAT-1 activity in Golgi-enriched microsomes, although the inhibition was greatest by the cysteine protease inhibitors. One dramatic effect, found in both cases, was the apparent activation of SAT-1 activity in the presence of ß-mercaptoethanol.

INTRODUCTION

Intracellular proteases perform a variety of highly controlled, necessary biological functions, such as the regulation of cellular proliferation (for review, 1). For example, in glycosphingolipid metabolism, specific hydrolases cleave bioreactive groups from the carbohydrate or lipid moieties. The catabolites formed alter the modulation of the cell surface. For example, the cell-dependent degradation of GM_3 ganglioside by sialidase to lactosylceramide (LacCer) (2-5) or by cerebrosidase to lyso-GM_3 (6), may relieve GM_3 inhibition of EGF-receptor tyrosine kinase autophosphorylation, thus allowing cell proliferation to continue (7,8). GM_3 and its catabolites also affect the protein kinase C (PK-C) signal transduction mechanism in a manner antagonistic to that observed for EGF-receptor (for review, 9). Catabolites of GM_3 ganglioside are postulated to be internalized and recycled back to the endoplasmic reticulum and Golgi for resynthesis of GM_3 (4,10).

Another function for proteolytic processing in complex carbohydrate metabolism has been proposed as a mechanism for the release of soluble, active forms of glycosyltransferases to the extracellular matrix through the site-specific cleavage of these enzymes within the "stem" region (11,12). Glycosyltransferases are the enzymes responsible for extending the carbohydrate chains of glycoproteins and glycolipids. The presence of endogenous proteases within the Golgi, responsible for these cleavages, complicate purification of intact glycosyltransferases from the Golgi. We report here our observations made during the purification of CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1), on the effects of various protease inhibitors on SAT-1 activity.

MATERIALS AND METHODS

Materials - Cytidine 5'-monophosphate sialic acid, (CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid, 286.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and its specific activity adjusted to 22,200 dpm/nmol with unlabeled CMP-sialic acid from Sigma (St. Louis, MO). The sphinganine-containing form of lactosylceramide was purchased from Sigma and used in activity assays. Ammonyx LO (lauryldimethylamine oxide, LDAO) was obtained from the Stepan Company (Northfield, IL). EDTA, azide and sodium cacodylate were purchased from Sigma Chemical Company (St. Louis, MO). PMSF, APMSF, Leupeptin, aprotinin, pepstatin, TPCK, TLCK-HCl, α_2-macroglobulin (and α_2-macroglobulin carrier-fixed), E-64 and dithiothreitol (DTT) were purchased from Boehringer Mannheim (Indianapolis, IN). Ep-459 and Ep-475 were gifts from Dr. Hanada of Taisho Pharmaceutical (Tokyo, Japan). Ultrapure electrophoretic-grade ß-mercaptoethanol was from Bio Rad (Richmond, CA). Sep Pak C_{18} cartridges were purchased from Waters Associates (Milford, MA). All other reagents were ultrapure or ACS grade from commercial sources.

Preparation of Golgi-enriched Microsomes. Golgi vesicles were isolated from rat liver by well-established procedures (13-17) as described in detail (18) in 25 mM sodium cacodylate (pH 6.5) containing 0.25 M sucrose in the absence of protease inhibitors.

Purification of CMP-sialic acid:lactosylceramide α2-3 Sialyltransferase. CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) was purified from rat liver Golgi by immunoaffinity chromatography using M12GC7 anti-SAT-1 monoclonal antibody coupled to Affi Gel 10 as previously described for the purification of SAT-1 from detergent-extracted rat liver Golgi vesicles (18).

CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) Activity Assay. SAT-1 assays, with radiolabeled CMP-sialic acid as the donor substrate and

lactosylceramide as the acceptor substrate, were carried out by adsorbing 5 nmol LacCer to the microtiter plates in 50% ethanol as described (19). The plates were washed with PBS and blocked with 5% (w/v) bovine serum albumin (BSA) for 30 min. After rinsing, 20 nmol of CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid (New England Nuclear, lot # 2655-018, specific activity adjusted to 20,000 dpm/nmol) was added. To the reaction wells were added sonicated mixtures containing the following components: 25 μl of assay buffer (200 mM sodium cacodylate (pH 6.5), 20 mM $MnCl_2$ and 0.3% (v/v) lauryldimethylamine oxide (LDAO) and 25 μl of SAT-1 enzyme or Golgi (0.005-0.1 mg protein). The plate was covered and incubated in a humidified environment at 37°C for 2 hr. Following the incubation period, the reaction mixture was removed and the microtiter wells washed 3 - 5 times with PBS. The wells were put into 5 ml Safety-Solve liquid scintillant and the amount of [^{14}C]-GM_3 formed counted in a Packard (model #460C) liquid scintillation counter.

RESULTS AND DISCUSSION

The homogeneity of the affinity purified SAT-1 was extremely difficult to verify because of the size heterogeneity of the enzyme in electrophoretic patterns (18 (see chapter 3)). The pattern generated could be varied depending on the temperature and time of solubilization in SDS-PAGE sample buffer.

While there are several explanations for these data, similar size heterogeneity has been observed with other purified sialyltransferases (Table 1). Proteolytic degradation and mechanical disruption of the enzymes from the Golgi during their purifications were considered as possible causes for the heterogeneity of these purified enzymes. Recently, this concept of proteolytic processing was addressed in a review by Paulson and Colley (12). The cDNA's from four known glycosyltransferases exhibit some homology with regard to gross structure. They all possess a cytosolic NH_2 terminus, a hydrophobic transmembrane domain, a "stem" region, and a Golgi lumenal COOH terminus. The "stem" region between the transmembrane and catalytic domain has been implicated as the necessary element in the anchoring and targeting of the glycosyltransferase in the protein transport between the endoplasmic reticulum and the Golgi. It is also the apparent site of proteolysis (26). The endogenous proteolytic activity, suggested to be a cathepsin D-like activity (27), releases the catalytically active C-terminus of Galß1-4GlcNAc α2-6 sialyltransferase (CMP-N-acetylneuraminate:ß-galactoside α2-6 sialyltransferase, EC 2.4.99.1) from the lumenal face of the *trans*-Golgi for transport out of the cell during acute-phase response.

Therefore, to minimize proteolytic degradation during the purification of SAT-1, anti-SAT-1 specific monoclonal antibody, M12GC7, (18) was used to immunoaffinity-purify the enzyme from lauryldimethylamine oxide-extracted

TABLE 1

SIZE HETEROGENEITY OF SIALYLTRANSFERASE

Reference	Specificity	Source	Fold Purification	Molecular weights (Kd)
Paulson *et al.* (1977) JBC 252, 2356.	Galß1-4GlcNAc α2-3ST	Bovine Colostrum	440,000-X	56[1] 43
Sadler *et al.* (1979) JBC 254, 4434.	Gal α2-3ST	Porcine Submaxillary Gland	92,200-X	49[1] 44
Sadler *et al.* (1979) JBC 254, 5934.	GalNAc α2-6ST	Porcine Submaxillary Gland	117,000-X	172 160 100 80 69 56
Weinstein *et al.* (1982) JBC 257, 13835.	Galß1-4GlcNAc α2-6ST	Rat Liver	23,000-X	47[1] 43
Weinstein *et al.* (1982) JBC 257, 13835.	Galß1-3(4)GlcNAc α2-3ST	Rat Liver	860,000-X	56[1] 44
Joziasse *et al.* (1985) JBC 260, 4941.	Galß1-3GlcNAc α2-3ST and α2-3SAT (SAT-4)	Human Placenta	20,000-X	65[1] 43 41 40
Gu *et al.* (1990) BBRC 166, 387-393.	NeuAcα2-3Galß1-4Glcß1-1Cer α2-8SAT (SAT-2)	Rat Liver	10,000-X	63[2] 59[2] 55[1] 51[2] 43[2]
Melkerson-Watson and Sweeley (1990) JBC, in press	Galß1-4Glcß1-1Cer α2-3SAT (SAT-1)	Rat Liver	42,800-X	60[1] 56 50 47

[1]Apparent molecular weights of the purified sialyltransferases.

[2]Molecular weight values were estimated form the SDS-PAGE pattern in Figure 3 of Gu, *et al* (25).

Golgi membrane proteins (28) in the presence of several protease inhibitors including leupeptin, pepstatin and E-64 (and its analogs Ep-459 and Ep-475, gifts from Dr. Hanada of Taisho Pharmaceuticals, Japan (29)), all of which are potent cathepsin inhibitors. The enzyme was purified to homogeneity with an apparent molecular weight of 60,000. The enzyme was inactivated, but was immunologically reactive on Western blots with M12GC7. We speculated that this inactivation was due to one or more of the cysteine (thiol) protease inhibitors (TLCK-HCl, TPCK, E-64, or Ep-459), which is consistent with the finding that a thiol (R-SH) group has been found in the region of the CMP-NeuNAc binding site of ß-galactoside α2-6 sialyltransferase (30).

A summary of the effects of these and other protease inhibitors, as well as some sulfhydryl reducing and antibacterial agents used in SDS-PAGE and protein chromatography column preservation, are listed in Table 2 along with their effect on SAT-1 activity. SAT-1 was immunoaffinity-purified from LDAO-extracted rat liver Golgi-enriched fraction as previously described elsewhere (18). No protease inhibitors were added to the purification buffer. Various protease inhibitors, at the concentrations specified, were added to immunoaffinity-purified SAT-1 and assayed for activity (Table 2). Of the inhibitors tested, only the serine protease inhibitor PMSF, common in most glycosyltransferase purifications (18,20-25), did not inhibit the enzyme. All other protease inhibitors significantly inactivated the purified SAT-1 48-87% under the conditions employed. The most inhibitory substances were the cysteine (thiol) protease inhibitors (leupeptin, TLCK-HCl, TPCK, E-64, Ep-459, and Ep-475), suggesting that SAT-1 may also contain a thiol group in or near its sugar nucleotide-binding site, as had been reported for ß-galactoside α2-6 sialyltransferase (30). Some sequence homology has been reported for the sugar-nucleotide binding region in other glycosyltransferase (for review, 12).

TABLE 2

EFFECTS OF PROTEASE INHIBITORS ON IMMUNOAFFINITY-PURIFIED SAT-1 ACTIVITY

Inhibitor	Inhibitor Specificity	Concentration	dpm [^{14}C]-GM_3	% of Control
Control	none	0.0	850±30	100%
PMSF	serine proteases	1000 μM	1120	132%
APMSF	serine proteases	20 μM	440	52%
Aprotinin	serine proteases	0.3 μM	220	26%
Leupeptin	serine and thiol proteases (e.g., cathepsin B & L)	1 μM	240	28%
TLCK-HCl	trypsin & thiol proteases	135 μM	230	27%
TPCK	chymotrypsin & thiol proteases	284 μM	280	33%
E-64	thiol proteases (e.g., cathepsin B)	2.8 μM	250	29%
Ep-459	thiol proteases (e.g., cathepsin D)	2.8 μM	210	25%
Ep-475	thiol proteases	2.8 μM	140	16%
Pepstatin A	acid proteases (e.g., cathepsin D)	1 μM	240	28%
α_2-Macroglobulin	general endoproteases	1 unit	280	33%
EDTA	metalloproteases	100 μM	250	29%
DTT	sulfhydryl reducing agent	100 μM	90	11%
ß-ME	sulfhydryl reducing agent	180 μM	3330	392%
Azide	antimicrobrial agent	1000 μM	110	13%

Pepstatin, a potent inhibitor of cathepsin D, also inhibited SAT-1. the enzyme. Cathepsin D, a thiol protease with a heavy metal requirement, exhibits a preference for peptides flanked by hydrophobic amino acid residues (1). Addition of EDTA can inhibit cathepsin D proteolytic degradation, but EDTA also inhibits SAT-1 activity, since SAT-1 has a divalent cation requirement for activity (18,31). A 10,000-fold purification of a related glycolipid sialyltransferase, GD_3 synthase (NeuAcα2-3Galß1-4Glcß1-1Ceramide α2-8 sialyltransferase, SAT-2), has recently been reported (25); this purification was carried out in the presence of 1 mM EDTA and 10 mM ß-mercaptoethanol (ß-ME). Analysis of SAT-2 by SDS-PAGE showed size heterogeneity. The predominant molecular weight was reported to be 55,000 daltons (25). The combination of EDTA and ß-ME in their purification buffer apparentlydid not inhibit proteolysis.

Our investigation of the effects of sulfhydryl reducing agents, ß-ME and dithiothreitol (DTT), commonly used in SDS-PAGE sample buffer, indicates that the addition of ß-ME at a final concentration of 0.18 mM enhances SAT-1 activity about 4-fold relative to control. Thus, there is potential use of ß-ME in concert with the appropriate concentration of protease inhibitors (e.g., EDTA or pepsatin) for the inhibition of cathepsin D proteolytic degradation of sialyltransferases during purification while maintaining active fractions for monitoring purification of these enzymes.

In a previous study, summarized in Table 3, are the results of adding the same concentrations of protease inhibitors to Golgi-enriched microsomal fraction. The inhibition of SAT-1 activity was not as dramatic. SAT-1 activity was stable in microsomes treated with 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, 1 unit α_2-macroglobulin, and 2.8 μM E-64. The cysteine (thiol proteinase inhibitors, TLCK-HCl, TPCK, Ep-459 and Ep-475 inhibited SAT-1

TABLE 3

EFFECTS OF PROTEASE INHIBITORS ON SAT-1 ACTIVITY IN GOLGI-ENRICHED MICROSOMES

Inhibitor	Inhibitor Specificity	Concentration	dpm [^{14}C]-GM_3	% of Control
Control	none	0.0	104±18	100%
PMSF	serine proteases	1000 μM	108±19	104%
APMSF	serine proteases	20 μM	86±11	83%
Aprotinin	serine proteases	0.3 μM	71±12	68%
Leupeptin	serine and thiol proteases (e.g., cathepsin B & L)	1 μM	220±36	212%
TLCK-HCl	trypsin & thiol proteases	135 μM	76±17	73%
TPCK	chymotrypsin & thiol proteases	284 μM	82±18	79%
E-64	thiol proteases (e.g., cathepsin B)	2.8 μM	155±87	149%
Ep-459	thiol proteases (e.g., cathepsin D)	2.8 μM	65±13	63%
Ep-475	thiol proteases	2.8 μM	90±13	86%
Pepstatin A	acid proteases (e.g., cathepsin D)	1 μM	154±63	148%
α_2-Macroglobulin	general endoproteases	1 unit	112±42	108%
EDTA	metalloproteases	100 μM	66±6	63%
DTT	sulfhydryl reducing agent	100 μM	66±6	63%
ß-ME	sulfhydryl reducing agent	180 μM	1170±155	1120%

20-40%. EDTA (100 μM) inhibited SAT-1. The greatest inhibition of SAT-1 activity when assayed in Golgi-enriched microsomes was observed at 63% of control following the addition of either Ep-459 or EDTA. Addition of 0.1 mM ß-ME resulted in an 11-fold enhancement of SAT-1 activity in intact Golgi.

Not only should the effects of protease inhibitors on the inhibition of proteolytic degradation and enzyme activity be considered, but so should the differential effects of detergents on both proteinase activity(ies) during purification as well as the detergent effects on the glycosyltransferase being purified. We recently reported the effects of lauryldimethylamine oxide (LDAO) on SAT-1 (28 (see chapter4)). SAT-1 activity and stability are dependent upon the concentration of LDAO used. Detergents have also been shown to stabilize and activate proteinase activity. Arribas and Castano (32) found that low concentrations of detergents (0.01%) such as Triton X-100, SDS and CHAPS activate the hydrolysis of protein substrates by proteases, while higher concentrations (0.1%) inhibit degradation. A similar argument may apply to LDAO. LDAO may stabilize the sialyltransferase activity and, in addition, stabilize (and potentially activate) the endogenous proteolytic activity associated with the purification of these enzymes. Another possibility is that the associated endogenous protease is a lysosomal proteinase (like cathepsin D) which co-purifies with SAT-1 in Golgi-enriched microsomes through the disruption and mixing of the Golgi vesicles with light lysosomes during sonication and detergent extraction. Dawson and coworkers (33) have studied the subcellular distribution of cathepsin D, as well as its pre-pro and pro forms, in human fibroblasts using Percoll density gradients. Subcellular fractionation of cathepsin D and its intermediates were found uniformly distributed throughout the gradient with a slight enrichment of the mature active form in more buoyant fractions. Further, treatment of pre-pro-forms of Cathepsin D with cysteine (thiol) proteinase inhibitors, Ep-459 or leupeptin, caused inhibition of cathepsin D processing. Treatment with Ep-475 had no effect.

Another important effect on proteinase activity in relation to SAT-1 and its purification is the stimulation of some proteases by ATP (for review, 1). Two classes of "ATP-dependent" proteases have been characterized; one requires ATP for stabilization, the second requires ATP for hydrolysis. ATP activation of cathepsins D and L has been reported (34,35). One mechanism reported for cathepsin L requires binding of ATP to the protein substrate, which increases susceptibility of the protein to proteolytic degradation (36).

Activation of SAT-1 through the phosphorylation of tyrosine residue(s) has been proposed as a mechanism of regulation of cell growth by the increase of cell surface GM_3 ganglioside during G_1 of the cell cycle (37 (see chapter 6)). ATP has been demonstrated to enter the Golgi (38). Potentially, ATP-stabilization of protease activity or ATP-dependent hydrolysis of enzyme by proteases may play a role in the mechanism for the proteolytic degradation associated with SAT-1 sialyltransferase during its purification.

CONCLUSIONS

From these observations and considerations, SAT-1 purifications are now carried out under the following conditions to inhibit proteolytic degradation during its purification. SAT-1 is typically immunoaffinity purified from 20 g (wet weight) rat liver which has been perfused with 25 mM sodium cacodylate (pH 6.5) containing 0.25 M sucrose, 1 mM PMSF, 1 μM leupeptin and 1 μM pepstatin A. Following homogenization in five volumes of the same buffer, the nuclei, mitochondria and cellular debris are removed by centrifugation at 5000 x g for 10 min. The Golgi-enriched microsomes are collected by centrifugation at 100,000 x g and detergent-extracted with LDAO as previously described (18). The LDAO-soluble proteins are reacted (batch method) with 2 ml carrier-fixed α_2-macroglobulin for 30 min and proteases liberated during detergent extraction are coupled to carrier-fixed α2-macroglobulin which is removed following centrifugation (as described by the manufacturer, BMB).

The relationship between the effects of protease inhibitors on the size heterogeneity of SAT-1 and on SAT-1 glycosyltransferase activity is unknown. The data are consistent, to a first approximation, with the hypothesis that proteolytic cleavage of SAT-1 during purification is primarily due to the action of a cathepsin-like thiol protease. Inhibition of SAT-1 glycosyltransferase activity by protease inhibitors, and activation by ß-mercaptoethanol, may occur by modification of thiol residues necessary for SAT-1 activity. Thus, attempts to inhibit protease activity generally lead to coincident inhibition of SAT-1 catalytic activity. The inhibition of SAT-1 catalytic activity by α_2 macroglobulin, however, does not fit this pattern, and remains to be explained.

The correlation between inhibition of SAT-1 proteolysis and catalysis may also imply a physiologically significant interaction between SAT-1 and a protease activity for which several speculative mechanisms can be envisioned: a) a specific protease

activity may be required for SAT-1 activity, and may be involved in the regulation of SAT-1 acivity during the cell cycle, b) an endogenous protease may form part of a complex with SAT-1, or c) SAT-1 may possess endogenous protease activity.

FOOTNOTES

[1]To whom correspondence should be addressed.

*This work was supported in part by a research grant from the National Institutes of Health (DK12434).

[2]The abbreviations used are:

APMSF, (4-amidino-phenyl)-methane-sulfonyl fluoride;

ß-ME, 2-mercaptonethanol;

CMP, cytidine 5' monophosphate;

DTT, dithiotheitol;

EGF, epidermal growth factor;

GD_3, NeuAcα2-8NeuAcα2-3Galß1-4Glcß1-1Ceramide;

GM_3, NeuAcα2-3Galß1-4Glcß1-1Ceramide;

GM_1, Galß1-3GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß1-1Ceramide;

GD_{1a}, NeuAcα2-3Galß1-3GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß1-1Ceramide;

Glycosphingolipid*

Kd, kilodalton;

LDAO, lauryl dimethylamine oxide, also known as Ammonyx LO;

PK-C, protein kinase C;

PMSF, phenylmethane-sulfonyl fluoride;

SAT-1, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase, also known as GM_3 synthase;

SAT-2, CMP-sialic acid:GM_3 α2-8 sialyltransferase, GD_3 synthase;

SAT-4, CMP-sialic acid:GM_1 α2-3 sialyltransferase, GD_{1a} synthase;

SDS-PAGE, sodium dodecyl sulfate polyacrylaminde gel electrophoresis;

ST, sialyltransferase;

TLCK-HCl, (L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl

TPCK, L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone

*All abbreviations for Glycosphingolipids are according to the Svennerholm nomenclature (39) and the IUPAC - IUB recommendations (40).

ACKNOWLEDGEMENTS

We thank Dr. Tom Deits for his comments and assistance in reviewing and preparing this manuscript.

We thank Carol Smith for her excellent secretarial support.

REFERENCES

1. Bond, J. S. and Bulter, P.E. (1987) Ann. Rev. Biochem. **56**, 333-364.
2. Usuki, S., Lyu, S.-C., and Sweeley, C.C. (1988) J. Biol. Chem. **263**, 6847-6853.
3. Usuki, S., Hoops, P., and Sweeley, C.C. (1988) J. Biol. Chem. **263**, 10595-10599.
4. Swamy, M.J. and Sweeley, C.C. (1989) Biochem. Biophys. Res. Commun. **162**, 1188-1193.
5. Melkerson-Watson, L.J., Ogura, K., Wiesner, D., and Sweeley, C.C. (1990) manuscript submitted.
6. Hanai, N., Nores, G.A., MacLeod, C., Torres-Mendez, C.-R. and Hakomori, S., (1988) J. Biol. Chem. **263**, 10915-10921.
7. Bremmer, E., Hakomori, S., Bowen-Pope, D.F., Raines, E., and Ross, R. (1984) J. Biol. Chem. **259**, 6818-6825.
8. Bremmer, E., Schlessinger, J., and Hakomori, S. (1986) J. Biol. Chem. **261**, 2434-2440.
9. Hakomori, S. (1990) J. Biol. Chem., in press.
10. Usuki, S. and Sweeley, C.C. (1988) Ind. J. Biochem. Biophys. **25**, 102-105.
11. Paulson, J. C. (1987) Trends Biochem. Sci. **14**, 272-276.
12. Paulson, J. C. and Colley, K. J. (1989) J. Biol. Chem. **264**, 17615-17618.
13. Morre, D.J., Merlin, L.M., and Keenan, T.W. (1969) Biochem. Biophys. Res. Commun. **37**, 813-819.
14. Morre, D.J. (1971) Methods Enzymol. **22**, 130-148.
15. Fleischer, S., and Kervina, M. (1974) Methods Enzymol. **31**, 6-41.
16. Carey, D.J., and Hirschberg, C.B. (1980) J. Biol. Chem. **255**, 4348-4354.
17. Leelavathi, D.E., Estes, L.W., Feingold, D.S. and Lombardi, B. (1970) Biochim. Biophys. Acta **211**, 124-138.

18. Melkerson-Watson. L.J. and Sweeley, C.C. (1990) JBC, in press.

19. Holmgren, J., Svennerholm, L., Elwing, H., Fredman, P. and Strannegard, O. (1982) Proc. Natl. Acad. Sci. **77**, 1947-1950.

20. Paulson, J.C., Beranek, W.E. and Hill, R.L. (1977) J. Biol. Chem. **252**, 2356-2362.

21. Sadler, J.E., Rearick, J.I., Paulson, J.C., and Hill, R.L. (1979) J. Biol. Chem. **254**, 4434-4443.

22. Sadler, J.E., Rearick, J.I., and Hill, R.L. (1979) J. Biol. Chem. **254**, 5934-5941.

23. Weinstein, J., de Souza-e-Silva, U., and Paulson, J.C. (1982) J. Biol. Chem. **257**, 13835-13844.

24. Joziasse, D.H., Bergh, M.L.E., ter Hart, H.G.J., Koppen, P.L., Hooghwinkel, G.J.M. and Van den Eijnden, D.H. (1985) J. Biol. Chem. **260**, 4941-4951.

25. Gu, X.-B., Gu, T.-J. and Yu, R.K. (1990) Biochem. Biophys. Res. Commun. **166**, 387-393.

26. Colley, K.J., Lee, E.U., Adler, B. Browne, J.K. and Paulson, J.C. (1989) J. Biol. Chem. **264**, 17619-17622.

27. Lammers, G. and Jamieson, J. C. (1988) Biochem. J. **256**, 623-631.

28. Melkerson-Watson and Sweeley, C.C. (1990) Biochem Biophys. Res. Comm. **172**, 165-171.

29. Hanada, K., Tamai, M., Adachi, T., Oguma, K., Kashiwagi, K., Ohmura, S., Kominami, E., Towatari, T., and Katunuma, N. (1983) in Proteinase Inhibitors: Medical and Biological Aspects (Katunuma, N., ed.) Japan Soc. Press, Tokyo, p.p. 25-36.

30. Baubichon-Cortay, H., Broquet, P., George, P. and Louisot, P. (1989) Glycoconjugate J. **6**, 115-127.

31. Burczak, J.D., Fairley, J.L., and Sweeley, C.C. (1984) Biochim. Biophys. Acta **804**, 442-449.

32. Arribas, J. and Castano, J. G. (1990) J. Biol. Chem. **265**, 13969-13973.

33. Johnson, K.F., Dawson, G. and Hancock, L.W. (JBC manuscript submitted 4-10-89 entitled "Proteolytic Processing of Lysosomal Enzymes in Cultured Human Fibroblasts".

34. Pillai, S. and Zull J.E. (1985) J. Biol. Chem. 260, 8384-8389.

35. Kirschke, H., Langer, J., Wiederanders, B., Ansorge, S., Bohley, P. (1977) Eur. J. Biochem. **74**, 293-301

36. McKay, M. J., Marsh, M.W., Kirschke, H. and Bond, J.S. (1984) Biochim. Biophys. Acta **784**, 9-15

37. Melkerson-Watson, L.J., Ogura, K., Wiesner, D.A. and Sweeley, C.C. (1990) manuscript in preparation, see dissertation chapter 6.

38. Capasso, J.M., Keenan, T.W., Abeijon, C. and Hirshberg, C.B. (1989) J. Biol. Chem. **264**, 5283-5240.

39. Svennerholm, L. (1964) J. Lipid Res. **5**, 145-155.

40. IUPAC-IUB Recommendations (1977) Lipids **12**, 455-468.

CHAPTER 6

EVIDENCE FOR THE REGULATION OF GM_3 GANGLIOSIDE BIOSYNTHESIS IN KB CELLS BY PHOSPHORYLATION OF CMP-SIALIC ACID:LACTOSYLCERAMIDE α2-3 SIALYLTRANSFERASE (SAT-1)

A Model for the Regulation of Cell Growth Via GM_3 Ganglioside Metabolism

L.J. Melkerson-Watson, K. Ogura, D.A. Wiesner and C.C. Sweeley

Dept. of Biochemistry
Michigan State University
E. Lansing, MI 48824-1319

October 29, 1990

Running Title: Studies on the Purification and Regulation of SAT-1

ABSTRACT

Treatment of KB cells with 4 mM butyrate synchronizes the cells in late M/early G_1 phases of the cell cycle. In monolayer, butyrate changes the morphology of these cells, which display long processes. Turnover of CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1), GM_3 and GM_3 sialidase in KB cells grown in monolayer culture appear to be cell-cycle dependent. The highest level of SAT-1 activity coincides with maximum levels of the GM_3 ganglioside. GM_3 levels are minimal when GM_3 sialidase activity is optimum. SAT-1 activity peaks 6-10 hr after the release of the KB cells from a butyrate block. This increase of SAT-1 activity occurs during early G_1, about 4-6 hr before the cells begin DNA synthesis. In monolayer, sialidase activity was found to be at a maximum late G_1 phase just prior to DNA synthesis and again throughout G_2 phase.

Regulation of GM_3 synthesis may involve the phosphorylation of KB cell SAT-1. Analysis of immunoaffinity-purified KB cell SAT-1, during different time points of expression within the G_1 phase, by SDS-PAGE and its immunodetection on Western blots with monoclonal specific for phosphotyrosine residues, indicates SAT-1 is a phosphotyrosine-containing protein. The expression of this phosphotyrosine form may regulate SAT-1 activity.

INTRODUCTION

Involvement of glycosphingolipids as tissue-specific antigens in differentiation and oncogenic transformation has been established (1,2). Several lines of evidence suggest a functional role for gangliosides in cellular proliferation as well. Specifically, exogenous addition of GM_3 (3,4) and the lyso and de-N-acetyl derivatives of GM_3 (5,6) have been shown to alter the mitogenic response to EGF-receptor kinase activity. Synthesis and degradation of endogenous GM_3 may be dependent upon cell cycle activation of SAT-1 (7-10), altered expression of the acidic and neutral GM_3 sialidase activities (11,12), and recycling of the LacCer (13). If incorporation of exogenous GM_3 into the plasma membrane can regulate EGF-receptor function, a similar role for endogenous GM_3 may be expected (13).

We have proposed a model for the regulation of cell growth based on the synthesis and degradation of endogenous GM_3 (13) which accommodates our results (7-14) and those of Hakomori and coworkers (3-6). According to our model, the inhibitory effect of GM_3 on the EGF-receptor kinase activity is abolished by a cell cycle-dependent sialidase-catalyzed conversion of GM_3 to LacCer (14). LacCer may be internalized (13) and recycled back to GM_3 within the Golgi by cell-cycle dependent activation of SAT-1 (7-10,15), possibly by a phosphorylation/dephosphorylation mechanism (15,16). GM_3 expression (7) and SAT-1 activity (8-10,15) are maximal late M/early G_1 of the cell cycle. Apparently, the cyclic accumulation of GM_3 at G_1, prior to DNA synthesis (9,15) may inhibit progression through the cell cycle (14).

KB cell growth can be modulated chemically with butyrate (7-10,15), phorbol esters, and retinoic acid (17,18). These compounds dramatically and reversibly alter the activity of SAT-1, the enzyme which is responsible for the synthesis of the GM_3 ganglioside and representing the first committed step of a multi-step pathway for the biosynthesis of more complex gangliosides. KB cells can be blocked chemically with butyrate in late M/early G_1, synchronizing the cells at a point (12-15 hrs) prior to

DNA synthesis (9). Butyrate treatment results in a 5-fold (9) up to a 10 to 15-fold (8) increase in SAT-1 activity; however, it remains to be determined whether this effect results in an increase of GM_3 per cell or an increase of cells expressing GM_3. We report here the cell-cycle dependent expression of GM_3, SAT-1 and sialidase for human KB cells in monolayer cultures. Determination of the cell cycle dependent expression of SAT-1, GM_3 sialidase and the level of GM_3 is necessary in substantiating our model of cell growth (14) through the recycling of GM_3 and the regulation of GM_3 synthesis by cell-cycle-dependent phosphorylation of SAT-1.

MATERIALS AND METHODS

Materials. Cytidine 5'-monophosphate sialic acid, (CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid, 286.5 mCi/mmol) was purchased from New England Nuclear (Boston, MA) and its specific activity adjusted to 22,200 dpm/nmol with unlabeled CMP-sialic acid from Sigma (St. Louis, MO). The sphinganine-containing form of lactosylceramide and bovine brain gangliosides, type II, were purchased from Sigma Chemical Co. (St. Louis, MO) and used in enzyme activity assays. Ammonyx LO (lauryldimethylamine oxide, LDAO) was obtained from the Stepan Company (Northfield, IL). Sodium cacodylate and thymidine were purchased from Sigma Chemical Company (St. Louis, MO). Eagle's modified minimal medium (EMEM), EMEM for suspension culture (EMEM-S), antibiotic/antimycotic solution composed of penicillin, streptomycin sulfate, and fungizone, HEPES, fetal bovine serum, non-essential amino acid solution, and L-glutamine were purchased from GIBCO (BRL) Laboratories (Grand Island, NY). [^{3}H]-Thymidine (thymidine [methyl-^{3}H], specific activity 60-90 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Anti-phosphotyrosine monoclonal antibody was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti-mouse IgG-alkaline phosphatase conjugate was from Boehringer Mannheim (Indianapolis, IN). Prestained molecular weight standards and ultrapure electrophoresis reagents were bought from BioRad (Richmond, CA). All other reagents were ultrapure or ACS grade from commercial sources.

Cells. Human epidermoid carcinoma strain KB cells (American Type Tissue Culture, Rockville, MD, ATCC #CCL17) were cultured in monolayer on plastic culture flasks (Corning, 150 cm^2, #25120) or in suspension in glass spinner flasks (Bellco Glass Co., Vineland, NJ) using Eagle's minimal essential medium (EMEM) (or EMEM-S for suspension) supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL, Grand Island, NY), 100 μg/ml penicillin, 100 units/ml streptomycin, fungizone (antibiotic/antimycotic solution from Gibco/BRL (Grand Island, NY), MEM

nonessential amino acid solution (Gibco/BRL, Grand Island, NY) (KB/EMEM complete). The cells were grown in a humidified environment with 7.5% CO_2 in air at 37°C. The stock cultures of KB cells were subcultured (1:8) every 2-3 days by trypsinization with 0.25% trypsin/EDTA (Versene) solution for 1-2 min at 22°C. An equal volume of KB/EMEM complete medium was added to inhibit the trypsin. The seeding density for the KB cells for monolayer cultures was 6.6 x 10^4 cells per cm^2 (with 92% survival following trypsinization). The passage numbers of the KB cells used for these experiments were from P_{381}-P_{394}. All chemical treatments were administered along with fresh medium at the time the KB cells were subcultured in the following concentrations: butyrate, 4 mM; and thymidine, 2 mM. Drug treatments were for 24 and 20 hr, respectively. For ganglioside and enzyme assays, KB cells were harvested by trypsinization followed by three washes in PBS, pH 7.2, (calcium and magnesium free) and stored at -70°C.

Methods

Cell Synchronization

Butyrate Block of KB Cells. KB cells (maintained as described above) were treated with fresh complete medium containing a final concentration of 4 mM butyrate (sodium butyrate, Sigma Chemical Co., St. Louis, MO lot #74F5063 or butyric acid (lot #105F-0613) neutralized with NaOH) for 24 hr as previously described by Chatterjee *et al.* (7), Macher *et al.*, (8) and Moskal *et al.* (9). Cells were released from butyrate by washing the cells three times with Hanks buffered saline (HBSS), centrifuging the cells at 500 x g for 10 min following each wash, and incubating with KB/EMEM complete.

Double-Thymidine Block of KB Cells. Synchronization of monolayer cultures of KB cells by a double thymidine block procedure (7,9) was performed as follows. KB cells at 6.6 x 10^4 cells/cm^2 were incubated in KB/EMEM complete for 20 hr in the presence of 2 mM thymidine (Sigma Chemical Co., St Louis, MO). The first-round

thymidine-treated cells were removed from the excess thymidine-containing media by trypsinization followed by centrifugation. These cells were resuspended in the appropriate fresh medium and cultured for 12 hr. Again, the cells were harvested and resuspended in fresh medium containing 2 mM thymidine for 20 hr. Following the second treatment with thymidine, the cells were harvested and the cell pellets washed three times with PBS. Viable cell counts in triplicate were made with trypan blue (Gibco/BRL, Grand Island, NY) and a hemocytometer.

Determination of DNA Synthesis.

KB cells were seeded at a concentration of 6.6 x 10^4 cells/cm^2 in monolayer culture. The cells were synchronized with 4 mM butyrate for 24 hr, after which butyrate was removed (as described above) and the cells were resuspended in KB/EMEM complete. Viable cells were counted in triplicate on a hemocytometer with trypan blue at two hour intervals following the release of the cells from the butyrate block. Each sample (a 150 cm^2 tissue flask) was washed three times with 20 ml of HBSS and incubated 1 hr at 37°C in HBSS containing 1 mCi/ml [^{3}H]-thymidine (ICN, lot # 4498167). The [^{3}H]-thymidine was removed. The cells were washed three times with phosphate-buffered saline (PBS), treated with 10 ml ice-cold 5% TCA at room temperature for 30 min, and then washed twice with 10 ml 5% TCA. The cells were treated overnight with 5 ml 1 N NaOH at room temperature. The amount of [^{3}H]-thymidine incorporated into DNA was determined on each time point in triplicate. 5.0 ml of scintillant (Safety-Solve, Research products, Inc. Mount Prospect, IL) was added to each 50 μl aliquot for counting in a Packard Model #640C Liquid Scintillation Counter.

Preparation of Cell Membranes

Cell lysates were prepared by equilibrating the cells (1 x 10^6 cells/ml) in 1 mM TRIS-HCl (pH 7.2) in a 100% N_2 atmosphere at 40 psi for 5 min, then lysing the cells by returning them to normal atmospheric conditions (N_2 bomb). Subcellular

membranes were prepared by differential centrifugation. The cell lysate (5-10 ml) was first centrifuged at 1000 x g for 10 min. The postnuclear supernatant was centrifuged at 10,000 x g for 20 min. The postmitochondrial supernatant was centrifuged at 100,000 x g for 1 hr. The cellular membrane pellet was resuspended in 0.5 ml of 1 mM TRIS-HCl (pH 7.2) and assayed for SAT-1 or GM_3 sialidase activity and membrane protein.

Purification of CMP-sialic acid:lactosylceramide α2-3 Sialyltransferase.

CMP-sialic acid:lactosylceramide α2-3 sialyltransferase (SAT-1) was purified from KB cells by immunoaffinity chromatography using M12GC7 anti-SAT-1 monoclonal antibody coupled to Affi Gel 10 as previously described for the purification of rat hepatic SAT-1 (19). KB cells were cultured as described above and synchronized with either a butyrate or double-thymidine block. Control cultures were asynchronous cultures. KB cells were harvested and membranes were prepared in 1 mM TRIS-HCl (pH 7.2) containing both protease inhibitors (pepstatin (0.5 μg/ml), leupeptin (0.7 μg/ml), aprotinin (0.25 μg/ml)) and phosphatase inhibitors (0.2 mM orthovandate, 8 mM sodium pyrophosphate, 1 mM α-DL-glycerol phosphate, 4 mM sodium molybdate, 6 mM sodium fluoride and 5 mM dithiothreitol).

Assays

Protein Concentration. The amount of membrane protein associated with each fraction was determined by the Bradford method (20) relative to a bovine serum albumin (BSA) standard.

SAT-1 Activity Assay. SAT-1 assays, with radiolabeled CMP-sialic acid as the donor substrate and lactosylceramide as the acceptor substrate, were carried out by a modification of a previously described procedure (21). Assay mixtures contained the following components in 100 μl: lactosylceramide, 0.1 μmoles; CMP-[$^{14}C_{4,5,6,7,8,9}$]-sialic acid (22,200 dpm/nmole), 0.04 μmoles; sodium cacodylate buffer (pH 6.5), 10 mmoles (100 mM final concentration); manganese chloride, 1

mmole (10 mM final concentration); 5.0% (v/v) LDAO and 0.005-0.1 mg of the protein. GM_3 reaction product was recovered by reverse phase chromatography on Sep Pak C_{18} cartridges as previously described (22).

GM_3 Sialidase Activity Assay. GM_3 sialidase activity present at each time point was quantitated by a colorimetric assay detecting free sialic acid. The assay mixtures contained 0.25 mg of bovine brain gangliosides, type II (Sigma Chemical Co. St. Louis, MO), 100 μl of the cellular membrane fraction and 100 μl of sialidase assay buffer (0.2 M sodium acetate (pH 4.6) containing 1% (w/v) Triton CF-54. Following incubation of the ganglioside with the sialidase for 3 hr at 37°C, 100 μl of 25 mM periodic acid in 0.125 N H_2SO_4 was added. After 30 min at 37°C, 1.0 ml of TBA reagent was added and the solution incubated at 96°C for 7.5 min. The tubes were cooled and 2.0 ml acetone/HCl (40:1, v/v) was added. The absorbance at 551 nm was read and the amount of sialic acid released was quantitated relative to a sialic acid standard.

Ganglioside Analysis.

Extraction of Gangliosides. Gangliosides were extracted by a modification of the method of Ladisch and Gillard (24). Briefly, the frozen cell pellets were placed in a 10 ml conical centrifuge tube and 5 ml methanol was added. The samples were sonicated and vortexed to break up the cell pellet, then 10 ml chloroform was added and the samples were again sonicated and vortexed. The extraction was carried out for 6 hr at 5°C. The mixtures were then centrifuged at 2000 x g for 15 min and the supernatants removed. The pellets were resuspended in chloroform-methanol (1:1, v/v) and the extraction was again carried out for 6 hr. These mixtures were centrifuged and the supernatants combined and reduced to one quarter volume in 8 ml conical centrifuge tubes and kept at -20°C overnight. After centrifugation at 2000 x g for 15 min, the supernatants were removed and taken to dryness under N_2 and any remaining solvent removed by lyophilization.

The total lipid extracts were taken up in 3 ml of diisopropyl ether/butanol (6:4, v/v) and dispersed by several minutes of sonication and vortexing. 1.5 ml of 100 mM NaCl was added and the solutions were sonicated and vortexed for approximately 2 min then centrifuged at 500 x g for 10 min. The upper organic phases were removed and the lower ganglioside-containing aqueous phases were re-extracted with 3 ml of fresh organic solvent. After sonication and vortexing and centrifugation, the organic phases were removed and the aqueous phases lyophilized overnight.

To remove salts and other low molecular contaminants, the samples were run over a 10 ml Sephadex G-50 column. Voided compounds were collected and solvent removed by lyophilization.. The samples were taken up in 200 μl chloroform-methanol (1:1, v/v) and centrifuged to remove residual insoluble impurities.

Ganglioside Quantitation. The gangliosides, extracted from the KB cells at each time interval following the release of the cells from butyrate, were applied in total onto a pre-run, pre-activated HPTLC plate in 5 mm bands. Known concentrations of GM_2 (by resorcinol assay (25) for lipid bound sialic acid, LBSA) were simultaneously chromatographed for quantitation of the gangliosides. The gangliosides were chromatographed on the HPTLC plate in a chloroform/methanol/0.2% $CaCl_2 \cdot 2H_2O$ (60:40:9, v/v/v) solvent system. Gangliosides were visualized by spraying with resorcinol-HCl reagent (25) and heating at 110°C for 12 min.

The plate was immediately scanned using a BioImage Visage 110 digital imager in the transmissive mode at an integration time of 126. Gangliosides were quantified by comparison with the GM_2 standard curve, where one fg of LBSA/cell equals integrated intensity/cell x 11.

RESULTS

Determination of SAT-1 Expression in KB Cells

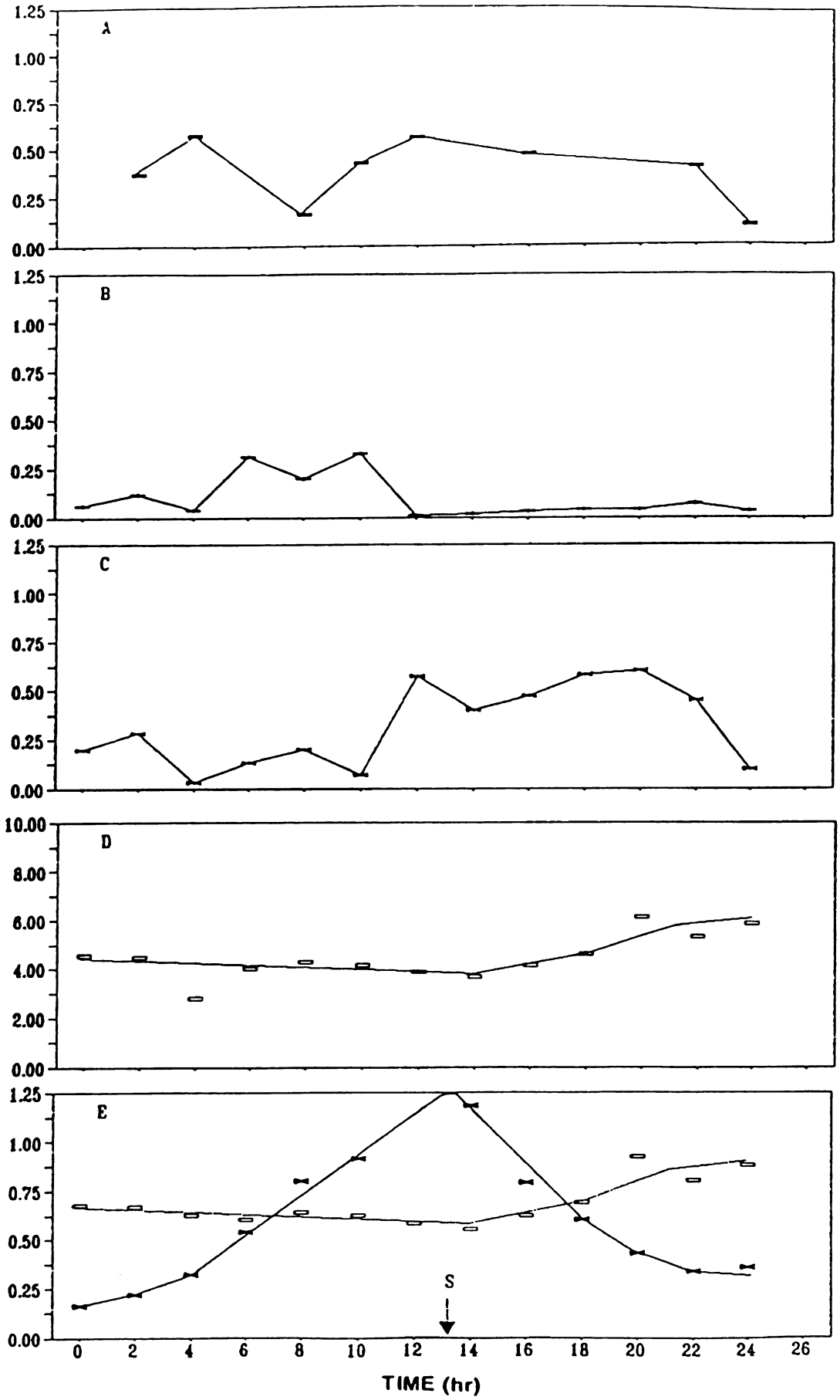
Turnover of GM_3 Ganglioside, SAT-1 and Sialidase Following Release of Butyrate Treatment. Under the conditions employed, KB cell SAT-1 was expressed maximally 6-10 hr following the release of the cells from the butyrate block. Monolayer cultures of KB cells contained maximal levels of GM_3 immediately upon release from butyrate decreasing to a minimum during early G_1 (8 hr after release of butyrate block) (Figure 1a). GM_3 increased to maximum levels mid to late G_1 (8-12 hr after release) coinciding with increased SAT-1 activity (Figure 1a and 1b). The amount of GM_3 decreased upon the activation of sialidase prior to S (Figure 1a and 1c). The sialidase activity was broad and showed two maxima. The first peak in sialidase activity was observed at 12 hr coincident with decreased GM_3 and just prior to the cells entering S phase (1e). The second peak was observed 16-22 hr after removal of butyrate and coincided with the appearance of more complex gangliosides (data not shown).

Additionally, GM_2 levels peaked with S phase and decreased during M phase and throughout the remainder of S phase there was a significant increase in GM_2 ganglioside as well as a proportional increase in the higher order gangliosides (data not shown). Thus, at the point in the cell cycle when monolayer KB cells commit to another round of replication or become quiescent, GM_3 is catabolized by GM_3 sialidase or GM_3 is metabolized to GM_2. Either pathway relieves the constraint of the cell for another round of replication.

Determination of DNA Synthesis. KB cells treated with 4 mM butyrate for 24 hr followed by growth in fresh KB/EMEM complete culture medium in the absence of butyrate, showed an increase in [^{3}H]-thymidine incorporation 12-14 hr after removal of the butyrate (Figure 1e). The peak was broad yet symmetrical, indicating that the butyrate treatment of these cultures synchronized the KB cells under culturing

Figure 1. KB Monolayer Culture Metabolism of GM_3 Ganglioside.

Human epidermoid carcinoma cells (KB cells) were grown in EMEM (Gibco/BRL, Grand Island NY) supplemented with 10% fetal bovine serum, non-essential amino acids, and Gibco's antibiotic/antimycotic solution at 37°C and 7.5% CO_2. KB cells were seeded at 6.6 x 10^4 cells/cm^2 in 150 cm^2 tissue culture flasks (Corning) containing 25 ml of fresh KB/EMEM complete medium. After establishing the cells for 24 hr, the cells were treated with 4 mM sodium butyrate in fresh KB/EMEM complete medium (pH 7.4) for 24 hr. The cells were released from the butyrate block by trypsinizing the cells and centrifuging at 500 x g for 10 min, washing three times with PBS (pH 7.2). The cells were cultured in fresh KB/EMEM complete medium. At each time interval indicated, the cells were harvested by trypsinization and centrifuging at 500 x g for 10 min, washing three times with PBS (pH 7.2). The viable cell density was determined by counting a 1:2 dilution of KB cells in trypan blue vital stain. The cell pellets were frozen at -70°C until assayed for either GM_3 content by the Ladisch procedure (24), or the microsomes assayed for SAT-1 and GM_3 sialidase activities (as described in Materials and Methods). Determination of DNA synthesis by incorporation of [^{3}H]-thymidine in KB cells grown in monolayer culture after release from butyrate is described in detail under methods. The data presented in this figure are: (A) GM_3 (nmol/cell); (B) Specific activity of SAT-1 (pmol/min/mg); (C) Specific activity of GM_3 sialidase (nmol/min/mg); (D) Cell density (cells/cm^2 x 10^4); (E) [^{3}H]-thymidine incorporation (dpm/cell) and number of cells per sample (x 10^7).


A
B
C
D
E
1.25
1.00
0.75
0.50
0.25
0.00
10.00
8.00
6.00
4.00
2.00
S
0
2
4
6
8
10
12
14
16
18
20
22
24
26
TIME (hr)

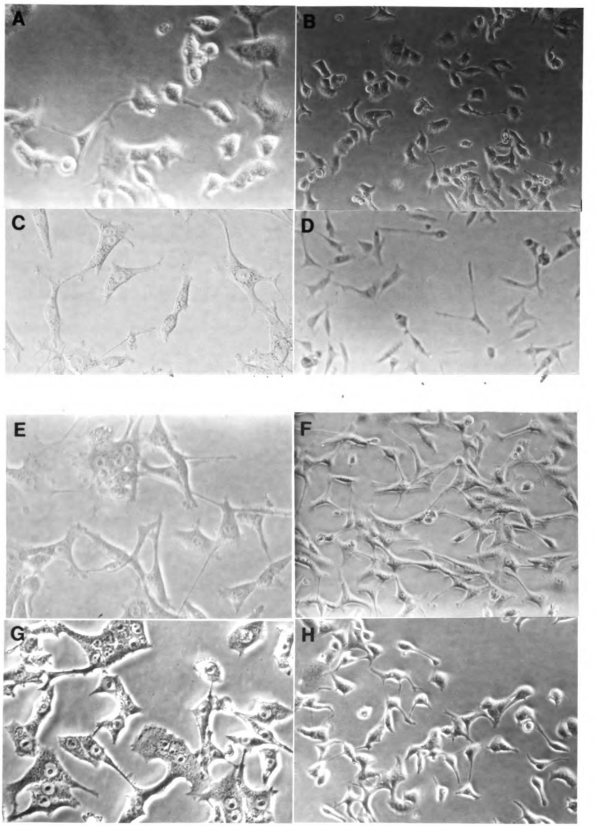
conditions employed.

Morphological Changes Induced by Butyrate and Thymidine Blocks in Monolayer Cultures of KB Cells. The morphology of KB cells treated with butyrate exhibit a drastic change in their morphology (8,9). As illustrated in Figure 2, KB cells change from round or polygonal shape (Figure 2a and 2b) to one that is more fibroblastic with long membranous processes (Figure 2c and 2d). This alteration of morphology is reversible as can be seen in photos taken of KB cells 6 hr following the removal of butyrate from the culture medium (Figure 2e and 2f). These observation correlate with previous reports from this laboratory (8,9). Treat of monolayer cultures of KB cells with a double thymidine block also cause distinct morphological changes. The cells, just prior to S phase, become more dense and the nuclei and nucleosomes very pronounced (Figure 2g and 2h). It is expected that these morphological changes of KB cells are associated with changes of the complex carbohydrates on the cell surface and the enzymes responsible for their synthesis. For example, GM_3 levels on the cell surface correlate well with the expression of SAT-1 activity.

Regulation of SAT-1 Activity in KB Cells by Phosphorylation.

SAT-1 Activity in Synchronized KB Cells. To investigate phosphorylation as a mode of regulating SAT-1, butyrate and thymidine were used to block KB cells at two different points in the G_1 phase of cell cycle. Butyrate treatment blocks KB cells just prior to maximal activity and thymidine blocks the cells when SAT-1 is inactive. The distribution of SAT-1 activity in KB cells treated with 4 mM butyrate, 6 hr after butyrate release, and 2 mM thymidine (double block) is illustrated in Figure 3. Cellular fractions of KB cells blocked 24 hr with butyrate gave SAT-1 activity levels slightly higher than control asynchronous cultures. Six hours following removal of butyrate SAT-1 activity was about the same (120% of control) as that of control cultures. KB cell SAT-1 activity from cells 6 hr after release from butyrate was three

Figure 2. Morphological Changes Induced by Butyrate and Thymidine Blocked Monolayer Cultures of KB Cells. KB Cells were grown in KB/EMEM complete medium without butyrate (A and B), with butyrate (C and D), six hours after the release of butyrate (E and F) and with thymidine (G and H) as described under Materials and Methods at a seeding density of 6.6×10^4 cells/cm^2 into 150 cm^2 tissue culture flasks. Panels A, C, E and G are 10X magnification of the cells and panels B, D, F and H show the cells at 20X.


A
B
C
D
E
F
G
H

times control (279%) KB cell SAT-1 from double-thymidine blocked cells showed diminished levels of activity (21% of control).

Immunoaffinity Purification of KB Cell SAT-1. Recently, we reported the purification of SAT-1 from rat liver Golgi to apparent homogeneity by immunoaffinity chromatography using a monoclonal antibody to rat liver SAT-1 (19). This monoclonal, M12GC7, specifically inhibited and could immunoprecipitate rat hepatic SAT-1 activity. M12GC7 can also immunodetect and immunoprecipitate KB cell SAT-1 (Figure 4). The multiple bands resolved were likely the result of proteolytic degradation of the sialyltransferase. Proteolytic activity and degradation has been reported associated with other glycosyltransferases (26-29) and has been postulated to play a mechanistic role in the release of glycosyltransferases from the Golgi (28).

Immunodetection of SAT-1 from KB Cells with Anti-Phosphotyrosine Monoclonal. SAT-1 from KB cells treated with 4 mM butyrate, 6 hr after butyrate treatment, or 2 mM double thymidine and control cells (asynchronous culture) was immunoaffinity-purified from detergent-extracted Golgi-enriched membranes as described previously for the rat liver enzyme (19). An antiphosphotyrosine monoclonal antibody detected a pattern of phosphotyrosine-containing polypeptides unique to each sample of immunoaffinity-purified SAT-1 (Figure 5). This may represent a turnover of the phosphorylated enzyme corresponding to different times of SAT-1 expression during the G_1 phase.

SAT-1 from the control cells contained a phosphotyrosine in the band corresponding to the 68 Kd mature form of SAT-1 with phosphotyrosine also being detected at the 60, 54, 37, 33 and 25 Kd. Butyrate-synchronized cells contained phosphotyrosine in the 68, 60, and 54 Kd polypeptides. Six hours following release of the butyrate from the cells, phosphotyrosine was immunodetected at 68, 60, 47, 37, 35, 33, 31, 25 and 18 Kd. SAT-1 immunoaffinity-purified from thymidine-blocked KB cells contained phosphotyrosine predominantly in the 68 Kd and 25 Kd bands with

Figure 3. SAT-1 Activity Associated with KB Cells. KB cells were synchronized with either butyrate or thymidine and their membranes recovered as described under the methods. SAT-1 activity was assayed as described under the experimental protocol. Controls were cellular fractions from asynchronous cultures of KB cells. CL is the specific activity associated with the cell lysate and M is the specific acitivity associated with the Golgi-enriched membrane fraction prepared as described under the methods section.

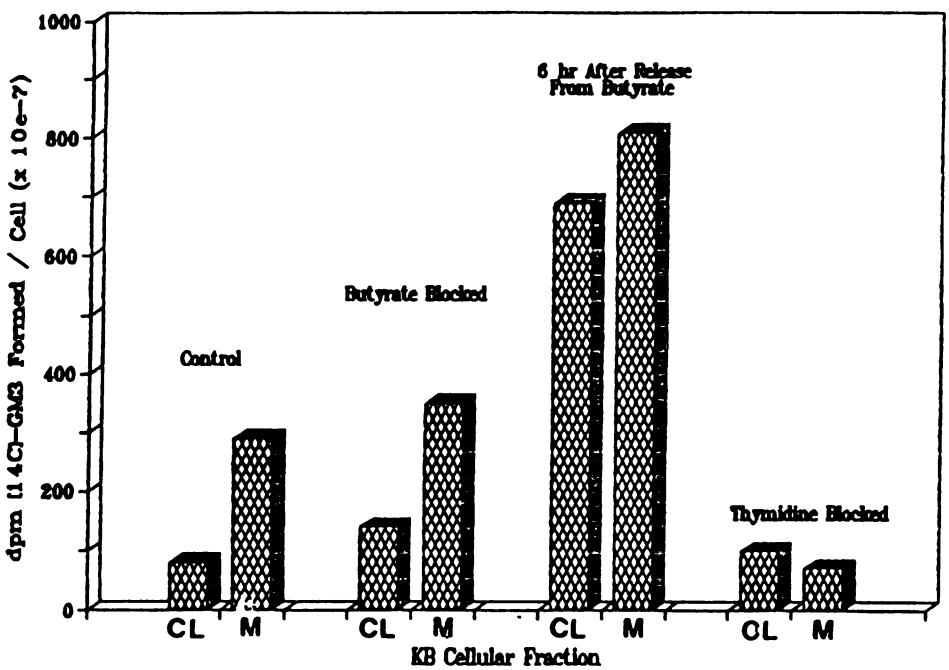

1000
800
600
400
200
0
dpm [14C]-GM3 Formed / Cell (x 10e-7)
6 hr After Release From Butyrate
Butyrate Blocked
Control
Thymidine Blocked
CL
M
CL
M
CL
M
CL
M
KB Cellular Fraction

Figure 4. Immunoprecipitation of SAT-1 from KB Cells. KB cell SAT-1 was immunoprecipitated from asynchronous cultures of KB cells with M12GC7 anti-SAT-1 specific monoclonal antibody bound to *Staph aureus* protein A-conjugated with rabbit anti-mouse IgG as previously described (31). The Staph aureus - rabbit anti-mouse IgG - M12GC7 - SAT-1 conjugate was pelleted by centrifugation, washed, and the SAT-1 released and analyzed by SDS-PAGE on 12% polyacrylamide. The *Staph aureus* cells were a gift from Dr. William Smith (Michigan State University, East Lansing, MI). The closed circles represent the molecular weight of the SDS-PAGE standards (BioRad) and the arrows indicate the molecular weights of the KB cell SAT-1 polypeptides.

KB Cell Polypeptides Detected by anti-SAT-1 12GC7 MAb in Western Blots of Total Cell Lysates

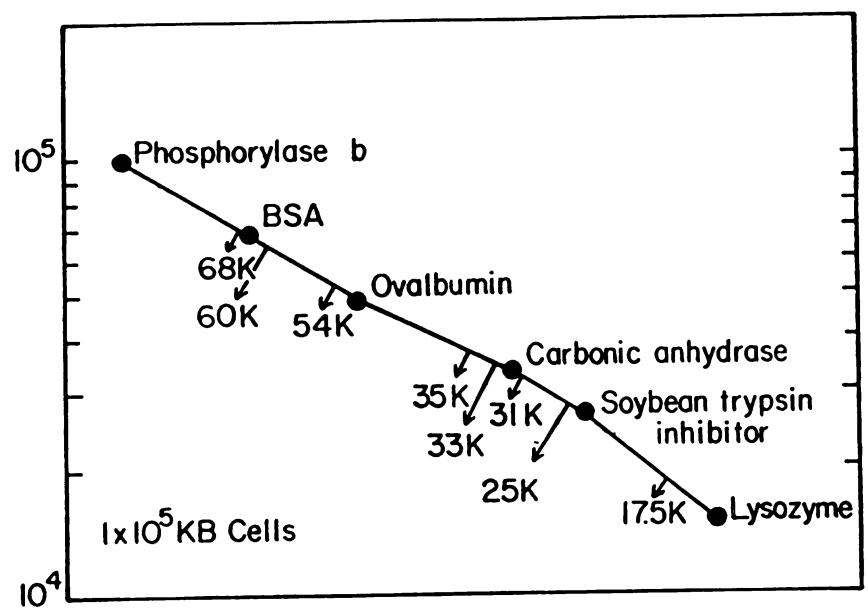
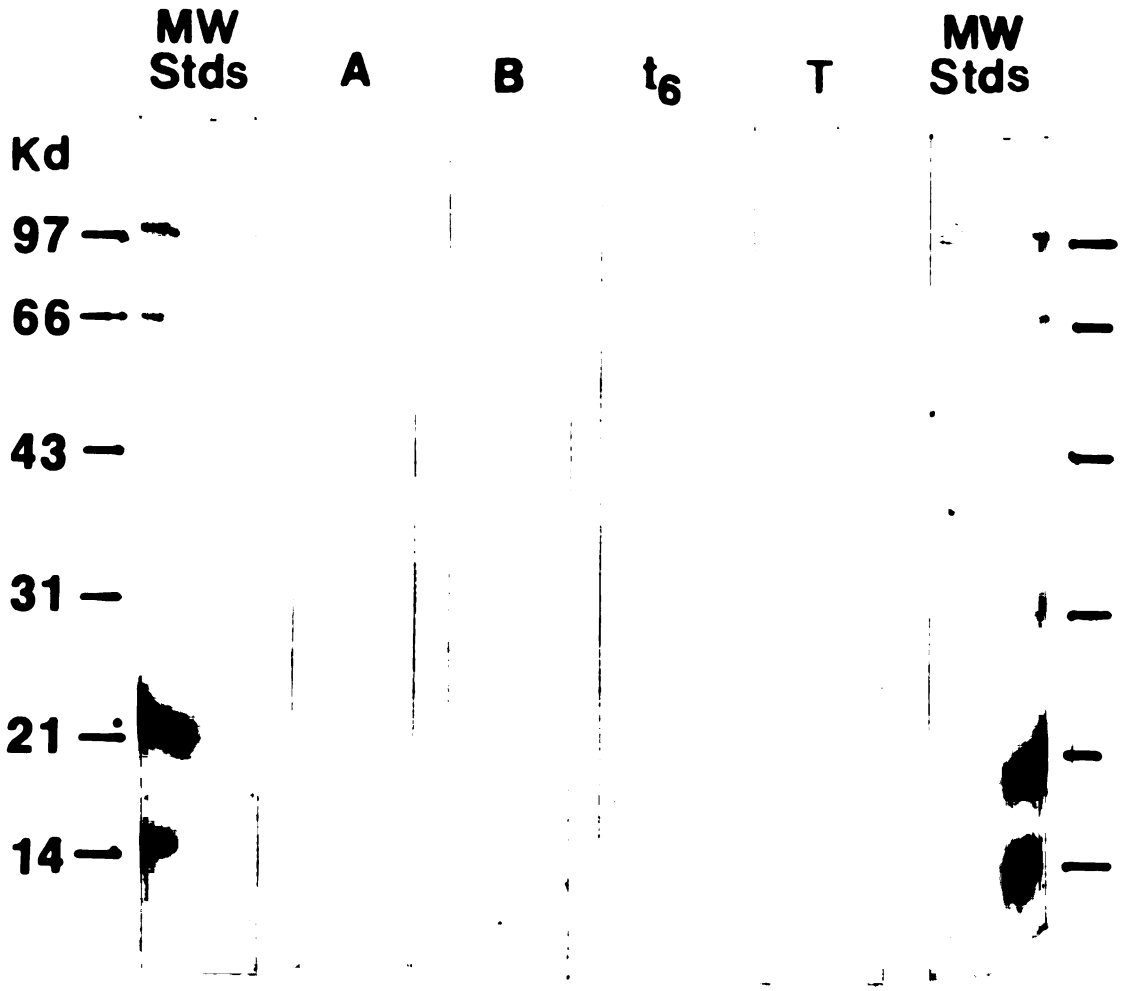


Figure 5. Immunodetection of Purified KB Cell SAT-1 by an Antiphosphotyrosine Monoclonal Antibody. SAT-1 was immunoaffinity purified with M12GC7-Affi Gel 10 from detergent-extracted Golgi-enriched membrane fraction (19) of KB cells that were blocked in G_1 phase with butyrate, 6 hours after release of butyrate, or blocked pre-S phase by a double-thymidine block, and control (asynchronous cells without chemical treatment). SAT-1 (10 μg) from each treatment was electrophoresed and transferred to Immobilon using 10 mM CAPS (pH 11.0) methanolic electrotransfer buffer with the BioRad Mini-Protean II system. The blot was blocked with 2% (w/v) gelatin in TBS (pH 7.4) for 1 hr at 37°C and incubated with a monoclonal antibody specific for antiphosphotyrosine (Upstate BioTechnology). Alkaline-phosphatase secondary antibody with NBT/BCIP reagent served as the detection method. Lane A shows phosphotyrosine-containing polypeptides of the asynchronous KB cells. Lane B shows the phosphotyrosine-containing SAT-1 polypeptides from butyrate-synchronized KB cells. Lane t_6 are the phosphotyrosine-containing SAT-1 polypeptides from KB cells six hours after they were released from butyrate. Lane T contains the SAT-1 polypeptides recovered from KB cells following a double-thymidine block.


MW Stds
A
B
t_6
T
MW Stds
Kd
97
66
43
31
21
14

minor bands detected at 60 and 47 Kd. These data may indicate that SAT-1 is activated by phosphorylation of specific tyrosine residue(s) and that this phosphorylated form of the enzyme may be proteolytically degraded as the KB cell progresses through the G_1 phase.

Control blots, performed following the pre-incubation of the antiphosphotyrosine monoclonal antibody with 50 mM soluble phosphotyrosine, resulted in no detection of SAT-1 with the antibody. Preincubation with 50 mM phosphoserine or phosphothreonine had no effect on the binding of the monoclonal (data not shown). These control experiments indicate this is a specific interaction between the monoclonal and the purified SAT-1 and the results demonstrate the presence of a phosphorylated form of SAT-1 corresponding to the active form of SAT-1, namely 6 hr following the release of butyrate from the cells. Control cultures also contained some detectable phosphotyrosine-containing SAT-1 at the 60,000 molecular weight.

DISCUSSION

KB cells treated with butyrate become synchronized late M / early G_1 of the cell cycle. These data and observations correlated well with previous reports from this laboratory (7-9). The morphology of these cells changes dramatically when in the presence of butyrate. There is an alteration of their cell surface complex carbohydrates and a change from polygonal-shaped cells to cells with a more fibroblastic appearance. Butyrate-treated cells characteristically have long membranous processes.

The turnover of GM_3 on the cell surface of KB cells was monitored as a function of time following the release of KB cells from butyrate and as a function of the enzymes responsible for its synthesis (CMP-sialic acid:lactosylceramide sialyltransferase, SAT-1) and its degradation (GM_3 sialidase). KB cells were chosen because they are easily and consistently synchronized with either a butyrate block (7-9) or a double-thymidine block (7,9). Previously, our laboratory reported an elevation is the levels of GM_3 and elevations in SAT-1 activity associated with treatment of these cells with butyrate (7-9). While cell culture-dependent expression of gangliosides has been demonstrated (7), neither the cell-cycle-dependent turnover of GM_3, nor the mechanism regulating GM_3 ganglioside biosynthesis and degradation were known.

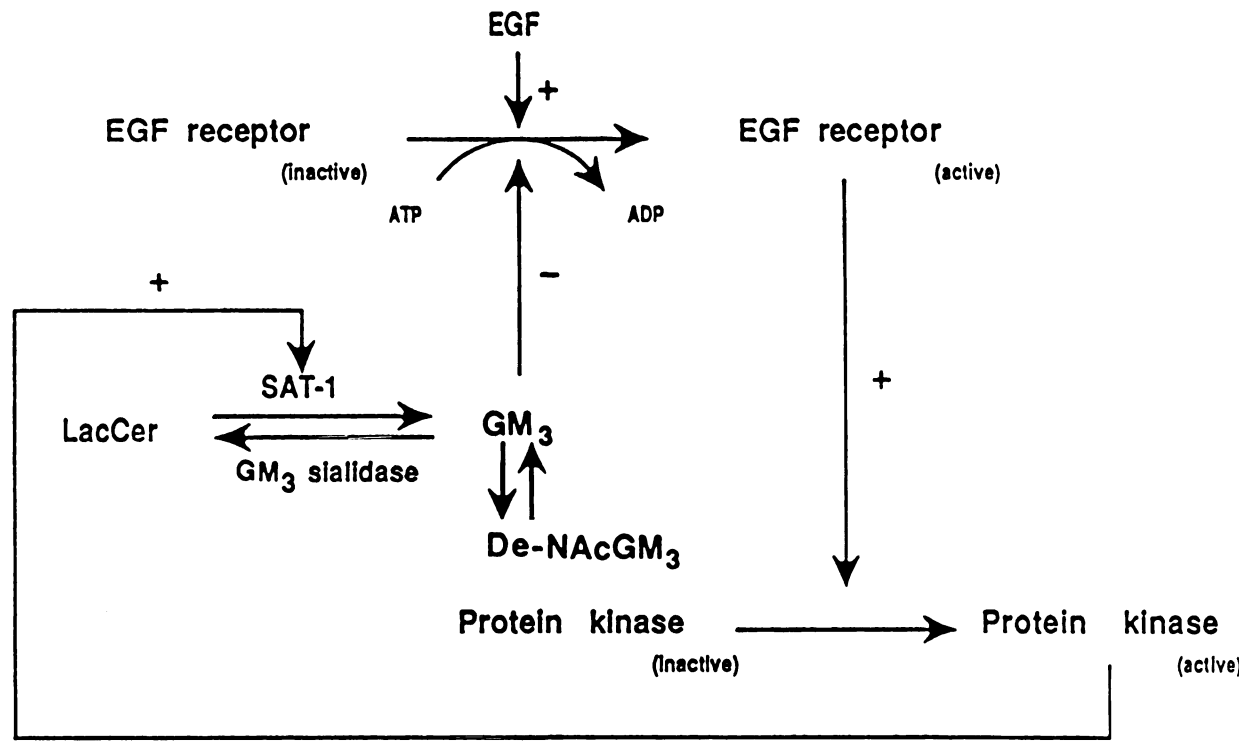
These results obtained for monolayer culture KB cells differ from those previously reported for KB cells in monolayer. Previously, in monolayer cultures, the SAT-1 activity and GM_3 levels of KB cells were highest immediately following release from butyrate (8,9). The data obtained for optimal GM_3 sialidase activity prior to S phase and during G_2 phase of the cell cycle agree with those reported for monolayers of normal human fibroblasts (11,12). The observed difference for SAT-1 activity and GM_3 levels may reflect the nature of the microtubule disruption caused by trypsinization and immediate treatment with butyrate.

While the regulation of glycosphingolipid metabolism and inhibition of cell proliferation with increased levels of GM_3 are largely correlational. GM_3 has been

demonstrated to modulate the protein kinase C and EGF-receptor tyrosine kinase signal transduction systems (3-6). We speculate that the regulation of GM_3 synthesis may involve phosphorylation of SAT-1. Previously, we reported increased SAT-1 activity following incubation of crude homogenates with a cAMP-dependent protein kinase and decreased activity following treatment with alkaline phosphatase (16). Immunodetection of immunoaffinity-purified SAT-1 with an anti-phosphotyrosine monoclonal indicates SAT-1 may contain one or more phosphotyrosine residues. We would like to propose an extension of our model (14) for the regulation of cellular proliferation by GM_3 ganglioside through the regulation of its synthesis by a phosphorylation mechanism as it potentially pertains to the EGF-R signal transduction (Figure 6). The model shown in Figure 6 is only hypothetical at this time, but it does accommodate the results reported by our laboratory (7-19) and those of Hakomori and coworkers (3-6, for review 30). Efforts in our laboratory continues to address the resolution of this cell growth regulatory mechanism involving the synthesis and degradation of GM_3 by SAT-1 and sialidase.

Figure 6. Proposed Model of Cell Growth Regulation. A working model for the regulation of cell growth based on the metabolism of GM_3, SAT-1 phosphorylation, and EGF-receptor phosphorylation. EGF-R phosphorylation is controlled by the presence of the GM_3 ganglioside. GM_3 is a negative effector of EGF-R autophosphorylation (3,4,30) and inhibits cell growth. The level of cell surface GM_3 and its turnover is regulated by extracellular sialidase (11-14), de-acetylase (5) and by SAT-1 (present study). SAT-1 (tyrosine) phosphorylation may be integrally related to the EGF-R tyrosine kinase signal transduction system.

Proposed Model for a Regulatory Mechanism of Cell Growth via the Metabolic Synthesis of GM3 by SAT-1



FOOTNOTES

[1]To whom correspondence should be addressed.

[2]This work was supported in part by a research grant from the National Institutes of Health (DK12434).

ACKNOWLEDGEMENTS

We thank Douglas Wiesner and Kiyoshi Ogura for their assistance with the quantitation of the KB cell GM_3 ganglioside and sialidase activity, respectively.

We thank Carol Smith for her excellent secretarial support.

ABBREVIATIONS

[3]The abbreviations used are:

CMP, cytidine 5'-monophosphate;

EGF, epidermal growth factor;

EMEM, Eagle's Minimal Essential Medium;

EMEM-S, Eagle's Minimal Essential Medium for Suspension Culture;

FBS, fetal bovine serum;

GD_{1a}, (NeuAcα2-3)Galß1-4GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß1-1Ceramide;

GD_3, NeuAcα2-8NeuAcα2-3Galß1-4Glcß1-1Ceramide;

GM_1, Galß1-4GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß1-1Ceramide;

GM_2, GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß1-1Ceramide;

GM_3, NeuAcα2-3Galß1-4Glcß1-1Ceramide;

GT_{1a},(NeuAcα2-8NeuAcα2-3)Galß1-4GalNAcß1-4(NeuAcα2-3)Galß1-4Glcß1-1Ceramide;

GT_{1b}, (NeuAcα2-3)Galß1-4GalNAcß1-4(NeuAcα2-8NeuAcα2-3)Galß1-4Glcß1-1Ceramide;

Gangliosides and Glycosphingolipid*

HBSS, Hank Buffered Salts Solution;

Kd, kilodalton;

LDAO, lauryldimethylamine oxide, also known as Ammonyx LO;

SAT-1, CMP-sialic acid:lactosylceramide α2-3 sialyltransferase, also known as GM_3 synthase;

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;

*All abbreviations for Glycosphingolipids are according to the Svennerholm nomenclature (32) and IUPAC-IUB recommendations (33).

REFERENCES

1. Hakomori, S. (1981) Ann. Rev. Biochem. **50**, 733-764.
2. Ledeen, R.W. (1989) in Neurobiology of Glycoconjugates, (Margolis, R.K. and Margolis, R.U., eds.) Plenum Press, NY, p.p.43-83.
3. Bremer, E.G., Hakomori, S., Bowen-Pope, D.F., Raines, E. and Ross, R. (1984) J. Biol. Chem. **259**, 6818-6825.
4. Bremer, E.G., Schlessinger, J. and Hakomori, S. (1986) J. Biol. Chem. **261**, 2434-2440.
5. Hanai, N., Dohi, T., Nores, G.A. and Hakomori, S. (1988) J. Biol. Chem. **263**, 6296-6301.
6. Hanai, N., Nores, G.A., MacLeod, C., Torres-Mendez, C.-R. and Hakomori S. (1988) J. Biol. Chem. **263**, 10915-10921.
7. Chatterjee, S., Sweeley, C.C. and Velicer, L.F. (1975) J. Biol. Chem. **250**, 61-66.
8. Macher, B.A., Lockney, M., Moskal, J.R., Fung, Y.K. and Sweeley, C.C. (1978) Exp. Cell Res. **117**, 95-102.
9. Moskal, J.R., Lockney, M.W., Marvel, C.C., Mason, P.A. and Sweeley, C.C. (1980) in ACS Symposium Series, No. 128, Cell Surface Glycolipids (C.C. Sweeley, ed.), p.p. 241-263.
10. Burczak, J.D., Fairley, J.L. and Sweeley, C.C. (1984) Biochem. Biophys. Acta **804**, 442-449.
11. Usuki, S., Lyu, S.-C., Sweeley, C.C. (1988) J. Biol. Chem. **263**, 6847-6853.
12. Usuki, S., Hoops, P. and Sweeley, C.C. (1988) J. biol. Chem. **263**, 10595-10599,
13. Swamy, M.J. and Sweeley, C.C. (1989) Biochem. Biophys. Res. Commun. **162**, 1188-1193.
14. Usuki, S. and Sweeley, C.C. (1988) Ind. J. Biochem. Biophys. **25**, 102-105.

15. Melkerson-Watson, L.J., Ogura, K., Wiesner, D.A. and Sweeley, C.C. (1990), submitted for publication.

16. Burczak, J.D., Soltysiak, R.M. and Sweeley, C.C. (1984) J. Lipid Res. **25**, 1541-1547.

17. Burczak, J.D., Moskal, J.R., Trosko, J.E., Fairley, J.L. and Sweeley, C.C. (1983) Exp. Cell Res. **147**, 281-286.

18. Moskal, J.R., Lockney, M.W., Marvel, C.C., Trosko, J.E. and Sweeley, C.C. (1987) Cancer Res. **47**, 787-790.

19. Melkerson-Watson, L.J. and Sweeley, C.C. (1990) "Purification to Apparent Homogeneity by Immunoaffinity Chromatography and Partial Characterization of the GM_3 Ganglioside Forming Enzyme, CMP-Sialic acid:Lactosylceramide α2-3 Sialyltransferase (SAT-1), From Rat Liver Golgi." submitted for publication (Chapter 3).

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

21. Burczak, J.D., Fairley, J.L., and Sweeley, C.C. (1984) Biochim. Biophys. Acta **804**, 442-449.

22. Melkerson-Watson, L.J., Kanemitsu, K. and Sweeley (1987) Glycoconjugate J. **4**, 7-16.

23. Holmgren. J., Svennerholm, L., Elwing, H., Fredman, P. and Strannegard, O. (19) Proc. Natl. Acad. Sci. **77**, 1947-1950.

24. Ladisch, S. and Gillard, B. (1987) Methods Enzymol. **138**, 300-306.

25. Ledeen, R.W. and Yu, R.K. (1982) Methods Enzymol. **83**, 139-191.

26. Weinstein, J., de Souza-e-Silva, U., and Paulson, J.C. (1982) J. Biol. Chem. **257**, 13835-13844.

27. Paulson, J.C. (1987) Trends Biochem. Sci. **14**, 272-276.

28. Paulson, J.C. and Colley, K.J. (1989) J. Biol. Chem. **264**, 17615-17618.

29. Lammers, G. and Jamieson, J.C. (1988) Biochem. J. **256**, 623-631.

30. Hakomori, S. (1990) J. Biol. Chem. **265**, 18713-18718.

31. DeWitt, D.L. and Smith, W.L. (1982) Methods Enzymol. **86**, 240-246.

32. Svennerholm, L. (1964) J. Lipid Res. **5**, 145-155.

33. IUPAC-IUB Recommendations (1977) Lipids **12**, 455-468.

CHAPTER 7

DISCUSSION

History and Perspectives

During the last five to ten years, several studies on glycosphingolipid function have suggested several possibilities: (a) as modulators of membrane receptors and transmembrane signal inducers, (b) as mediators of cell-cell recognition in development and differentiation, and (c) as membrane receptors. The term "bimodal" has been used to describe the role of one particular glycosphingolipid, GM_3 ganglioside (1) as it primarily functions as a membrane modulator of membrane-associated protein kinases and mediator of cell adhesion. This duality of function by GM_3 at the cell surface appears to be dependent on whether the cell is quiescent or in a proliferative state (for review, 1,2).

The concept that GM_3 ganglioside, as well as other gangliosides, regulate cell growth was initially based on changes in glycosphingolipid synthesis associated with oncogenic transformation, cell cycle and "contact inhibition" of cell growth (for review, 2). The idea that GM_3 may influence cell proliferation was suggested by the close association between the level of GM_3 on the cell surface, the level of SAT-1 activity and cell growth (3-10; for review, 1).

The effect of gangliosides on cell growth modulation through modified signal transduction has been shown to occur in two of the three major transmembrane signaling systems operating in most eukaryotic cells, growth-factor receptor-associated protein kinases, i.e., the EGF-R autophosphorylation (11,12) and protein kinase C (13,14). Modulation of these two mechanisms by GM_3 ganglioside and its naturally occurring catabolites, lyso-GM_3 (13, 14), de-N-acetyl-GM_3 (15) and N,N-dimethylsphingosine (16), was work accomplished through the efforts of Hakomori and coworkers (for review, 1). They have demonstrated that exogenously added GM_3

(11,12) and lyso GM_3 (13,14) strongly inhibit EGF-R tyrosine autophosphorylation, while de-N-acetyl GM_3 (15) and N,N-dimethylsphingosine (16) increase this EGF-R kinase activity. Lyso-GM_3 and N,N-dimethylsphingosine inhibit protein kinase C activity. A major criticism of these studies has been whether or not these observations with exogenously added GM_3, or its derivatives, is merely a pharmacological effect (17) or reflects an actual role of endogenous GM_3 (9).

Recently, Weis and Davis (17) designed a series of experiments to test the hypothesis that expression of GM_3 at physiological levels modulates the signal transduction of the EGF-R in mutant CHO cells which possess a reversible defect in glycosylation. In the absence of galactose these cells cannot synthesize gangliosides cannot perform terminal glycosylations of glycoprotein (an experimental problem). Weis and Davis (17) demonstrated that the state of glycosylation did not affect the ability of the EGF-R to bind EGF and function as a receptor. Using these cells, Weis and Davis (17) demonstrated that decreased expression of gangliosides there was increased EGF-R autophosphorylation and increased EGF-associated cellular proliferation. The converse was also true. Thus, their findings for endogenously synthesized ganglioside modulation of EGF-R were consistent with those reported for exogenously added GM_3 (11,12), adding support to the hypothesis that EGF-R function is modulated by gangliosides. However, the level of *in vitro* autophosphorylation of EGF-R isolated from the mutant CHO cell membranes was independent of galactose. They concluded from these results that the inhibition of tyrosine autophosphorylation of the EGF-R in these cells was not due to the direct interaction of the EGF-R with GM_3. They proposed alternative mechanisms for the observed inhibition of EGF-R tyrosine kinase activity in these galactose-dependent cells: i.e., other galactose-regulated molecules may modulate EGF-R autophosphorylation; inhibition by GM_3 on EGF-R autophosphorylation may involve the state of threonine phosphorylation of the receptor or the oligomeric state of the receptor, as previously suggested by Schlessinger (18).

This study by Weis and Davis (17) is in contrast to findings by Wedegaertner and Gill (19), who examined the effect sphingosine on the activation of an immunoaffinity-purified protein tyrosine kinase domain of the EGF-R. Their results demonstrated that the addition of sphingosine induced a conformational change that mimics the effect of the conformational change of the holo-receptor when EGF binds, inducing a fully active tyrosine kinase activity. Further, the GM_3 ganglioside has been shown to co-precipitate with the EGF-R using EGF-R specific monoclonal antibody (20). Clearly, the mechanism of the functional role played by GM_3, and its catabolites, on the autophosphorylation of the EGF-R, and concomitantly cell growth regulation, is controversial and needs to be defined.

Work in our laboratory on the modulation of cell proliferation by GM_3 focuses on the two enzymes responsible for the synthesis and degradation of GM_3. Our model (9) suggests that when cells are in a proliferative state, the inhibition of cell proliferation by GM_3 is relieved by the dissociation of GM_3 from the EGF-R through the catabolism of GM_3 by GM_3 sialidase. Loss of the sialic acid from GM_3 gives lactosylceramide (LacCer) and relieves the constraint on the EGF-R, permitting subsequent phosphorylation reactions and continued cell growth. We speculate that LacCer, or its catabolites (glycosylceramide, ceramide, sphingosine) is internalized by the cell to the endoplasmic reticulum or Golgi (10), where it (or they) serve as substrates in resynthesizing GM_3. SAT-1 catalyzes the addition of sialic acid to LacCer forming GM_3 ganglioside.

A logical point where SAT-1 may be regulated is post-translational modification. Early evidence by Dawson and coworkers (21) suggested a phosphorylation/dephosphorylation mechanism in the regulation of GM_2 synthase. Supportive are the findings of various phosphoprotein and protein kinases have been found within the Golgi, as well as evidence for ATP translocation (22). Further, Strous *et al.* (23) have identified a serine-linked phosphate on the cytoplasmic amino

terminus of a Golgi galactosyltransferase in HeLa and HepG2 cells. They speculated that this posttranslational modification may be involved in membrane targeting of Golgi-resident glycosyltransferases.

Considerations on the Regulation of SAT-1

Recently, Dumont et al. (24) speculated about the coexistence of cAMP-dependent and independent mitogenic pathways. Two-dimensional electrophoretograms of ^{32}P-labeled proteins in dog thymocytes indicated that the proteins phosphorylated due to the growth factor receptor stimulated tyrosine kinases or phorbol esters or other activators of the phosphatidylinositol cascade, which in turn stimulated PK-C and resulted in the phosphorylation of some common proteins. In contrast, cAMP acting via protein kinase A resulted in a different phosphoprotein pattern, indicating that even though transmembrane signaling systems are initially distinct they do seem to act in a convergent manner to control the phosphorylation/dephosphorylation of proteins involved during the progression of a cell through G_1.

SAT-1 activity is cell cycle-dependent (see Chapter 6). SAT-1 activity peaks about 4-6 hours before DNA synthesis, during early G_1 of the cell cycle. SAT-1 activity can be stimulated chemically by butyrate (3-5), retinoic acid and phorbol esters (25). Phorbol esters are known modulators of phosphorylation acting through a DAG mechanism on PKC. Other agents such as Prostaglandin E (21), enkephalins (21), the B-subunit of cholera toxin (26), and GQ_{1b} gangliosides (27) are activators of cAMP-dependent protein kinases (cAK), which also stimulate glycosyltransferase activity. Further, stimulation of SAT-1 activity correlates with increased cAMP-dependent protein kinase and decreases in the presence of alkaline phosphatase (28).

I have proposed that the regulation of SAT-1 is integrally involved with membrane signal transduction and have suggested a model whereby SAT-1 activity is regulated by a bimodal system with protein kinase C and epidermal growth factor receptor signal transduction systems (See Chapter 1 Figure 7). Much like the

"bimodal" function of GM_3 (the SAT-1 enzyme product) the proposed "bimodal" regulation of SAT-1 is dependent on whether the cells are proliferating or quiescent and that the site of phosphorylation is dependent on which signal transduction mechanism operates to regulate the production of GM_3. As a model for cell growth regulation (Chapter 6 Figure 8), the receptor-mediated tyrosine kinase, operating possibly via a kinase cascade, may act as a positive effector on SAT-1 through phosphorylation of specific tyrosine residue(s). An antiphosphotyrosine antibody specifically blots purified SAT-1 from rat liver (Figures 1 and 2) and from KB cells (Chapter 6). The phosphorylation pattern of phospho-tyrosine(s) on KB cell SAT-1 differs among samples taken during different times within the G_1 phase of the cell cycle. Over the last year the several studies on the effects of GM_3 catabolites on PK-C and EGF-R show an antagonist/protagonist effect (reviewed, 1). Thus, it seems plausible that SAT-1 is similarly affected by PK-C and EGF-R mediated kinase activities. Clearly, such a proposed requires detailed investigation into the protein sequence and isolation of the gene transcribing SAT-1.

In vitro activation of PK-C and of SAT-1 can be stimulated by phorbol esters. Phorbol esters presumably act as substitutes for diacylglycerol (DAG) (29). Hydrolysis of membrane-bound enzymes, anchored by glycosyl-phosphatidylinositol (GPI), by phospholipase C (GPI-PLC) releases DAG (30). SAT-1 is a membrane-associated enzyme. The mechanism through which it is anchored in the Golgi is unknown. We investigated the possibility that SAT-1 was GPI-anchored and postulated a potential mechanism for PK-C activation of SAT-1 through its release from the Golgi by GPI-PLC.

Intact and "leaky" rat liver Golgi vesicles were treated with GPI-PLC (a gift from Dr. Martin Low) and the membrane pellets and supernatants were examined for SAT-1 activity. These data are summarized in Table 1. SAT-1 activity was found to be associated with the supernatants in both the intact and permeabilized Golgi. This

Figure 1. Immunodetection of SAT-1 with Antiphosphotyrosine Monoclonal Antibody. Immunoaffinity-purified rat hepatic SAT-1 (10 μg) was analyzed by two dimensional gel electrophoresis (2D SDS-PAGE), electrotransferred to Immobilon (Millipore), and immunodetected with an antiphosphotyrosine monoclonal antibody (UpState Biotechnology, Inc.). First dimension tube gels were performed according to the method of O'Farrell (42) with an ampholyte mixture of 1 part pH 3-10 and 4 parts pH 5-8. The second-dimension (2D) slab gels were standard 12% Laemmli (43) SDS-PAGE. The 2D blot containing 10 μg SAT-1 was immunodetected with antiphosphotyrosine monoclonal (1:1000, 10μg /ml), alkaline phosphatase-conjugated secondary antibody (BMB, 1:6000) and BCIP/NBT reagent and the blot analyzed by the BioImage Visage 110 computerized digital imager (BioImage/Millipore, Ann Arbor, MI) to estimate the molecular weight and pI of the protein. SAT-1 is indicated by the arrow. Its apparent molecular weight was about 60,000 daltons and the pI in the range of pH 6.2. The carbamylated IEF standards are (A) creatine phosphokinase, M.W. 40 Kd and pI range: pH 4.9-7.1, (B) glyceraldehyde-P dehydrogenase, M.W. 36 Kd and pI range: pH 4.7-8.3, and (C) carbonic anhydrase, M.W. 30 Kd and pI range: pH 4.8-6.7. The SDS-PAGE molecular weight standards are phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (43 Kd), carbonic anhydrase (31 Kd), soybean trypsin inhibitor (21 Kd) and lysozyme (14 Kd).

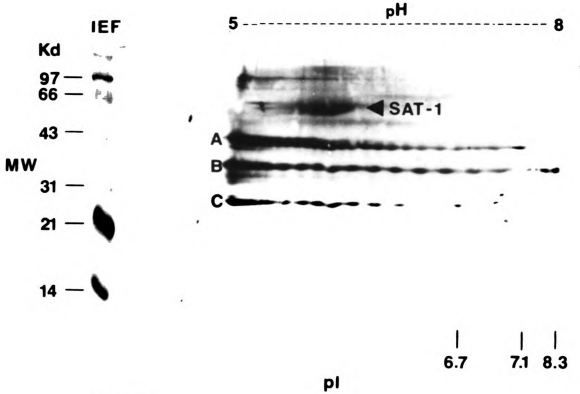

IEF
Kd
97
66
43
MW
31
21
14
pH
5
8
SAT-1
A
B
C
6.7
7.1
8.3
pI
A = CPK
B = GADPH
C = CA

Figure 2. Specificity of the Immunodetection of SAT-1 with Antiphosphotyrosine Monoclonal Antibody. Immunoaffinity-purified SAT-1 (10 μg) was analyzed following SDS-PAGE (43) and Western blot with an antiphosphotyrosine monoclonal antibody (UpState Biotechnology, Inc.). Unbound sites on the Western blots were blocked with TRIS-buffered saline (pH 7.4) containing 2% (w/v) gelatin for 30 min at 37°C. Prior to immunodetection of SAT-1 with the antiphosphotyrosine monoclonal antibody, the monoclonal antibody was preincubated with 50 mM phosphotyrosine (A) and 50 mM phosphothreonine (B) or no pretreatment (C). The immunodetection was carried out in TRIS-buffered saline (pH 7.4) containing 1% gelatin and 0.05% Tween 20 (ELISA grade). Specific detection of rat hepatic SAT-1 (lane 2) was demonstrated at an apparent molecular weight of about 60 Kd (denoted with an arrow). The molecular weight standards (lane 1) were phosphorylase b (97 Kd), bovine serum albumin (66 Kd), ovalbumin (43 Kd), carbonic anhydrase (31 Kd), soybean tryspin inhibitor (21 Kd) and lysozyme (14 Kd).

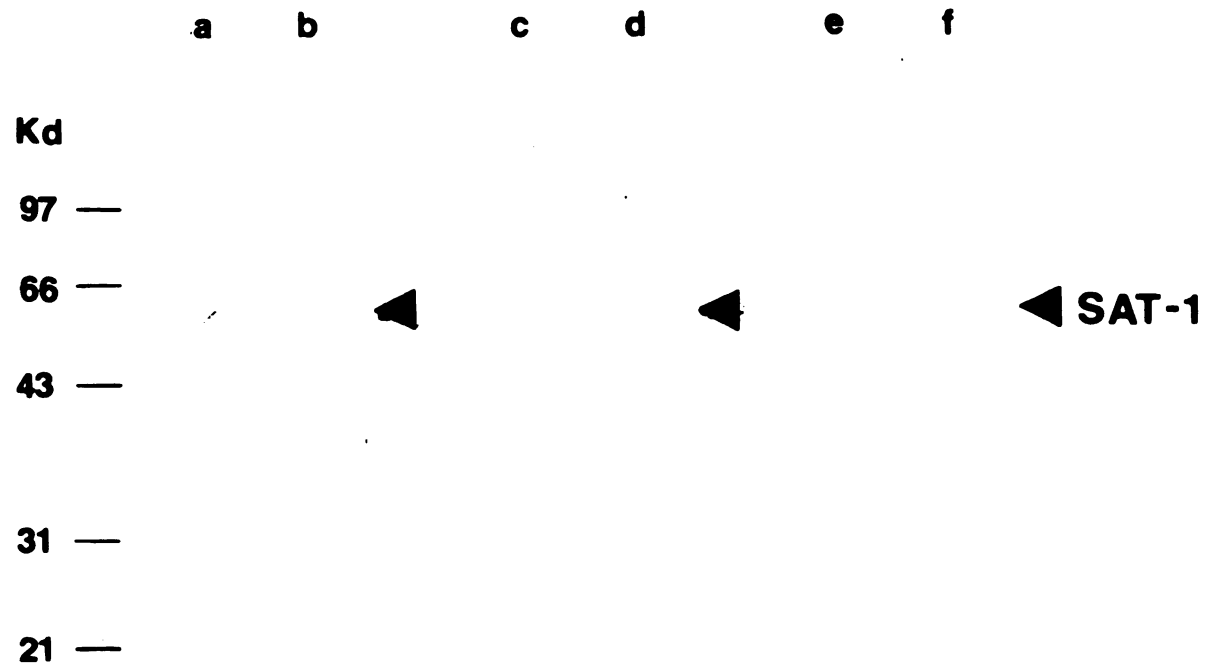

a
b
c
d
e
f
Kd
97
66
43
31
21
SAT-1

Table 1. Phosphatidyl Inositol as a Potential Membrane Anchor for SAT-1. Golgi vesicles were prepared from rat liver (as described in Chapter 3) based on well-established procedures. Like other glycosyltransferases, SAT-1 has a lumenal topography. Assay of SAT-1 activity requires the Golgi to be permeabilized with detergent. The Golgi were made "leaky" with Triton CF-54 (final concentration, 0.3%) according to the method of Carey and Hirschberg (31). Intact and leaky Golgi (1.4 mg) were incubated with *S. aureus* phosphatidylinositol phospholipase C (a gift from Dr. Martin Low, Columbia University, New York, NY) at a final concentration of 20 μg/ml for 2 hr at 37°C. Total volume was 2.0 ml. Golgi-enriched membranes were recovered by centrifugation at 150,000 x g for 90 min at 4°C. The supernatants and pellets were assayed for SAT-1 activity as previously described (32,33; see Chapters 2 and 3). Verification of GPI-PLC activity was the hydrolysis of 1000 μg of PI resuspended in 25 mM sodium cacodylate (pH 7) and GPI-PLC (20 μg/ml), final volume 1.0 ml. The reaction conditions were as described for treatment of the Golgi. Aliquots from the control reactions for enzyme activity (25 μl) were chromatographed on HPTLC plates with a chloroform/method/H_2O (65:35:5, v/v/v) solvent system or on C_{18}-HPTLC in methanol/H_2O (2:1, v/v). DAG standards, sn-1-2,dioctanoylglycerol and sn-1-oleoyl-2-acetylglycerol, were run in parallel. The lipids were detected with 50% sulfuric acid. The experimental protocol was performed twice and assayed in duplicate each time.

Table 1

Phosphatidyl Inositol as a Potential Membrane Anchor for SAT-1

	Fraction	GPI-PLC (20 μg/ml)	Specific Activity SAT-1 ($pmol \cdot min^{-1} \cdot mg^{-1}$)
Intact Golgi			
	Supernatant	+	10.0
	Pellet	+	2.7
	Supernatant	-	15.2
	Pellet	-	5.3
"Leaky" Golgi[1]			
	Supernatant	+	4.1
	Pellet	+	4.6
	Supernatant	-	14.6
	Pellet	-	7.0

[1] The term "leaky" refers to Golgi which were made permeable through the addition of Triton CF-54 to a final concentration of 0.3%.

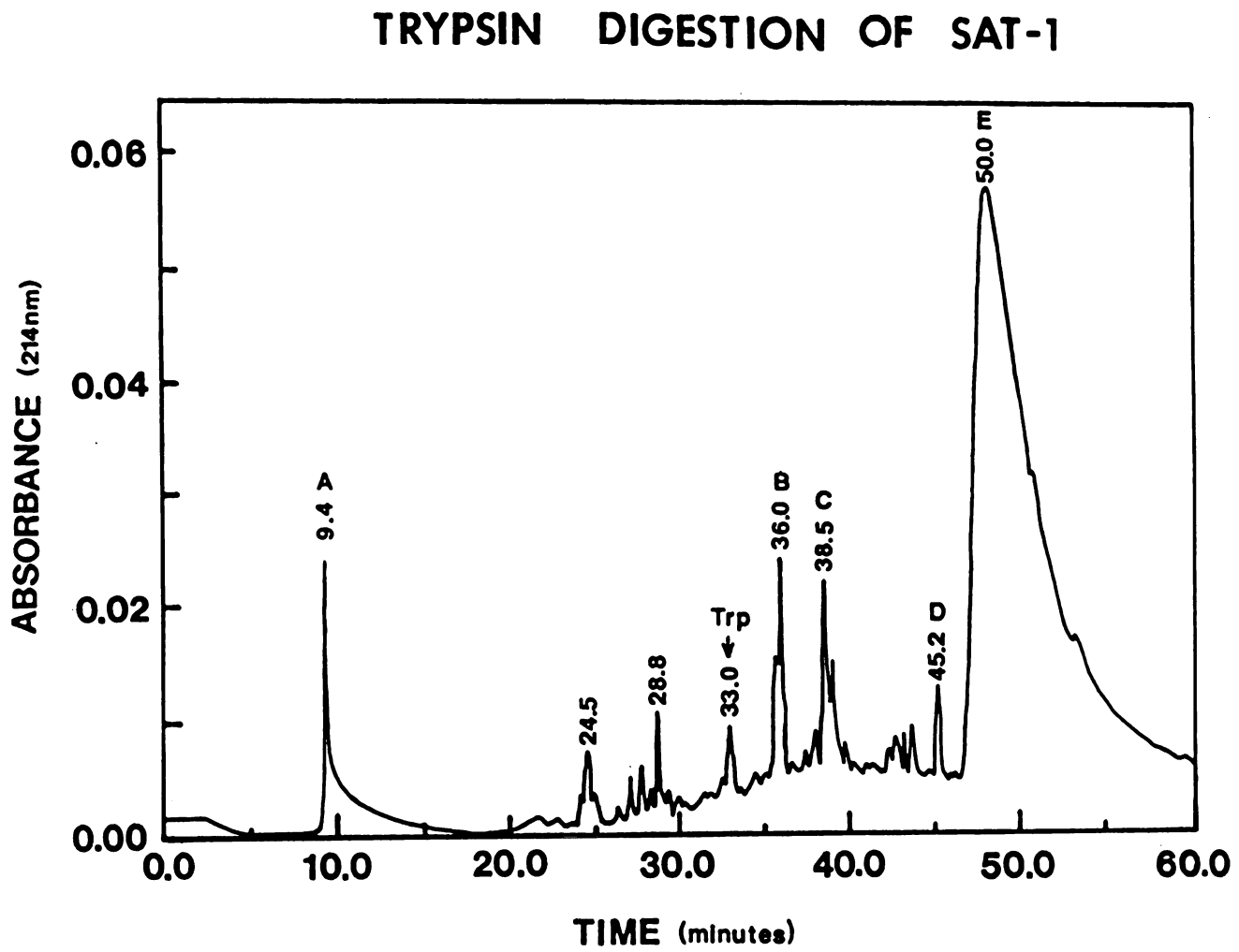
may have resulted from the mechanical disruption and proteolytic degradation during purification. This investigation on GPI as a potential membrane anchor for SAT-1 was performed about one year prior to the detection of proteolytic degradation of SAT-1 and was performed without thiol protease inhibitors (see chapter 5). Further, GPI-PLC has been demonstrated to release alkaline phosphatase (APase) and phosphodiesterase, enzymes which are negative effectors of sialyltransferase activities (21,28). In consideration of these factors, there is no direct evidence for or against GPI being involved in SAT-1 anchoring within the Golgi. Therefore, to explore the relationship between this novel lipid anchoring system and SAT-1, a repeat of the experiment is needed with appropriate protease and APase inhibitors.

Protein Sequencing of SAT-1

Studies employing antiphosphotyrosine monoclonal antibody are only suggestive about the regulation of SAT-1 activity (see Chapter 6). However, this work adds to other data implicating a role for phosphorylation in the regulation of SAT-1 (or other glycosyltransferases). Identification of potential phosphorylation sites within the protein sequence would support our findings. During the last year, many attempts have been made to obtain protein sequence information. The approach has come full circle and the end result to date, three residues: **Ser-Tyr-Gly**.

SAT-1 appears to be N-terminally blocked. Therefore, SAT-1 was electrophoresed and electrotransferred to Immobilon for enzymatic degradation with trypsin. Digestion was incomplete, perhaps due to the high degree of glycosylation of the enzyme (See Chapter 3), as evidenced by the broad peak in the C_8-HPLC profile (Figure 3). This hydrophobic peak, estimated by A_{214nm} to be 7-8 μg, gave no sequence information. We also attempted cyanogen bromide (CNBr) and enzymatic deglycosylation of SAT-1 with N-glycanase followed by trypsinization as alternative approaches.

Figure 3. Reverse-Phase C_8-HPLC of SAT-1 Tryptic Peptides. Reverse-phase HPLC was performed on an Aquapore RP-300 C_8 column (Applied Biosystems) (250 x 1.0 mm x 7 μm). Individual peptides were eluted from the microbore column on a 60 min linear gradient established between 0.1% trifluoroacetic acid and 90% acetonitrile. Sample volume injected was 250 μl. No sequence information was obtained for the major peak.


TRYPSIN DIGESTION OF SAT-1
ABSORBANCE (214nm)
0.06
0.04
0.02
0.00
0.0
10.0
20.0
30.0
40.0
50.0
60.0
TIME (minutes)
9.4 A
24.5
28.8
33.0 Trp
36.0 B
38.5 C
45.2 D
50.0 E

Analysis of CNBr Digests. No internal amino acid sequence was obtained from the major peaks of SAT-1 (ethanol/acetone precipitated) digestion with CNBr (Figure 4). The three major peaks were rechromatographed to resolve individual species. The recovery of total protein was 10-20 % of starting material for each run. We expected some loss during the CNBr digestion, but another probability was protein loss from adsorption to the plastic collection tubes during C_8-HPLC. To minimize loss of SAT-1, the sample was loaded directly onto the reverse-phase HPLC column along with appropriate buffer controls containing LDAO. Comparison of the profiles gave two unique peaks at the hydrophobic end of the run; the remainder of the peaks generated were attributed to LDAO (Figures 5a and 5b). These peaks were analyzed on SDS-PAGE and verified to be the 60 Kd SAT-1 and a 56 Kd proteolytic fragment (See Chapter 4). Both appeared to be N-terminally blocked. Comparison of these chromatograms to the first CNBr digests indicated the peaks which generated "no sequence" were in fact LDAO.

Two-dimensional SDS-PAGE was employed to recover SAT-1 without LDAO contamination. LDAO protonates at $pH \leq 7$. The pI of SAT-1 was estimated in the range of pH 5.7-6.2 by 2D-SDS-PAGE (See Chapter 3). Theoretically, under optimal IEF conditions for SAT-1, LDAO should be charged and proceed through the tube gel. SAT-1 resolved by 2D SDS-PAGE (40 μg) was electrotransferred to Immobilon and digested with CNBr (Figure 6). This has proved to be the most successful approach to date. Amino acid sequencing of three peaks (A, B, E) has been tried but sequence information could not be obtained from B because of a technical error. Peak A gave the following internal sequence information: **Ser-Tyr-Gly**. No sequence information was obtained from Peak E. The remaining peaks have not been tried.

Figure 4. Reverse-Phase C_8-HPLC Following CNBr Digestion of SAT-1. Reverse-phase HPLC of SAT-1 peptides generated following CNBr digestion was on a C_8 microbore HPLC Aquapore RP-300 column (Applied Biosystems) (250 mm x 1.0 mm x 0.7 μm). Elution was preformed using a 90 min linear gradient established between 0.1% TFA and 90% acetonitrile. No sequence information was obtained from the labeled peaks.

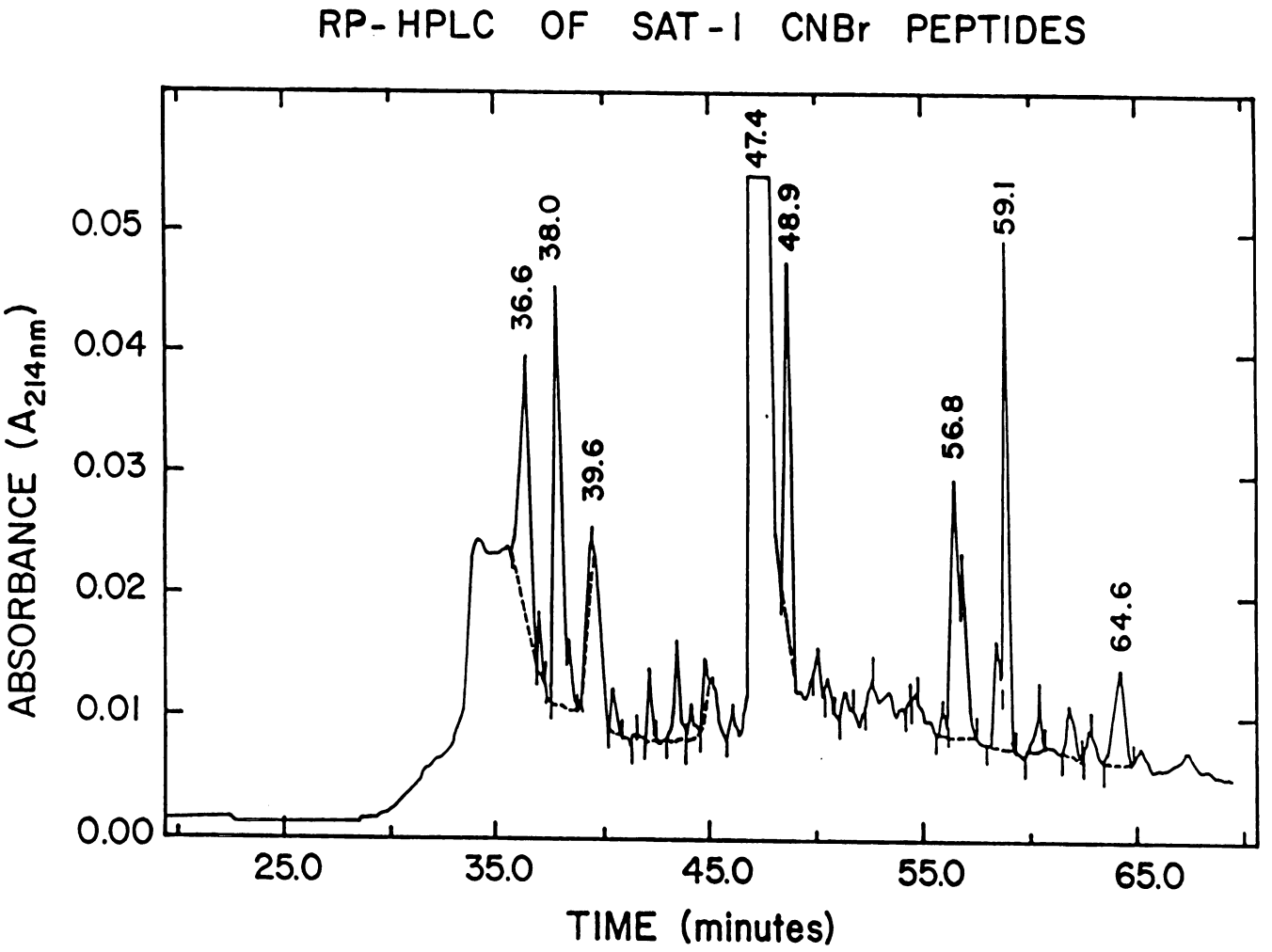
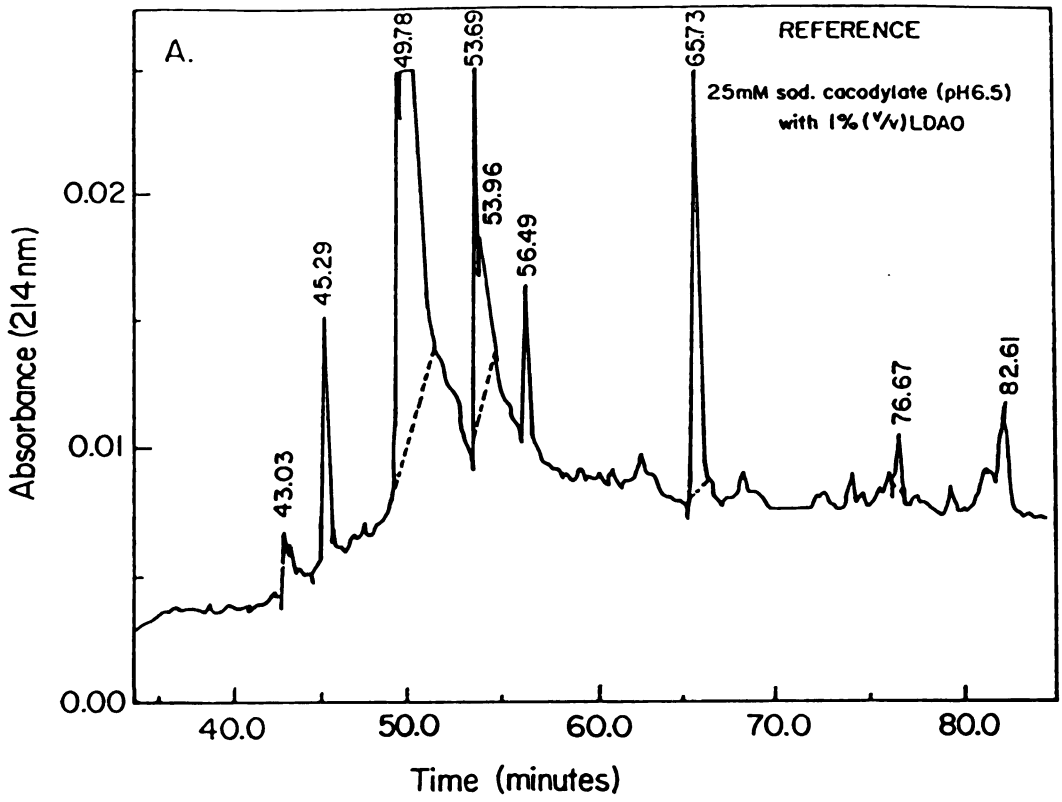

RP-HPLC OF SAT-1 CNBr PEPTIDES
ABSORBANCE (A_{214nm})
0.00
0.01
0.02
0.03
0.04
0.05
25.0
35.0
45.0
55.0
65.0
TIME (minutes)
36.6
38.0
39.6
47.4
48.9
56.8
59.1
64.6

Figure 5. Reverse-Phase C_8-HPLC of SAT-1 and Buffer Containing LDAO. Resolution of purified SAT-1 sialyltransferase from LDAO was achieved on a C_8 microbore HPLC system (Applied Biosystems). SAT-1 in 25 mM sodium cacodylate (pH 6.5) containing 5% (v/v) LDAO was applied onto an Aquapore C_8, RP-300 (250 x 1.0 mm x 7 μm) reverse-phase microbore column and eluted on a 90 min linear gradient established between 0.1% (v/v) trifluoroacetic acid and 90% (v/v) acetonitrile. Sample volume injected was 250 μl. Panel A illustrates a typical chromatogram obtained from buffer containing LDAO. Panel B shows the resolution of unique SAT-1 proteins from the same LDAO containing buffer. No sequence information was obtained from the 56 or 60 Kd proteins.


A.
REFERENCE
25mM sod. cacodylate (pH6.5)
with 1% (v/v) LDAO
49.78
53.69
65.73
53.96
56.49
45.29
43.03
76.67
82.61
Absorbance (214 nm)
0.02
0.01
0.00
40.0
50.0
60.0
70.0
80.0
Time (minutes)

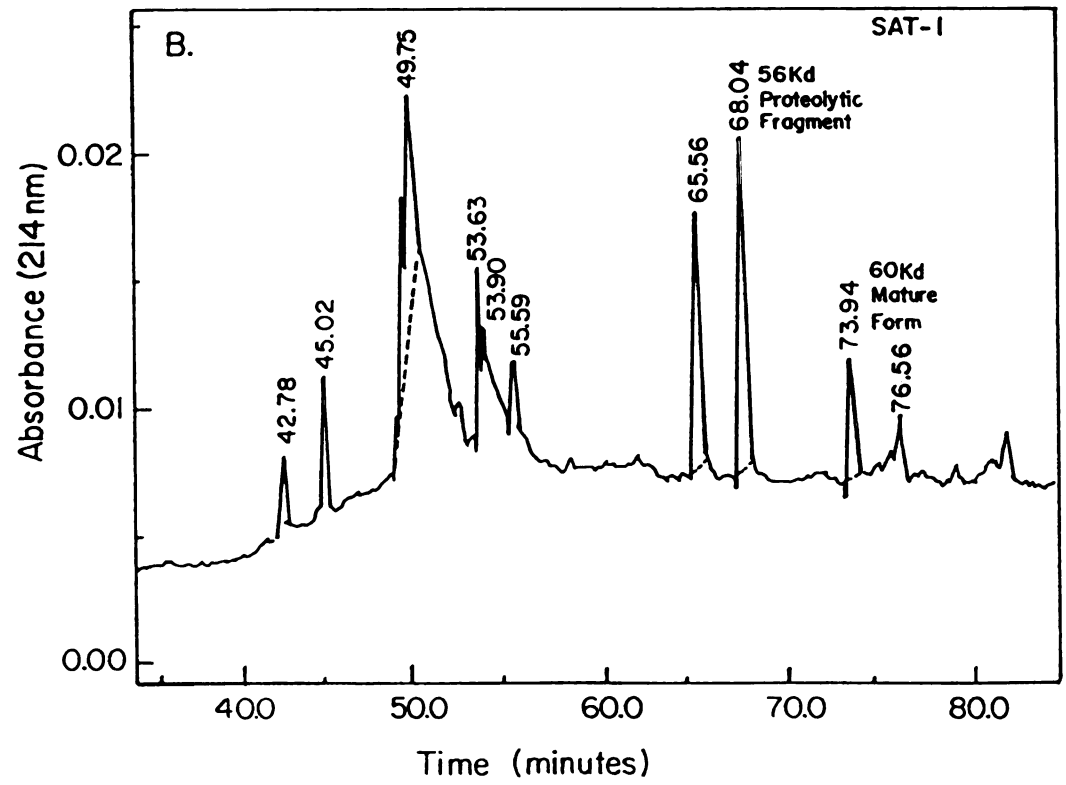
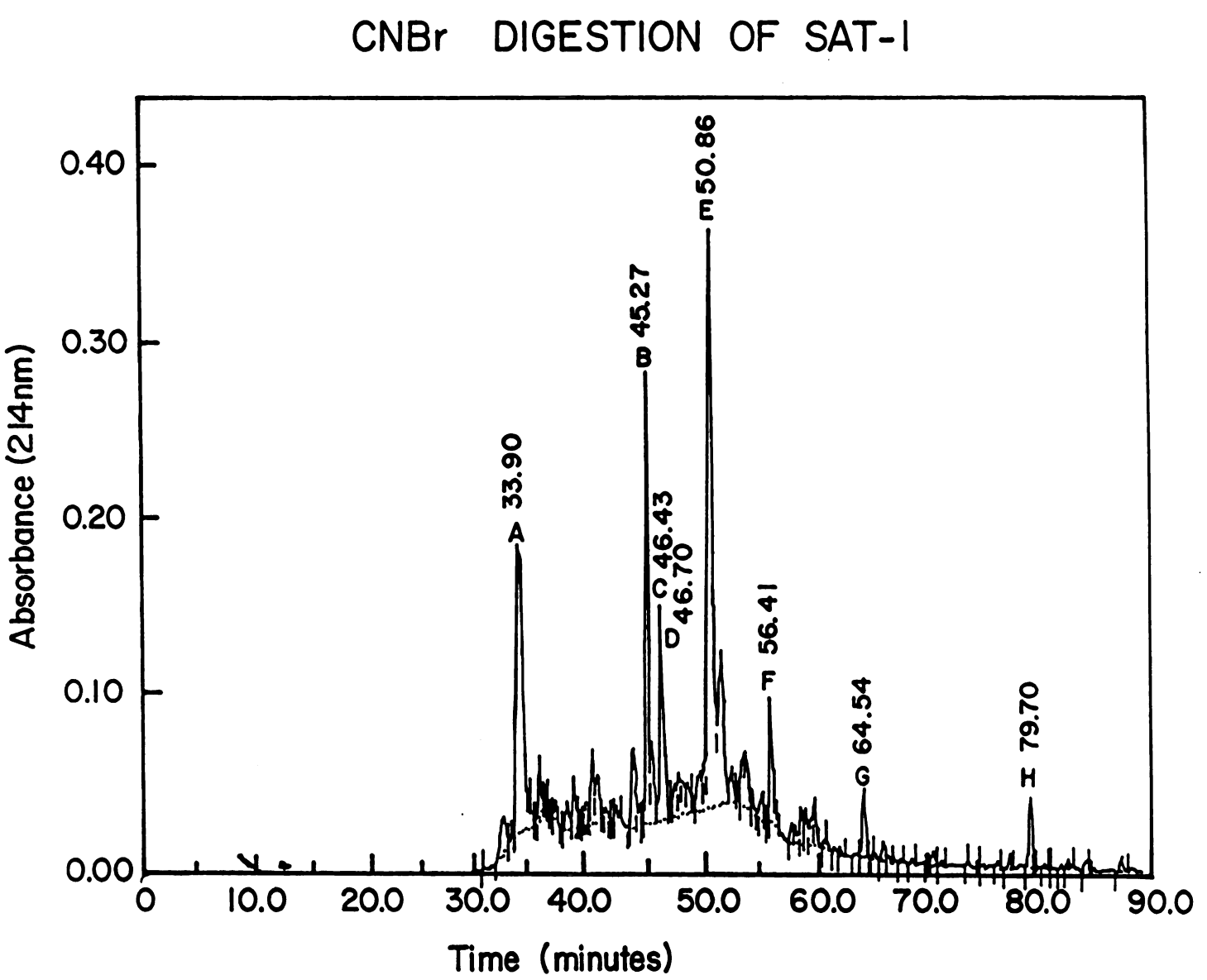

B.
SAT-1
49.75
56Kd
Proteolytic
Fragment
68.04
65.56
53.63
53.90
55.59
45.02
42.78
60Kd
Mature
Form
73.94
76.56
Absorbance (214 nm)
0.02
0.01
0.00
40.0
50.0
60.0
70.0
80.0
Time (minutes)

Figure 6. Reverse-Phase C_8-HPLC of SAT-1 Following 2D SDS-PAGE and CNBr Digestion. Reverse-phase HPLC of SAT-1 peptides generated following CNBr digestion was on a C_8 microbore HPLC Aquapore RP-300 column (Applied Biosystems) (250 mm x 1.0 mm x 0.7 μm). Elution was preformed using a 90 min linear gradient established between 0.1% TFA and 90% acetonitrile. No sequence information could be obtained from B because of technical error. No sequence information was obtained from E. Sequence obtained from peak A was Ser-Tyr-Gly. The remaining peaks have not been tried.


CNBr DIGESTION OF SAT-1
Absorbance (214nm)
0.00
0.10
0.20
0.30
0.40
0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
Time (minutes)
A 33.90
B 45.27
C 46.43
D 46.70
E 50.86
F 56.41
G 64.54
H 79.70

Analysis of Tryptic Digests. Deglycosylation of SAT-1 with N-glycanase was accomplished as described by the manufacturer (Genzyme). The deglycosylated SAT-1 was recovered by SDS-PAGE. The tyrpsinization was performed directly on SAT-1 within the gel according to the method of Matsudaira (34) and the resulting peptides were resolved on by C_8-HPLC. The profile is shown in Figure 7. The chromatogram from the deglycosylated enzyme gave better resolution than that generated from the first tryptic digest (Figure 3). A series of extremely hydrophobic residues, eluting with 90% acetonitrile, were recovered and may correspond to the transmembrane domain. None of these peaks have been analyzed for amino acid sequence.

Thus, after one year of obtaining no sequence information from these conventional approaches, we have sent 4 nmol of affinity-purified rat liver SAT-1 to Dr. Donald Hunt (University of Virginia, Charlottesville, VA) to have SAT-1 sequenced by protein mass-spectrometry.

Cloning a cDNA Encoding SAT-1

Rabbit anti-rat hepatic SAT-1 polyclonal antibodies have been produced (see discussion below) and protein A-purified. Immunodetection of SAT-1 on Western blots by these antibodies is sensitive in the 0.5 ng range. These antibodies have been used in a primary screen of a human hepatoma cDNA expression library containing about 1 x 10^6 clones. It has been reported that there is sufficient homology within the catalytic domain between species to permit antibody (and oligonucleotide) probes to detect glycosyltransferases in more that one species (35,36). About 30-40 putative positives were detected on each of 5 x 150 mm blots (Figure 8). The positives need to be plaque-purified, the phage isolates cultured and rescreened with antibody. While false positive exist, these results indicate that sequence information of SAT-1 may be obtained through the cloning of this cDNA expression library. Rescreening should be done with polyclonal monospecific anti-SAT-1 (deglycosylated form) antibodies. These can be prepared by affinity purification using deglycosylated SAT-1 coupled to

Figure 7. Reverse-Phase C_8-HPLC of Tryptic Peptides from Deglycosylated SAT-1. Reverse-phase HPLC was performed on an Aquapore RP-300 C_8 column (Applied Biosystems) (250 x 1.0 mm x 7 μm). Individual peptides were eluted from the microbore column on a 60 min linear gradient established between 0.1% trifluoroacetic acid and 90% acetonitrile. Sample volume injected was 250 μl. To date, none of these peaks have been sequenced.

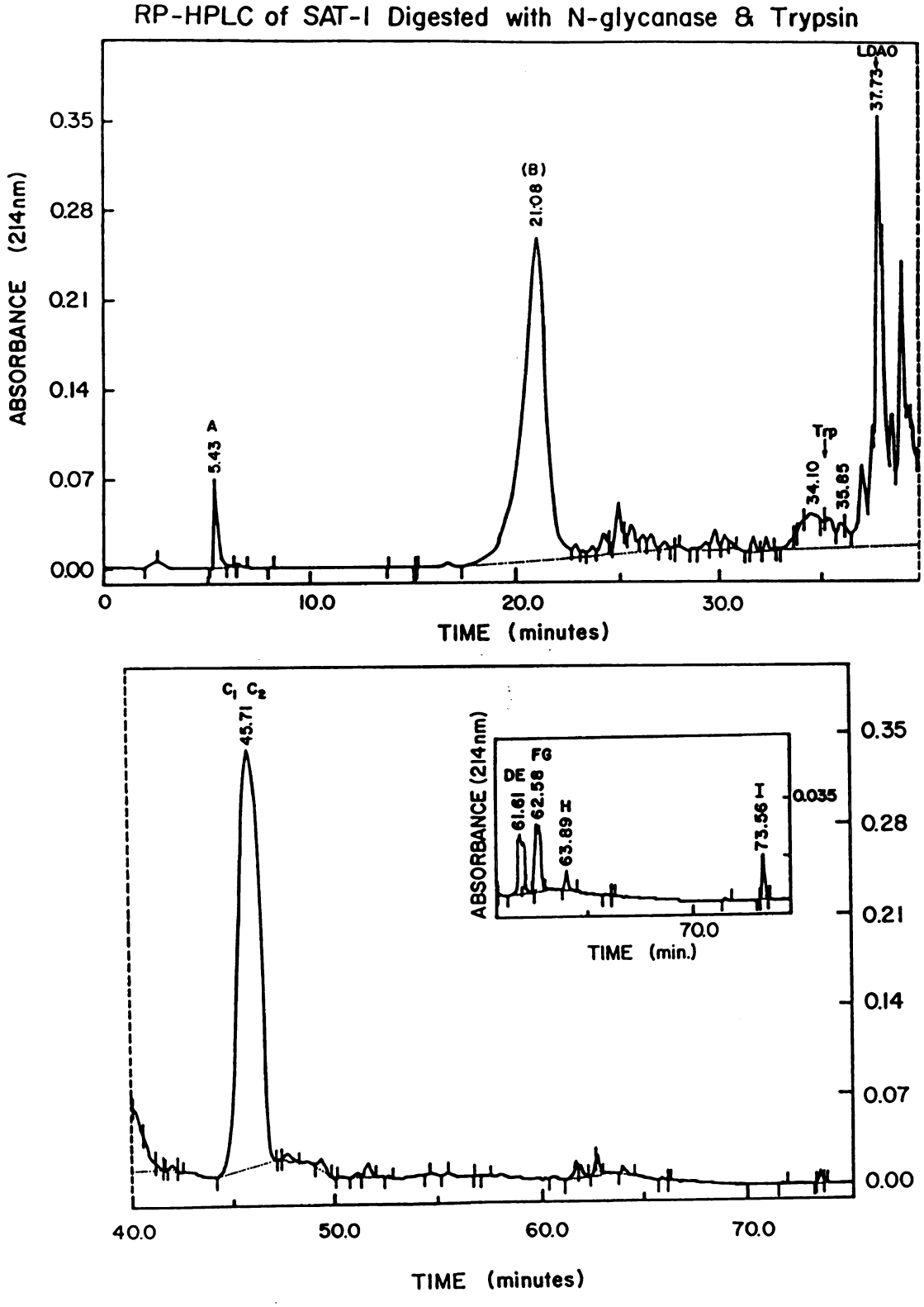
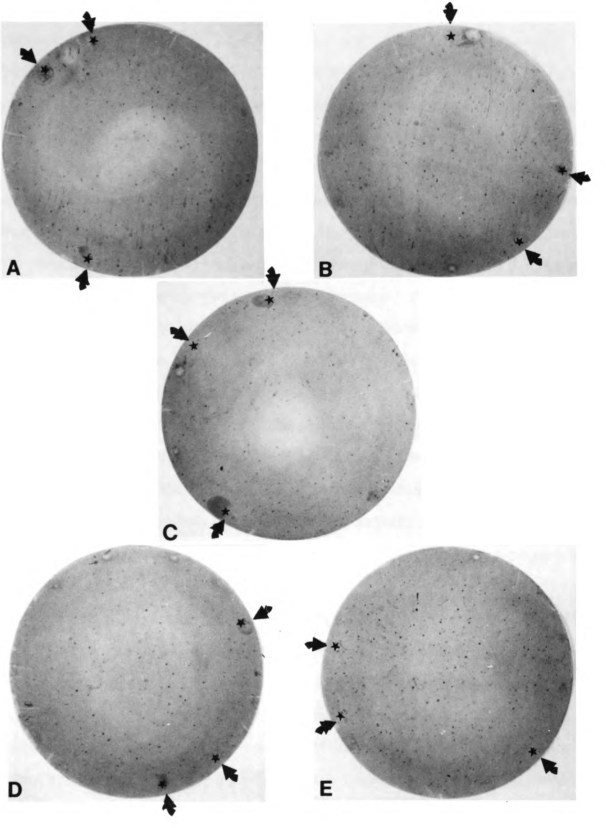

RP-HPLC of SAT-I Digested with N-glycanase & Trypsin
ABSORBANCE (214nm)
0.35
0.28
0.21
0.14
0.07
0.00
A
5.43
(B)
21.08
LDAO
37.73
34.10
35.85
0
10.0
20.0
30.0
TIME (minutes)
C1 C2
45.71
DE
61.61
62.58
FG
63.89
H
73.56
I
0.035
70.0
TIME (min.)
40.0
50.0
60.0
70.0
TIME (minutes)

Figure 8. Primary Screen of a cDNA Expression Library with Rabbit Anti-SAT-1 Polyclonal Antibodies. About 1 x 10^6 clones from a human hepatoma cDNA expression library were plated onto five 150 mm plates (A - E). The library filters were screened with rabbit anti-SAT-1 IgG polyclonal antibodies and detected by the alkaline-phosphatase conjugated secondary antibody system. Approximately 30-40 putative positives were detected per plate.


A
B
C
D
E

Affi-Gel 10. About 500 μg of SAT-1 has been deglycosylated with N- and O-glycanase for this purpose.

Intracellular Localization of SAT-1

SAT-1 resides within the Golgi. SAT-1 activity and that of thiamine pyrophosphatase, a Golgi membrane-marker enzyme, were recovered together in our Golgi-enriched membrane fraction following differentiation centrifugation and discontinuous sucrose gradient. SAT-1 distributed through the 10-32% interfaces and most concentrated at 25-32% (see Chapter 2). Using a similar centrifugation protocol, Trinchera and Ghindoni (37) localized SAT-1 activity within the *cis* Golgi using an immunolocalized glycosyltransferase as an enzyme marker. The addition of sialic acid is considered a terminal glycosylation and as such, SAT-1 would be localized within the *trans* Golgi where other sialyltransferases have been immunolocalized (38). However, GM_3 also serves as percursor for other gangliosides and presuming a precursor-product relationship which during glycolipid synthesis transverse would lend support to the presence of SAT-1 in the denser fractions of the Golgi stack. The best method for identifying the location of SAT-1 will be immunolocalized with a rabbit anti-SAT-1 polyclonal antibody specific for the deglycosylated form of SAT-1.

I have enzymatically digested SAT-1 with N- and O-glycanases (Genzyme) in preparation for the immunolocalization of SAT-1 in rat liver thin sections. Administration of this as an antigen was delayed pending an necropsy report on a rabbit used for preparing anti-SAT-1 polyclonal antibodies. The rabbit died as the result of a systemic lymphosarcoma. The report from the veterinarian appears in Appendix A.

Speculation as to the cause of our rabbit's demise is intriguing. The potential exists that this disease state may have resulted from administering SAT-1 to the animal. Serum is rich in shed gangliosides (39,40) has been shown suppress the immune response during tumorgenesis. LacCer, the acceptor substrate for SAT-1, is a major

component of the hemopoetic system. While it is generally presumed that antigen in adjuvant is inactive. The possibility that SAT-1 is active exists. RIBI ImmunoChem (Hamilton, MT) MPL^{TM} + TDM (0.5 mg trehalose dimycolate (TDM) and 0.5 mg monophosphoryl lipid A (MPL) in 2% oil-Tween 80-H_2O; lot #060-124), which employs a detergent-lipid emulsion, was used as the adjuvant. It is unknown if SAT-1 (at 50-200 μg/ml) would be active in such an adjuvant system. SAT-1 exhibits differential activity depending on the detergent used to solubilize the Golgi (see Chapter 4).

Another possible consideration was an autoimmune response. Glycosyltransferases share greater than 85% sequence homology between species (35). The rabbit elucidated a good immune response to the rat liver SAT-1. After the third inoculation, her condition began to quickly decline after 7 days.

A third possibility is that the antigen may have contained some residual lauryldimethylamine oxide (LDAO). LDAO can be toxic and HPLC analyses from our protein work indicate that while ethanol and acetone precipitation minimize the amount of detergent in a sample, some LDAO still remains (see Chapter 4). In raising monoclonal antibodies to SAT-1 in mice, it was necessary to do a phosphatidylcholine:LDAO exchange (see Chapter 3). This approach was also employed in raising the polyclonals.

To define a causal relationship, i.e., active SAT-1, autoimmune response, LDAO toxicity, or an inherent defect of the animals health, to the death of the rabbit. Another control rabbit should be immunized with a control sample in RIBI. The control would be a similar starting volume of 25 mM sodium cacodylate (pH 6.5) containing 1.0 M NaCl and 0.3% (v/v) LDAO (approximately 20-25 ml), concentrated using a centriprep 10 to about 1.0 ml, and ethanol and acetone precipitated (as described, 41). The resulting pellet should be resuspended in PBS containing 1 mg PC and dialyzed. (Note: The immunoaffinity purification of SAT-1 has been modified to

reduce the amount of LDAO in the enzyme fraction. Detergent extraction and immunoaffinity purification is carried out as described (Chapter 3), but an additional wash step changes the column buffer to 25 mM sodium cacodylate (pH 6.5) containing 0.1 M NaCl and 0.3% LDAO. The enzyme is eluted in the same buffer with 1 M NaCl.)

Closing Statement

Research in our laboratory focuses on the chemistry and metabolism of glycosphingolipids as it pertains to the modulation of cell proliferation by GM_3 ganglioside. Our working hypothesis for cells in a proliferative state is that inhibition of cell growth by GM_3 ganglioside is relieved through the dissociation of GM_3 from the epidermal growth factor receptor (EGF-R) by degradation of GM_3 to lactosylceramide (LacCer) by GM_3 sialidase. Loss of the sialic acid from GM_3 relieves the constraint on EFG-R autophosphorylation and cell growth continues. LacCer, once internalized to the Golgi, serves as substrate by CMP-sialic acid:LacCer α2-3 sialyltransferase (SAT-1) to reform GM_3 and repopulate the cell surface to modulate another round of the cell growth cycle.

The studies described in this dissertation were specifically aimed at purifying SAT-1 to address the question of the regulation of GM_3 ganglioside biosynthesis, more specifically how SAT-1 is regulated. Purification and characterization of SAT-1 generates information which will prove most useful to others investigating glycolipid glycosyltransferases. SAT-1 activity is unique among glycosyltransferases. It is specific for a particular carbohydrate-lipid structure and as such reflects its potential as a key regulatory step in ganglioside biosynthetic cascade. The results of the cell-cycle dependent expression of SAT-1, potentially modulated by tyrosine phosphorylation, when considered with a variety of other studies on GM_3 metabolism and function, may enable others to continue to define the mechanism played by GM_3 ganglioside in the regulation of cell growth and ultimately mechanisms controlling oncogenesis.

REFERENCES

1. Hakomori, S. (1990) J. Biol. Chem. **265**, 18713-18716.
2. Hakomori, (1985) Cancer Res. **45**, 2405-2414.
3. Chatterjee, S., Sweeley, C.C. and Velicer, L.F. (1975) J. Biol. Chem. **250**, 61-66.
4. Macher, B.A., Lockney, M., Moskal, J.R., Fung, V.K. and Sweeley, C.C. (1978) Exp. Cell Res. **117**, 95-102.
5. Moskal, J.R., Lockney, M.W., Marvel, C.C., Mason, P.A. and Sweeley, C.C. (1980) in ACS Symposium Series, No. 128, Cell Surface Glycolipids (C.C. Sweeley, Ed., p.p. 241-263.
6. Burczak, J.D., Soltyziak, R.M. and Sweeley, C.C. (1984) J. Lipid Res. **25**, 1541-1547.
7. Usuki, S., Lyu, S.-C. and Sweeley, C.C. (1988) J. Biol. Chem. **263**, 6847-6853.
8. Usuki, S., Hoops, P. and Sweeley, C.C. (1988) J. Biol. Chem. **263**, 10595-10599.
9. Usuki, S. and Sweeley, C.C. (1988) Ind. J. Biochem. Biophys. **25**, 102-105.
10. Swamy, M.J. and Sweeley, C.C. (1989) Biochem. Biophys. Res. Commun. **162**, 1188-1193.
11. Bremmer, E.G., Hakomori, S., Bowen-Pope, D.F., Raines, E. and Ross, R. (1984) J. Biol. Chem. **259**, 6818-6825.
12. Bremmer, E.G., Schlessinger, J., and Hakomori, S. (1986) JBC **261**, 2434-2440.
13. Hanai, N., Nores, G., Torres-Mendez, C.R., and Hakomori, S. (1987) Biochem. Biophys. Res. Commun. 127-134.
14. Hanai, N., Nores, G.A., MacLeod, C., Torres-Mendez, C.-R. and Hakomori, S. (1988) J. Biol. Chem. **263**,10915-10921.

15. Hanai, N., Dohi, T., Nores, G.A. and Hakomori, S. (1988) J. Biol. Chem. **263**, 6296-6301.

16. Igarashi, Y., Kitanura, K., Toyokuni, T., Dean, B., Fenderson, B.A., Ogawa, T., and Hakomori, S. (1990) J. Biol. Chem. **265**, 5385-5389.

17. Weis, F.M.B. and Davis, R.J. (1990) JBC **265**,12059-12066

18. Schlessinger, J. (1988) J. Biol Chem. **263**, 3119-3123.

19. Wedegaertner, P.B. and Gill, G.N. (1989) JBC **264**, 11346-11353.

20. Hanai, N., Nores, G.A., MacLeod, C., Torres-Mendez, C.R. and Hakomori, S. (1988) J. Biol. Chem. **263**, 10915-10921.

21. Dawson, G., McLawhon, R. and Miller, R.J. (1980) J. Biol. Chem. **255**, 129-137.

22. Capasso, J.M., Keenan, T.W., Abeijon, C. and Hirschberg, C.B. (1989) J. Biol. Chem. **264**, 5283-5240.

23. Strous, G.J., van Kerhoff, P., Fallon, R.J. and Schwartz, A.L. (1987) Eur. J. Biochem. **169**, 307-311.

24. Dumont, J.E., Jauniaux, J.-C. and Roger, P.P. (1989) TIBS **14**, 67-71.

25. Moskal, J.R., Lockney, M.W., Marvel, C.C., Trosko, J.E. and Sweeley, C.C. (1987) Cancer Res **47**, 787-790.

26. Spiegel, S. (1989) in *Gangliosides and Cancer* (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 17-30.

27. Nagai, Y. and Tsuji, S. (1989) in *Gangliosides and Cancer* (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 33-39.

28. Burczak, J.D., Soltyziak, R.M., and Sweeley, C.C. (1984) J. Lipid Res. **25**, 1541-1547.

29. Bishop, W.R. and Bell, R.M. (1988) Oncogene Res. **2**, 205-218.

30 Low, M.G. and Saltiel, A.R. (1988) Science **239**, 268-275.

31. Carey and Hirschberg, C. (1981) J. Biol. Chem. **256**, 989-993.

32. Melkerson-Watson, L.J., Kanemitsu, K. and Sweeley, C.C. (1987) Glycoconjugate J. **4**, 7-16.

33. Melkerson-Watson, L.J. and Sweeley, C.C. (1991) J. Biol. Chem., in press (see Chapter 3).

34. Matsudaira, P. (1990) Methods Enzymol **182**, 602-613.

35. Lance, P., Lau, K.M. and Lau, J.T.Y. (1989) Biochem. Biophys. Rec. Commun. **164**, 225-232.

36. Paulson, J.C. and Colley, K.J. (1989) J. Biol. Chem. **264**, 17615-17618.

37. Trinchera, M. and Ghidoni, R. (1989) J. Biol. Chem. **264**, 15766-15769.

38. Weinstein, J., Lee, E.U., McEntee, K., Lai, P.H. and Paulson, J.C. (1987) J. Biol. Chem. **262**, 17735-17743.

39. Ladisch, S. (1989) in *Gangliosides and Cancer* (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 217-230.

40. Portoukalian, J. (1989) in *Gangliosides and Cancer* (Oettgen, H.F., ed.) VCH Publishers, New York, NY, p.p. 165-171.

41. Melkerson-Watson, L.J. and Sweeley, C.C. (1990) Biochem. Biophys. Res. Commun. **172**, 165-171.

42. O'Farrell, P.H. (1975) J. Biol. Chem. **250**, 4007-4021.

43. Laemmli, U.K. (1970) Nature **277**, 680-685,

APPENDIX A

REPORT OF LABORATORY EXAMINATION FINAL

ANIMAL HEALTH DIAGNOSTIC LABORATORY
P.O. Box 30076
Lansing, MI. 48909
Phone (517) 353-1683

PRIVILEGED INFORMATION
NOT FOR PUBLICATION

GROSS NECROPSY

Case Number: 1008571
Reported : 08/09/90
Received : 07/30/90
Pathologist: JAR
Case Origin: NECROPSY

Client Account: 9016

STEIN, SUSAN
UNIVERSITY LAB ANIMAL RESOURCES
C-111 CLINICAL CENTER
UNIV. ACCT. #21-3019
CAMPUS MI 48824

Owner:

BIOCHEMISTRY

Name : BETH
Breed : RABBIT DOMESTIC

Age: UNKNOWN
Sex: FEMALE

History : The rabbit arrived at MSU on 1/10/90. The animal was inoculated three times with CMP sialic acid in an adjuvant of rat liver origin. This enzyme catalyzes the synthesis of GM3 ganglioside. The inoculations began 6/18/90. Shortly after the inoculations began, the rabbit was intermittently anorectic. On 7/27/90, the rabbit was inappetent and was not defecating. Shortly afterwards, a purulent ocular nasal discharge was observed. The rabbit was forcefed a cereal-based diet on 7/27 through 7/29. The animal was euthanatized by a terminal sanguination under anesthesia.

Specimen: CARCASS

GROSS LESIONS, < 200 LBS

The carcass weighed 2.1 kg and was judged to be in good condition. The stomach contained a poorly formed hairball. Portions of the jejunum and cecum were distended with gas (postmortem change). The gallbladder was distended, and the contents were inspissated (alteration possibly secondary to the presence of a gastric hairball). Both kidneys were similar in appearance, and were tan with multiple coalescing white, non-raised foci. The foci were up to a centimeter in diameter and appeared to be confined to the cortex. All skeletal muscles contained yellow/white streaks (indication of degeneration, necrosis and/or mineralization). Both the white and red muscles were affected, especially the white muscles. The heart was unremarkable.

LABORATORY FINDINGS

Nutritional examination: Tests are in progress, and results will be forwarded in a supplemental report when available.

*DENOTES ADDITIONAL TEST RESULTS

Specimen: FIXED TISSUES

HISTOPATHOLOGIC EXAMINATION

Multiple tissues were examined. Skeletal muscle fibers consisted of severe degeneration and necrosis. Affected muscle fibers were scattered throughout muscle fascicles. Myofiber alterations included loss, vacuolation, hyalinization, and mineralization. In regions in which myofibers were lost, there was an accumulation of macrophages. The heart contained focal aggregates of lymphocytes within the endocardium of the right ventricle and the myocardium of the left ventricular papillary muscle. The lymphocytes were pleomorphic, and occasional mitotic figures were observed. The kidney contained numerous multifocal interstitial, coalescing aggregates of pleomorphic lymphocytes. Within the region of interstitial lymphocytic infiltration, there was loss of tubules. In other tubules, there were protein casts. The glomeruli had no thickening of the mesangium or basement membranes. The glomeruli did appear slightly hypercellular, due to the presence of cells resembling lymphocytes. The distribution of the alterations within the kidney appeared to be cortical. Numerous lymphocytes were present within the region adjacent to the renal transitional epithelium. The liver had prominent portal areas, due to the presence of numerous lymphocytes. The lymphocytes were encircling bile ducts and extended into the adjacent hepatic parenchyma. The lymphocytes were markedly pleomorphic, and mitotic figures were present. Centrilobular hepatocytes had mild vacuolar change. The gallbladder contained inspissated bile, which was amorphous and basophilic. Numerous lymphocytes were present within the wall of the gallbladder. Mild hemosiderosis was present in the spleen. Mild peribronchiolar lymphocytic aggregation was present within the lung. The adrenal cortex capsule and medulla were infiltrated by lymphocytes. The gut-associated lymphoid tissue was prominent, and some macrophages were present. The lamina propria of the duodenum contained a focal accumulation of lymphocytes. Other tissues were unremarkable.

CONCLUSION

Disseminated lymphosarcoma; disseminated skeletal muscle degeneration and necrosis, suggestive of a nutritional myopathy.

Comments:

James A. Render 8/9/90
Pathologist (517) 353-5275

sae

Additional copies to: Dr. Render, +1