Studies on the Modified Macromolecular Bovine Glycoprotein Acceptors for ST3Gal I and ST3Gal III and the Preliminary Measurements of Their Levels in Renal Transplant Patient Serum, Pre- and Post-Transplant

By

Khristofer Garcia

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

Department of Chemistry
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

There are . . .

no heroes too small,
nor mountains too large,
no journey too far,
nor conquest too grand,

if it drives your PASSION and steers your path.

K.S., K.G. 2004

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Throughout my entire graduate studies process, there have been several people that I have encountered who without would not make my wonderful and fulfilling experience possible. To those individuals, I say thank you!

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ABBREVIATIONS

Carbohydrate Subunits

Fuc	D-fucose
Gal	D-galactose
GalNAc	D-*N*-acetylgalactosamine
Glc	D-glucose
GlcNAc	D-*N*-acetylglucosamine
Man	D-mannose
NeuAc	D-*N*-acetyl-neuraminic acid

Glycosyltransferases

GT	Glycosyltransferase
ST	Sialyltransferase
GlcT	Glucosyltransferase
FucT	Fucosyltransferase
GalT	Galactosyltransferase
ManT	Mannosyltransferase
GlcNAcT	N-acetylglucosaminyltransferase
GalNAcT	N-acetylgalactosaminyltransferase
ST3Gal I	β-D-Galβ1,3GalNAcβ-R α2,3-sialyltransferase
ST3Gal III	β-D-Galβ1,3(4)GlcNAcβ-R α2,3-sialyltransferase
ST6Gal I	β-D-Galβ1,4GlcNAcβ-R α2,6-sialyltransferase
ST6GlcNAc I	NeuAcα2,3Galβ1,3GlcNAcβ-R α2,6-sialyltransferase

Acceptor Designations

As	Asialylated acceptor
(-O)	O-linked oligosaccharide chains removed
(+O)	O-linked oligosaccharide chains still attached
(-G)	β1,4-linked galactose residues removed.
As(-O)	Asialylated acceptor, O-linked oligosaccaride chains removed

Materials and Methods

N	Normality
Δ Activity	Change in activity = (dpm_{test} – $dpm_{control}$)
Da	Dalton
kDa	Kilodalton
dpm	disintegrations per minute
MWCO	Molecular weight cutoff
PTA	Phosphotungstic acid
RPM	Revolutions per minute
TCA	Trichloroacetic acid

General text

et al.	et alii (and others)
α_1-AGP	α_1-acid glycoprotein
Asn	Asparagine
Asp	Aspartic acid
cDNA	Complementary deoxyribonucleic acid
CMP	Cytidine monophosphate
CTS	Cytoplamic, transmembrane, and stem regions
DGF	Delayed graft function
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid

ER	Endoplasmic Reticulum
GDP	Guanidine diphosphate
Gly	Glycine
HPLC	High performance liquid chromatography
ICAM-1	Intercellular adhesion molecule 1
K_m	Michaelis-Menten constant
LNT	Lacto-N-tetraose
M-M	Michaelis-Menten
MS	Mass spectrometry
MUC1	Mucin 1
NMR	Nuclear magnetic resonance
Pro	Prolne
Ser	Serine
Sialyl-Le^x	Sialyl-$Lewis^x$
Sialyl-Le^a	Sialyl-$Lewis^a$
Thr	Threonine
Trp	Tryptophan
V_{max}	Maximum velocity
VCAM-1	Vascular cell adhesion molecule 1
UDP	Uridine diphosphate
NH(2)-GNWWWW	NH(2)-Gly-Asn-Trp_4

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ABSTRACT

A recent study demonstrated the elevation of ST6Gal I levels in kidney transplant patient serum, pre-transplant, which was correlated with post-transplant events such as delayed graft function, and in turn, predisposes to poor long term outcome (Thorne-Tjomsland *et al.*, 2000). As an extension of this study, an enzyme assay system is developed for measuring the levels of two other well-characterized sialyltransferases, ST3Gal I and ST3Gal III, in human serum utilizing modified bovine glycoproteins, α_1-AGP and fetuin, as acceptors. Subsequent to specific chemical and enzymatic treatments of bovine α_1-AGP and bovine fetuin, the modified glycoproteins were tested against a selected panel of commercially available sialyltransferases to determine the presence of proper acceptor sites. Various kinetic studies were performed on the modified acceptors, assaying them in human control serum, followed by their employment in one set of serum samples obtained from a selected kidney transplant patient for preliminary examination of the levels of particular sialyltransferases. Our results indicated that the acceptors, *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP, produced high activity from recombinant ST3Gal III, while recombinant ST3Gal I displayed high activities towards *As(+O,-G)* fetuin. Kinetic analysis of the acceptor for ST3Gal I in human serum demonstrated activity with a Km = 0.48±0.14 mM, and Vmax = 1.47±0.30 pmol NeuAc/min. No significant ST3Gal III activity was detected with *As(-O,-G)* fetuin or *As(-G)* bovine α_1-AGP when employed in human serum. Results obtained from preliminary examination of ST6Gal I, ST3Gal III, and ST3Gal I levels from the longitudinal serum samples of a renal transplant patient employing the modified acceptors revealed the presence of ST6Gal I activity at high levels, with the ST3 enzymes displaying low levels, similar to the results

found in human control serum. In conclusion, there were no significant differences found in ST3Gal III and ST3Gal I levels in the renal transplant patient versus a healthy individual. Furthermore, ST3 serum levels in the kidney transplant patient appeared insignificant in comparison to the ST6Gal I activities detected.

INTRODUCTION

CHAPTER 1. Glycobiology and Protein Glycosylation: A Brief Review

Carbohydrates, or sugars, are often associated with processes such as metabolism and energy generation. However, these molecules have also served as integral components for signaling and structural purposes. Sugars are ubiquitous in nature, often found attached to proteins or lipids. As conjugates of proteins, they modulate structure and/or function. When linked to lipids, their contribution is relevant for cell-cell recognition and signaling. These molecules are prominently located at the surface of all cells, serving as tags to the outside world. Carbohydrates, whether they exist freely in the extra-cellular matrix, or conjugated to proteins or lipids, generally hold a great deal of structural information that is exploited in many forms of biological recognition. Cell interactions with cytokines, hormones, toxins, antibodies, lectins, bacteria, viruses, and other cells have all demonstrated the involvement of carbohydrate recognition (Varki, 1993).

Apart from their obvious importance, the scientific advancements in carbohydrate biology and chemistry have been both bitter and sweet. In a remarkable era led by the genomes and the proteins, glycans and glycoconjugate studies have often been overshadowed and consequently have lagged behind in research. Part of the problem can be attributed to the complexity of sugars. Whereas DNA and proteins essentially exist in linear sequences, sugar chains, or saccharides, have the potential to branch and contain many isomers, allowing a multitude of possible structures. In addition, DNA and proteins have fewer basic building blocks (4 and $\geq$20, respectively) in comparison to sugars, which have over 30 different units. The complexity and diversity of saccharides

have posed challenges that have been quite labor-intensive, often frustrating to researchers throughout carbohydrate history. Furthermore, the lack of tools to analyze carbohydrate structures and sequences in nature has made elucidation of their biosynthesis and function difficult. Even so, their significance as signalling macromolecules has spurred developments of new technologies to explore and characterize these sugar chains, opening up a new frontier of molecular biology known as "glycobiology". It was in 1988, when Rademacher, Parekh, and Dwek introduced this term in order to integrate carbohydrate studies with various aspects of science, including chemistry, biochemistry, cellular and molecular biology. Glycobiology is thus the study of structure, biosynthesis, and biology of saccharides (sugar chain or glycans) that are widely distributed in nature (Rademacher *et al.*, 1988). Currently, glycobiology is one of the most rapidly growing fields in science. A special issue of Science (Hurtley *et al.*, 2001) had described glycobiology as the "Cinderella field, having to compete with its cousins, the genome and proteins". Science recruited glycobiology experts to address the relevance of carbohydrate research, entailing the most recent discoveries and applications in this field. The areas focused on included the emerging analytical tools, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR), which have been successful in elucidating glycan structure (Dell and Morris, 2001). In addition, improved developments on carbohydrate synthesis, chemical or enzymatic, have allowed amplified production of glycoconjugates in sufficient quantities for further characterization (Sears and Wong, 2001). More importantly, a great deal of attention has been focused on protein glycosylation and subsequent modifications, which include sugar trimming and addition, transport within the secretory system (Helenius and Aebi, 2001), and

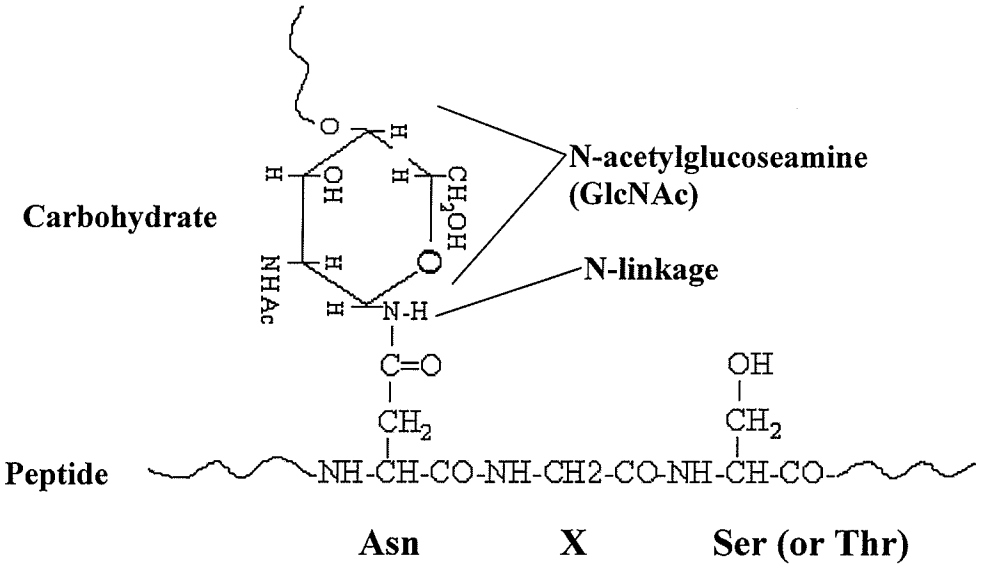
involvement in the immune system (Rudd *et al.*, 2001). Nevertheless, a great deal of research is still required in order to stand at par with the knowledge attained for genomes and proteins. As a contribution to this newly expanding field, the primary aim of this thesis is to measure the levels of particular enzymes, called sialyltransferases, involved in the biosynthesis of glycoconjugated molecules, particularly glycoproteins, in human systems. Thus, it is imperative to briefly overview protein glycosylation and the large family of enzymes that sialyltransferases belong to, known as glycosyltransferases, responsible for the biosynthesis of these glycoconjugates.

Protein Glycosylation

Glycosylation, the process involving the addition of sugar units or glycan chains onto proteins, is one of the most important post-translational modifications. Proteins require carbohydrates for correct folding, final sub-cellular localization, biological activity, and metabolic stability. Compared with other forms of protein modifications, which may include sulfation, acetylation, or phosphorylation to name a few, glycosylation is significantly more complex, and involves intricate cellular machinery dedicated to synthesis and modulation. Thus, carbohydrates have the potential to change or add new properties to the protein without changing the actual protein sequence.

The oligosaccharides found on proteins are typically attached in two distinct ways, either N- or O-linked as shown in Figure 1. Carbohydrate structures that are covalently bonded to the oxygen atom of a serine (Ser) or threonine (Thr) residue of the peptide backbone are known as O-linked oligosaccharides. N-linked oligosaccharides have their carbohydrate structure covalently linked to the nitrogen atom of an asparagine

A)



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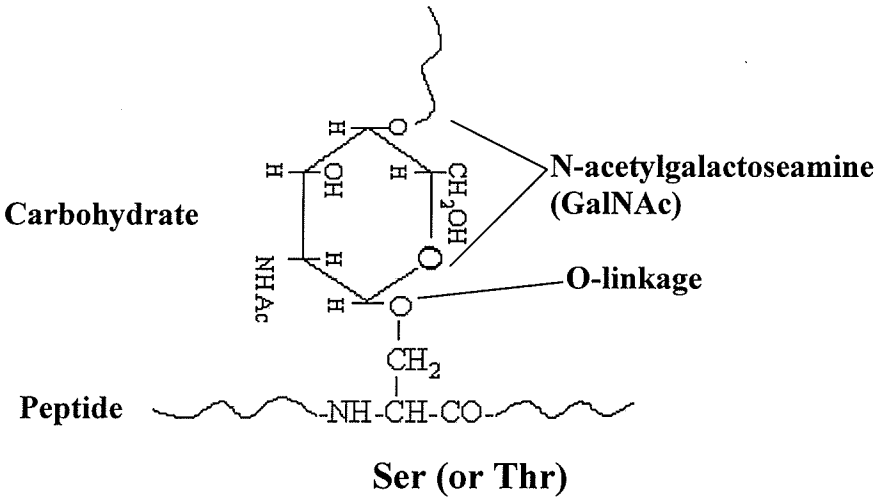


Figure 1. N- and O-linked Glycans.

Schematic diagrams of the 2 ways glycans are conjugated to the peptide backbone. A) N-linked glycans are represented by a covalent bond via nitrogen atom between N-acetylglucosamine (GlcNAc) and an asparagine (Asn) residue that is part of the sequon Asn-X-Serine/Threonine (Ser/Thr), where X can be any amino acid with the exception of aspartic acid and proline. B) O-linked glycans are represented by a covalent bond via oxygen atom between N-acetylgalactosamine (GalNAc) and a Ser or Thr residue. (Reference: Ion Source) http://www.ionsource.com/Card/carbo/nolink.htm

(Asn) residue in an Asn-X-Ser/Thr sequence, where X may be any amino acid, with the exception of proline (Pro), due to steric hinderance (Kornfeld and Kornfeld, 1985) and aspartic acid (Asp), because of its negative charge on the side chain. During the O-glycosylation process, sugars are added in a reasonably straightforward stepwise fashion, varying structurally from a single N-acetylgalactosamine (GalNAc) residue to oligosaccharides containing many residues. On the other hand, the process of N-glycosylation is comparably more complicated (reviewed by Helenius and Aebi, 2001). The biosynthesis of N-linked oligosaccharides starts with the addition of sugars onto the lipid intermediate precursor embedded in the endoplasmic reticulum (ER) membrane, known as dolichol-phosphate. Initial steps occur on the cytosolic side of the ER, where typical sugars (Figure 2), starting with N-acetylglucosamines (GlcNAc), are added one by one in the beginning stages of the dolichol-phosphate cycle (Figure 3). Upon the addition of two GlcNAc and five mannose (Man) residues to dolichol-phosphate by N-acetylglucosaminyltransferases (GlcNAcT) and mannosyltransferases (ManT), the structure is flipped into the lumenal ER, where further elongation occurs with the addition of 7 more sugar residues (4 Man and 3 glucoses or Glc by glucosyltransferases). The final structure of the precursor, $Glc_3Man_9GlcNAc_2$-P-P-Dol, is recognized by an oligosaccharyltransferase enzyme, which transfers the oligosaccharide precursor from dolichol onto an Asn residue that is part of the glycosylation sequon Asn-X-Ser/Thr. If transfer onto the nascent polypeptide is successful, the trimming and processing of the protein-bound glycan occurs. At the outset, the three Glc residues and two Man residues are excised in the ER by several different glucosidases and mannosidases at pH 6, in contrast to the glucosidases and mannosidases in lysozymes that work optimally at pH

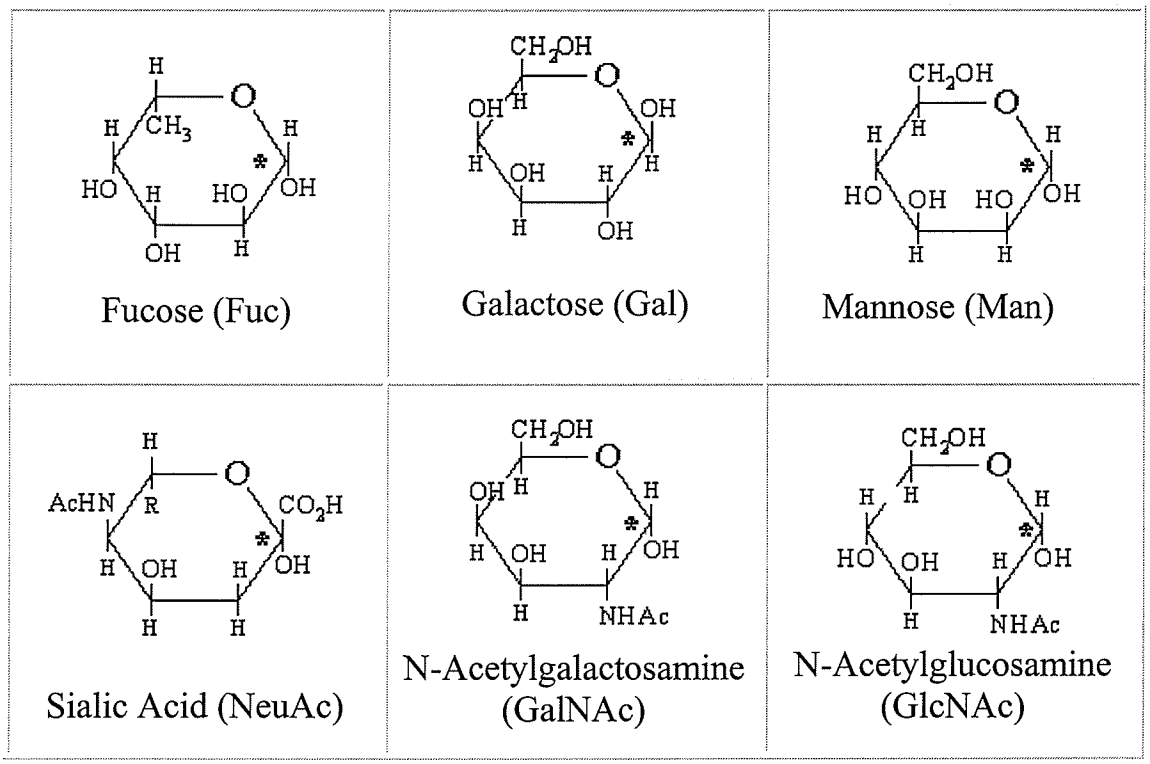


Figure 2. Common Monosaccharides Utilized in the Synthesis of Glycoproteins.

Carbons at the 'starred' (*) position are known as 'anomeric' carbons. The orientation of the hydroxyl groups of a sugar residue determines the configuration: α-configuration (when below the plane of the ring) or β-configuration (when above the plane of the ring), with the exception of fucose, since all sugars shown are D-sugars and fucose is an L-sugar. The link between 2 sugar residues are also determined by which carbons are involved (Diagram modified from Ion Source).
http://www.ionsource.com/Card/carbo/sugar.htm

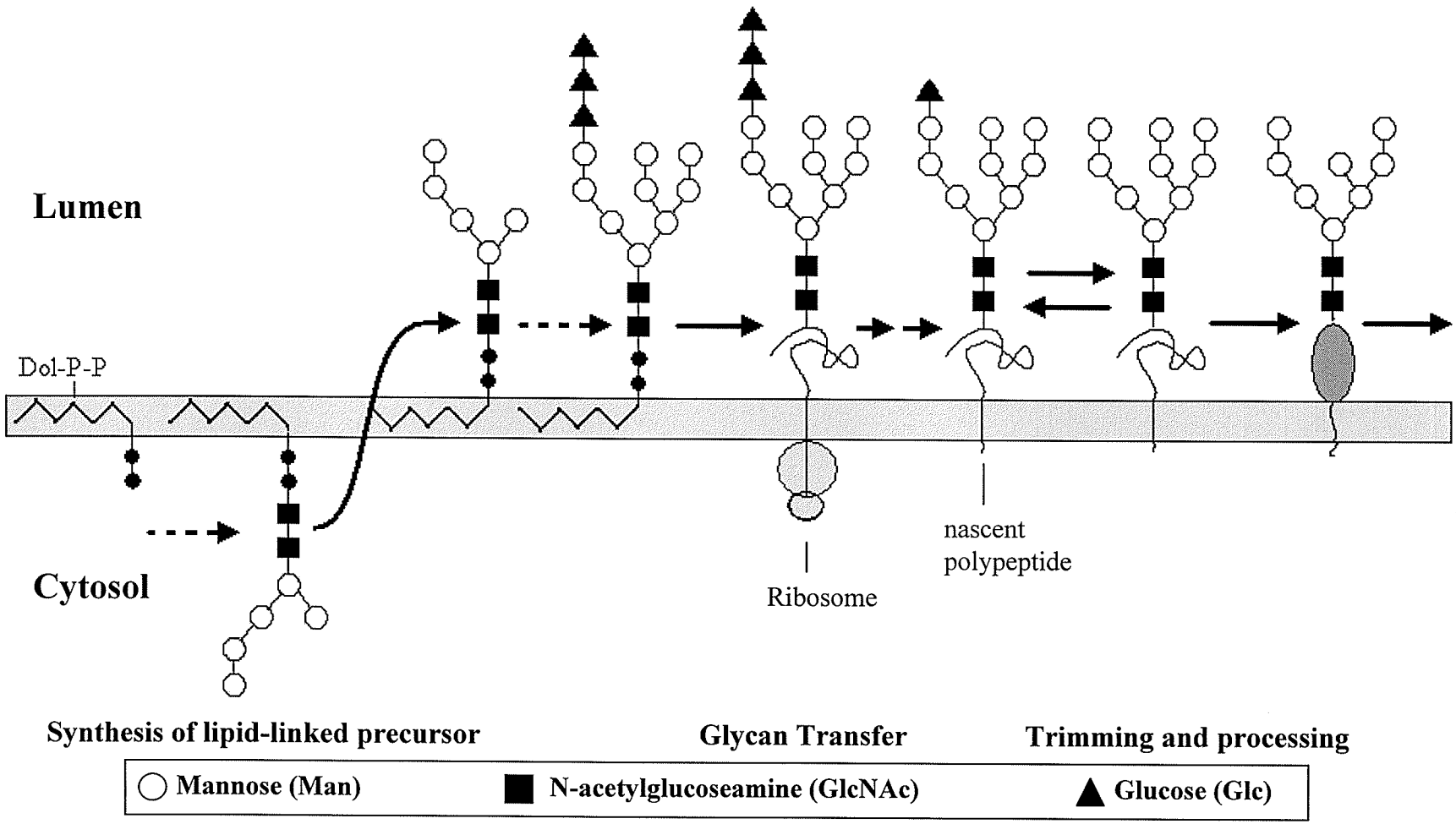


Figure 3. Dolichol Phosphate Cycle in the Endoplasmic Reticulum (ER).

The biosynthesis of N-linked core oligosaccharides begins on the cytosolic side of the ER, where the initial addition of sugar residues is attached to the lipid precursor, dolichol-phosphate. The oligosaccharide is flipped onto the lumenal side and further addition occurs. The structure, $Glc_3Man_9GlcNAc_2$-P-P-Dol, is transferred onto a nascent peptide with additional trimming and processing prior to entry into the Golgi (Diagram adapted from Helenius and Aebi, 2001).

~4. Upon initial trimming, the resultant glycoprotein is transferred to the Golgi apparatus. The Golgi apparatus is a highly complex, compartmentalized system, where both O- and N-linked glycans are further glycosylated and differentiated (Figure 4). As the glycoprotein travels through each of the membrane-bound compartments, particularly the *cis*-Golgi network, the *cis*, *medial*, and *trans* cisternae, and the trans-Golgi network, carbohydrate residues are removed or attached based on the type of enzyme that the protein encounters, resulting in a completed glycoprotein. Additionally, the final N-linked glycan can be classified into one of three distinct groups: 1) high mannose type, 2) hybrid type, and 3) complex type (Figure 5). All three groups consist of the same pentasaccharide core.

Glycosyltransferases

Significant portions of the glycosylation process in the ER-Golgi pathway are enzymatically-mediated reactions. The primary enzymes responsible for the glycosylation machinery in living cells are called glycosyltransferases. Specifically, they transfer a sugar molecule, or monosaccharide, from high energy donors, one at a time, onto specific positions of specific precursors, also known as acceptors (Figure 6) (Varki *et al.*, 1999). The sequential action of glycosyltransferases functionally creates an end product that is a favorable precursor for the next glycosyltransferase along the pathway. In other words, glycan structure is dependent on the subsequent acting enzymes, based on their localization in the ER-Golgi pathway and the linkage-specific precursor that is recognized. The end product is a polymer of sugar chains arranged in a linear or branched manner (Paulson and Colley, 1989).

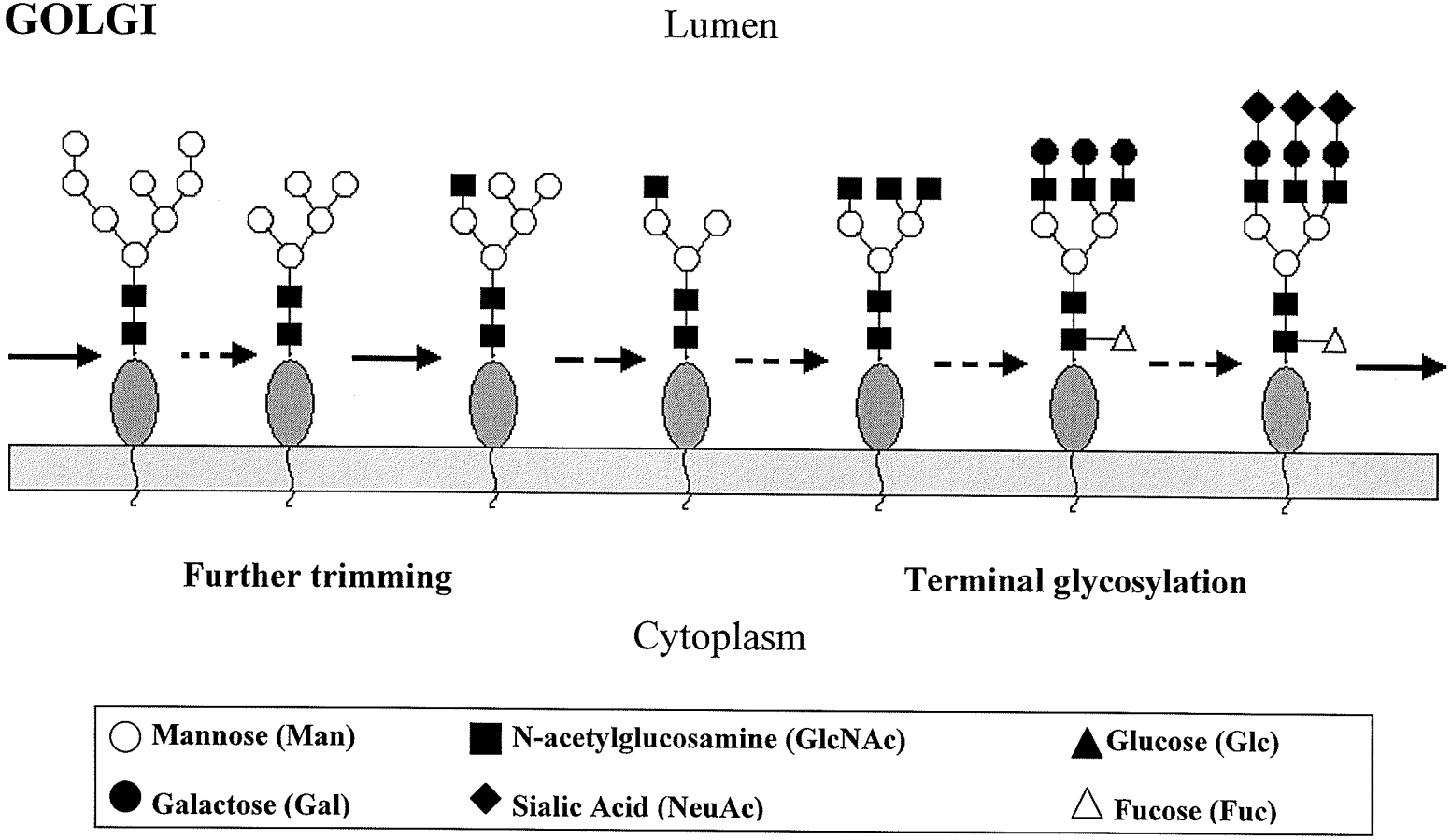


Figure 4. Trimming, Differentiation, and Further Glycosylation in the Golgi.

Further trimming of Man residues occurs upon initial entry into the Golgi. Terminal glycosylation is carried out as the glycoprotein passes through the *cis*, *medial*, and *trans* Golgi networks, passing several different glycosyltransferases. The above diagram is only one of many possible terminal glycosylation pathways existing, which can lead to several different types of glycoproteins (Diagram adapted from Helenius and Aebi, 2001).

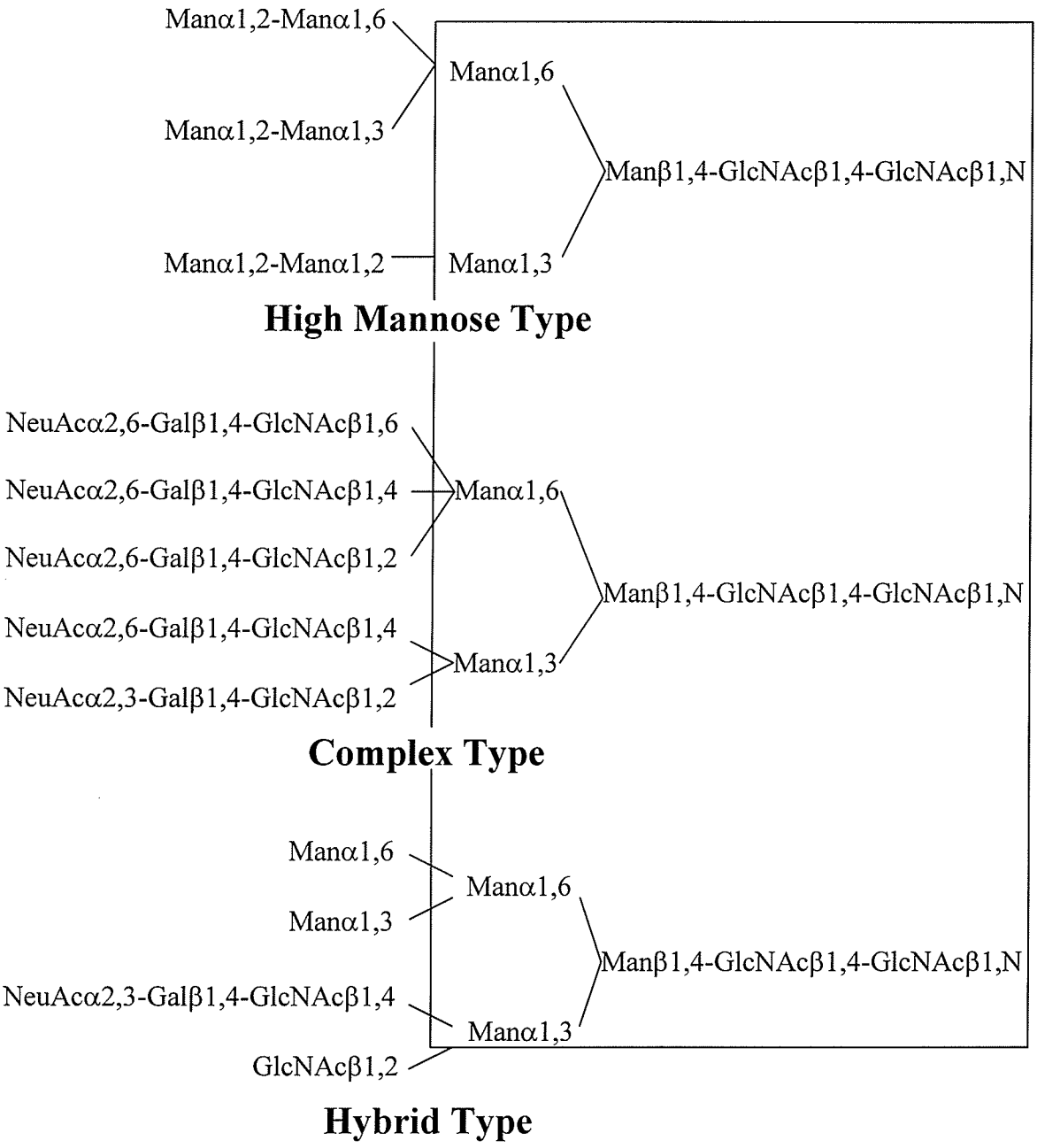
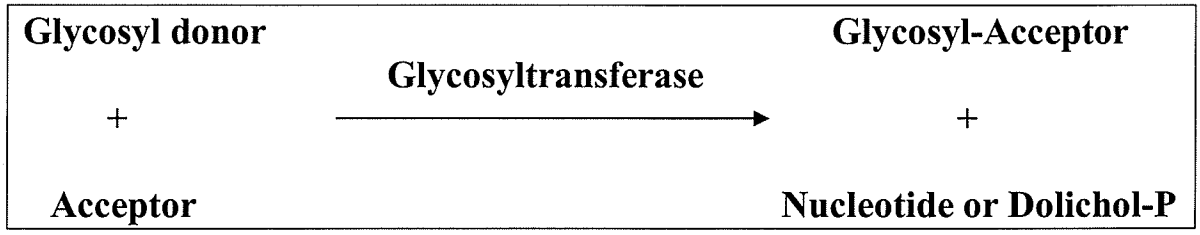


Figure 5. The 3 Sub-Groups of N-linked Glycans.

One example from each of the 3 different groups of N-linked oligosaccharides: high mannose-type, complex-type, and hybrid-type, are shown above. All types share a common pentasaccharide core, shown within the solid lined box. Those sugars shown outside of the box are subjected to variation (Kobata, 1992).



Glycosyl donors	Acceptors
CMP-Sialic acid	Oligosaccharides
GDP-Fucose	Monosaccharides
GDP-Mannose	Proteins
UDP-Galactose	Lipids (Ceramides)
UDP-N-acetylgalactosamine	
UDP-N-acetylglucosamine	
UDP-Glucose	
UDP-Glucuronic acid	
UDP-Xylose	
Dolichol-P-Glucose	
Dolichol-P-Mannose	
Dolichol-P-($Glucose_3$-$Mannose_9$-N-$acetylglucosamine_2$)	

Figure 6. Typical Glycosyltransferase Reaction.

A typical glycosyltransferase reaction is shown above, along with a list of some common glycosyl donors and acceptors that are commonly found in mammals (Varki *et al.*, 1999).

More than 150 different glycosyltransferases are known to exist, but there may be at least double this number to be characterized to account for all the wide-ranging carbohydrate structures present in nature (Varki *et al.*, 1999). Glycosyltransferases, being glycoproteins themselves, may be grouped into families depending on sequence similarities in peptide domains essential for structure and biological function. These families show high sequence homology between species but low sequence homology between families within one species (Paulson and Colley, 1989). Within each family, the donor substrate is typically only one nucleotide activated monosaccharide which determines the nomenclature for glycosyltransferases; i.e. galactosyltransferase (GalT) transfers a galactose (Gal) molecule, sialyltransferase (ST) transfers a sialic acid (NeuAc) molecule, fucosyltransferase (FucT) transfers a fucose (Fuc) molecule, etc.

Glycosyltransferases normally require a divalent cation as a co-factor for activity, e.g. Mg^{+2} or Mn^{+2}, and prefer a pH range of 5.0 to 7.0, i.e. the pH range detected within the ER-Golgi network (Varki *et al.*, 1999). They also exhibit Michaelis-Menten constants (K_m) for both their substrates; the nucleotide sugar in the low micromolar value range, and the acceptor substrate in the range of low micromolar to low millimolar values. These enzymes of eukaryotic cells are typically type II membrane proteins with a short N-terminus in the cytoplasm, a transmembrane domain, a stem region and a longer catalytic domain at the C-terminus protruding into the Golgi lumen (Paulson *et al.*, 1987). However, the typically membrane-bound enzymes also exist in soluble forms, being detected in milk, serum, and various other bodily fluids (El-Battari *et al.*, 2003; Beyer *et al.*, 1981; Sadler, 1984). Furthermore, elevated levels of soluble glycosyltransferases have been found in serum during disease (Kim *et al.*, 1972) and inflammation (Lammers

and Jamieson, 1988). The mechanism of release occurs at the stem region, where they can be secreted from the cell in soluble forms by way of proteolytic cleavage *via* endogenous proteases, while the catalytic subunit remains active and intact (Paulson *et al.*, 1987; Lammers and Jamieson, 1988).

Up until the early 1990s, it was widely believed that the Golgi glycosyltransferases were segregated into distinct compartments within the secretory system, with the early-acting glycosyltransferases being localized in the *cis* and *medial* compartments of the Golgi, and late-acting enzymes generally localized in the *trans*-Golgi cisternae and the *trans*-Golgi network (Figure 7). The information that led most groups to believe this localization scheme was obtained from studies done on individual glycosyltransferases (Berger and Hesford, 1985; Bergeron *et al.*, 1985; Roth, 1987; Duncan and Kornfeld, 1988). The early acting Golgi- enzyme, N-acetylglucosaminyltransferase I (GlcNAcT I), was found to concentrate in the medial-Golgi compartment, while the terminal glycosyltransferases, α2,6-sialyltransferase (ST6Gal I) and β1,4-galactosyltransferase (GalT I) were expected to, as well as shown to, be localized in the *trans*-Golgi cisternae and *trans*-Golgi network. However, the distributions of glycosyltransferases have also greatly varied from the traditional belief with respect to differing cell types (Colley, 1997), where if glycoproteins were not glycosylated successfully in one compartment, a supplementary opportunity would be provided for proper glycosylation.

Although the mechanism for glycosyltransferase retention to specific Golgi subcompartments has eluded researchers, it was suspected earlier on that the membrane proteins of the Golgi are comprised of specific retention signals, which are nonexistent in

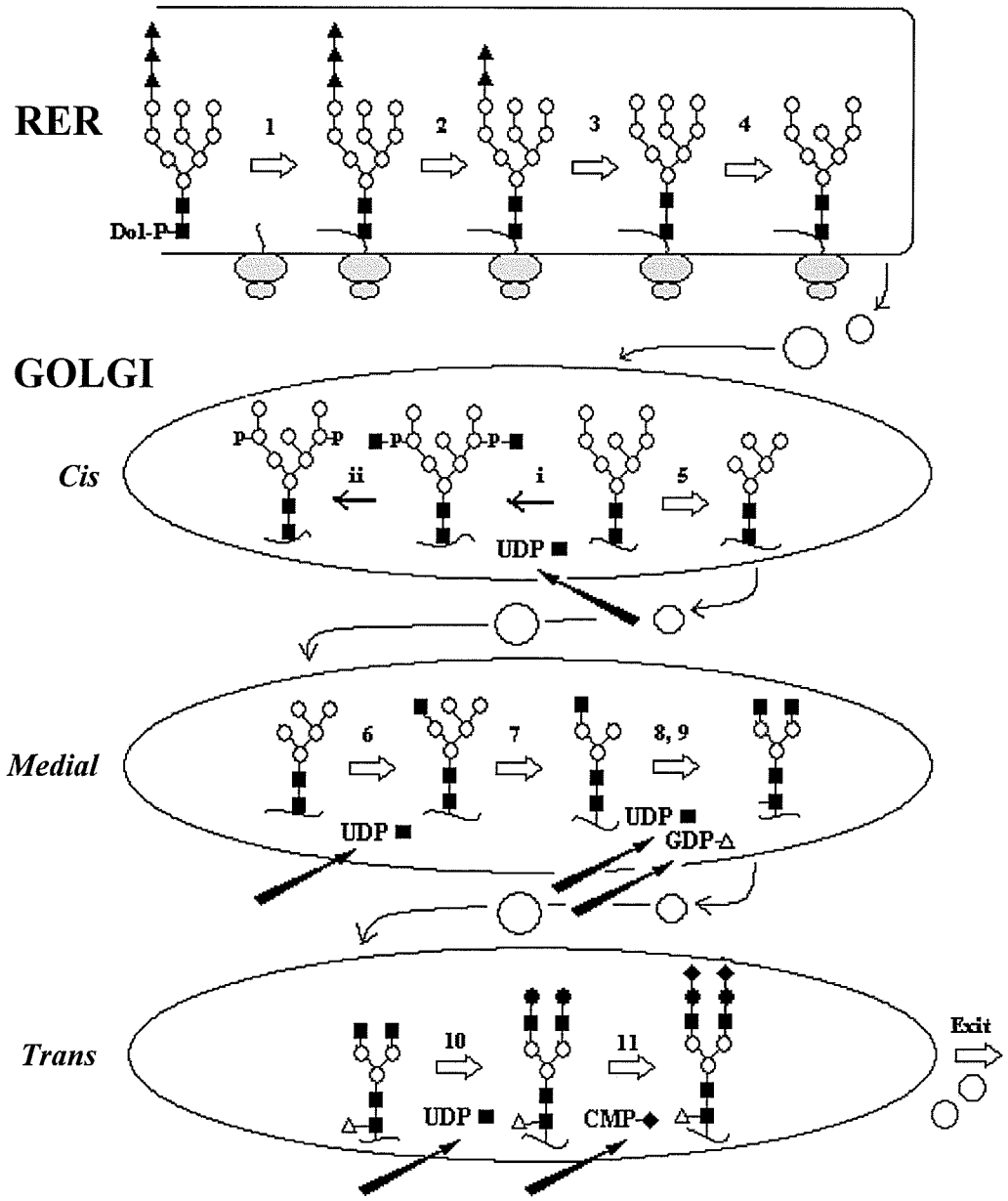


Figure 7. Oligosaccharide Processing in the ER and Golgi.

Upon movement through the oligosaccharide processing pathway, the glycoprotein encounters different glycosyltransferases or other enzymes in different compartments. The type of enzyme the glycoprotein comes into contact with is dependent on the localization of these enzymes within the ER and Golgi; i.e. enzymes 1-4 in the ER, 5, i, and ii in the *cis* Golgi, 6-9 in the *medial* Golgi, and 10-11 in the *trans* Golgi. The enzymes are: 1) OST; 2) α-glucosidase I; 3) α-glucosidase II; 4)ER α1,2-mannosidase; i) GlcNAc phosphotransferase; ii) GlcNAc-1-phoshodiester α-N-acetylglucosamidase; 5) Golgi α-mannosidase I; 6) GlcNAcT I; 7) Golgi α-mannosidase II; 8) GlcNAcT II; 9) FucT; 10) GalT; 11) ST. The monosaccharides are: GlcNAc(■); Man(o); Glc(▲); Fuc(Δ); Gal(•); NeuAc(♦) (Kornfeld and Kornfeld, 1985).

other proteins, that are either secreted or embedded in the membrane (Rose and Doms, 1988). There are now two models that have been introduced to account for retention of glycosyltransferases: the kin-recognition model and the cholesterol concentration gradient model. Briefly, the former model suggests the initial homodimer formation of a set of the same glycosyltransferases upon synthesis in the ER. The homodimer moves through the Golgi apparatus until coming across "kin" enzyme oligomers that are of specific cisternae, causing the formation of a multimeric complex through interactions involving the transmembrane segment and/or stem region (Nilsson *et al.*, 1994a; 1994b). The production of the large subunit consequently prevents further movement of the enzymes in the Golgi apparatus, resulting in retention within the particular subcompartment. The latter model, also known as the bilayer thickness model, bases its theory on the presence of a cholesterol concentration gradient in the secretory pathway, where there is an increase in cholesterol content progressing from the ER to the cis, medial, and trans Golgi compartments. Hence, a particular glycosyltransferase will be localized in its correct compartment based on the length of its transmembrane segment (Bretcher and Munro, 1993; Masibay, et al., 1993). Although ample evidence has been shown to support both theories, several examples have been opposing (Colley, 1997), where neither model has been shown to fully elucidate the actual retention mechanism of the glycosyltransferases. In fact, recent studies have suggested that the cytoplasmic, transmembrane, and stem (CTS) regions of glycosyltransferases all contribute to sorting and retention (Grabenhorst and Conradt, 1999). In addition, the cytoplasmic tail of α1,2-fucosyltransferase has been shown to contain a sequence for Golgi localization (Milland

et al., 2001). In any case, further studies are required for determination of the actual mechanism of Golgi glycosyltransferase retention.

Accordingly, glycosyltransferases are significant for glycosylation to occur, requiring a high degree of organization. However, it is not unusual to report elevated levels of glycosyltransferases in mammals during disease states, as abberant glycosylation is fairly indicative of certain cancers and diseases (Petretti *et al.*, 1999; Petretti *et al.*, 2000). Oligosaccharide structures can dramatically change during development and oncogenesis, becoming more highly branched and sialylated during these phenomena (Dall'Olio and Chiricolo, 2001). Hence, the next section will introduce a group of enzymes within the glycosyltransferase family called sialyltransferases that have shown significant differential expression during cell development and along with disease states.

CHAPTER 2. Essentials in Sialyltransferases

The sialic acid family is comprised of a group of closely related nine carbon carboxylated sugars. All have a distinct negative charge and normally reside at the non-reducing terminal end of glycoproteins or glycolipids, enabling their involvement in many major biological processes (Varki, 1992; Harduin-Lepers *et al.*, 1995). Diversity in sialic acids arise from the way they are attached onto their corresponding glycoconjugate, being found in an α2,3- or α2,6-linked orientation to a Gal residue, or in an α2,6-linked orientation to a GlcNAc or GalNAc residue. Less commonly, α2,8-linked sialic acids have also been discovered in gangliosides and in α2,8-homopolymer forms. All these sialic acids are attached to glycoconjugates by a group of enzymes called sialyltransferases. Forming a subset of the glycosyltransferase family, sialyltransferases are a group of functional enzymes that catalyze the transfer of NeuAc from the high energy donor cytidine-5'-monophosphate (CMP) onto a terminal non-reducing end of an oligosaccharide chain of a glycoprotein or glycolipid, also known as acceptors.

CMP-NeuAc + Acceptor → NeuAc-Acceptor + CMP

In the past decade, there has been elevated attention focused on sialyltransferases, since they sialylate significant glycoconjugates involved in biological processes such as cell-cell interaction and recognition (Harduin-Lepers *et al.*, 2001; Collins *et al.*, 2002). There are at least 18 different members, all of which have been cloned and characterized (Table 1). In humans, only 15 sialyltransferase cDNAs have been discovered, but there should be more than 20 different enzymes to account for all known sialyl-oligosaccharide

Sialyltransferases	Structures formed	cDNA source/gene
ST6Gal I SIAT 1	**NeuAcα2-6**Galβ1-4GlcNAcβ-	Placenta gene
ST6GalNAc I Sia7a	(NeuAcα2-3)$_{0-1}$(Galβ1-3)$_{0-1}$GalNAc-Ser **NeuAcα2-6**	Pyloric mucosa
ST6GalNAc II Sia7b	(NeuAcα2-3)$_{0-1}$Galβ1-3GalNAc-Ser **NeuAcα2-6**	MDA-MB 231 epithelial cells
ST6GalNAc III Sia7c	NeuAcα2-3Galβ1-3GalNAc-R **NeuAcα2-6**	N/A
ST6GalNAc IV Sia7d	NeuAcα2-3Galβ1-3GalNAc-R **NeuAcα2-6**	HepG$_2$ 3 last exons
ST6GalNAc V $G_{D1\alpha}$ synthase	$G_{D1\alpha}$	EST
ST6GalNAc VI	$G_{D1\alpha}$, ($G_{T1\alpha}$)	Liver
ST3Gal I Siat 4	**NeuAc5α2-3**Galβ1-3GalNAc-	Placenta gene
ST3Gal II Siat 5	**NeuAc5α2-3**Galβ1-3GalNAc-	Liver CEM
ST3Gal III Siat 6	**NeuAc5α2-3**Galβ1-3/4GlcNAcβ-	Placenta
ST3Gal IV Siat-4c, STZ	**NeuAc5α2-3**Galβ1-4GlcNAc- (preferred) **NeuAc5α2-3**Galβ1-3GalNAc-	Placenta gene
ST3Gal V G_{M3} synthase Siat 9	**NeuAc5α2-3**Galβ1-4Glc-Cer	HL-60
ST3GalVI	**NeuAc5α2-3**Galβ1-4GlcNAcβ-	SK-MEL-37
ST8sia I SAT-II G_{D3} synthase	**NeuAc5α2-8**NeuAcα2-3Galβ1-4Glc-Cer	Melanoma
ST8Sia II SIAT 8a, STX	**NeuAc5α2-8**NeuAcα2-3Galβ1-4GlcNAc	Placenta
ST8Sia III SIAT 8b	**NeuAc5α2-8**NeuAcα2-3Galβ1-4Glc-Cer	Brain
ST8Sia IV PST-1, SIAT 8d	**NeuAc5α2-8**(NeuAc5α2-8)$_n$NeuAcα2-3Galβ1-R	Fetal brain
ST8Sia V SAT-V	G_{D1c}, G_{T1a}, G_{Q1b}, G_{T3}	brain

Table 1. A List of All Human Sialyltransferases Cloned Since 2001.

A complete list of cloned human sialyltransferase cDNAs, collected up until 2001, are listed, including their current and former names, the type of linkages produced and the source from which their clones were made. For a further clarification of abbreviations, refer to Harduin-Lepers *et al.*, 2001.

structures. The difference between sialyltransferases can be attributed to their unique features based on ability to utilize a specific acceptor substrate, and to the orientation in which they attach sialic acid onto these substrates. As well, their expression depends on the type of species, tissue, cell, or developmental stage being examined. Expression of recombinant sialyltransferases from their cDNA counterparts has also allowed elucidation on the substrate specificity of each enzyme *in vitro.* For a deeper examination of similarities and differences in characteristic traits, primary attention will be focused on some common features found in all sialyltransferases, including structure and localization.

Firstly, examination of protein sequences from the first few cloned sialyltransferases provided evidence that they all share a Type II membrane protein topology, a common feature found in all members of the glycosyltransferase family. This topology, shown in Figure 8, consists of a short NH_2-terminal cytoplasmic domain, which currently has no identifiable function. A hydrophobic signal-anchor domain, approximately 16-20 amino acids in length, acts as an uncleavable signal peptide, as well as a membrane-spanning region, orienting the stem region and COOH-terminal catalytic domain into the lumen of the Golgi (Paulson and Colley, 1989). The stem region is sensitive to proteolysis, which is the suggested mechanism for the liberation of soluble forms (Lammers and Jamieson, 1988), while the COOH-terminus catalytic domain is responsible for the catalytic activity of sialyltransferases.

Despite the similar topological features shared by glycosyltransferases, further analysis at the primary amino acid sequence level showed little homology within the family, with the exception of short consensus sequences found amongst

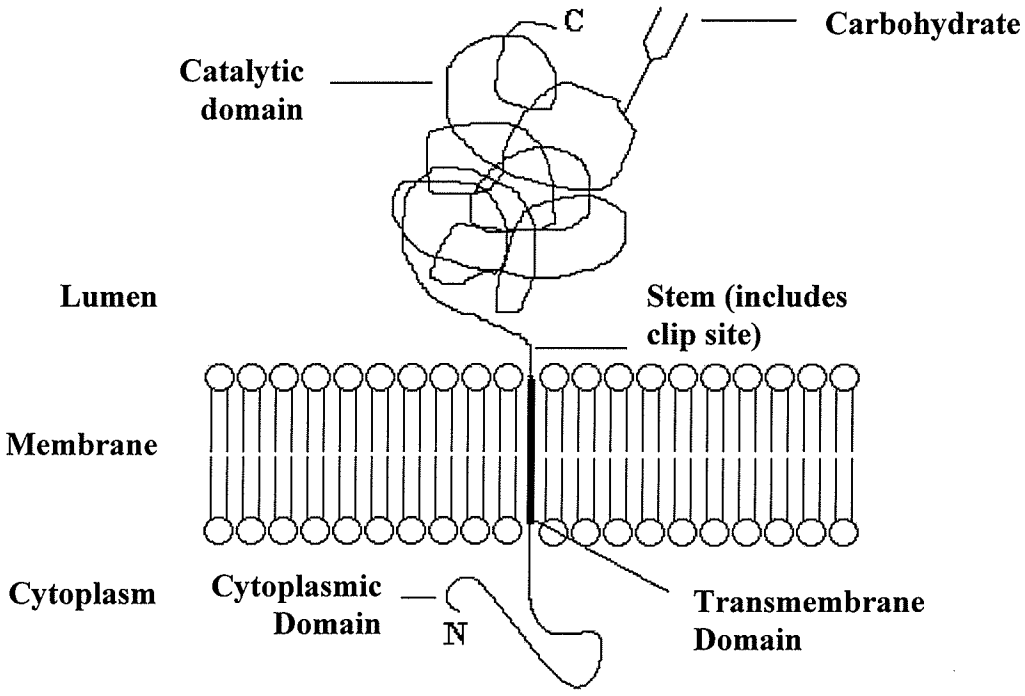


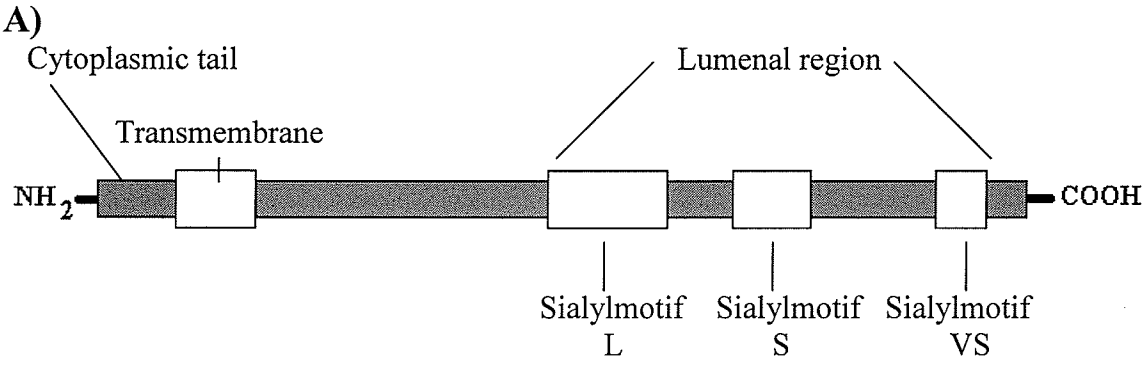
Figure 8. Sialyltransferase Type II Membrane Protein Topology.

The N-terminus consists of the cytoplasmic tail or domain, residing in the cytoplasm. The transmembrane domain is hydrophobic in nature and is embedded into the Golgi membrane. The C-terminus consists of the catalytic domain and the stem region, both typically residing in the lumen of the Golgi. The stem region contains a clip site that is sensitive to proteases, allowing soluble forms to be produced. (Adapted from Field and Wainwright, 1995)

sialyltransferases. The early 1990s introduced the discovery of a few highly conserved protein sequences located in the lumenal region of all sialyltransferases cloned to date, known as "sialylmotifs" (Livingston and Paulson, 1993; Drickamer, 1993). Within the middle of the lumenal catalytic domain lies the L (or large)-sialylmotif, the largest of the conserved sequences consisting of 8 invariant amino acids in a 48 amino acid stretch. The shorter S (or short)-sialylmotif, located closer to the C-terminal end, typically stretches about 23 amino acids in length and contains 2 invariants. Another conserved region called the VS (very short)-sialylmotif was identified, residing near the COOH-terminus (Geremia *et al.*, 1997). In order to determine the functionality of these conserved regions, studies were carried out involving site-directed mutagenesis targeting the invariant amino acids in the L-and S-sialylmotifs, using ST6Gal I as the model. The results demonstrated high binding affinity of the L-sialylmotif towards CMP-NeuAc, while the S-sialylmotif showed a high binding affinity for both CMP-NeuAc and the glycoprotein acceptor (Datta and Paulson, 1995; Datta *et al.*, 1998). Figure 9 consists of a diagram of these sialylmotifs in 4 different sialyltransferases along with the invariant amino acids shown in bold and identified with arrows. One of the invariant amino acids in both motifs has been identified as a cysteine residue, which may be involved in disulfide bonding (Drickamer, 1993).

Extensive studies with regards to sialyltransferase subcellular localization have revealed their location to be restricted to the *trans* cisternae and *trans* Golgi network, acting as the final step in the sequence of glycosyltransferase enzymes adding sugar residues onto glycan structures (Carey and Hirschberg, 1981; Warren and Malhotra, 1998). However, these membrane-bound sialyltransferases have also been found to be

Conserved regions, sialylmotif L, S, and VS in the sialyltransferases



B)

Sialylmotif L		RESIDUES
ST3Gal I	RCAVVGNSGNLKDSSYGPEIDSHDFVLRMNKAPTGG-FEADVGSRD	(138-182)
ST6Gal I	RCAVVSSAGSLKNSQLGREIDNHDAVLRFNGAPTDN-FQQDVGTKT	(180-224)
ST6GalNAc I	TCAVVGNGGILNDSRVGREIDSHDYVFRLSGAVIKG-TEQDVGTRT	(292-336)
ST8Sia I	KCAVVGNGGILKMSGCGRQIDEANFVMRCNLPPLSSEYTRDVGSKT	(136-181)

Sialylmotif S		RESIDUES
ST3Gal I	PSTGILSIIFSIHICDEVDLYGF	(264-286)
ST6Gal I	PSSGMLGIIIMMTLCDQVDIYEF	(318-340)
ST6GalNAc I	PTTGALLLLTALJLCDKVSAYGF	(447-469)
ST8Sia I	LSTGLFLVSAALGLCEEVSIYGF	(272-294)

Sialylmotif VS		RESIDUES
ST3Gal I	TGVHDGDFEYNIT	(309-321)
ST6Gal I	GAYHPLLFEKNMV	(363-374)
ST6GalNAc I	YINHDFRLERMVW	(492-503)
ST8Sia I	SGYHAMPEEFLQL	(304-316)

Figure 9. Distribution of the 3 Sialylmotifs in 4 Different Sialyltransferases.

A) Diagram of a typical sialyltransferase displaying the locations of the L-, S-, and VS-sialylmotifs. In general, they are found in the catalytic domain on the luminal side of the Golgi. **B)** The amino acid sequences of the L-, S-, and VS-sialylmotifs from 4 different sialyltransferases, with the conserved amino acids shown in bold along with arrows for easier distinction. (Reference: glycoforum) http://www.glycoforum.gr.jp/index.html.

diffusely distributed in other subcompartments of the Golgi apparatus and have been discovered to be oriented both lumenally and cytoplasmically (Berger and Hesford, 1985; Roth, 1987; Colley, 1997). Other evidence has also revealed the presence of these membrane bound enzymes in soluble forms detectable in mammalian serum (Kaplan *et al.*, 1983; Sherblom *et al.*,
1986; Lammers and Jamieson, 1988; Maguire *et al.*, 1996, Kitazume *et al.*, 2001).

Recent developments in sialyltransferase enzymology, in addition to other glycosyltransferases in general, can be attributed to the progression in molecular biology and cloning aspects of these enzymes. In the beginning, characterization of enzyme activity and acceptor substrate specificity was limited to purified sources of sialyltransferases from several mammalian and avian sources. Further advances were made difficult based on the low abundance of these enzymes in tissue, affecting their availability for purification and molecular cloning purposes. Regardless of the fact, several groups were able to utilize classical purification procedures to obtain sialyltransferases purified to homogeneity (Sadler *et al.*, 1979; Weinstein *et al.*, 1982a; Gu *et al.*, 1990; Melkerson-Watson and Sweeley, 1991; Preuss *et al.*, 1993). Purification of the sialyltransferase proteins eventually led to successful cloning of three sialyltransferase cDNAs (Weinstein *et al.*, 1987; Gillespie *et al.*, 1992; Wen *et al.*, 1992) using initial knowledge of the amino acid sequence in the three respective purified enzymes. Sequence comparison of the three cloned sialyltransferases revealed the presence of the two sialylmotifs described earlier, which were utilized to obtain cDNA clones of additional sialyltransferases without the requirement of purification or knowledge of the clone itself. In fact, these sialylmotifs, through the use of a polymerase

chain reaction (PCR) based method involving degenerate synthetic primers targeted to the conserved regions, has allowed the discovery and successful cloning of several new members to this expanding gene family, which would have been next to impossible to purify nonetheless (Tsuji *et al.*, 1996).

Because of the growing number of sialyltransferases being discovered through cloning, several groups of authors were applying different names for the same sialyltransferase. In 1996, Tsuji and colleagues introduced a systematic nomenclature due to the growing complexity in distinguishing one sialyltransferase from another. Their goal for a nomenclature system was to provide a specific name for the sialyltransferase gene product, but not provide complete functional properties of the enzyme. The system was composed of four elements: ST, x, y, and z, where ST denotes the sialyltransferase family, x represents the carbon on the acceptor sugar to which the NeuAc is transferred (e.g. 6 for NeuAcα2,6Gal), y expresses the acceptor sugar to which NeuAc is transferred (e.g. Gal, GlcNAc, NeuAc) and z is the roman numeral representation given, in chronological order, to each new distinct gene in the subgroup (Tsuji *et al.*, 1996). Therefore, these enzymes have now been divided into 4 sub-families based on their substrate specificity: ST6Gal-, ST3Gal-, ST6GalNAc-, and ST8Sia (Harduin-Lepers *et al.*, 2001). Presently, there are several studies being done on all types of sialyltransferases. However, additional research with regards to secondary protein structure, mechanism of catalysis, and cellular regulation of transcription is imperative for further comprehension of these sialyltransferases. The next section will emphasize some background information on a few well-characterized sialyltranferases, ST6Gal I, ST3Gal III, and ST3Gal I, as these enzymes are mainly focused on in my thesis.

i) ST6Gal I

The ST6Gal I enzyme attaches a sialic acid residue from CMP onto a terminal galactose residue in an α2,6-linked orientation.

CMP-NeuAc + Galβ1-4GlcNAc-R → NeuAcα2-6Galβ1-4GlcNAc-R + CMP

ST6Gal I exhibits a strict specificity for transferring sialic acid onto a type 2 substrate acceptor (Galβ1-4GlcNAc), either as a free disaccharide or as a terminal N-lactosamine of an N- or O-linked oligosaccharide chain, but cannot utilize type 1 (Galβ1-3GlcNAc) or type 3 (Galβ1-3GalNAc; ie. T-antigen) oligsaccharide chains (Weinstein *et al.*, 1982b). However, other *in vitro* studies have shown evidence of ST6Gal I transferring sialic acid onto structures such as lactose (Paulson *et al.*, 1977b), GalNAcβ1-4GlcNAc (Nemansky and van den Eijnden, 1992), and Manβ1-4GlcNAc (Van Pelt *et al.*, 1989). In fact, it has been shown that only the 6-hydroxyl group in the β-Gal and the 2-acetamide group in GlcNAc are required by ST6Gal I for the reaction to effectively occur, while the other hydroxyl groups also accept modifications (Wlasichuk *et al.*, 1993). Further studies have concluded that ST6Gal I favor type 2 terminal structures, which are typically found in N-glycans, on the Galβ1-4GlcNAcβ1-2Manα1-3 branch, as opposed to the Manα1-6 branch in a bi-antennary structure (Joziasse *et al.*, 1985, 1987). The same studies also displayed that the presence of an additional Manα1-6 branch in tri- and tetra-antennary structures can decrease the transfer rates of sialic acid onto both branches.

ST6Gal I can also be found in a wide variety of mammals and is typically expressed in several different animal livers and hepatoma cells, from which it has been easily purified to homogeneity in large quantities (Harduin-Lepers *et al.*, 1995). As a result of its abundance, ST6Gal I was the first sialyltransferase to have its cDNA clone isolated from rats (Weinstein *et al.*, 1987). In addition, the ST6Gal I enzyme was the first cDNA cloned in humans (Grundmann *et al.*, 1990). ST6Gal I has also been purified from bovine colostrum in soluble form, where the normally membrane-bound enzyme has been released via proteolytic cleavage at the non-catalytic N-terminal domain (Paulson *et al.*, 1977a; Hesford *et al.*, 1984).

Prior studies have also shown the presence of soluble ST6Gal I enzymes in a variety of mammalian sera (Kaplan *et al.*, 1983; Van Dijk *et al.*, 1986; Lammers and Jamieson, 1988). Several factors such as bacterial infections, chemical inflammatory agents, and a variety of other pathological conditions induce inflammation in humans and animals, leading to significant biochemical and physiological changes known as the acute phase response (Koj, 1974; Kaplan *et al.*, 1983). One significant change involves the elevation of various circulating serum glycoproteins, and because of their enhanced synthesis during the acute phase response, they are referred to as acute phase reactants. A few examples of acute phase reactants include such glycoproteins as α_1-acid glycoprotein, fibrinogen, and haptoglobin (Jamieson *et al.*, 1983; Jamieson *et al.*, 1987) where they are mainly synthesized in the liver as secretable glycoproteins. ST6Gal I also exhibited properties as an acute phase-reactant (Kaplan *et al.*, 1983; Jamieson *et al.*, 1993), displaying elevated levels (~ 5 fold) in rat serum during inflammation. In addition, a cathepsin D-like protease, induced by inflammation, was shown to be

responsible for the release of ST6Gal I, from its membrane bound form in the liver to its free form in serum (Lammers and Jamieson, 1988). Pro-inflammatory cytokines have also induced the release of ST6Gal I from endothelial cells and hepatocytes (Woloski *et al.*, 1985; Hanasaki *et al.*, 1994).

ii) ST3Gal III

ST3Gal III preferentially transfers NeuAc onto type 1 (Galβ1-3GlcNAc) glycan structures, but can also transfer onto type 2 (Galβ1-4GlcNAc) structures less efficiently (Weinstein *et al.*, 1982b).

CMP-NeuAc + Galβ1-3(4)GlcNAc-R → NeuAcα2-3Galβ1-3(4)GlcNAc-R + CMP

Type 1 and type 2 terminal sequences are recognized by ST3Gal III when their oligosaccharide chains are N-linked to glycoconjugates. Indeed, with the use of modified type 1 and type 2 acceptors, Wlasichuk and colleagues (1993) determined that ST3Gal III requires the 3-, 4-, and 6-hydroxyls of the terminal β-Gal, as well as the sub-terminal sugar for precise recognition, which may explain the enzyme's cross-reactivity for both types of acceptors. Galβ1-3[NeuAcα2-6]GlcNAcβ sequences cannot be utilized by ST3Gal III, though it contains the type 1 structure (Chandrasekaran *et al.*, 1995). It was then concluded that side-arm α2-6 sialylation of the GlcNAc residue must occur after α2-3 terminal sialylation of the β-Gal residue, in order to synthesize NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ oligosaccharides. The same was concluded for the sialyl-Le^a structure, (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAcβ-R), where α2-3 sialylation by ST3Gal

III precedes fucosylation. ST3Gal III is also said to be involved in the synthesis of sialyl-Le^a and sialyl-Le^x, the levels of which are elevated in tumor cells and carcinomas (Harduin-Lepers *et al.*, 1995).

ST3Gal III has been found in several mammals, but first purified to homogeneity from rat liver (Weinstein *et al.*, 1982a) and later from human colorectal carcinoma cells (Liepkans *et al.*, 1988). Studies on the distribution of ST3Gal III mRNA has been shown to be tissue specific rather than development stage dependent, being highly expressed in brain, kidney, heart, spleen, and colon, and low in thymus and submaxillary glands (Tsuji, 1996). The cDNA clones of the enzyme were introduced from rat liver sources (Wen *et al.*, 1992) and human placenta (Kitagawa and Paulson, 1993). Variable levels of ST3Gal III expression and activity have also been reported, where elevated levels were observed in breast carcinomas (Recchi *et al.*, 1998) and malignant gliomas (Yamamoto *et al.*, 1997) as compared to their normal counterparts, while a decrease in expression was observed in ptyergium cells versus normal conjunctiva (Creuzot-Garcher *et al.*, 1999).

There has been no evidence found in the literature regarding the presence or detection of ST3Gal III in human serum. However, it has been detected in rat serum (Ratnum, *et al.*, 1987), where a 1.7-fold increase was observed following colchicine treatment using a modified high-pressure liquid chromatographic method. In addition, another study confirmed an elevation of the product 3`-sialyllactose in bovine serum of 2-day-old calf sera versus 20-day-old calf sera, suggesting ST3Gal III as the main contributor (Sherblom *et al.*, 1986). Release of ST3Gal III was suggested to involve a cathepsin D-like proteinase during turpentine induced inflammation, a release mechanism similar to that of ST6Gal I (Richardson and Jamieson, 1995).

Several different K_m values have been reported for ST3Gal III, which may be due to differing tissue sources and acceptor types (oligosaccharide acceptors versus macromolecular acceptors) (Williams *et al.*, 1995). Kinetic studies were performed comparing native and recombinant forms of ST3Gal III, where the cloned counterpart displayed lower affinity for small molecular weight oligosaccharide acceptors (i.e. lacto-N-tetraose) as compared to their preferred large molecular weight macromolecular acceptor (i.e. rat and human asialo-α_1-acid glycoprotein). However, this would not compromise the ability of the recombinant form to properly glycosylate its respective acceptor (Williams *et al.*, 1995).

iii) ST3Gal I

The ST3Gal I enzyme is responsible for the catalytic addition of NeuAc α2,3-linked onto type 3 (Galβ1-3GalNAc) terminal sequences.

CMP-NeuAc + Galβ1-3GalNAc → NeuAcα2-3Galβ1-3GalNAc + CMP

The Galβ1-3GalNAc unit can typically be found at the terminal position of several types of glycoproteins, glycolipids, and oligosaccharides such as asialo-mucins, antifreeze glycoprotein, asialofetuin, ganglioside G_{M1}, etc (Harduin-Lepers *et al.*, 1995). Studies have shown that the 3-hydroxyl group of β-Gal is specifically required by ST3Gal I for addition of NeuAc (Kuhns *et al.*, 1993). The Galβ1-3GalNAc unit <u>α-linked</u> to phenyl, benzyl, *p*- and *o*-nitrophenyl groups were preferred as acceptor substrates for ST3Gal I, as opposed to being <u>β-linked</u> to aromatic organic groups (Klohs *et al.*, 1981;

Kuhns *et al.*, 1993). In addition, ST3Gal I cannot utilize oligosaccharides, such as lactose (Galβ1-4Glc) or substrates containing terminal Galβ1-3GlcNAc and Galβ1-4GlcNAc, as acceptor substrates (Harduin-Lepers *et al.*, 1995). A similar sialyltransferase, ST3Gal II, catalyzes the same reaction, exhibiting the same acceptor substrate specificity. These two enzymes were shown to be distinguishable in a kinetic parameter and acceptor competition experiment, revealing that oligosaccharides of type 2 structures and O-linked oligosaccharide glycoproteins were preferred by ST3Gal I, where as ST3Gal II displayed preference towards gangliosides (Tsuji, 1996).

ST3Gal I has shown high expression in a wide variety of tissues in several types of mammals, being purified to homogeneity from tissues such as the submaxillary gland (Rearick *et al.*, 1979; Sadler *et al.*, 1979), liver (Conradt *et al.*, 1988), and human placenta (Jozaisse *et al.*, 1985), and has been weakly detected in other tissues such as the kidney, spleen, and brain (Lee *et al.*, 1994; Harduin-Lepers *et al.*, 1995; Tsuji, 1996). Other studies have shown an over expression of ST3Gal I mRNA as well as elevated activity in human colorectal (Schneider *et al.*, 2001), breast (Burchell *et al.*, 1999), and gynecological (Wang *et al.*, 2002) carcinomas.

Variations in K_m values reported for ST3Gal I also differ from one source to another, where ST3Gal I from human placenta was observed to display a 20-fold lower affinity towards the donor substrate CMP-NeuAc as compared to its porcine submaxillary gland counterpart (Joziasse *et al.*, 1985). However, the Km for recombinant ST3Gal I, (Williams *et al.*, 1995) compared to the native form (Rearick *et al.*, 1979), showed little difference when using asialo-antifreeze glycoprotein as an acceptor, suggesting recombinant ST3Gal I activity is retained.

CHAPTER 3. Introduction to Current Work

i) ST6Gal I Levels in Renal Transplant Patient Serum, Pre-transplant

The main focus of my thesis targets the development of an assay system to specifically measure two well-characterized sialyltransferases, ST3Gal III and ST3Gal I, in human serum. Recently, studies done in our laboratory by Thorne-Tjomsland *et al.* (2000) have demonstrated elevated ST6Gal I levels in kidney transplant patient serum pre-transplant. The significance of this observation was suggested to adversely affect post-transplant events such as delayed graft function (DGF), which in turn predisposes to poor long term outcome. In addition, adhesion molecules are up-regulated during ischemia/reperfusion, a condition of sudden obstruction and restoration of blood supply to an organ, upon transplantation. E-selectin, ICAM-1 and VCAM-1 are adhesion molecules that are α2,6-sialylated (Hanasaki *et al.*, 1994), which could possibly explain the elevated ST6Gal I levels in renal transplant patient serum pre-transplant. Some of the findings from the study are reported here. Serum samples were obtained from a total of 70 patients immediately before their transplant, i.e. upon cross matching and immunosuppressant administration, and from 19 healthy, sex-, and age-matched control patients, used for comparison. Utilizing a modified version of an assay used to measure rat sialyltransferase levels (Lammers and Jamieson, 1988), the results showed several significant findings. Higher ST6Gal I mean serum levels were obtained in the 70 renal patients (3162±97 U) as opposed to the 19 control patients (2569±125 U). In addition, although there were no significant differences in ST6Gal I serum levels between patients on hemodialysis (3198±137 U) and peritoneal dialysis (3353±153 U), these patients displayed higher activity than patients who weren't on dialysis (2470±116 U). A

significantly higher ST6Gal I serum level was found in the 20 patients (3735±228 U) with DGF, where dialysis was necessary, as compared to those who did not require it (2933±83 U). Typically, about 20 to 40% of renal transplant patients develop DGF, a condition characterized by the requirement for dialysis and a tendency toward acute rejection (Samaniego *et. al.*, 1997). Correlations were also observed between ST6Gal I levels and DGF. An elevation in serum ST6Gal I levels pre-transplant and cold ischemic time, another risk factor for DGF, were shown to be independent risk factors for DGF.

Present studies in our laboratory involve the measurement of ST6Gal I levels longitudinally through the period when patients are most likely prone to develop DGF. Hence, development of an assay system for ST3Gal III and ST3Gal I in human serum would also allow measurement of their levels in kidney transplant patient serum, taking into consideration the significant role of sialyltransferases and sialic acids in recognition and adhesion processes. Furthermore, an extension of this study can be explored where the levels of the three sialyltransferases can be measured longitudinally through the period when patients are most likely prone to develop DGF. Potentially, the information obtained will allow us to better establish correlations between different peak levels of different sialyltransferases, or between peak levels of a specific sialyltransferase and clinical parameters signaling DGF.

ii) History of Bovine Glycoproteins

In order to assay a particular sialyltransferase, acceptors with precise carbohydrate configurations preferably recognized by one sialyltransferase are required. These acceptors can be glycoproteins, where the sugar components have been modified

or remodeled (macromolecular acceptor), or they can be simple oligosaccharides of defined structures (oligosaccharide acceptors). Over the years, oligosaccharide acceptors have been successfully utilized in facilitating the substrate specific determination of several sialyltransferases *in vitro*. However, to effectively assay for a particular sialyltransferase in human serum, the best substrate for these enzymes are macromolecular acceptors, since they most accurately depict the substrates that sialyltransferases will encounter and utilize in nature or *in vivo*. ST6Gal I assays in human serum are a modified version of the rat ST6Gal I assay system, carried out by methods routinely employed in our laboratory (Lammers and Jamieson, 1988). The acceptor, asialo-human α_1-AGP, which contains type 2 terminal oligosaccharide structures (Galβ1-4GlcNAc-R) N-linked to an asparagine, was employed to assay serum ST6Gal I (Thorne-Tjomsland *et al.*, 2000). However, the same acceptor can also be utilized to assay for ST3Gal III, since both sialyltransferases recognize type 2 structures. Therefore, it was difficult to determine which sialyltransferase was being assayed for when employing asialo-human α_1-AGP. In animal models studied to date, ST6Gal I was shown to be much more abundant, easier to purify, and displayed higher activities when measured up to ST3Gal III levels, which were shown to be relatively lower by comparison (Weinstein *et al.*, 1982a, 1982b). In spite of this fact, exclusive measurement of ST3Gal III levels in human serum would be ideal, as to avoid intentional assaying of two sialyltransferases with one acceptor. This was achieved by exploiting the fact that ST3Gal III recognizes type 1 (Galβ1-3GlcNAc-R) terminal structures as well. Therefore, asialylated glycoproteins with only type 1 structures N-linked to asparagine or those with both type 1 and type 2 structures, where type 2 structures were removed, would be the

best possible macromolecular acceptors employed for the specific assaying of ST3Gal III. A macromolecular acceptor for ST3Gal I should contain type 3 terminal structures (Galβ1-3GalNAc-R), O-linked to serine or threonine of a glycoprotein. A similar sialyltransferase, ST3Gal II, also recognizes type 3 sequences, but prefers them on gangliosides (Tsuji, 1996). Hence, it can be very difficult, yet significant, to choose a suitable glycoprotein candidate for exclusive assaying of these sialyltransferases.

Potential glycoprotein candidates, that contain type 1 and Type 2 terminal sugar structures, include bovine prothrombin (Mizuochi *et al.*, 1979), and rat α_1-AGP (Turchen *et al.*, 1977; Jamieson, 1977). ST3Gal I has previously been assayed for in mammals, employing the antifreeze glycoprotein as the macromolecular acceptor, (DeVries *et al.*, 1970; Williams, *et al.*, 1995). Although these glycoproteins would serve best for assaying the ST3 enzymes, they are economically straining if used in large amounts. In contrast, the bovine glycoproteins, fetuin and α_1-AGP, deemed to be especially promising as acceptor substrates, as they are more affordable in abundant quantities.

Prior research by another graduate student in our laboratory utilized a modified form of bovine fetuin as a macromolecular acceptor for ST6GlcNAc I in rat liver Golgi preparations (Szidonya, 2001). The ST6GlcNAc I enzyme recognizes NeuAcα2-3Galβ1-3GlcNAc- structures, which is the resulting product of action by the ST3Gal III enzyme. The carbohydrate structures of the glycoprotein, bovine fetuin, have been extensively studied and characterized (Green *et al.*, 1988). The 48,000 kDa protein, a major component of fetal calf serum, has been used as a model for studies on glycoprotein structure and biosynthesis and shows structural and biological relations to the human plasma glycoprotein α_2-HS (Yoshioka *et al.*, 1986; Dziegielewska *et al.*, 1987; Christie *et*

al., 1987; Reynolds *et al.*, 1987). In total, fetuin contains 6 carbohydrate chains; three oligosaccharide structures are N-linked to asparagine, making up ~80% of the total carbohydrate content, and the other 3 are O-linked to serine or threonine, making up the remaining 20%. Majority of the oligosaccharides are tri-antennary, but have also been known to exist in di-, and tetra-antennary chains in low amounts. More importantly, bovine fetuin contains sialylated sugar chains with Galβ1-4GlcNAc- and a small fraction of Galβ1-3GlcNAc- terminal structures, which are the glycan sequences required for recognition by ST3Gal III (Green *et al.*, 1988). Furthermore, the O-linked oligosaccharide chains of fetuin, which comprise the terminal sequence Galβ1-3GalNAc- can serve as acceptors for the ST3Gal I enzyme. With proper modifications of the fetuin glycan structures, exclusive acceptors for both sialyltransferases can be developed.

Another potential glycoprotein of interest, α_1-acid glycoprotein (α_1-AGP) of bovine origin, is affordable in large quantities. The human and rat forms of α_1-AGP have been used in our laboratory by previous researchers to assay sialyltransferase levels in animal models (Kaplan *et al.*, 1983; Lammers and Jamieson, 1988; Richardson and Jamieson, 1995). Furthermore, in addition to their abundance in the plasma of humans and other animals, the amino acid sequence and carbohydrate compositions have been well characterized (Tamura *et al.*, 1989). Unfortunately, very few studies have been performed on the carbohydrate structures of the 42,000 kDa bovine form of α_1-AGP (Hunter and Games, 1995). Bovine α_1-AGP can potentially serve as an acceptor for a sialyltransferase, as the bovine glycoprotein has high carbohydrate content of ~26.6% (Hunter and Games, 1995). Large portions of the carbohydrates were found to contain sialic acids (Yoshima *et al.*, 1981). In addition, other properties that would make bovine

α_1-AGP a suitable acceptor for a sialyltransferase include a very acidic iso-electric point, and high solubility in water and other polar solvents (Tamura *et al.*, 1989). Properties, like the presence of O-linked or N-linked chains, the types of sugars present and their terminal sequences (α- or β-linked), and the number of each terminal sequences, are unknown.

iii) Modification of Macromolecular Acceptors

Subsequent to choosing suitable glycoproteins for assaying ST3Gal III and ST3Gal I, in particular, producing the acceptors was the next logical step. This involved remodeling of the glycoproteins, bovine α_1-AGP and bovine fetuin, through various chemical and enzymatic treatments. Firstly, sialic acid residues were removed to make available the acceptor sites for sialyltransferases. This was carried out by dilute acid hydrolysis treatment, a procedure that has been successfully utilized in our laboratory. Secondly, specific removal of the glycans O-linked to a Ser or Thr of the peptide backbone was necessary, but only for glycoprotein acceptors of ST3Gal III to prevent recognition of the glycoprotein acceptor by endogenous ST3Gal I. The release of O-linked carbohydrate structures was performed using a chemical-based method previously designed by Likhosherstov *et al.* (1990). This method involves a modified alkaline sodium borohydride treatment in the presence of Cd^{+2} salts, which offered maximal hydrolysis of the O-linked glycans while providing minimal harm to the rest of the glycoprotein. Lastly, β1,4-linked galactose residues were exclusively removed enzymatically employing *Streptococcus pnuemoniae* β1,4-galactosidase. The purpose for this procedure was to allow retention of acceptor sites for ST3Gal III, or ST3Gal I, while

removing sites recognizable by ST6Gal I. Following each treatment, the resulting modified glycoproteins were analyzed with recombinant or purified sialyltransferases. Exploitation of purified rat liver ST6Gal I would give insight on the successful action of the β1,4-galactosidase, where *Spodoptera frigoperda* recombinants of ST3Gal III and ST3Gal I would confer the presence of their respective acceptor sites, as well as impart information regarding the success of O-linked chain removal by the latter enzyme. The results obtained from these assays should also provide ample information on whether the modified glycoprotein is a suitable acceptor for the sialyltransferase under consideration.

iv) Kinetic Studies

Upon the completion and analysis of macromolecular acceptors utilized to assay for ST3Gal III and ST3Gal I, various kinetic tests were performed to optimize the determination of these sialyltransferase levels in human systems. These tests included varying the amount of glycoprotein acceptor added, varying length of incubation period, and varying the volume of serum added. Other kinetic parameters have been studied previously, where the optimum pH has been shown to be ~6.4 for both of the α2,3-sialyltransferases, at an optimum reaction temperature of 37°C (Kono *et al.*, 1997). Human serum samples presumed to contain ST3Gal III and ST3Gal I were used as the enzyme source. All reaction conditions were kept constant throughout each test except for the variable being examined. Potentially, the data obtained from these kinetic analyses will provide insight on how well these acceptors are for assaying their respective sialyltransferase as well as what type of sialyltransferase levels are present in human control serum, whether they are non-existent or exist at high levels. Furthermore,

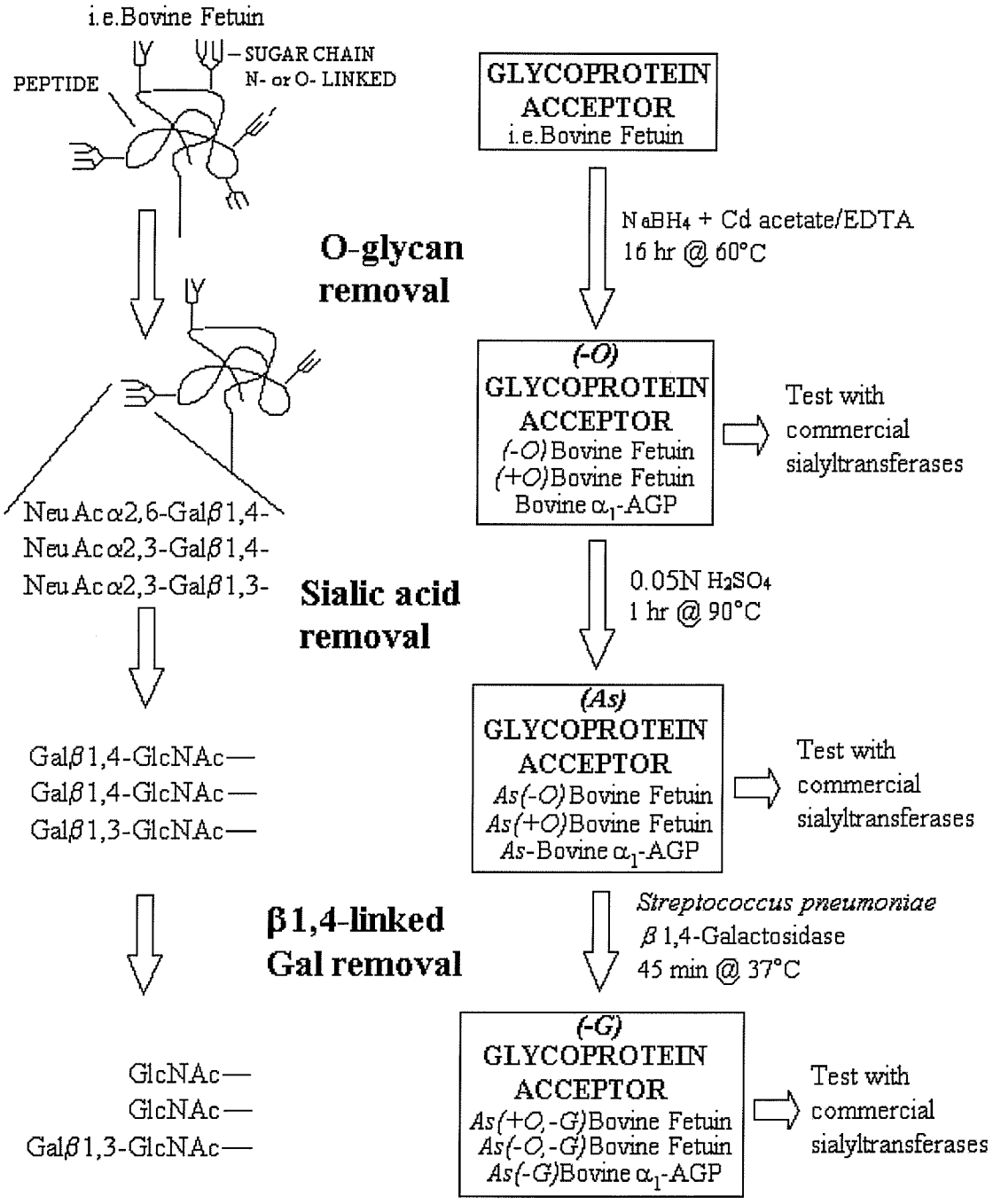


Figure 10. Chemical and Enzymatic Modification of Bovine Glycoproteins.

On the left hand side is a schematic diagram of bovine fetuin undergoing all modification steps. These steps are paralleled with the flow diagram on the right hand side, showing a summarized version of the chemical and enzymatic modifications (ie. O-linked glycan removal, sialic acid removal, β1,4-linked galactose removal), the designation given subsequent to a particular modification (*-O*, *As*, and *-G*), and when testing with commercial sialyltransferases will occur.

K_m and V_{max} values can be determined for each acceptor, along with estimations on optimal conditions required to carry the out the assays.

v) Preliminary Examination of ST3 Enzyme Levels in Renal Transplant Patient Serum

Finally, the macromolecular acceptors were employed against a set of longitudinal serum samples obtained from a specifically selected renal transplant patient. For comparison purposes, the acceptor for ST6Gal I, *As*-human α_1-AGP, was also assayed against the serum samples. The significance of this initial investigation might provide indication on how well the modified glycoprotein acceptors can detect their sialyltransferase counterpart. In addition, an initial assessment on ST3Gal III and ST3Gal I levels in renal transplant patient serum can be made in comparison to ST6Gal I levels. Furthermore, the preliminary examination might offer insight that may mimic results in the future study on the longitudinal analysis of sialyltransferase levels.

THESIS OBJECTIVES

The following is a list of the primary goals and issues addressed in my thesis:

1) To modify and analyze macromolecular glycoproteins in order to assay for specific sialyltransferases, particularly ST3Gal III and ST3Gal I in human serum.

2) To perform kinetic analysis of macromolecular acceptors in human systems, emphasizing the effects of acceptor concentration, incubation time, and serum content (enzyme concentration) on serum sialyltransferase activity. In addition, K_m and V_{max} values are to be determined where applicable.

3) To employ the final glycoprotein acceptors in the serum samples obtained from a renal transplant patient for preliminary examination of ST3Gal III and ST3Gal I levels, pre- and post-transplant, and to compare these levels with the levels of ST6Gal I.

MATERIALS

Renal transplant patient serum and human control serum samples were collected on the basis of patient approval with signed documents. They were obtained from the Transplant Immunology Laboratory at the Health Science Centre, and stored at –20°C. Purified commercially available recombinant enzymes ST3Gal III and ST3Gal I (both derived from *Spodoptera frugiperda*) came from Calbiochem-Novabiochem Corporation (San Diego, CA) while purified rat liver ST6Gal I and recombinant *Streptococcus pneumoniae* β1,4-galactosidase came from Sigma Chemical Company (St. Louis, MO). Human α_1-acid glycoprotein and bovine α_1-acid glycoprotein were also purchased from Sigma while bovine fetuin prepared using the Pederson method was purchased from Life Technologies (Gibco BRL) (Burlington, ON). Cytidine 5'-monophospho-N-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid, ammonium salt, was purchased from Amersham Corporation (Oakville, ON). BD Vacutainer™ SST™ Tubes for serum determinations were purchased from Becton Dickinson (Mississauga, ON). Spectrum molecular-porous dialysis tubing (MWCO ranging from 10,000 – 14,000 Da) came from Fisher Scientific, while Millipore Corporation (Bedford, MA) provided the Amicon ultrafiltration membranes (MWCO 30,000 Da). Aqueous Counting Scintillant (ACS) was purchased from Amersham. Whatman No. 1 filter paper discs (2.5 cm in diameter) came from Fisher Scientific (Edmonton, AB). Chemicals were obtained from the following companies: EDTA-Na_4 came from Aldrich Chemical Company Incorporated (Milwaukee, WI); cadmium acetate and sodium cacodylate were from J.T. Baker Chemical Company (Phillipsburg, NJ); NaOH came from Mallinckdrodt Specialty

Chemicals Company (Paris, KY); phosphotungstic acid, Lacto-N-tetraose, CMP-NeuAc, bovine serum albumin, and sodium borohydride were from Sigma; trichloroacetic acid, glacial acetic acid, H_2SO_4, ethanol, ethyl ether, butanol, and triton CF-54 came from Fisher Scientific.

METHODS

1) Development of Macromolecular Acceptor Substrates

i) Procedure for Sialic Acid Removal

Removal of sialic acid residues from bovine fetuin, human α_1-AGP, and bovine α_1-AGP was performed as previously described (Kaplan *et al.*, 1983). The procedure involved incubation of ~25 mg of the given glycoprotein acceptor with 5 ml of 0.05 N H_2SO_4 in a heated water bath of 90°C for 1 hour. Following incubation, the solution was cooled on ice and extensively dialyzed for a few days, using 12,000-14,000 Da MWCO molecular-porous dialysis tubing at 4°C against deionized water. Dialysis tubing was washed in deionized water to remove any sodium azide preservative present. The dialyzed product, i.e. Asialo-fetuin, was allowed to freeze for lyophilization. The freeze-dried product was weighed and tested against a selected amount of commercially available recombinant and purified sialyltransferases.

ii) Procedure for Modified O-linked Glycan Chain Removal

A modified alkaline sodium borohydride treatment in the presence of a Cd^{+2} salt for the removal of O-linked glycan chains in bovine fetuin and bovine α_1-AGP was utilized to provide maximal hydrolysis of the O-linked glycans and allowing minimal harm to the rest of the glycoprotein (Likhosherstov *et al.*, 1990). A 1:1 volume ratio of a cadmium acetate (23 mg/ml)-EDTA·Na_4 (0.1 M) solution was prepared and titrated to a pH of ~7-7.5 with 4 N acetic acid and 0.5 M NaOH. Approximately 10 mg of each glycoprotein acceptor was employed with the following reaction mix: 1.6 ml of deionized water, 0.29 ml of cadmium acetate-EDTA·Na_4 solution, 0.11 ml of NaOH (0.5 M), 152

mg of sodium borohydride, and 0.1 ml of butanol. The reaction mixture was stoppered and incubated at 60°C for 16 hours. Following incubation, the mixture was cooled in an ice water bath, brought back to a pH of ~7-7.5 with 4 N acetic acid or glacial acetic acid, and then adjusted to a final volume of 10 ml with deionized H_20. All measurements were scaled up or down, depending on the amount of the glycoprotein acceptor used. The mixture was then dialyzed extensively for a few days at 4°C using pre-washed 12,000-14,000 MWCO molecular-porous dialysis tubing against deionized H_2O. The solution, if in excess volume, can be concentrated by subjection to 20 lb/in of N_2 gas pressure through an Amicon ultrafiltration membrane with a 30,000 MWCO. The concentrated solution was divided into 2-3 ml aliquots and placed into glass test tubes. The glycoprotein acceptor was freeze-dried and tested against commercial sialyltransferases, using the same procedure as previously described.

iii) Procedure for β1,4-Galactose Sugar Removal

As(-O) bovine fetuin and *As(-O)* bovine α_1-AGP was then treated with the recombinant enzyme *Streptococcus pneumoniae* β1,4-Galatosidase, which specifically cleaves galactose residues that are β1,4-linked (Zeleny *et al.*, 1997). Treatment employed reconstituting ~25 mg of the given glycoprotein acceptor into 2.5 ml of cacodylate buffer (1 M, pH 6.0). After the addition of 100 µl of β1,4-Galactosidase, and incubation of the total solution at 37°C for 45 minutes, the reaction was terminated and cooled on ice. For a couple of days, extensive dialysis of the mixture against deionized water at 4°C was carried out using pre-washed 10,000 MWCO molecular-porous dialysis tubing. The dialyzed product was freeze dried and also tested against commercial

sialyltransferases.

2) Analysis of Macromolecular Acceptor Substrates Utilizing Commercial Sialyltransferases

Following each step during modification of the glycoproteins bovine fetuin, bovine α_1-AGP, and human α_1-AGP, the acceptors were assayed with selected commercially available sialyltransferases. Approximately 25 µl (~500-600 µg) of recombinant ST3Gal III, ST3Gal I, or purified ST6Gal I, in a 1:100 dilution of 18% BSA/cacodylate buffer (1 M, pH 6.0), was used as the enzyme source. The rest of the assay mixture contained the glycoprotein acceptor being analyzed, at a specified concentration (typically 150 µg), and other components described in the next section. The rest of the assay is carried out as described in the following section as well.

3) General Assay System

A modified version of a rat sialyltransferase assay system was used to measure the activity of sialyltransferases in human serum as summarized here (Lammers and Jamieson, 1988). Briefly, all reaction mixtures (65 µl), unless otherwise indicated, contained 25 µl (500-600 µg) of one type of enzyme source whether it be human serum or one of the commercial sialyltransferase, the glycoprotein acceptor (ie. asialo human α_1-AGP) at a specified concentration (0 – 250 µg), 0.69 mM bovine serum albumin, 46 mM Na cacodylate buffer, 0.073 mM CMP-[4,5,6,7,8,9-14C] NeuAc (20nCi/nmol), 0.060 mg/ml of CMP-NeuAc, and 0.5% Triton CF-54. Assays were performed in duplicate; control assays, to which no acceptor was added (instead, deionized water) were also prepared. Control assays were required to account for activity from endogenous

substrates or enzymes present in serum assays, as well as non-specific interactions between recombinant enzymes. All assays were incubated at 37°C for 2 hours, and terminated/cooled on ice. The assay solution (50 µl) was spotted onto a Whatman No. 1 filter paper disc. The discs were allowed to dry overnight or in a 37°C oven for at least 1 hour. The discs then underwent a series of washes in order to precipitate the final reaction product. The first step consisted of a wash in a 10% TCA/1% PTA solution for 10 minutes. Then, washing in a 5% TCA/1% PTA solution (twice for 5 minutes each) was performed. Additional washings included a rinse with a 2:1 v/v ratio of an ethanol/ethyl ether mixture for 10 minutes and rinsing in pure ethyl ether for 5 minutes. The discs were allowed to air dry and then were individually placed into scintillation vials. Approximately 5-10 ml of ACS scintillation fluid was added to each vial, after which the activity of each disc was determined by a liquid scintillation counter (detects the ^{14}C-radioisotope and expresses activity in disintegrations per minute or dpm). Following activity measurements, an average is taken for duplicate values. Control values were then subtracted from the average test value for determination of change (Δ) in activity. Reaction velocity values were calculated for K_m and V_{max} determinations, utilizing the ratio 11.34 dpm/pmol NeuAc transferred.

4) Patient Data

***NOTE: All blood samples obtained from renal transplant patients and human controls were collected with their approval along with signed documents stating that their blood samples were being utilized in this study. In addition, the Ethics Committee approved the blood/serum research*.**

i) Demographics

For a specific study, human serum was collected from a total of 35 patients with renal transplants immediately before and at various time points following transplant (up to one year). Almost all of the patients involved in the study failed to provide all required samples, due to lack of accessibility to the patient, but only 4 patients were excluded from statistical analysis studies due to either severe lack of samples or because the exact times of sample collection were poorly recorded. Because serum samples from the 35 patients are limited in supply and are also being utilized in other studies, I did not want to waste any of the serum samples. Thus it was ideal to utilize serum samples obtained from one of the 4 patients being excluded, as it would allow a preliminary examination on what type of ST3 levels are present in human serum and prevent waste of serum samples that are precious. Serum samples from one out of these four patients (whose name is disclosed for patient confidentiality) were utilized for analysis of macromolecular acceptors in this study. For pre-transplant samples, collection occurred upon final cross-match and prior to administration of any immunosuppressive medications. Samples obtained after transplant were taken at particular time points, ideally at 1, 4, 8, 12, 16, 20, and 24 hours post-transplant, as well as on days 2, 3, 4, 5, 6, and 7 post-transplant. In addition, 3 more samples were taken at weekly interval (weeks 2, 3, and 4) in the first month post-transplant, and then one sample was taken at 2, 3, 4, 5, 6, 7, 12, and 16 months post-transplant. The levels of particular sialyltransferases in human serum were then measured in the selected patient sample, for preliminary examination. Furthermore, because the study is a blind study, several parameters were not disclosed, i.e. gender, prior transplants, age, whether the selected patient was prone to

DGF, etc.

ii) Immunosupression

All patients were subjected to a standardized post-transplant triple therapy of cyclosporine, azathioprine, and prednisone.

iii) Delayed Graft Function (DGF) and Clinical Rejection

Delayed Graft Function is defined by the requirement of dialysis by the patient in the immediate post transplant period. Elevated levels in serum creatinine levels diagnose clinical rejection.

5) Preparation of Human Serum Samples

Blood was collected from renal transplant patients and human control patients and placed into BD Vacutainer™ SST™ Tubes, containing a clot activator and gel for serum separation and serum determination. Isolation of serum was obtained by inversion (5 times) and then by centrifugation of blood at 2500 G for 10 minutes at room temperature. Human serum, the top liquid layer above the blood coagulate, was then aliquoted into microcentrifuge tubes and stored at -20°C.

RESULTS

A) Modification and Analysis of Macromolecular Acceptors for Assaying ST3Gal I and ST3Gal III

Although oligosaccharide acceptors are more suited for the substrate specific determination of sialyltransferases *in vitro*, effective assaying of a particular sialyltransferase in human serum requires a macromolecular acceptor, which can be specifically targeted by a sialyltransferase, *in vivo*. To produce macromolecular acceptors, glycoproteins are utilized, which comprise the proper sugar molecules with the desired configuration. They can then be modified by the addition or removal of specific sugars, chemically or enzymatically, thus being recognizable to an exclusive sialyltransferase. The bovine glycoproteins, α_1-AGP and fetuin, were chosen and subjected to various treatments. Firstly, a dilute acid hydrolysis treatment was performed for cleavage of sialic acid residues, giving rise to asialylated forms of the glycoprotein acceptors and labeled as (*As*). Secondly, and only if necessary, O-linked carbohydrate structures were removed by using the modified alkaline-borohydride hydrolysis discussed previously (Likhosherstov *et. al.*, 1990). The glycoprotein acceptors that underwent this type of treatment were labeled with (-*O*). Finally, *Streptococcus pnuemoniae* β1,4-galactosidase allows the specific removal of β1,4-linked galactose residues. The (-*G*) label was given to glycoprotein acceptors that received the latter treatment. Three final macromolecular acceptors were prepared: *As(-O,-G)* bovine fetuin and *As(-G)* bovine α_1-AGP, which were modified to specifically assay for ST3Gal III, and *As(+O,-G)* bovine fetuin, which should be recognizable by ST3Gal I, and less efficiently by ST3Gal III. Following each procedure, the modified glycoproteins were employed against

recombinant or purified sialyltransferases, purified rat liver ST6Gal I, and *spodoptera frigoperda* recombinants of ST3Gal III and ST3Gal I. The information obtained is likely to provide evidence for the absence or presence of an exclusive acceptor site for a specific sialyltransferase.

i) *As(-O,-G)* Bovine Fetuin

Modification of bovine fetuin into *As(-O,-G)* bovine fetuin required all 3 treatments in sequence. Subsequent to each treatment, the following products were obtained: *As*-bovine fetuin (after sialic acid removal), *As(-O)* bovine fetuin (after O-linked chain removal), and *As(-O,-G)* bovine fetuin (After β1,4-galactosidase treatment). Each product reacted differently to the 3 commercial sialyltransferases. As shown in Table 2, the activities of each commercial sialyltransferase towards *As*-fetuin were relatively high, with ST6Gal I demonstrating the highest activity out of the three at 22780±2278 dpm, preceding ST3Gal I (11350±1135 dpm) and ST3Gal III (3253±325 dpm). For *As(-O)* fetuin, the activity of purified rat liver ST6Gal I (19100±1910 dpm) displayed a slightly lower activity compared to its activity towards *As*-fetuin, showing little difference upon release of the O-linked glycans (Figure 11). However, low activities were obtained for *As(-O,-G)* fetuin when used as an acceptor for ST6Gal I (35.5±3.6 dpm), demonstrating the greatest decrease in activity upon removal of β1,4-linked Gal residues. The final ST6Gal I activity retained towards the *As(-O,-G)* fetuin acceptor was less than 0.2 % when compared with its *As*-fetuin counterpart. In Figure 12, recombinant ST3Gal I activities towards the 3 modified fetuin substrates were shown to vary greatly. Removal of the O-linked glycans from *As*-fetuin greatly diminished the

action of ST3Gal I, displaying a decrease greater than 10,000 dpm towards *As(-O)* fetuin substrate (1150±115 dpm). ST3Gal I also exhibited a further decrease in activity towards *As(-O,-G)* fetuin as compared to the *As(-O)* form, resulting in an approximate final activity of 434±44 dpm, which also represented only 3.8±1.0% of the activity produced by the *As*-form. Finally, recombinant ST3Gal III activities were shown to be retained relatively well for each of the modified bovine fetuin products (Figure 13). Little difference in activity was observed between *As*-fetuin (3253±325 dpm) and *As(-O)* fetuin (2976±298 dpm), suggesting that removal of the O-linked glycans had no major affect on the activity of recombinant ST3Gal III. Furthermore, although ST3Gal III displayed low values towards the final acceptor substrate (representing just under 50% of the ST3Gal III activity produced by *As*-fetuin), *As(-O,-G)* fetuin was still able to act as an acceptor exclusively for ST3Gal III, with a final activity of 1516±152 dpm.

Recombinant or purified sialyltransferase	Δ Activity (dpm)			
	As-Fetuin	*As(-O)* fetuin	*As(+O,-G)* fetuin	*As(-O,-G)* fetuin
ST6Gal I	22780	19100	6.4	35.5
ST3Gal III	3253	2976	1920	1516
ST3Gal I	11350	1151	9330	434

Table 2. Activity of Commercial Sialyltransferases Towards All Four Modified Bovine Fetuin Glycoproteins.

Selected sialyltransferases (at 500-600 μg concentration) include purified ST6Gal I from rat liver, and recombinant ST3Gal III and ST3Gal I, both from *Spodoptera frugiperda*, while *As*-fetuin, *As(-O)* fetuin, and *As(-O-G)* fetuin are the acceptor substrates used at 150 μg of protein. *As(-O,-G)* fetuin is the final glycoprotein product used to assay for ST3Gal III in human serum, while *As(+O,-G)* fetuin is used to assay ST3Gal I. Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values (which are assays that did not contain the modified glycoprotein and replaced by deionized water to account for non-specific self-sialylation of the recombinant or purified sialyltransferase, or sialylation of endogenous acceptors in human serum).

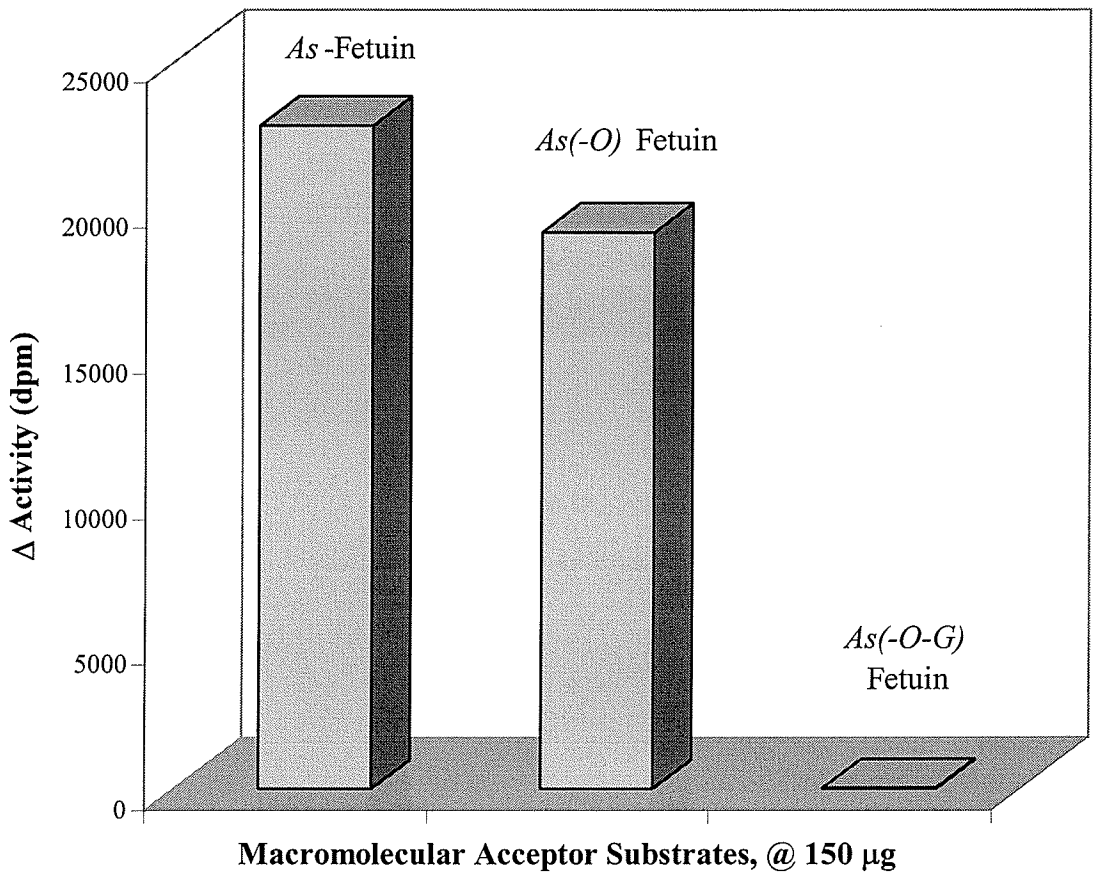


Figure 11. Graphical Depiction of Purified Rat Liver ST6Gal I Activity Assayed with 3 of the Modified Bovine Fetuin Glycoproteins.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

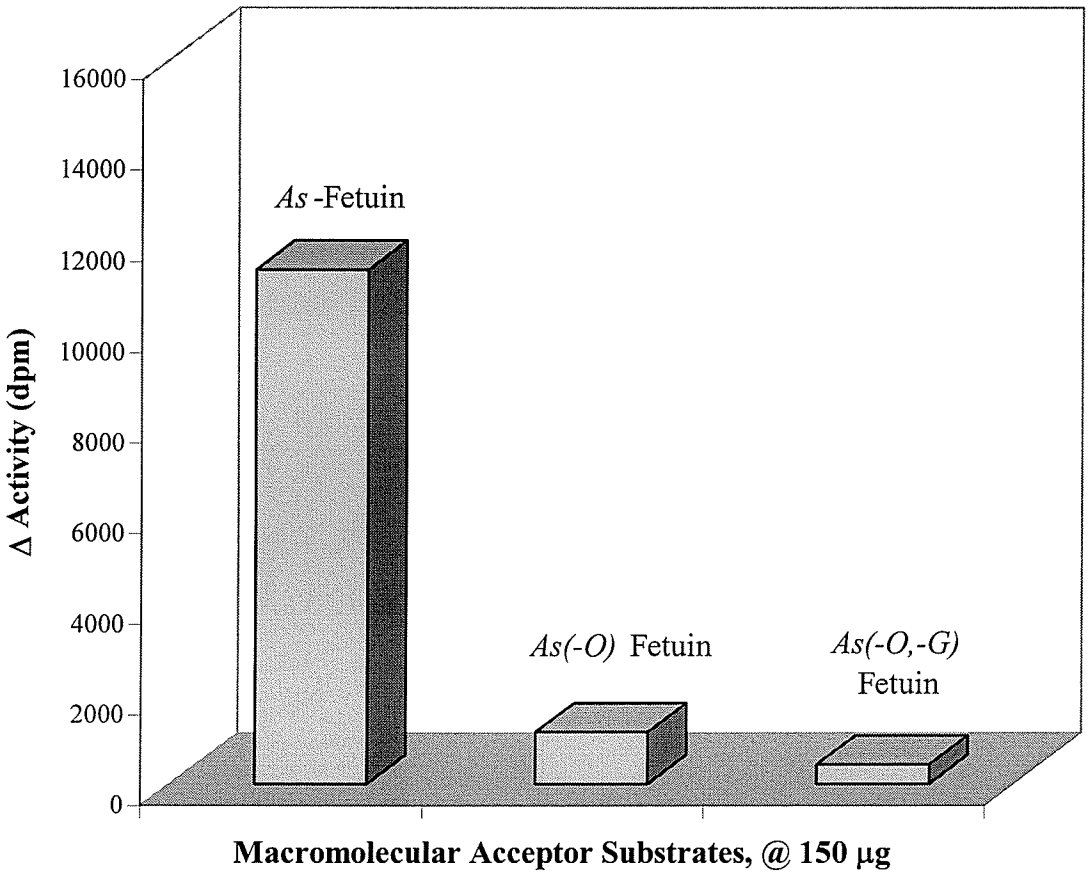


Figure 12. Graphical Depiction of *Spodoptera Frugiperda* Recombinant ST3Gal I Activity Assayed with the 3 Modified Bovine Fetuin Glycoproteins.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

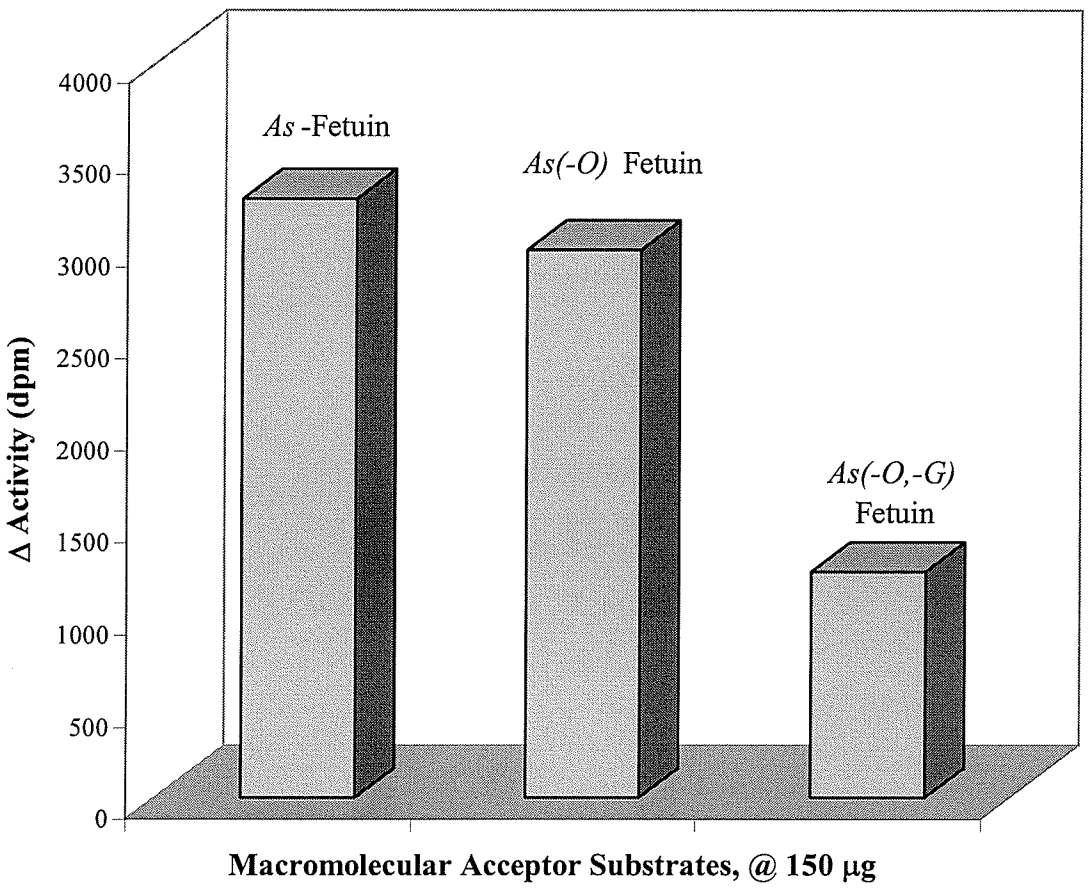


Figure 13. Graphical depiction of *Spodoptera Frugiperda* recombinant ST3Gal III activity assayed with the 3 modified bovine fetuin glycoproteins.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

ii) *As(+O,-G)* Bovine Fetuin

Removal of all sialic acid and β1,4-linked galactose residues from bovine fetuin results in the macromolecular acceptor, *As(+O,-G)* bovine fetuin. In Table 2, ST6Gal I activity towards the *As(+O,-G)* form reduced drastically to 6.4±1.2 dpm subsequent to removal of β1,4-linked galactose residues, in comparison to *As*-fetuin (22780±2278 dpm) (Figure 14). On the contrary, ST3Gal III (Figure 15) and ST3Gal I (Figure 16) activities did not diminish as drastically towards the *As(+O,-G)* substrate, retaining approximately 59 ±6% and 82±8% of the activity produced by their *As*-counterparts, respectively. Furthermore, *As(+O,-G)* bovine fetuin was able to act as an acceptor for both recombinant ST3Gal I and ST3Gal III, with final activities of 9330±933 dpm and 1920±192 dpm. In addition, the ST3Gal I activity was shown to be higher in comparison to the activity produced by ST3Gal III.

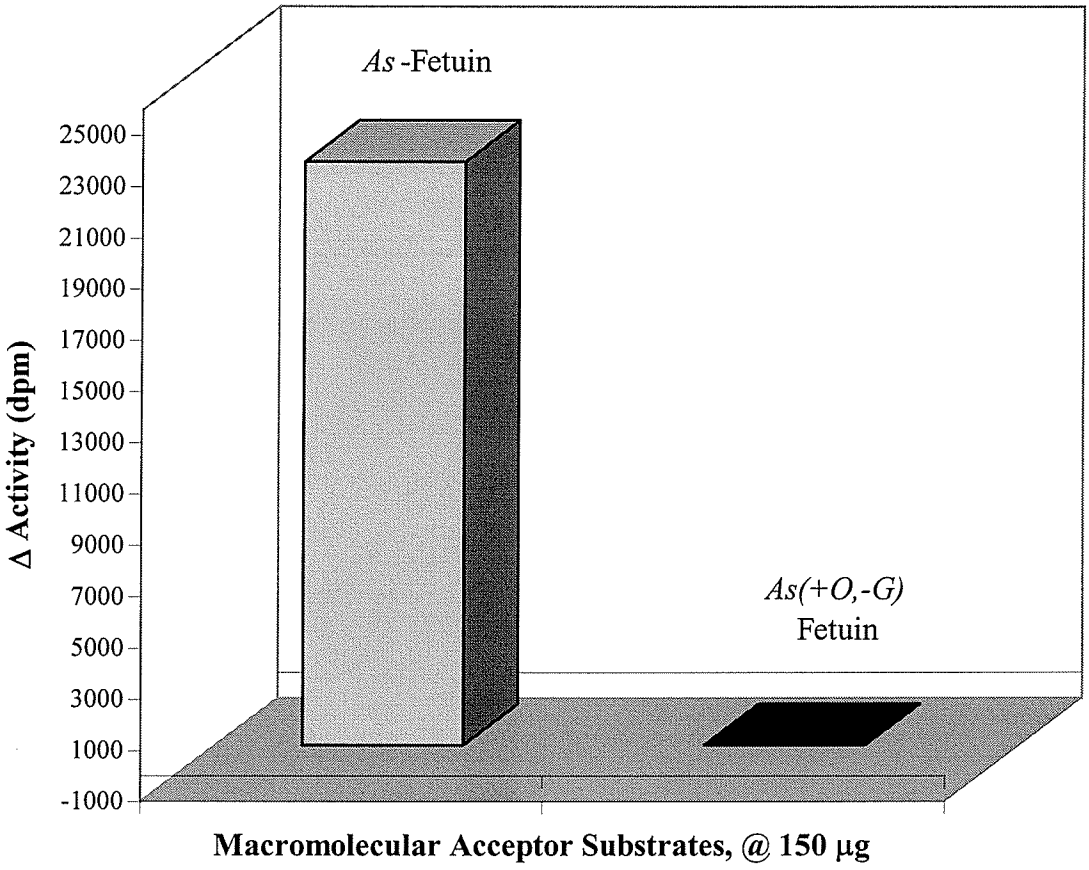


Figure 14. Graphical Depiction of Purified Rat Liver ST6Gal I Activity Assayed with the 2 Modified Bovine Fetuin Glycoproteins.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

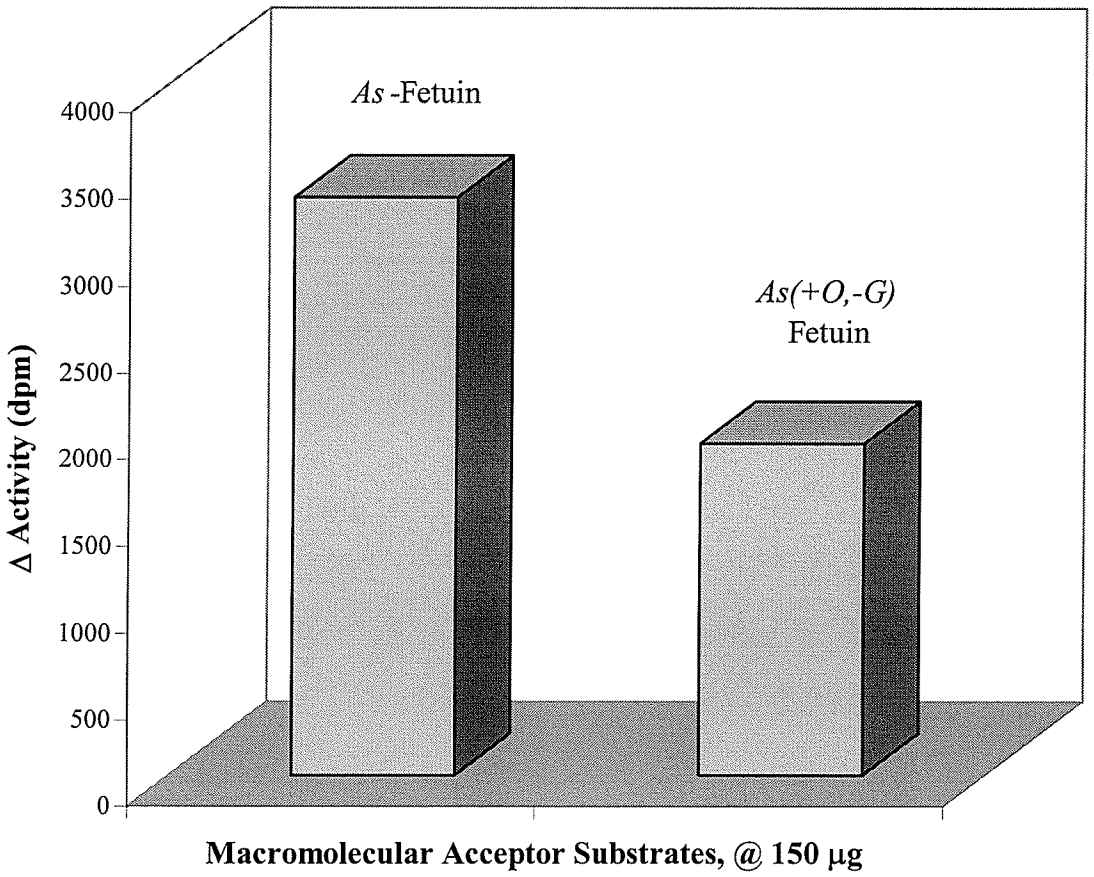


Figure 15. Graphical Depiction of *Spodoptera Frugiperda* Recombinant ST3Gal III Activity Assayed with the 2 Modified Bovine Fetuin Glycoproteins.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

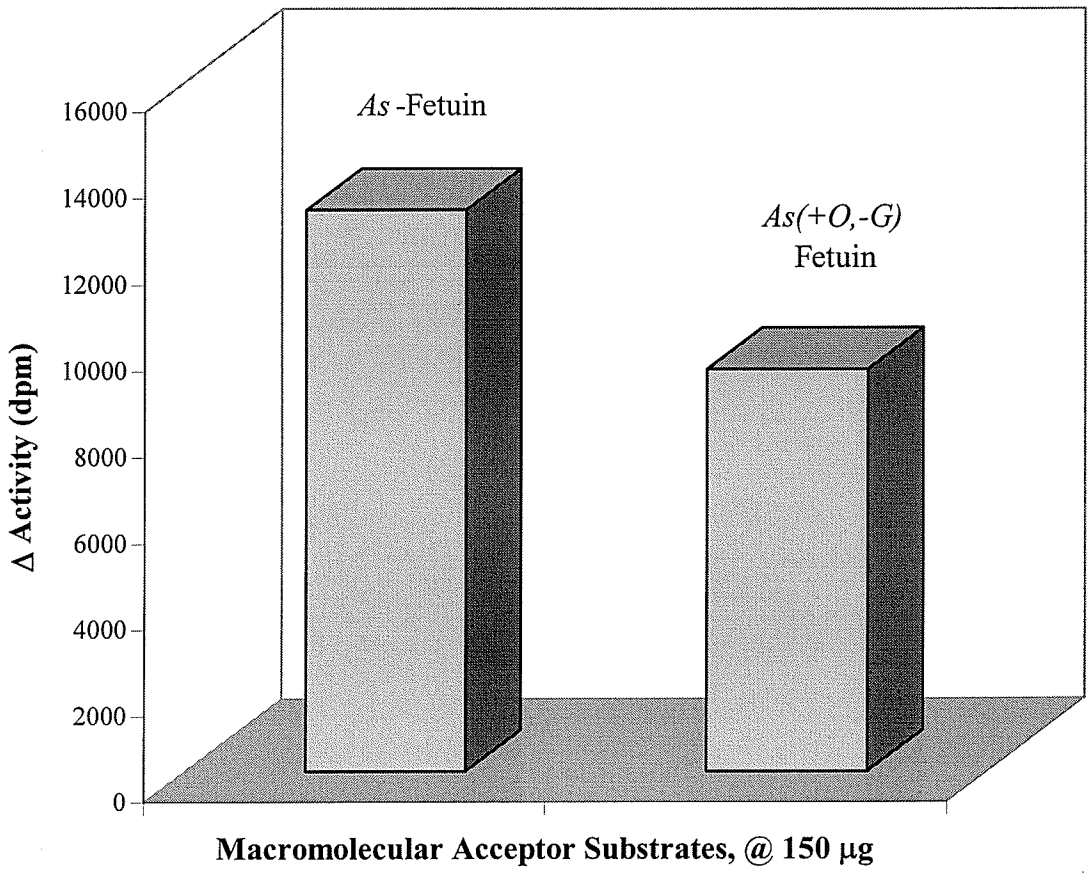


Figure 16. Graphical Depiction of *Spodoptera Frugiperda* Recombinant ST3Gal I Activity Assayed with the 2 Modified Bovine Fetuin Glycoproteins.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

iii) *As(-G)* Bovine α_1-AGP

As shown in Table 3, *As*-bovine α_1-AGP was able to act as an acceptor for ST6Gal I and ST3Gal III, displaying high activities of 19250±1925 dpm and 5717±572 dpm, respectively. Interestingly, ST3Gal I showed no significant activity towards the asialylated substrate (23.8±2.5 dpm). Furthermore, similar results were obtained when ST3Gal I was assayed against *As(-G)* form of bovine α_1-AGP (9.3±1.0 dpm). As shown in Figure 17 and 18, *As*- and *As(-G)* bovine α_1-AGP produced recombinant ST3Gal I activities close to 0 dpm, when compared to activities produced by purified ST6Gal I and recombinant ST3Gal III . As well, these results demonstrated *As*- and *As(-G)* forms of bovine α_1-AGP to be poor acceptor substrates for the ST3Gal I enzyme. Following the removal of β1,4-linked galactose residues from *As*-bovine α_1-AGP, the ST6Gal I activity greatly diminished to ~0 dpm (Figure 17 and 18). In contrast, *As(-G)* bovine α_1-AGP was still able to act as an acceptor for ST3Gal III, displaying an activity of 5314±530 dpm. Furthermore, there was only a slight reduction in ST3Gal III activity obtained towards the β1,4-galactosidase treated form versus the non-β1,4-galactosidase treated bovine α_1-AGP, retaining 93±10% of the activity attained by *As*-bovine α_1-AGP.

Recombinant or purified sialyltransferase	Δ Activity (dpm)	
	As bovine α_1-AGP	*As(-G)* bovine α_1-AGP
ST6Gal I	19250	-106
ST3Gal III	5717	5315
ST3Gal I	23.8	9.3

Table 3. Activity of the Commercial Sialyltransferases Towards the Two Modified Glycoproteins of Bovine α_1-AGP.

The following sialyltransferases selected were purified ST6Gal I from rat liver, and recombinant ST3Gal III and ST3Gal I, both from *Spodoptera frugiperda* (at 500-600 μg concentration), while *As*-bovine α_1-AGP and *As(-G)* bovine α_1-AGP were the acceptor substrates used at 150 μg of protein. *As(-G)* b. α_1-AGP is the final glycoprotein product used to assay for ST3Gal III in human serum. Activity values, represented as disintegrations per minute or dpm, were obtained from various trials, n (n=3 or 4) and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values (which are assays that did not contain the modified glycoprotein and replaced by deionized water to account for non-specific self-sialylation of the recombinant or purified sialyltransferase, or sialylation of endogenous acceptors in human serum).

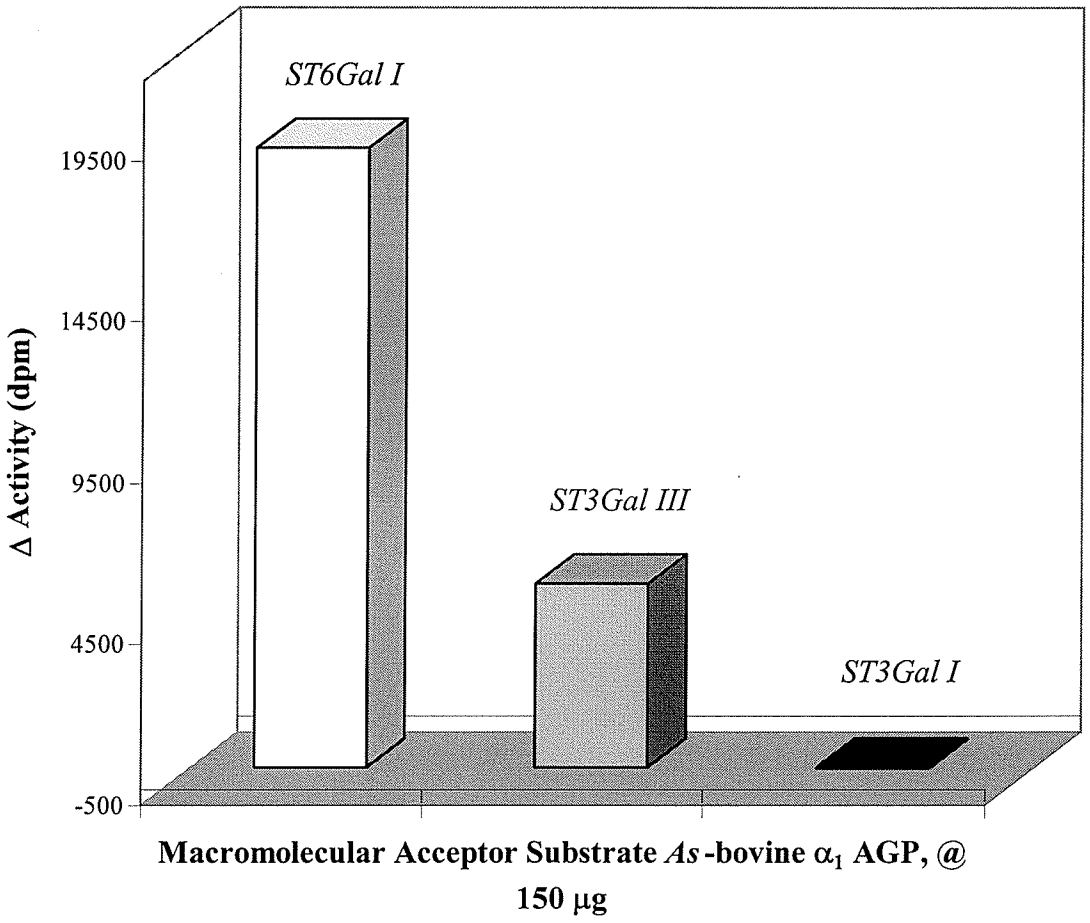


Figure 17. Graphical Depiction of Purified Rat Liver ST6Gal I, *Spodoptera Frugiperda* Recombinant ST3Gal III and ST3Gal I Activities Assayed with *As*-bovine α_1-AGP.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

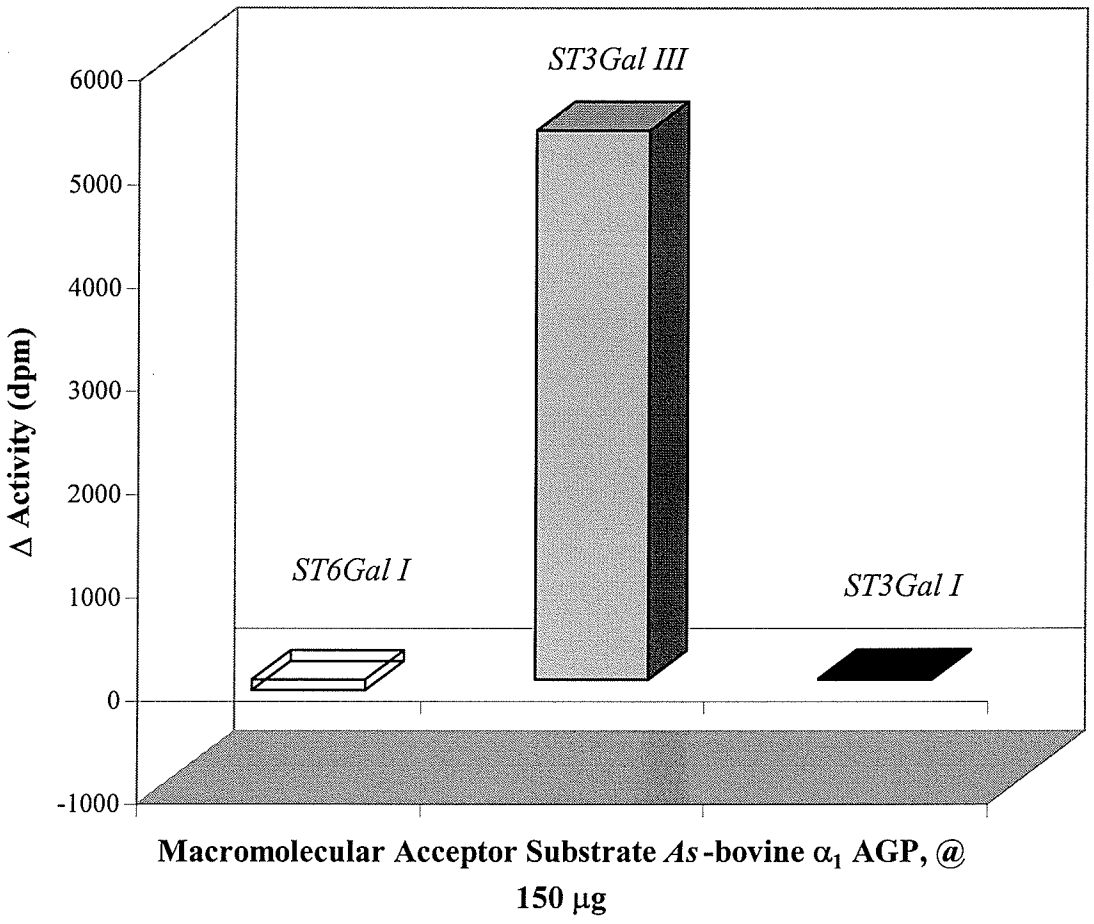


Figure 18. Graphical Depiction of Purified Rat Liver ST6Gal I, *Spodoptera Frugiperda* Recombinant ST3Gal III and ST3Gal I Activities Assayed with *As(-G)* Bovine α_1-AGP.

Activity values, represented as disintegrations per minute (dpm), were obtained from various trials, n (n = 3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average. The Δ activity is the activity value subtracted by the control values.

B) Kinetic Studies on Macromolecular Acceptors for ST3Gal I and ST3Gal III in Human Control Serum

Upon completion and analysis of macromolecular acceptors utilized to assay for ST3Gal III and ST3Gal I, kinetic analysis was performed on the acceptors. Also, investigations were made on sialyltransferase levels in human serum. In mammalian systems, certain sialyltransferases have been detected in serum (Kaplan *et al.*, 1983; Maguire *et al.*, 1996) and previous studies in our laboratory have detected the presence of ST6Gal I in human serum (Thorne-Tjomsland *et al.*, 2000). Therefore, assaying ST3Gal III and ST3Gal I with the modified bovine glycoproteins is based on the assumption that they are detectable in human serum as well. Three kinetic parameters were investigated for each glycoprotein acceptor, *As(+O,-G)* bovine fetuin, *As(-O,-G)* bovine fetuin, and *As(-G)* bovine α_1-AGP, while keeping all other reaction conditions constant throughout each test. Such parameters include the effect of incubation time, acceptor concentration, and varying volume of serum used. Furthermore, K_m and V_{max} values were determined where applicable.

i) Varying Incubation Time

In order to examine the effects of varying incubation time on the activity of serum sialyltransferases, the reaction mixture was measured at 37°C for 1, 2, 3, and 4 hours (Table 4). An acceptor concentration of 150 μg was used, while the rest of the assay components were held at the constant standard measurements discussed in the Methods section. The effect of incubation time on serum sialyltransferase activity, shown in Figure 19, displayed a linear relation with time and pmol of NeuAc transferred for *As(+O,-G)* fetuin. Activities produced by the acceptors *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP with increasing incubation period also exhibited linear relations between time and pmol of NeuAc transferred, but not to the extent of *As(+O,-G)* fetuin. The slope of each line was determined, also allowing determination of sialyltransferases activity rates for each acceptor in human serum. The ST3Gal I acceptor, *As(+O,-G)* fetuin, produced an activity rate of 10.30±2.10 pmol NeuAc transferred per hour (pmol NeuAc/hour) @ 150 μg, while the fetuin and α_1-AGP acceptors (150 μg) for ST3Gal III were 0.78±0.16 pmol NeuAc/hour and 0.45±0.09 pmol NeuAc/hour, respectively. The low activity rates obtained for the ST3Gal III acceptors may suggest that this enzyme is not present in human control serum. A longer incubation period was not chosen for future studies as to avoid loss of acceptor integrity with prolonged exposure at 37°C and possible detection of any non-specific activity present in serum. In addition, ST6Gal I assays in previous study (Thorne-Tjomsland *et al.*, 2000), were performed at 2 hours. Hence, 2 hours of incubation was used as the standard for further experiments, as comparisons between sialyltransferase peak levels in future longitudinal studies involving the renal transplant patient data analysis would be uniform.

Incubation Period (Hours)	NeuAc transferred, pmol (acceptor concentration @ 150 μg)		
	As(-O,-G) fetuin	*As(+O,-G)* fetuin	*As(-G)* bovine α_1-AGP
1	0.40	10.93	0.74
2	1.61	23.88	0.06
3	2.42	24.78	0.57
4	3.14	44.24	2.74

Table 4. The Effect of Varying the Incubation Time Period on Sialyltransferase Activity Employing the 3 Modified Glycoprotein Acceptors.

Length of incubation periods were varied over a range of 0-4 hours. Human control serum (25 μl) was used as the source for sialyltransferases. Assays were performed at a standard of 37°C, employing *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, and *As(-G)* bovine α_1-AGP as the glycoprotein acceptors at 150 μg, while CMP-NeuAc concentration is held in excess. The data collected was obtained from various trials, n (n=3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average.

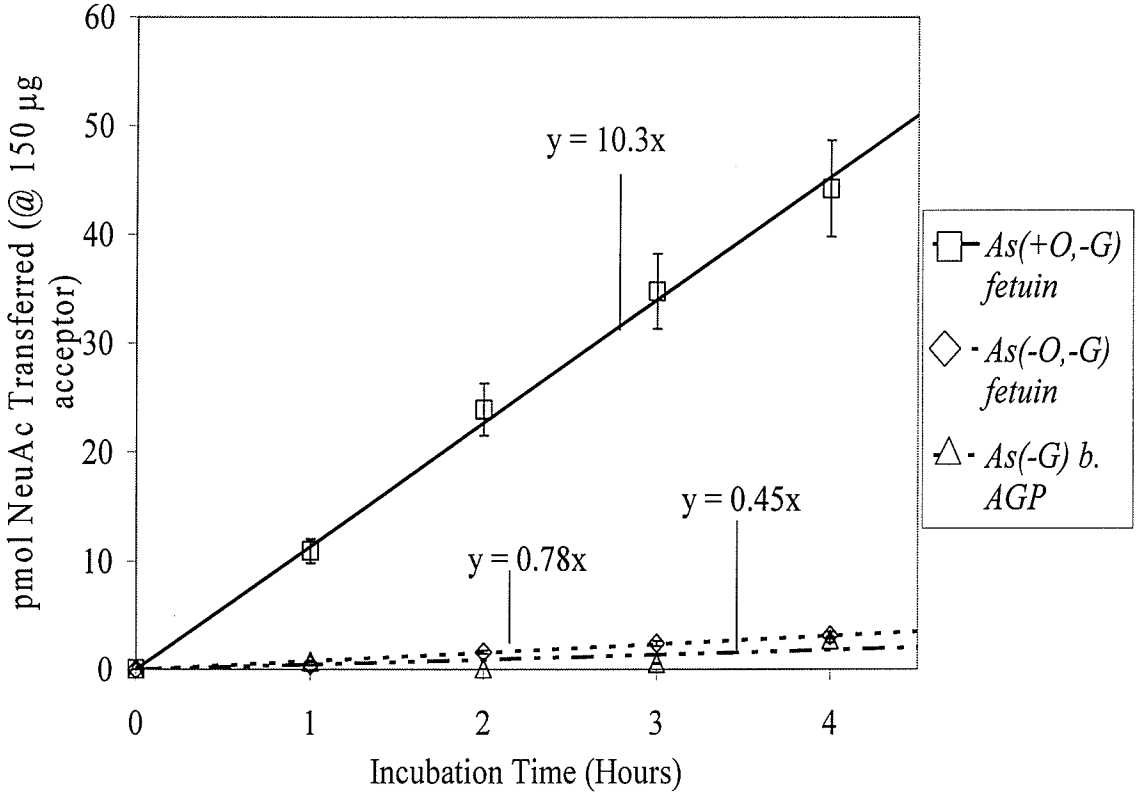


Figure 19. The Effect of Varying Incubation Time on Sialyltransferase Activity.

Length of incubation periods were varied over a range of 0-4 hours. Human control serum (25 µl) was used as the source for sialyltransferases. Assays were performed at a standard of 37°C, employing *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, and *As(-G)* bovine α_1-AGP as the glycoprotein acceptors at 150 µg, while CMP-NeuAc concentration is held in excess. The data collected was obtained from various trials, n (n=3 or 4), and an average was taken. Each trial came within ± 10% error as compared to the average.

ii) Varying Acceptor Concentration

Human serum sialyltransferase activity was measured using varied acceptor concentration ranging between 0 to 250 μg (Table 5). Other reaction mixture components were held at the constant standard concentrations, as discussed in the Methods section, and the solution was brought to a final volume of 65 μl. The assay mixture was allowed to incubate at 37°C for 2 hours. As shown in Figure 20, the sialyltransferase activity in human serum employing *As(+O,-G)* fetuin increased in a linear fashion at lower concentrations (0 μg - 200 μg) of acceptor, displaying first order reaction kinetics. At higher concentrations of acceptor substrate (200 μg- 250 μg), the transfer rate appears to slightly plateau, but does not reach zero order reaction kinetics, suggesting that acceptor substrate saturation might occur at higher concentrations. Sialyltransferase activities utilizing concentrations higher than 250 μg for *As(+O,-G)* fetuin was difficult to determine because the acceptor could not stay in solution. This may be due to degradation of the protein. The other acceptor substrates, *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP, exhibited lower activity relative to *As(+O,-G)* fetuin.

Michaelis-Menten kinetics were determined only for *As(+O,-G)* fetuin, because it was the only acceptor out of all three to produce significant activity and a nearly hyperbolic curve in Figure 21, allowing determination of K_m and V_{max} values. The information obtained for *As(+O,-G)* fetuin was re-plotted as the parameters of concentration of acceptor, (presuming a MW of 45,000 Da) ranging between 0.017 – 0.085 mM, versus reaction velocity in pmol of NeuAc transferred per minute. Although Lineweaver-Burke plots, used to find K_m and V_{max} shown in Figure 22, can be less precise, as greater weighing is placed on lower concentrations, it was the method

commonly used in our laboratory and allows uniform comparison with K_m and V_{max} values of other acceptors developed in our lab. It was determined that the equation of the best fit line was $y = (0.29\pm0.06)x + (0.68\pm0.14)$. K_m value obtained was 0.48±0.14 mM, while the V_{max} value was 1.47±0.30 pmol of NeuAc transferred/min. (Table 6 and 7). A concentration of 150 μg (0.051 mM) of acceptor was used, since it lies on the linear portion of the Michaelis-Menten plot and prior to any potential substrate saturation concentration. In addition, the fetuin acceptor consistently stayed soluble at this concentration, where the acceptor would occasionally come out of solution at higher concentrations (200 or 250 μg), Since *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP did not produce significant activities over varied concentration and did not produce a graphical hyperbola, Michaelis-Menten kinetics were not calculated. Higher concentrations of acceptor were also tested. Although the bovine α_1-AGP acceptor was readily soluble at concentrations higher than 250 μg, significant activity was not detected (data not shown). On other hand, the *As(-O,-G)* fetuin acceptor would not stay in solution, which was also observed with the *As(+O,-G)* fetuin acceptor. Thus, no K_m or V_{max} values were obtained for *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP (Table 7). In the case of the latter two acceptors, the same concentration employed for *As(+O,-G)* fetuin was used for simpler comparison during preliminary examination of ST3Gal III and ST3Gal I levels in renal transplant patient serum.

Amount of Acceptor Added (μg)	Sialyltransferase activity (pmol of NeuAc transferred/min) towards the following acceptors:		
	As(-O,-G) fetuin	*As(+O,-G)* fetuin	*As(-G)* bovine α_1-AGP
50	0.012	0.061	0.0010
100	0.020	0.093	-0.013
150	0.028	0.20	0.017
200	0.020	0.230	-0.00011
250	0.018	0.250	-0.0036

Table 5. The Effect of Varying Amounts of Acceptor Added on Sialyltransferase Activity Employing the 3 Modified Glycoprotein Acceptors.

Human control serum (25 μl) was used as the source for sialyltransferases. *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, and *As(-G)* b. (bovine) α_1-AGP were used as the glycoprotein acceptors at varying amounts of 0-250 μg while CMP-NeuAc concentration was held in excess. Assays were performed at a standard of 37°C for 2 hours. Various trials, n, were performed (n=3 or 4) and an average was taken. Each trial came within ± 10% error as compared to the average.

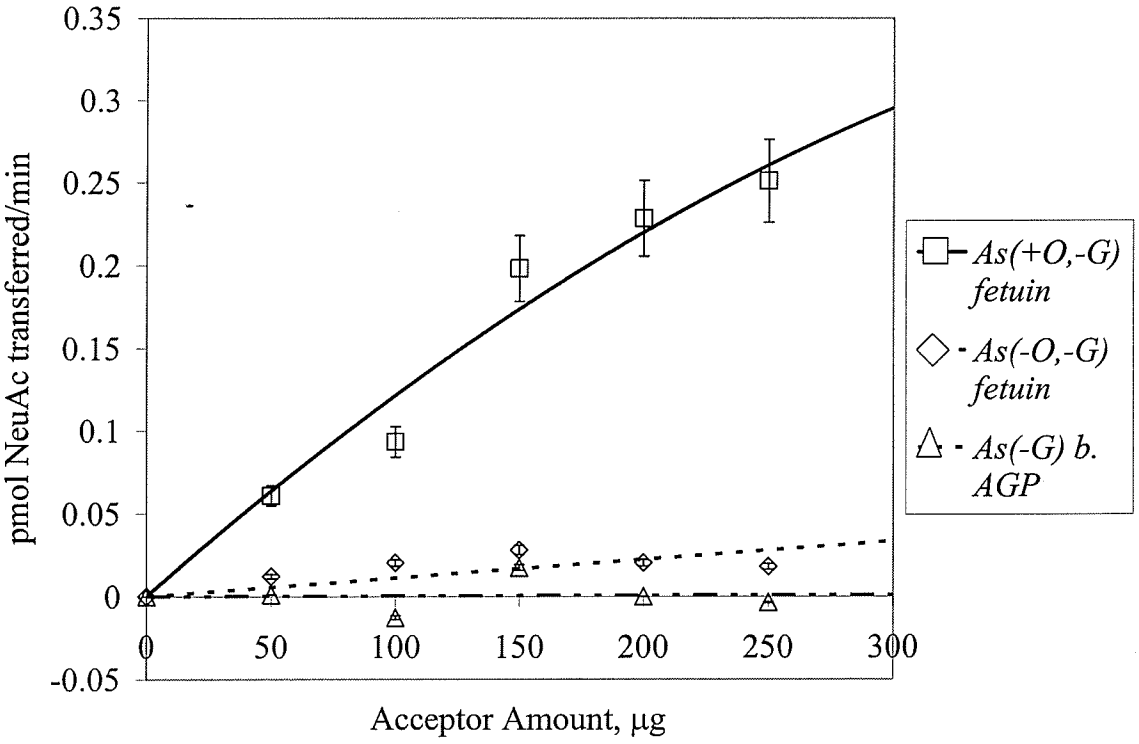


Figure 20. The Effect of Varying Glycoprotein Acceptor Concentration on Sialyltransferase Activity.

Human control serum (25 μl) was used as the source for sialyltransferases. *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, and *As(-G)* b. (bovine) α_1-AGP were used as the glycoprotein acceptors at varying amounts of 0-250 μg while CMP-NeuAc concentration was held in excess. Assays were performed at a standard of 37°C for 2 hours. Various trials, n, were performed (n=3 or 4) and an average was taken. Each trial came within ± 10% error as compared to the average.

[Acceptor], mM	Reaction Velocity, pmol NeuAc transferred per min.	1 / [Acceptor], mM^{-1}	1 / Reaction Velocity, $(pmol\ NeuAc / min)^{-1}$
0.017	0.061±0.006	59	16±3
0.034	0.093±0.010	30	11±2
0.051	0.20±0.02	19	5.1±1.1
0.068	0.23±0.03	15	4.4±1.0
0.085	0.25±0.03	12	4.0±0.8

Table 6. Data for Michaelis-Menten and Lineweaver-Burke Plots

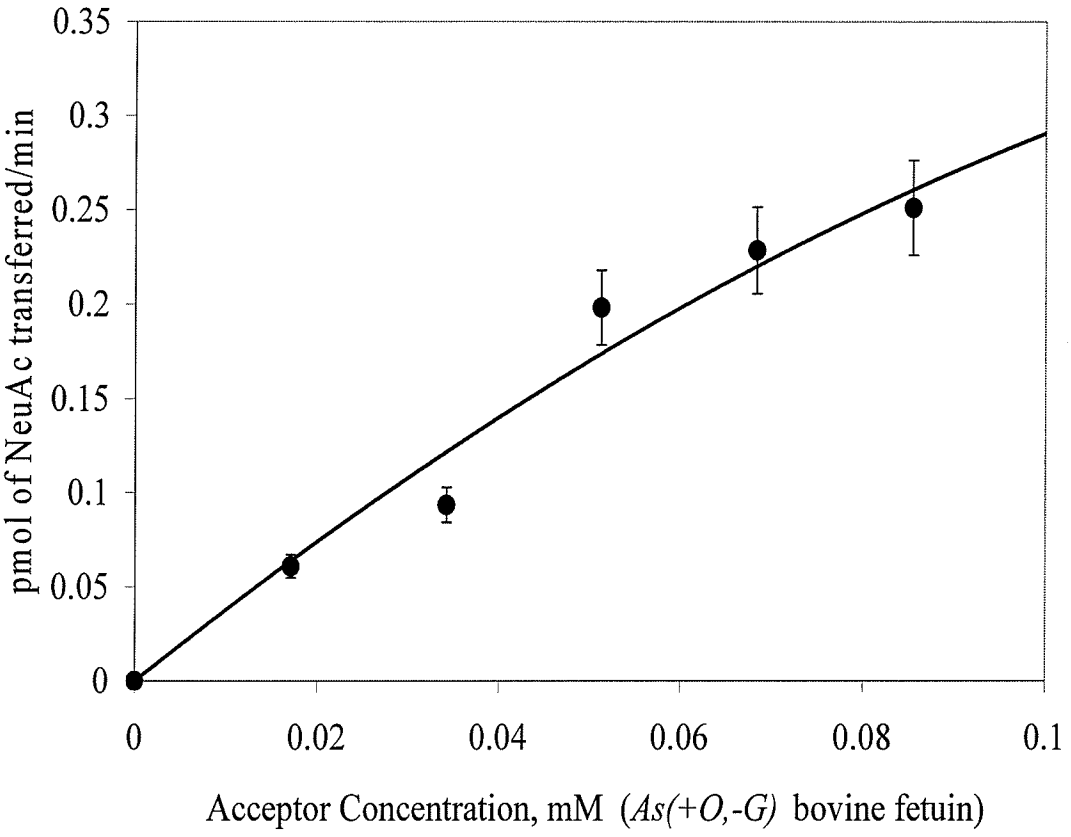


Figure 21. Michaelis-Menten Plot of Varying Amount of Acceptor, *As(+O,-G)* Fetuin, Added.

Human control serum (25 μl) was used as the source for sialyltransferases. *As(+O,-G)* fetuin was used as the glycoprotein acceptors at varying amounts of concentration (0.017 – 0.085 mM) while CMP-NeuAc concentration was held in excess. Assays were performed at a standard of 37°C for 2 hours. Various trials, n, were performed (n=3 or 4) and an average was taken. Each trial came within ± 10% error as compared to the average.

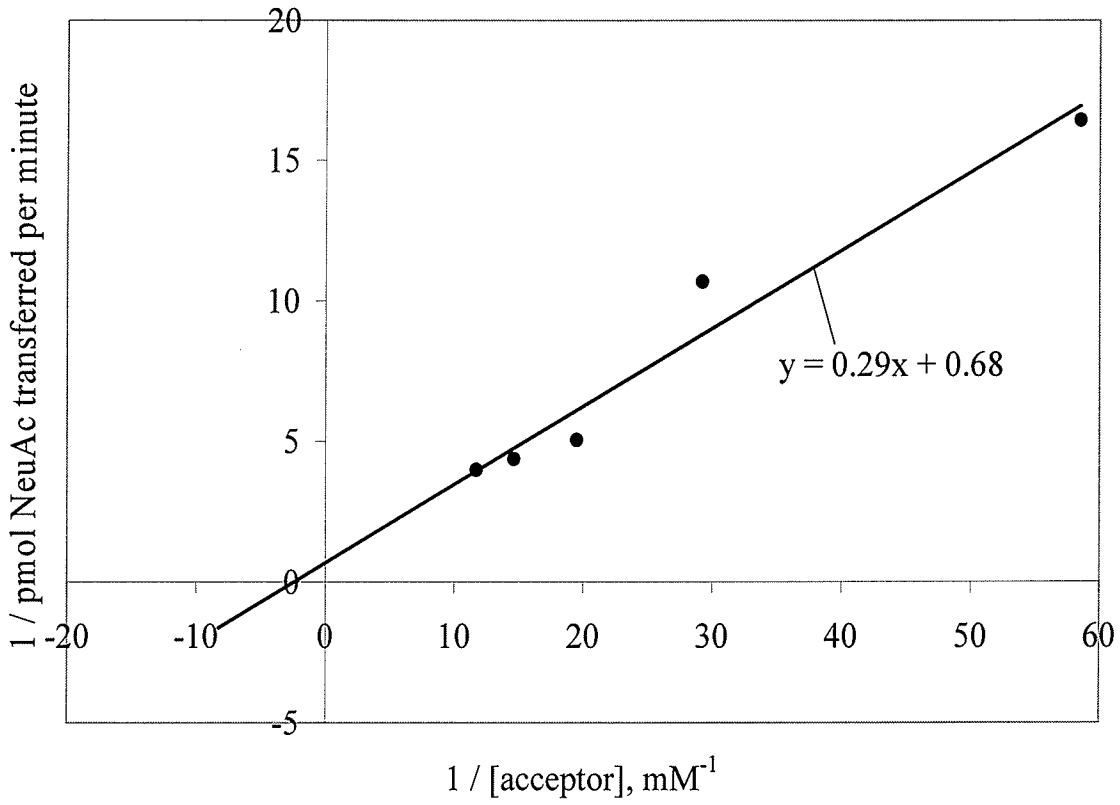


Figure 22. Lineweaver-Burke Plot of Varying Amount of Acceptor, *As(+O,-G)* Fetuin, Added.

K_m and V_{max} values of *As(+O,-G)* fetuin were determined by Lineweaver-Burke plot, where the best fit line obtained produced the equation shown above. Errors values are shown in Table 6 and 7.

Macromolecular Acceptor	K_m (mM)	V_{max} (pmol NeuAc transferred /min)
***As(+O,-G)* bovine fetuin**	0.48±0.14	1.47±0.30
***As(-O,-G)* bovine fetuin**	N/A	N/A
***As(-G)* bovine α_1-AGP**	N/A	N/A

Table 7. K_m and V_{max} Values for Macromolecular Acceptors Developed for ST3Gal I and ST3Gal III in Human Serum.

iii) Varying Serum Content

The amount of human serum used is also significant because it affects the quality of the assay system used to assay for sialyltransferases in renal transplant patients. Since human serum contains many unknown components, exact enzyme concentrations of a particular sialyltransferase are difficult to quantify. Even so, in order to examine the effects of enzyme concentration on reaction rate, known concentrations of enzyme are essential. Instead, the concentration of serum sialyltransferases is represented as volume of serum or serum content. Amounts of serum used varied from 0 μl to 55 μl, keeping constant the acceptor substrate concentration at 150 μg and the incubation period at 2 hours (Table 8). The rest of the assay components were once again held at constant standard measurements as discussed in the Methods section. As shown in Figure 23, activity produced by *As(+O,-G)* fetuin increased linearly at lower amounts of serum (0 μl – 30 μl), demonstrating first order kinetics. Zero order kinetics was observed at higher amounts of serum (30 μl – 55 μl), shown by the plateau in activity. From the hyperbolic plot, it was evident that saturation of the enzyme was achieved at approximately 30 μl of serum. Furthermore, the results confirm that 25 μl of serum used in all serum sialyltransferase assays was the suitable and optimal amount that should be employed. In contrast, the acceptors *As(-G)* b. α_1-AGP and *As(-O,-G)* fetuin did not demonstrate hyperbolic relationships. Furthermore, the results displayed here for the latter acceptors are similar to results obtained for analysis of the other kinetic parameters tested.

Volume of Serum Added (μl)	Sialyltransferase activity (pmol of NeuAc transferred/min) towards the following acceptors (@ 150 μg concentration):		
	As(-O,-G) fetuin	*As(+O,-G)* fetuin	*As(-G)* bovine α_1-AGP
0	0	0	0
15	-0.011	0.18	0.010
25	0.028	0.22	-0.003
35	0.009	0.31	0.004
45	-0.024	0.35	-0.009

Table 8. The Effect of Varying Serum Content on Sialyltransferase Activity Employing the 3 Modified Glycoprotein Acceptors.

Volumes of serum used varied over 0-55 μl while keeping other reaction conditions at a.standard. Human control serum samples were used as the source for sialyltransferase. Assays were performed at a standard of 37°C for 2 hours, employing *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, and *As(-G)* bovine α_1-AGP as the glycoprotein acceptors at 150 μg, while CMP-NeuAc concentration is held in excess. Each trial came within ± 10% error as compared to the average.

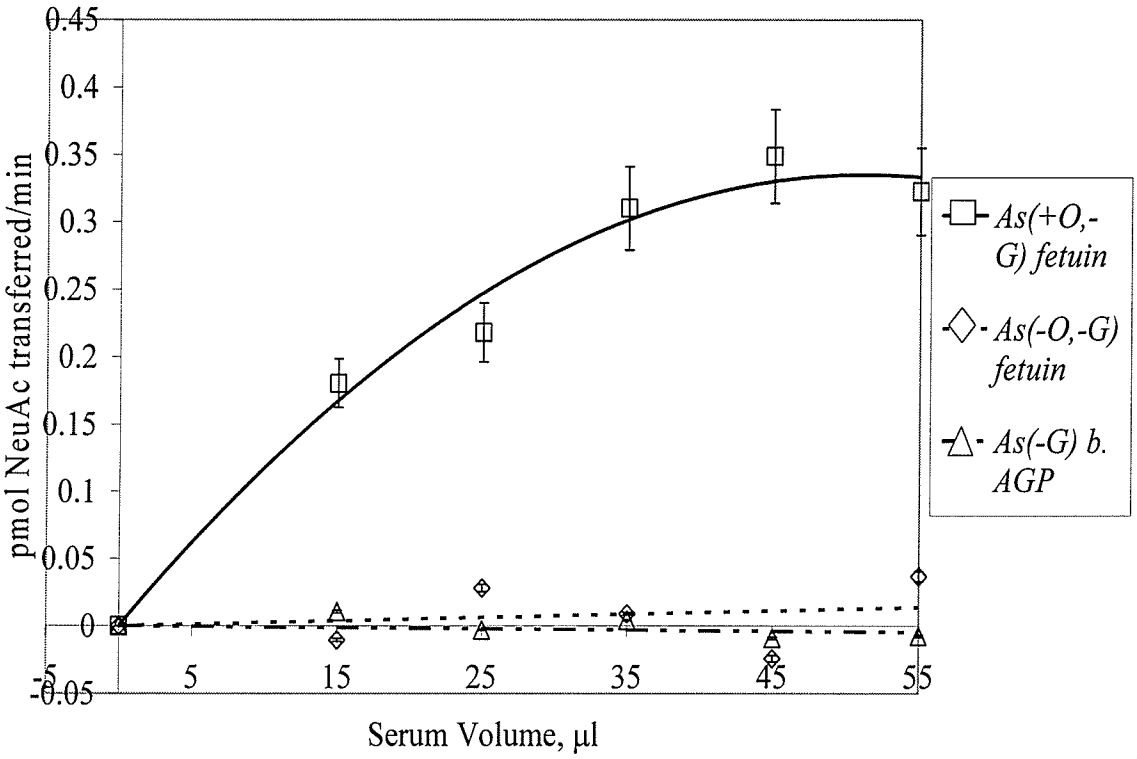


Figure 23. The Effect of Varying Serum Volume on Sialyltransferase Activity.

Volumes of serum used varied over 0-55 µl while keeping other reaction conditions at a.standard. Human control serum samples were used as the source for sialyltransferase. Assays were performed at a standard of 37°C for 2 hours, employing *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, and *As(-G)* bovine α_1-AGP as the glycoprotein acceptors at 150 µg, while CMP-NeuAc concentration is held in excess. Each trial came within ± 10% error as compared to the average.

C. Preliminary Measurements of ST3Gal I and ST3Gal III Levels in Renal Transplant Patient Serum, Employing the Bovine Glycoprotein Acceptors.

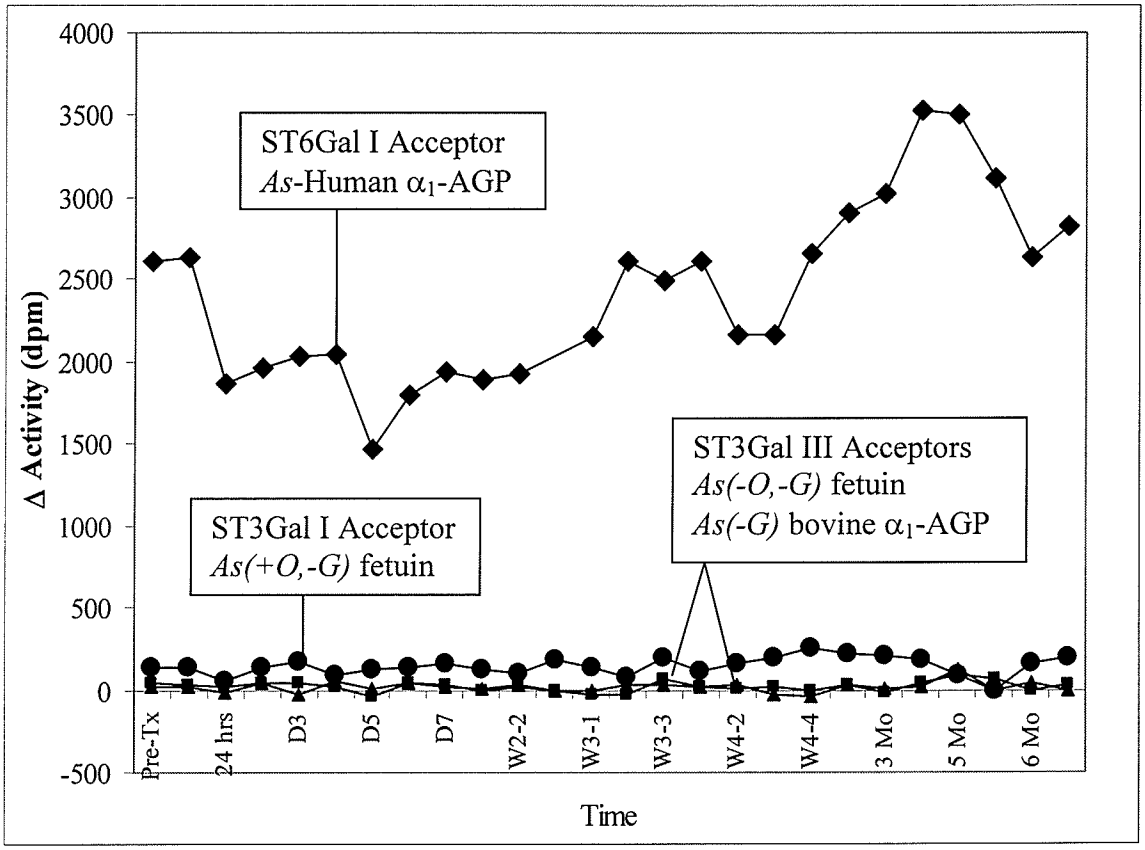
Following the kinetic analysis made on the macromolecular acceptors, determination of K_m and V_{max} values, and determination of their optimal reaction conditions in human systems, the acceptors were employed in serum obtained from a renal transplant patient, while monitoring sialyltransferase levels. A total of 35 sets of serum samples were available to use, which were obtained from 35 renal transplant patients for longitudinal analysis of ST6Gal I levels. All serum samples were limited in supply and of great value, being utilized in the analysis of other sialyltransferases and glycoproteins. In addition, further analysis of ST3 enzymes in all these patients would not only be laborious and time consuming, but may prove to be expensive and ineffective, as it is unknown what type of ST3 enzyme levels are present. With these factors to consider, I was limited to choose only one patient to perform my assays on for a preliminary examination on what type of ST3 enzyme levels to expect.

The samples obtained from four of the patients were excluded from statistical analysis studies, because the exact times of sample collection was poorly recorded, or the amount of serum samples collected was insufficient for proper analysis. However, choosing one patient out of the four, to employ the modified acceptors in, would be ideal, since they would not be used in other studies, and would allow preliminary measurements of ST3Gal I and ST3Gal III levels in renal transplant patient serum. The following patient chosen (whose name was not disclosed due to patient confidentiality) had enough samples collected but actual times of when the samples were collected was not properly recorded. However, since this study was for preliminary purposes, actual collection

times were not relevant and instead, an estimate was made based on the information received from the Transplant Immunology Laboratory. Essentially, serum samples, for the chosen patient, were collected at the following time points: Pre-transplant, 12 and 24 hours, day 2, 3, 4, 5, 6, and 7, post-transplant. In addition, three samples were obtained during weeks 2 and 3, four samples from week 4, and one sample each from month 2, 3, 4, 5, 6, and 7, post-transplant.

Initial tests on *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP showed that these acceptors were good for assaying ST3Gal III, while *As(+O,-G)* fetuin was recognized by ST3Gal I and ST3Gal III less efficiently. These acceptors, along with the acceptor for ST6Gal I, *As*-human α_1-AGP, which will be used for comparison purposes, were employed for preliminary determination of ST3Gal III, ST3Gal I, and ST6Gal I levels longitudinally in renal transplant patient serum. Assays were performed at a standard of 37°C for 2 hours. *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, *As(-G)* bovine α_1-AGP, and *As*-human α_1-AGP, were employed as the glycoprotein acceptors at 150 μg, in 25 μl of serum while CMP-NeuAc concentration is held in excess. Assays were done in duplicate, where each sample displayed ± 5% deviation from the average. Upon examination, ST6Gal I activities appear greatly elevated in comparison to activities exhibited by ST3Gal I and ST3Gal III (Figure 24). The activities reported for the ST3Gal III acceptors, *As(-O,-G)* fetuin (-40.8 to 91.6 dpm) and *As(-G)* bovine α_1-AGP (-41.1 to 119.9 dpm), even displayed negative Δ activity values, essentially detecting little to no significant ST3Gal III activity. On the other hand, the ST3Gal I acceptor, *As(+O,-G)* fetuin (0.4 to 255.1 dpm) did not yield negative Δ activity values, producing low ST3Gal I activity, in comparison with activities produced by *As*-human α_1-AGP, which ranged

between 1472.7 to 3525.8 dpm. Peak activities were observed at the 4-5 month range for *As*-human α_1-AGP, with minimum activities at Day 5. For *As(+O,-G)* fetuin, maximum activities were found at the 1-3 month range and the lowest at ~6 months post-transplant. Sialyltransferase activities produced by the ST3Gal III acceptors were treated as insignificant based on the results obtained in the kinetic studies and the low activities produced in this particular section of study when compared to activities produced by the other acceptors.



LEGEND

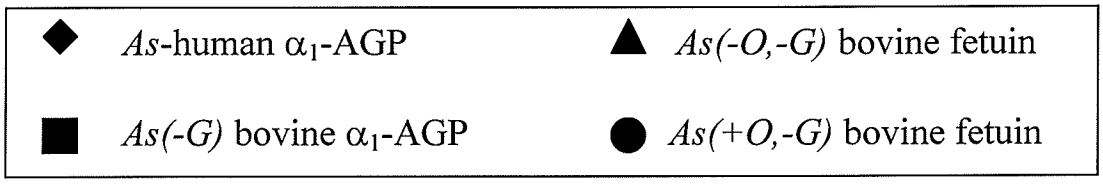


Figure 24. Longitudinal Analysis of Sialyltransferase Levels, Particularly ST6Gal I, ST3Gal III and ST3Gal I, Employing the Acceptors *As*-Human α_1-AGP, *As(-O,-G)* Bovine Fetuin, *As(-G)* Bovine α_1-AGP, and *As(+O,-G)* Bovine Fetuin.

The first time point represents the sialyltransferase activity from a serum sample obtained prior to the patient's transplant. Time representations are coded as follows: D3, D5, D7 = Day 3, 5, and 7; W2-2 = Week 2, sample 2; W3-1 = Week 3, sample 1; W3-3 = Week 3, sample 3; W4-2 = Week 4, sample 2; W4-4 = Week 4, sample 4; 3 Mo = 3rd month; 5 Mo = 5th month; 6 Mo = 6th month. Samples were tested in duplicate, where each sample displayed ± 5% deviation from the average. Assays were performed at a standard of 37°C for 2 hours. *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, *As(-G)* bovine α_1-AGP, and *As*-human α_1-AGP, were employed as the glycoprotein acceptors at 150 µg, in 25 µl of serum while CMP-NeuAc concentration is held in excess. Assays were done in duplicate, where each sample displayed ± 5% deviation from the average.

DISCUSSION

A) Studies on the Development of Macromolecular Acceptors for ST3Gal I and ST3Gal III

In most mammalian systems, assaying a specific sialyltransferase, whether the source is from rat liver Golgi preparations or in this case, human serum, requires a suitable acceptor which contains the precise carbohydrate structural configuration and which can preferably be found in nature. Thus, the glycoproteins, bovine fetuin and bovine α_1-AGP, were modified to specifically assay for ST3Gal III and ST3Gal I in human serum. There are several ways to modify oligosaccharide chains, involving either enzymatic or chemical treatments. The reasons for utilizing the particular methods chosen were focused on providing the most efficient and cost-effective way of modifying carbohydrates. The initial modification step requires the removal of sialic acid residues, which can either be done enzymatically *via* neuraminidase enzyme as before (Paulson *et al.*, 1977a), or chemically *via* dilute acid hydrolysis (Turchen *et al.*, 1977; Jamieson, 1977). The latter method was exploited, because it's inexpensive and has been a standard successful procedure often utilized in our laboratory. Release of O-linked glycans was carried out using a chemically modified version of the alkaline-borohydride hydrolysis method (Likhosherstov *et al.*, 1990). Enzymatic cleavage of oligosaccharides O-linked to a Ser/Thr residue of the peptide backbone is also possible by the enzyme O-glycanase. While it may be a less detrimental process to the integrity of the protein, the latter procedure was avoided, as it is costly, especially when applied in large quantities. In addition, the treatment for O-linked oligosaccharide removal was not required when developing a macromolecular acceptor for ST3Gal I in human systems. The final

modification step for developing macromolecular acceptors for ST3Gal III and ST3Gal I prevent recognition of the acceptors from ST6Gal I through removal of β1,4-linked Gal residues by β1,4-galactosidase treatment. The β1,4-galactosidase enzyme specifically cleaves Gal residues that are β1,4-linked, leaving the ST3Gal III acceptor site, β1,3-linked Gal residues, intact. β1,4-galactosidase treatment was chosen because it was specific and cost effective.

Following each treatment, the resulting modified glycoprotein was assayed against a selected panel of commercially available sialyltransferases, which include purified rat liver ST6Gal I, and recombinant forms of *Spodoptera frigoperda* ST3Gal I and ST3Gal III. This was performed to verify that each treatment was successfully completed. Removal of sialic acid residues from the bovine glycoproteins was successful, as indicated by the Δ activity produced by the commercial sialyltransferases towards *As*-fetuin and *As*-bovine α_1-AGP. *As*-fetuin appeared to contain acceptor sites for all the selected sialyltransferases, with the following Δ activities being obtained for ST3Gal III (3253±325 dpm), ST3Gal I (11350±1135 dpm), and ST6Gal I (22780±2278 dpm). In contrast, *As*-bovine α_1-AGP reacted well with only ST3Gal III (Δ activity = 5717±572 dpm) and ST6Gal I (Δ activity = 19250±1925 dpm), but not ST3Gal I (Δ activity = 23.8±2.4 dpm). The results suggest an absence of the terminal sugar sequence Galβ1-3GalNAc-R, which should be recognized by ST3Gal I. Similar results were observed subsequent to removal of O-linked oligosaccharides from bovine α_1-AGP (data not shown), which was expected, because the treatment removes the ST3Gal I acceptor sites. In addition, previous studies by another graduate student in our laboratory, utilizing bovine α_1-AGP to develop a macromolecular acceptor for the ST6GlcNAc I enzyme, had

produced the same result (Szidonya and Jamieson, 2001). The resemblance between the Δ activity results obtained for the O-linked glycan-cleaved and non-cleaved bovine α_1-AGP implied that O-linked chain removal was an unnecessary procedure and thus, not performed for this particular glycoprotein. Although there has been no published evidence of bovine α_1-AGP containing acceptor sites for ST3Gal I, our results also show the absence of Galβ1,3GalNAc- acceptor sites and possibly O-linked glycans altogether.

On the other hand, fetuin is known to contain O-linked sugars. Subsequent to removal of the sialic acids, *As*-fetuin was subjected to alkaline-borohydride treatment, which should result in decreased reactivity with recombinant ST3Gal I. As predicted, successful cleavage of oligosaccharide chains O-linked to fetuin's polypeptide backbone was verified by the decrease in Δ activity exhibited by ST3Gal I (1150±115 dpm) towards *As(-O)* fetuin, representing only 10% of the activity prior to O-chain removal. In addition, acceptor sites for ST3Gal III and ST6Gal I were kept intact, as confirmed by the Δ activity produced by their commercial counterparts (2976±298 dpm, and 19100±1910 dpm, respectively).

Because *As(-O)* fetuin, and potentially *As*-bovine α_1-AGP, contain β1,4- and β1,3-linked Gal residues, the specific removal of β1,4-linked Gal is necessary for these bovine glycoproteins to act as acceptors exclusively for ST3Gal III. The removal of β1,4-linked galactose terminal residues should result in a loss of purified ST6Gal I activity, the acceptor site that is recognized by this sialyltransferase. Furthermore, ST3Gal III activity should exhibit a decrease in activity as well, since terminal β1,4-linked galactose residues are also recognized by this particular sialyltransferase, but should also retain some activity, as the Galβ1-3-R terminal site is potentially intact.

The results obtained show data as predicted, with a complete loss of ST6Gal I activity and a partial loss of ST3Gal III activity towards the *–G* forms of fetuin (ST6Gal I = 6.4±0.6 dpm, ST3Gal III = 1920±192 dpm) and bovine α_1-AGP (ST6Gal I = -106±11 dpm, ST3Gal III = 5315±532 dpm). Accordingly, β1,4-galactosidase treatment was an effective and successful method for the removal of β1,4-linked galactose residues without affecting the β1,3-linked Gal acceptor site for ST3Gal III. Overall, the final glycoprotein acceptors, *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP, were shown to be poor acceptor substrates for recombinant ST3Gal I and purified ST6Gal I, after all treatments, and still managed to remain a fine acceptor for recombinant ST3Gal III.

The acceptor specifically developed for ST3Gal I, *As(+O,-G)* fetuin, was also assessed. As expected, the results indicated that this molecule could act as an acceptor substrate to ST3Gal I (9330±933 dpm), and less efficiently, ST3Gal III (1920±192 dpm), as the O-linked oligosaccharide chains were kept intact. No ST6Gal I activity was observed upon removal of the β1,4-linked galactose residues, as predicted. In addition, the modifications performed on fetuin's N-linked glycans were geared towards assaying ST3Gal III.

In a comparison between bovine fetuin and bovine α_1-AGP, the results suggest that one of them may display higher acitivites, based on carbohydrate content and number of acceptor sites available. Furthermore, one glycoprotein may act as a better acceptor for one particular sialyltransferase, and not the other. For example, when comparing the Δ activity produced by *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP, the acceptors for recombinant ST3Gal III, the bovine α_1-AGP acceptor displayed a higher activity. These results appear plausible, as bovine α_1-AGP consists of a larger portion of carbohydrates

as compared with most other glycoproteins (Yoshima *et al.*, 1981). Inferring that the carbohydrates of bovine α_1-AGP are only N-linked to the peptide backbone, α_1-AGP should reasonably give a higher Δ activity than fetuin, which contains O-linked glycans as well, as ST3Gal III prefers to act on N-linked glycans. Thus, when assayed for recombinant ST3Gal III, our results demonstrated what was predicted. Furthermore, bovine fetuin has been shown to contain only a small fraction of Galβ1-3GlcNAc terminal structures (Green *et al.*, 1988). Only a few studies have been done on the terminal carbohydrate structures of bovine α_1-AGP, none of which have indicated the presence of Galβ1-3GlcNAc and in what amount. Based on the results, bovine α_1-AGP achieved a Δ activity that was approximately 4.34X fold greater than bovine fetuin, suggesting that bovine α_1-AGP contains 4.34X fold more of the Galβ1-3GlcNAc terminal structures than bovine fetuin. Taking into account all the information obtained, results point towards bovine α_1-AGP being more adequate than bovine fetuin as a suitable acceptor for ST3Gal III. On the other hand, bovine fetuin was shown to be a more suitable candidate as an acceptor for ST3Gal I, as studies have shown that fetuin possesses Galβ1,3GalNAc- terminal sequences on O-linked oligosaccharides (Green *et al.*, 1988). In addition, the results obtained in this study have shown that *As(+O,-G)* fetuin reacts well with recombinant ST3Gal I, whereas the bovine α_1-AGP acceptor does not.

Although the procedure for testing the macromolecular acceptors provided a rapid and convenient method for analyzing the presence of specific carbohydrate acceptor sites for a particular sialyltransferase, further structural elucidation of these modified glycoproteins is still required. Hence, there is no guarantee that the structure of the

protein or carbohydrate portions have been salvaged after all the chemical and enzymatic treatments. For example, the chemical procedure for O-linked oligosaccharide chain removal, the modified sodium borohydride treatment, involves 16 hours of incubation at 60°C, that may affect the integrity of the glycoprotein, structurally or functionally, although there is no evidence of this in the literature. The same can be said for the procedure for chemical removal of sialic acid residues, involving incubation at 90°C for one hour. However, this procedure has often been utilized in the past and has been a successful method for removal of sialic acid residues by many research groups. Analytical tools, such as MS and NMR, have been useful in elucidating the structural detail of the carbohydrates attached to several proteins (Dell and Morris, 2001), including bovine fetuin (Ishii-Karakasa *et al.*, 1997) and bovine α_1-AGP (Hunter and Games, 1995). This is especially important for the modified glycoproteins used here, because the chemical and enzymatic treatments do have the potential to affect the structure and function of the glycoproteins. The information obtained from using the commercial sialyltransferase test assays is limited to determining the presence of the acceptor site specific for the recombinant or purified sialyltransferase being used. Furthermore, there are very few of these purified or cloned sialyltransferases, along with the high prices of these enzymes, leaving them at disadvantage for application. Thus, it is imperative that the next step be to clarify the structural detail in *As(-O,-G)* fetuin, *As(+O,-G)* fetuin, and *As(-G)* b. α_1-AGP to verify the studies performed here.

Other methods, such as O-glycanase treatment instead of sodium-borohyride hydrolysis for O-linked glycan chain removal, or use of neuraminidase instead of chemical removal of sialic acid residues, may provide milder approaches for glycan

refashioning of the glycoproteins. As it was just mentioned earlier, the chemical versions for removal of O-linked glycans or sialic acids are performed at extreme enough conditions which may affect the structure of the protein. An enzymatic route would allow specific elimination of the unwanted sugar sites while maintaining an environment that is less denaturing and more favourable. Although these enzymes would prove to be more costly for large-scale systems as compared to chemical versions for removal of sugars, it would be interesting to see how chemically treated versus enzymatically treated acceptors would compare for assaying their respective sialyltransferase.

Bovine fetuin and bovine α_1-AGP were shown to be suitable glycoproteins for modification into acceptors for ST3Gal III and ST3Gal I, although other glycoproteins may prove to be more suitable. Rat asialo-α_1-AGP and bovine prothrombin are known to contain terminal carbohydrate sequences recognized by ST3Gal III, while the antifreeze glycoprotein has often been utilized to assay ST3Gal I, as discussed in the introduction. Although these glycoproteins are rather expensive for use in large amounts, they may prove better at measuring sialyltransferase levels in human systems.

B) Kinetic Studies on Macromolecular Acceptors for ST3Gal I and ST3Gal III

Following completion of the specifically designed bovine fetuin and α_1-AGP macromolecular acceptors and verification of their glycan structures, it was determined that *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP were strict acceptors for ST3Gal III, where *As(+O,-G)* fetuin primarily reacted with ST3Gal I. These acceptors were then prepared for use in human serum to detect and measure ST3Gal I and ST3Gal III levels. Furthermore, to facilitate efficient usage of these macromolecular acceptors in human systems, various kinetic tests were performed to determine the optimal condition parameters for these enzymatic assays. In this particular study, emphasis has been placed on studying the effects of varied incubation time, acceptor concentration, and serum volume, since the other kinetic parameters, e.g. pH, temperature, and CMP-NeuAc concentration optimums have been predetermined, as discussed earlier.

Upon analysis on the effect of incubation time on sialyltransferase activity (pmol NeuAc transferred), linear relationships were observed for all three modified acceptors. The ST3Gal I acceptor, *As(+O,-G)* fetuin, produced an activity rate of 10.30±2.10 pmol NeuAc transferred/hour @ 150 μg concentration. On the other hand, the fetuin and α_1-AGP acceptors for ST3Gal III displayed lower activity rates (0.78±0.16 pmol NeuAc/hour and 0.45±0.09 pmol NeuAc/hour, respectively) suggesting that ST3Gal III may not be present in human serum. A 2 hour incubation period was also used for further test, as previous enzymatic assays on sialyltransferases have utilized 2 hours as the incubation period at 37°C. As well, ST6Gal I assays in human serum were also incubated at 2 hours. Hence, it was concluded that 2 hours of incubation would detect enough

sialyltransferase activity to prevent loss of acceptor integrity and allow uniform comparison between differing sialyltransferases (specifically ST6Gal I). Furthermore, components of serum can be rather ambiguous, resulting in false positives and non-specific reactions, e.g. assaying other sialyltransferases.

Upon initial analysis of the kinetic data obtained for all the macromolecular acceptors, it was evident that *As(+O,-G)* fetuin was the only acceptor that produced significant activity from the human serum samples, ranging from a minimum of 0.061 pmol NeuAc transferred/min (@ 50 μg) to a maximum of 0.250 pmol NeuAc transferred/min (@ 250 μg). In contrast, *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP did not produce significant Δ activities for any concentration of acceptor added. The same result was observed for these acceptors when assessing the effects of varied incubation periods and varied serum content on serum sialyltransferase activity, where maximum activity values did not even reach 0.30 pmol NeuAc transferred/min. In some cases, the activity of the control assays, where the modified glycoprotein acceptor was replaced with deionized water, exceeded values obtained for the test assays, which included acceptors, resulting in negative activity values. Overall, Figures 19, 20, and 23, show that the activities produced by *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP do not reach much above the zero baseline, which may suggest that ST3Gal III is absent in human control serum, as discussed later. Nevertheless, utilization of the ST3Gal III acceptors in the serum samples of renal transplant patients was still performed, as the acceptors could potentially produce differing results such as an elevation in levels pre- or post-transplant. Assessment of the data obtained for *As(+O,-G)* fetuin suggests the presence of endogenous ST3Gal I in human control serum, but at lower levels than that of ST6Gal I.

For comparison purposes, previous studies have utilized *As*-human α_1-AGP, an acceptor for ST6Gal I, in human serum, obtaining Δ activities of 2569 dpm (Thorne-Tjomsland, *et al.*, 2000). The maximum activity produced by *As(+O,-G)* fetuin, exhibited only a small fraction (~19.51%) of the activity produced by asialo-human α_1-AGP, where the *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP acceptors demonstrated even smaller fractions (1.48 % and 0.92 % respectively).

Further analysis was emphasized on the *As(+O,-G)* fetuin acceptor, where the Δ activity maximized at 250 μg of glycoprotein added (0.250 pmol NeuAc transferred/min), which was the highest concentration used to measure the activity. At higher concentrations, the Δ activity seemed to approach a plateau, almost resembling a typical Michaelis-Menten plot. Higher concentrations of acceptor were used, which unfortunately results in the fetuin acceptors coming out of solution. Therefore 150 μg of acceptor was utilized for further tests, as it was the concentration at which modified fetuin stays in solution. Although slopes of Lineweaver-Burke plots are largely influenced by smaller concentrations, this method was used in this study, since it was commonly used in our lab and allows uniform comparison between K_m and V_{max} values of other acceptors used in our lab. The Lineweaver-Burke plot concurred K_m to be 0.48±0.14 mM with a V_{max} of 1.47±0.30 pmol of NeuAc transferred per minute. In comparison to K_m values previously reported for a different ST3Gal I acceptor, antifreeze glycoprotein (Rearick *et al.*, 1979; Williams *et al.*, 1995), the K_m value obtained for *As(+O,-G)* fetuin was more comparable to the 0.39 mM values obtained by Rearick *et al.* (1979). Higher K_m values (0.84 and 1.18 mM) were obtained for native and recombinant forms of ST3Gal I, respectively (Williams *et al.*, 1995). Variations in K_m values reported

in this study versus literature sources are most likely due to the utilization of differing glycoprotein acceptor substrates, where fetuin was used here in comparison with the antifreeze glycoprotein used in the previous studies. In addition, our acceptor was employed against human serum, where the literature K_m determination was based on employment in porcine submaxillary gland.

The effect of serum volume added on serum sialyltransferase activity was also examined, with As*(+O,-G)* fetuin as the macromolecular acceptor, since exact concentrations of a particular serum sialyltransferase would be difficult to determine, as the contents of human serum can be rather ambiguous, containing a variety of components. Previous studies in our laboratory have often involved the addition of 25 μl of serum to each sample assay, which seemed a large amount for measuring the Δ activity of the serum sialyltransferase. Figure 23 displayed hyperbolic relations between serum sialyltransferase activity and serum volume for *As(+O,-G)* fetuin, where the Δ activity started to approach zero order kinetics at approximately 30 μl of serum added. These results also show that using a 25 μl sample of serum would be optimal for assaying serum sialyltransferases. In order to obtain optimal serum sialyltransferase activity, an enzyme concentration at the higher end of the first order kinetics portion must be chosen, which was shown to be 25 μl obtained by my results.

Although the results obtained here acquired indicate low circulating levels or the absence of the ST3 enzymes in human serum, several studies led us to infer a dissimilar outcome. There are currently no studies that indicate the presence of ST3Gal III in human serum, but several research groups have measured α2,3-sialylation in the sera of other mammals, including bovine (Sherblom *et al.*, 1986) and rat (Kaplan *et al.*, 1983;

Ratnum *et al.*, 1987; Maguire *et al.*, 1996) systems. Previous reports have shown an increase of ST6Gal I in the liver and serum of rats during inflammation or the acute phase response (Kaplan *et al.*, 1983), where the release mechanism has been shown to be via Cathepsin D-like proteinase and to act as an acute phase reactant (Lammers and Jamieson, 1988). Release of ST6Gal I involving Cathepsin D has also been observed in the mouse and guinea-pig (Lammers and Jamieson, 1990), and the rat and mouse in whole-cell systems (McCaffrey and Jamieson, 1993). More importantly, similar studies have been extended to other sialyltransferases, where the same release by Cathepsin D-like proteinase has been demonstrated for ST3Gal III in rats (Richardson and Jamieson, 1995). Recently, active secreted forms of ST3Gal I have been detected when hST3Gal I is expressed in COS-7 cell lines (Vallejo-Ruiz *et al.*, 2001). Further evidence has shown secretion of ST3Gal I and ST6Gal I in culture cell media when expressed in Chinese hamster ovary cells (El-Battari *et al.*, 2003).

It is still uncertain whether ST3Gal III and/or ST3Gal I are present as soluble forms in human serum, though the results obtained do seem to indicate their absence. Another explanation for their lack of detection in the serum may be attributed to the regulation of sialyltransferase expression and/or inhibition. ST6Gal I has been found to exist as an active monomer or as a dimer held by the disulfide bond of the conserved cysteine residues within the Golgi (Ma and Colley, 1996). The dimer form was shown to exhibited weak catalytic activity, yet still retained the ability to bind galactose or galactose-terminated substrates, thus providing another control level of cellular enzyme activity. It is possible to suggest that ST6Gal I, due to what is said in the previous statement and to its abundance in serum, may engulf the acceptor substrates of ST3Gal

III and ST3Gal I, leading to the low detection level of the ST3 enzymes. Another study provided evidence suggesting over expression of one particular sialyltransferase might affect the sialylation potential of the cell (Georgopoulou and Breen, 1999). Since ST6Gal I is expressed in elevated levels in renal transplant patient serum, the sialylation action of the ST3Gal III and ST3Gal I may be hindered. Aside from control by other sialyltranferases, inhibition may be due to other components in serum. Some common sialyltransferase inhibitors tend to mimic the substrates that they recognize, where studies have shown 5'-cytidine diphosphate (similar to CMP-NeuAc, which is the naturally occurring donor substrate for these enzymes) was the best inhibitor for α2,3-sialyltransferases (Kleineidam et al., 1997). A hexapeptide inhibitor, NH_2-GNWWWW (NH_2-Gly-Asn-Trp_4), known to strongly inhibit ST3Gal I, can act as a generic inhibitor of the N- and O-glycan-specific sialyltransferases in mammalian cells, which may result in *in vivo* reduced NeuAc expression of cellular glycoproteins (Lee *et al.*, 2002). Other inhibitors include Ca^{+2}/calmodulin antagonists and phosphatase inhibitors (Reboul et al., 1992), and peroxisome proliferators (Fayos and Bartles, 1994).

C) Preliminary Studies on ST3Gal I and ST3Gal III levels in Renal Transplant Patient Serum

There are several advantages for developing macromolecular acceptors for ST3Gal I and ST3Gal III in humans systems, but the primary motive is to allow measurement of these sialyltransferases in renal transplant patient serum. Previous studies in our laboratory (Thorne-Tjomsland *et al.*, 2000) have demonstrated elevated levels of a specific sialyltransferase, ST6Gal I, in renal transplant patient serum, pre-transplant, which has been implicated with adverse post-transplant events such as DGF and poor survival outcome. Post-transplant serum samples have now been collected from a group of 35 patients, at specified time intervals subsequent to transplantation. The principal goal, for efficient usage of these samples, is to track the levels of ST6Gal I, ST3Gal III, and ST3Gal I longitudinally during DGF-prone periods, to correlate peak levels between different sialyltransferases or between levels of individual sialyltransferases and clinical parameters signalling DGF. It is possible that ST6Gal I affects the sialylation of adhesion molecules which are up-regulated during ischemia/reperfusion, particularly α2,6 sialylated E-selectin, ICAM-1, and VCAM-1 (Hanasaki *et al.*, 1994) . However, it cannot be stated with complete certainty that increased levels of ST6Gal I contribute to the development of DGF. ST3Gal III may also play a role in the progression of DGF, as sLe^x, a ligand for the selectins, is α2,3-sialylated. ST3Gal I may also contribute to α2,3-sialylation of O-linked oligosaccharides on glycoproteins up-regulated during ischemia/reperfusion. Therefore, with the production of ST3Gal III and ST3Gal I macromolecular acceptors, beneficial information can be obtained.

Given that the primary intention for developing these glycoprotein acceptors was to allow measurement of the α2,3-sialyltransferases in renal transplant patient serum, it only seems logical to provide a preliminary evaluation on what kind of levels are present, since there has been no literature evidence of ST3Gal III and ST3Gal I being assayed for in human serum. Hence, the acceptors that were modified in this study, along with the ST6Gal I acceptor, *As*-human α_1-AGP, were all assayed against one patient's set of serum samples, where one sample was obtained prior to transplantation and the rest of the samples were collected at various time points subsequent to the patient's renal transplant event. Greater attention was focused on the activity levels of ST3Gal III and ST3Gal I, where as the ST6Gal I activity was measured mainly for comparison purposes, even though the information obtained from these preliminary tests would give insight on when ST6Gal I peak levels would occur. In addition, correlations were not made on peak levels between the different sialyltransferases, as this early examination may be biased to this particular patient. Furthermore, the longitudinal analysis is a blind study; hence, whether the patient has developed DGF or other poor post-transplant event outcomes is unknown. Initial analysis of ST3Gal III and ST3Gal I activity in human control serum, discussed in the previous section, suggests a lack of these enzymes in human serum, especially when compared to the literature value reported regarding ST6Gal I activity. These results may further predict the possibility of their low level detection or absence in the renal transplant sera.

As expected, elevated levels of ST6Gal I were observed, displaying activities ranging from 1472.7 to 3525.8 dpm. In contrast, ST3Gal III and ST3Gal I activities were much lower comparatively, as shown in Figure 24. Furthermore, higher activities were

detected by *As(+O,-G)* fetuin, as compared to the ST3Gal III acceptors, indicating that ST3Gal I would be present in higher quantities than ST3Gal III. Both *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP produced the lowest activity when assayed against renal transplant patient sera, but did not differ from the levels detected in human control serum, reinforcing the assumption that ST3Gal III is absent in human serum altogether, even after the potential up-regulation of proinflammatory cytokines and adhesion molecules surrounding transplantation. The employment of *As(+O,-G)* fetuin in the patient's set of serum samples demonstrated similar enzyme activities detected in human control serum as well, suggesting that there was also no elevation of ST3Gal I levels observed in renal transplant patients. As well, the activities of ST3Gal I seem insignificant when compared to the abundance of circulating ST6Gal I, but may provide some sialylation contribution to molecules up-regulated during ischemia/reperfusion injury, DGF, or other poor post-transplant outcomes.

Although the results obtained for ST6Gal I levels are difficult to decipher, especially as the results may be exclusive to this particular patient and the patient parameters are unknown, the information obtained is still very interesting. Peak activities were detected at the 4-5 month range (~3500 dpm), about a 35% increase in activity in comparison with the pre-transplant sample (2605.1 dpm). Although it cannot be stated with certainty, the peak activity may represent a possible rejection episode. At day 5, ST6Gal I activity is lowest (1472.7 dpm), almost a 50% decrease in activity from pre-transplant. This low peak, although inconclusive, may be due to immunosuppressant treatments, but it is difficult to determine without patient parameters.

When comparing the measured ST3 enzyme activity to the levels detected for ST6Gal I, it almost appears as though the activities of ST3Gal I and ST3Gal III are relatively insignificant. However, these preliminary tests do provide information on what kind of ST3 levels to expect. Furthermore, ST6Gal I has been shown to be the most abundant and easiest to purify of all the sialyltransferases (Weinstein *et al.*, 1982a; 1982b). Hence, comparison of ST3 activities to ST6Gal I may give an impression that the ST3 enzymes are non-existent in human serum. The measurement of these enzymes utilizing the modified bovine glycoproteins, as well as correlating peak levels between the different sialyltransferases may still give insight on the variations of sialylation patterns occurring in the kidney transplant patients, and may or may not indicate poor prognosis of post-transplant events.

Although this preliminary test provides only minimal information on the differential expression of soluble sialyltransferases in the serum of kidney transplant patients, aberrant sialylation is not uncommon. ST6Gal I, ST3Gal III, and ST3Gal I, or the presence of the terminal sialic acid that they catalyze, have all been shown to be expressed at variable degrees. It has long been known that cancerous and disease states often express more heavily sialylated glycans on their surface and that this feature sometimes correlates with invasion (Dall'Olio and Chiricolo, 2001). For example, poor survival has been linked to elevations in α2,6-linked associated sialic acids and sialyltransferase levels in oral cavity cancer patients as opposed to healthy individuals (Raval *et al.*, 2003). Furthermore, enhanced or differential expression of ST6Gal I has been detected in several carcinomas, including cirrhotic liver and hepatocellular carcinoma (Cao *et al.*, 2002), gastric cancer (Gretschel *et al.*, 2003), colorectal cancer

(Xu *et al.*, 2003), and cervical squamous cell carcinoma (Wang *et al.*, 2003). Variable levels of ST3Gal III expression and activity have also been reported, where gastric carcinomas (Gretschel *et al.*, 2003), breast carcinomas (Recchi *et al.*, 1998) and malignant gliomas (Yamamoto *et al.*, 1997) demonstrated elevated levels in comparison to their non-cancerous counterparts, while ptyergium cells displayed decrease levels when compared to normal conjunctiva (Creuzot-Garcher *et al.*, 1999). Similarly to the case of ST3Gal III, there have been no studies in the literature that indicate the presence of ST3Gal I in a membrane-free soluble form. However, elevated ST3Gal I levels were detected in primary breast carcinomas, due to the aberrant glycosylation of MUC1 mucin, which is normally expressed on the lumenal surface of endothelial cells and contain acceptor sites for ST3Gal I. In addition, overexpression of ST3Gal I mRNA has been observed in human colorectal (Schneider *et al.*, 2001), breast (Burchell *et al.*, 1999), and gynecological (Wang *et al.*, 2002) carcinomas.

Moreover, several research groups have applied variable methods to measure differential sialyltransferase levels, ranging from serum sialic acid spectrophotometric measurements and sialyltransferase activity using radiometric assay techniques (Raval *et al.*, 2003) to observe changes in sialyltransferase mRNA expression between malignant and healthy tissue cell cultures (Wang *et al.*, 2003). Antibodies that react well with particular sialyltransferases have also been employed to measure their presence or expression (Gangopadhyay *et al.*, 1998; Lise *et al.*, 2000). Although each technique may prove advantageous for a particular study, disadvantages may arise from utilizing these techniques for other types of research. However, it would be interesting and potentially

beneficial to apply these techniques here in future studies to observe whether similar or dissimilar results are obtained.

CONCLUSION

In conclusion, the results indicate that the development of macromolecular acceptors for ST3Gal III and ST3Gal I has been successfully attained. *As(-O,-G)* fetuin and *As(-G)* bovine α_1-AGP reacted well with recombinant ST3Gal III, while *As(+O,-G)* fetuin was a good acceptor for ST3Gal I and less efficiently, ST3Gal III, as expected. The utilization of glycoproteins as acceptors more closely mimics *in vivo* conditions. Furthermore, the chemical and enzymatic treatments of the bovine glycoproteins, fetuin and α_1-AGP, specifically targeted to remove unwanted sugars while retaining essential acceptor sites, provided a simple and rapid technique for remodelling precise acceptors for the sialyltransferase being assayed. Employment of commercially available recombinant or purified sialyltransferases also offered a prompt and qualitative means for testing the macromolecular acceptors, allowing determination for the presence or absence of particular acceptor sites as compared to traditional analytical methods. Results obtained also reaffirmed initial speculations made by a prior graduate student in our laboratory regarding the absence of O-linked carbohydrates in bovine α_1-AGP.

Kinetic tests revealed the potential levels of ST3Gal III and ST3Gal I in human control serum, revealing a greater presence of ST3Gal I. In comparison to ST6Gal I levels, results from kinetic studies performed on the glycoprotein acceptors implied low levels of ST3Gal I (~1.48 %) in human control serum by a lack of activity produced by *As(+O,-G)* fetuin, while ST3Gal III levels (~0.92 %) were shown to be lower and potentially non-existent, with *As(-O,-G)* fetuin and *As(-G)* α_1-AGP producing poor Δ activity in the same serum samples. Unlike the acceptors for ST3Gal III, K_m and V_{max} values were achievable for *As(+O,-G)* fetuin. Furthermore, optimal assay conditions

were determined for these acceptors in human systems, which were useful when assayed against renal transplant patient serum.

The modified glycoprotein acceptors, when employed against a set of serum samples obtained from a renal transplant patient pre- and post-transplant event, exhibited similar Δ activities produced in human control serum. Poor ST3 levels were obtained, in comparison to ST6Gal I, which reinforced their possible absence in secreted, soluble form in human serum altogether. These preliminary studies may indicate the possible outcome for employing these acceptors in the longitudinal study of sialyltransferase levels in the serum of renal transplant patients.

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