Development of Chemical Tools to Discover and Characterize Sialic Acid Mediated Interactions

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Glycosylation refers to the addition of carbohydrates onto proteins, lipids, and other biomolecules. Proteins and lipids on the cell surface are frequently found to be glycosylated. This glycosylation can have a number of functions, one of which is to mediate specific binding interactions between the glycans of glycoconjugates and glycan binding proteins. Such glycan-mediated interactions are implicated in numerous cell-signaling processes. Many of these glycan-mediated interactions involve a class of carbohydrates known as sialic acids. The presence of sialic acid on a glycan can recruit specific sialic acid binding partners. Alternatively, sialic acids can mask underlying glycan epitopes, thereby preventing protein binding. There are several challenges in studying sialic acid mediated interactions. **Chapter 1** describes the importance of sialic acids as well as the challenges in studying their interactions.

Sialidases are enzymes that hydrolytically cleave sialic acid from its underlying glycan. In this regard, sialidases have the potential to impact numerous cellular processes in a dynamic fashion. Relatively little is known about the substrates of mammalian sialidases. **Chapter 2** describes a novel assay that takes advantage of chemical labeling of sialic acids to characterize sialidase substrate specificity towards various complex glycans. **Chapter 3** focuses on efforts to develop assays to identify the protein components of sialidase substrates in a cellular context. Finally, **Chapter 4** describes the development of a series of probes to discover novel sialic acid mediated interactions. Taken together, this work describes several new techniques that will allow for better understanding of sialic acid mediated interactions.

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Prior Publications

Research articles

- **Parker, R. B.,** McCombs, J.E. and Kohler, J. J. Sialidase specificity determined by chemoselective modification of complex sialylated glycans. *ACS Chemical Biololgy*, 2012, 21;7(9), pp 1509-14.

Review articles

- **Parker, R. B.** and Kohler, J. J. Regulation of intracellular signaling by extracellular glycan remodeling, *ACS Chemical Biology,* 2010, 5(1), pp 35-46
- Pham, N. D., **Parker, R. B.**, Kohler, J .J. Photocrosslinking approaches to interactome mapping, *Current Opinion in Chemical Biology*, 2013, 17(1) pp 90-101

Chapter 1: Introduction to sialic acids

Introduction

The functions of proteins are tightly regulated by post-translational modifications. One of the most abundant modifications is glycosylation, which is the covalent addition of glycans to the protein. In fact, it is estimated that over 50% of proteins are glycosylated. (*1*) Broadly speaking, glycosylation can affect proteins in one of two ways: by altering its intrinsic structure or by recruiting specific glycan binding proteins. (*2*)

Most glycosylation takes place in the secretory pathway, which is composed of the endoplasmic reticulum (ER) and the Golgi apparatus. Proteins destined for secretion are translated into the ER and transported to the Golgi on their way to the cell surface. The ER and Golgi contain a diverse set of enzymes responsible for the glycosylation of these proteins. Along with these enzymes are the carbohydrates destined for addition onto the growing glycan chain on these proteins. Before these carbohydrates are added onto glycans they must first be activated by conjugation with a high-energy molecule such as CTP, UTP, or GTP. This conjugation is the source of energy for the transfer reactions and the resulting sugar-nucleotide is referred to as the “activated sugar”.

Glycans are divided onto two broad categories, *N*-linked and *O*-linked. This designation is based on the amino acid side chain to which they are attached. *N*-linked glycans are linked to asparagine residues. The consensus sequence for *N*-linked glycans is NxS/T. *N*-linked glycans can affect protein stability and activity. For example, the presence of *N*-linked glycans is critical for the activity of the hormone erythropoietin, which regulates red blood cell production. Mutation of *N*-linked glycosylation sites decreases the affinity of erythropoietin for its receptor and dramatically decreases its serum half-life. (*3*) *N*-linked glycans are first synthesized as a glycolipid: $Glc_3Man_9GlcNAc_2$-P-P-Dolichol. The glycan portion of this glycolipid is transferred *en masse* from the lipid to the destination

protein by a complex of proteins termed oligosaccharide transferase (OST). See Figure 1.1a for examples.

Unlike *N*-linked glycans, *O*-linked glycans are attached to serines or threonine residues. While there is no consensus sequence for *O*-linked glycosylation several trends in amino acid composition are frequently found around these sites; adjacent amino acids tend to include proline and tend to lack acidic or bulky amino acids. (*4*) One common class of *O*-linked glycans is the mucin type, which is critical for development (*5, 6*) and leukocyte extravasation. Granulocytes express the carbohydrate antigens sialylLewis X (Figure 1.1b) and these cells are recruited to activated endothelium by interactions between the endothelial selectin (E selectin) and the sialylLewis X antigen. (*7*) These glycan-protein interactions allow the leukocyte to bind to the endothelium as a prerequisite for crossing into the surrounding tissue.

Once the initial *N*- or *O*-linked glycans are in place additional modifications can be made by glycosidases and glycosyltransferases. These ER and Golgi resident enzymes trim or add monosaccharides to generate a diverse repertoire of glycan structures. (*8-10*) See Figure 1.1 for examples.

Glycosylation is a regulated process with implications for cellular signaling

The concept of biological regulation frequently gets misapplied because there is no universally accepted definition of what constitutes regulation. Therefore, if one wishes to describe a process as regulated it is helpful to first define the term. One definition of regulation is a process that modulates the frequency, extent, or rate of another process. (*11*) In this sense, glycosylation is a regulated process. The regulation of glycosylation takes place at several levels. The frequency, rate, and extent of glycosylation is dictated by the presence of specific sets of glycosyltransferases and glycosidases that dictate the possible combinations of carbohydrates that can be added to the growing glycan chains on proteins as

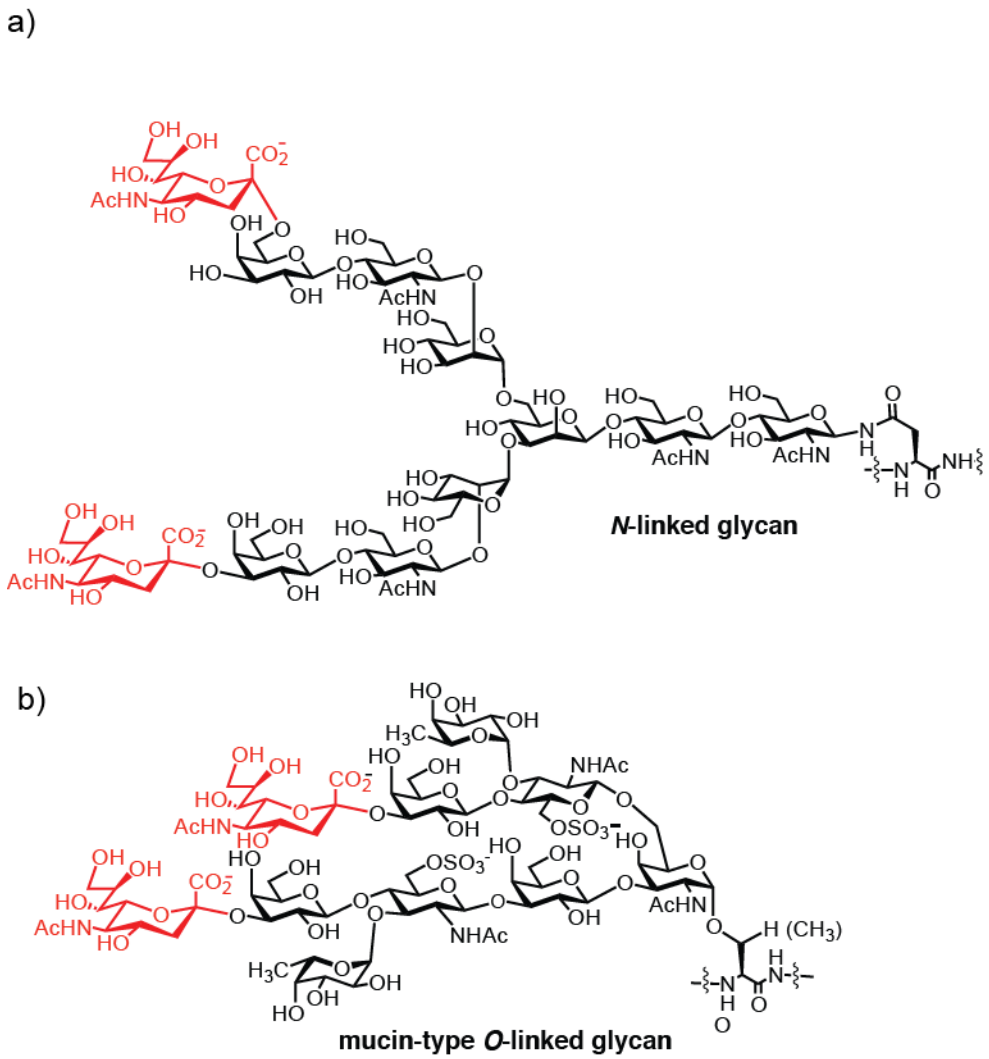

Figure 1.1 Multiple types of glycans present sialic acid. a) An *N*-linked glycan displaying sialic acid in both an α2,6 linkage (top) and an α2,3 linkage (bottom). b) A mucin-type *O*-linked glycan presenting sialic acid in two α2,3 linkages. Sialic acids are highlighted in red.

they pass through the secretory pathway (Figure 1.2). The extent of glycosylation can be influenced by subtle changes in the concentration of the activated sugar donors. *N*-acetylglucosamine (GlcNAc) is a very common carbohydrate found at the core of all *N*-linked glycans as well as the branch points of glycan chains. UDP-GlcNAc is the activated form of this sugar and the concentration of UDP-GlcNAc influences the degree of branching on glycans, which leads to very different glycan structures. (*12*) These altered glycan structures have potential

functional implications as increased branching of GlcNAc offers protection against proteolysis to certain proteins, including those of the integrin family. (*13*) Whether other glycan structures are regulated by the concentration of their constituent activating sugars is unclear at this point but it represents an intriguing possibility.

Even once a mature glycoprotein exits the secretory pathway and arrives at its final destination it is still subject to regulation by hydrolytic enzymes that reside alongside the glycoprotein. (*14*) (Figure 1.2). Heparan sulfate proteoglycans (HSPG) are regulatory components of Wnt signaling that reside in the extracellular matrix. Wnt binds directly to HSPG, which sequesters it from its receptor. The affinity of Wnt for HSPG is determined in part by the extent of sulfation of HSPG. A class of extracellular enzymes known as sulfatases cleaves the sulfate residues from glucosamine-6-sulfate in HSPGs, thereby modulating the affinity for Wnt and consequently its signaling capacity. (*15, 16*)

Sialic acids are important mediators of protein and cellular interactions

One of the key carbohydrates found in glycans is sialic acid. Sialic acids are 9-carbon, α-keto acids typically found at the non-reducing end of glycan chains. The terminal position of sialic acid puts it in a prime position to mediate binding events. Sialic acids are essential for development as knockout of the enzyme responsible for the first committed step in sialic acid biosynthesis causes embryonic lethality in mice. (*17*)

Sialic acids are a family of fifty structurally related members. In mammals, the major forms of sialic acids are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) (Figure 1.3). Structural variations can also occur in the form of the linkage between the sialic acid residue and the underlying glycan (Figure 1.3) leading to many possible combinations of sialylated structures.

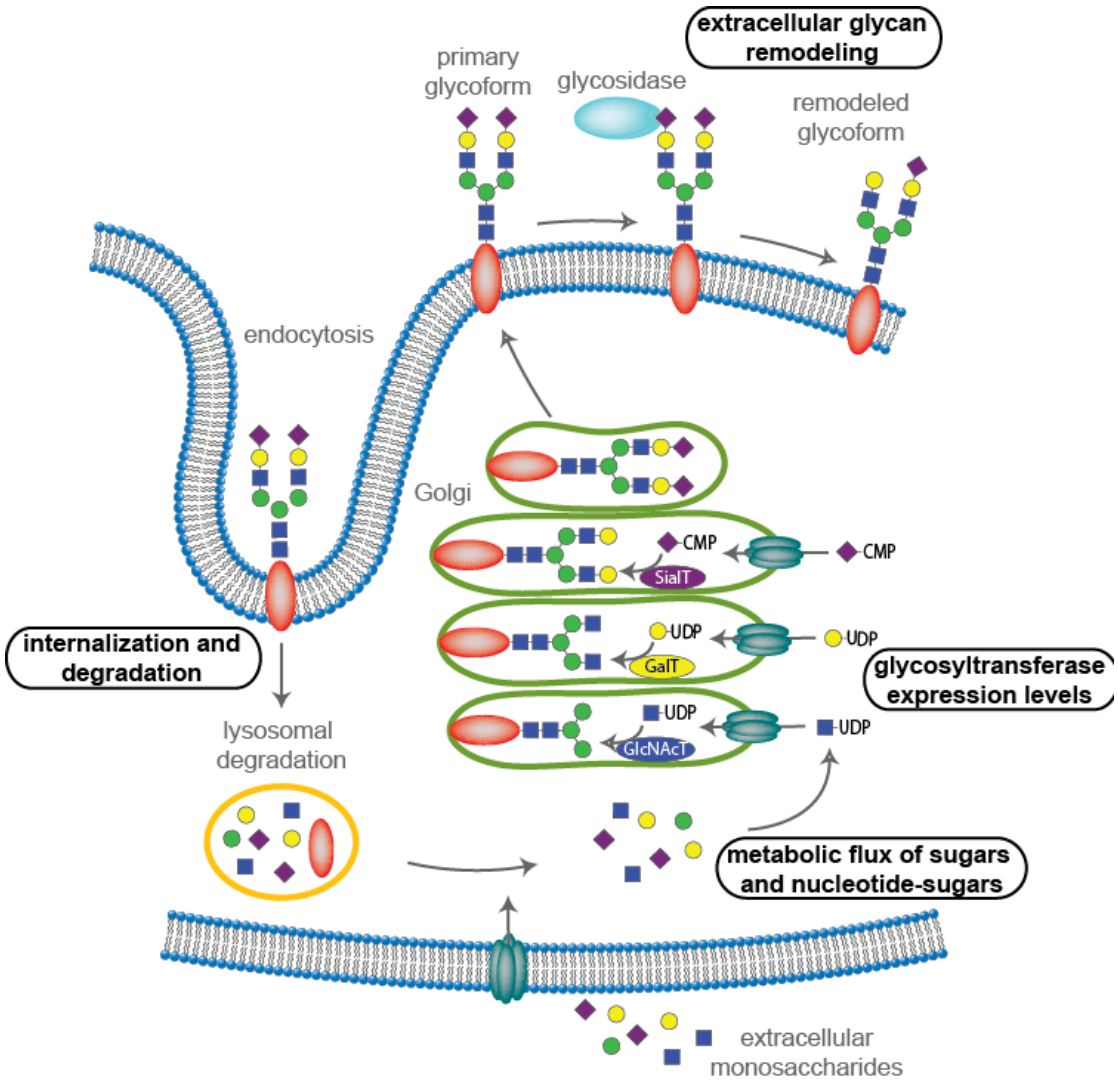

Figure 1.2. Glycosylation is a regulated process. Glycosylation can be regulated at multiple points. Monosaccharides need to be activated by conjugation to high-energy molecules prior to being used as a substrate for glycosyltransferases. The metabolic flux of these activated sugars can influence the structures of glycans produced. The expression levels of glycosyltransferases dictate the possible combinations of monosccharides that can be added to the glycan. Glycosidases can dynamically remodel glycans in response to various stimuli.

The importance of sialic acid linkage in biological function is perhaps best exemplified by the interaction between sialic acids and influenza virus. Influenza viruses express coat proteins called hemagglutinin (HA) that bind sialic acids and are necessary for infecting host cells. (*18*) Humans predominantly express sialic

acids in α2,6 linkage in their upper respiratory tract epithelium. HA from human specific influenza bind preferentially to α2,6 linked sialic acids. Birds, meanwhile, express predominantly α2,3 linked sialic acids on their upper respiratory tract epithelium and HA from avian specific influenza prefer binding to α2,3 linked sialic acids. This sialic acid linkage specificity of HA is one of the major determinants of the species barrier preventing cross-species transmission of influenza. (*18*)

The presentation of sialic acid is regulated by two classes of enzymes in mammals: sialyltransferases and sialidases. Sialyltransferases reside in the Golgi apparatus where they catalyze the addition of sialic acids onto the glycan components of glycoproteins. Sialidases exist in numerous cellular compartments where they hydrolyze sialic acids exposing the underlying glycan.

Sialic acid mediated interactions in mammals

Sialic acids contain several chemical properties that combine to make them unique amongst carbohydrates. With a nine-carbon framework, they are the largest of the commonly occurring carbohydrates. The acidic carboxylate gives them a negative charge at physiological pH. These features combined with their terminal position on glycan chains puts sialic acid in a prime position to mediate interactions on the molecular and cellular level.

Structural variations to the sialic acid can impact its biological function. SIGLECs (sialic acid–recognizing Ig-superfamily lectins) are a class of immunomodulatory receptors that bind sialic acid to influence immune response through their ITIM (immunoreceptor tyrosine-based inhibition motif) or ITAM (immunoreceptor tyrosine-based activating motif) domains. (*19*) CD22 is a SIGLEC that both binds to and presents sialic acid, which leads to sialic acid dependent oligomerization. This oligomerization of CD22 creates an inhibited state, thereby preventing B-cell signaling. Acetylation of the 9-position of Neu5Ac on CD22 disrupts the binding

of adjacent CD22 molecules. Therefore acetylation of sialic acid can act as an activating signal leading to immune activation. (*20*)

Myelin-associated glycoprotein (MAG) is one of the most conserved members of the SIGLEC family. MAG is critical for the stability of myelin as evidenced by defects in mice lacking it. (*21*) MAG binds to sialylated glycolipids known as gangliosides and inhibits neuronal outgrowth. Intriguingly, ablation of MAG's interaction with gangliosides by treatment with sialidase enhances neuronal outgrowth and improves recovery of mice with spinal cord injury. (*22*)

One common structural variant of sialic acid in mammals is Neu5Gc, which is the hydroxylated form of sialic acid. Neu5Gc is synthesized by hydroxylation of the precursor Neu5Ac. Intriguingly, humans have lost the enzyme responsible for this hydroxylation step and therefore, do not make Neu5Gc. This enzyme remains in our closest evolutionary relatives, the chimpanzees. Why the loss of enzyme occurred in humans is unexplained but it is noteworthy that Neu5Gc from dietary sources (such as red meat) can be incorporated into the cell surface. Indeed, antibodies against Neu5Gc are found in humans and are hypothesized to play a role in chronic inflammation as a result of excessive red meat consumption. (*19*)

While many important functions have been assigned for sialic acid mediated interactions, it is likely that many more are yet to be discovered. Many of the reported sialic acid mediated binding events in the literature were discovered using the so-called 'neuraminidase effect'. This occurs when sialic acid is enzymatically removed by the researcher and interactions between components are observed to be either established or ablated. (*23*) This approach yields no information about the identities of the interacting partners and is of little use in discovering novel interactions. One of the aims of my research is to develop chemical tools to allow for the discovery of novel sialic acid mediated interactions.

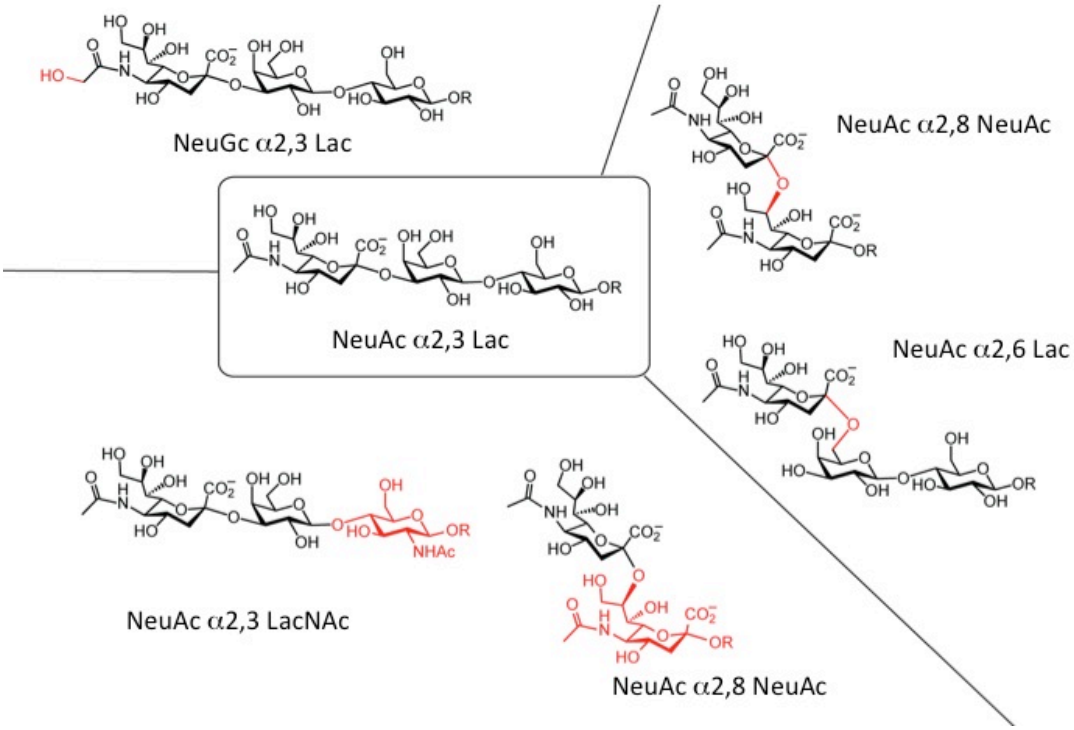

Figure 1.3. Sialic acids occur in diverse presentations. A prototypical sialic acid-containing glycan is shown at center. Modifications such as hydroxylation of the *N*-acyl position are common (top). Sialic acids are found linked to their underlying glycans in a number of different linkage patterns (right). The glycan to which sialic acid is attached is also highly variable (bottom). These variations occur in a combinatorial fashion, leading to myriad possible presentations of sialic acid. The red highlights denote the structural variations mentioned above.

Mammalian sialidases are implicated in numerous signaling pathways

Sialidases are a class of enzyme responsible for hydrolyzing sialic acids from the underlying glycan. Sialidases are expressed widely in nature. While viral and bacterial sialidases have been studied since the 1940s, mammalian sialidases have only recently begun to be characterized. Sialidase activity was detected in mammalian cells as early as the 1960s but the identity of the enzymes responsible remained unknown. In the 1980s biochemical characterization placed the number of distinct sialidases at three, each with distinct subcellular localizations: lysosomal, cytosolic and plasma membrane. (*24*)

The first sialidase to be cloned was neuraminidase 1 (Neu1). (*25*) Further molecular cloning revealed a total of 4 genes encoding sialidases known as NEU1-4 in humans. Of these, NEU1 is the best characterized. It forms an obligate protein complex with Protective Protein/Cathepsin A (PPCA) and beta-galactosidase (*26*) in the lysosome where it hydrolyzes sialic acids from proteins and lipids destined for degradation. Intriguingly however, this is not its only role. NEU1 has been found on the cell surface as well where it participates in cellular signaling events. NEU1 desialylation of Toll-like receptor 4 appears to enhance receptor activity (*27*) This finding was made possible by the fact that Neu1 appears unique among sialidases in that it forms stable protein complexes with its substrates allowing for co-immunoprecipitation experiments to be performed.

Defects in NEU1 in humans result in the lysosomal storage disorder known as sialidosis. (*28*) This disease is caused by abnormal accumulation of sialylated glycoproteins and lipids in the lysosome. Sialidosis is divided into two types: I and II. Sialidosis type I symptoms include bilateral macular cherry-red spots, progressive vision impairment and myoclonus syndrome. Type II sialidosis patients present skeletal dysplasia, mental retardation and hepatosplenomegaly. Patients typically present symptoms at birth or in the first year of life. Type II sialidosis is usually fatal within the second decade of life while type I is not

usually fatal. Current treatments are limited and rely on treating the symptoms of sialidosis such as anti-seizure and anti-inflammatory medications. (*28*)

The observation that the severity of the disease is correlated with residual sialidase activity suggested the possibility that restoration of the enzyme or its activity might be an effective cure for the disease. Enzyme replacement therapy (ERT) has been investigated for a number of lysosomal storage disorders. Because of their lysosomal localization exogenously added enzymes are endocytosed and some of the activity eventually reaches the lysosome. Neu1 exogenously added to *Neu1*-/- mice reduced the lysosomal burden in several tissues. Unfortunately, after several weeks of treatment the mice generated an immune response against the exogenous protein and long-term treatment could not be evaluated. (*29*)

NEU2 is known as the cytosolic sialidase due to its unique localization. Unlike the other three mammalian sialidases, NEU2 is not membrane-associated but is expressed in a diffuse, soluble pattern in the cytosol. NEU2 is expressed predominantly in muscle tissue where it is implicated in muscle development. NEU2 expression correlates with myoblast differentiation into myotubes and is inversely correlated with muscle hypertrophy and atrophy. (*30*)

It was surprising to find a sialidase with exclusively cytosolic localization as sialic acid-containing glycoconjugates are generally thought of in the extracellular space. However there is some recent evidence that such glycans may exist in the cytosol as well. There is evidence of glycosylated proteins entering the cytosol as a function of ER-associated degradation (ERAD). In this way, misfolded glycoproteins are transported out of the ER and into the cytosol to await degradation by the proteasome. These proteins are derived from the ER so they may or may not contain sialic acid as sialylation typically occurs in the Golgi (*31*) However, there are also examples of free oligosaccharides in the cytosol and indeed, some of these oligosaccharides contain sialic acid. Recently it was

shown that the stomach cancer cells MKN7 and MKN45 contain sialylated free oligosaccharides that reside in the cytosol. It is likely that these oligosaccharides are leaked out of the lysosome as these cell types are known to contain lysosomes with porous membranes. (*32*) In addition to glycoproteins and free oligosaccharides there are also reports of sialylated lipids (known as gangliosides) occurring in the cytosol. (*31*) It is conceivable that there may be more glycans in the cytosol than previously acknowledged and NEU2 may be involved in their life cycle.

NEU2 is implicated in signaling events in cancer cells. Overexpression of murine Neu2 in the highly invasive B16-BL6 mouse melanoma cell line causes a dramatic decrease in metastasis. (*33*) Additionally overexpression of NEU2 in the chronic myelogenous leukemia cell line K562 restores sensitivity to apoptotic stimuli by decreasing the anti-apoptotic proteins Bcl-XL and Bcl-2. (*34*) How NEU2 exerts these effects is unknown because the substrate(s) of NEU2 in these contexts remains unidentified. Intriguingly however, this group demonstrated that the substrate of NEU2 in K562 cells might be a sialylated protein. After performing a sialic-acid-specific lectin blot against the cytosolic fractions of K562 cells expressing NEU2 they observe a decrease in several bands relative to samples not expressing the enzyme. This implies that there are cytosolic sialylated proteins being desialylated by NEU2 in the cytosol. However, the identity of these proteins remains unknown.

NEU3 is also known as the ganglioside sialidase because it is highly specific for sialic acid-containing glycosphingolipids. (*35*) NEU3 localizes generally to the plasma membrane and is found concentrated around membrane ruffles in response to growth factors such as EGF. Interestingly, NEU3 has been shown to hydrolyze gangliosides on adjacent cells thereby mediating cell-cell communication.

NEU3 has been shown to have several roles in cell signaling. NEU3 is a positive regulator of neurite formation in mice (*36*) and in human and is involved in the regulation of regeneration of rat hippocampus neurons.

NEU3 is implicated in the pathogenesis of cancer. It is found to be upregulated in a number of carcinomas including colon, renal and prostate. (*37*) Additionally, NEU3 has been shown to activate Interleukin-6 (IL-6) and NEU3 mediated IL-6 activation leads to PI3K/Akt signaling pathway and thus may contribute to a malignant phenotype, including suppression of apoptosis and enhanced cell motility. Silencing of NEU3 in HeLa cells results in decreased Bcl-XL and overexpression results in the opposite effect. The cancer cell lines HT-29 and MCF-7 showed similar effects upon NEU3 silencing but the non-cancerous WI-38 and NHDF cell lines showed no such changes.(*38*) This suggests that NEU3 has cancer specific effects.

Relatively little is known about NEU4. Neu4 was originally identified from database searches based on its homology to the murine sialidase Neu3 and has since been found in humans as well. (*39*) NEU4 has two splice forms, the long and the short form. There is discrepancy as to the subcellular localization of this enzyme. Based on overexpression studies, the long form has been reported to localize to the lysosome (*40*) or to the mitochondria, while the short form has been observed in the ER (*41, 42*). Unlike NEU3, expression of NEU4 appears to downregulate neurite formation. (*43*)

Klotho is often referred to as the anti-aging hormone. Mice deficient in *Klotho* demonstrate a syndrome similar to enhanced human aging; presenting symptoms including impaired angiogenesis and increased arteriosclerosis. (*44*) Klotho has recently been shown to have sialidase activity as well. (*23, 45*) Treatment of cells with the extracellular domain of Klotho leads to rapid desialylation of the ion channel TRPV5. This desialylation exposes underlying galactose residues that can bind to galectins on the cell surface to retain TRPV5

on the cell surface. The discovery of Klotho's sialidase activity was unexpected, as Klotho has no homology with any of the other sialidases. This raises the intriguing possibility that other sialidases are out there waiting to be discovered.

Evidence has mounted that mammalian sialidases have a significant role in both normal and pathological processes. However, this remains a largely unexplored field of research due in part to the low expression levels and instability of the enzymes. This, in combination with the relatively poor labeling approaches for labeling sialic acids has left the field bereft of mechanistic information as to how these enzymes exert their functions.

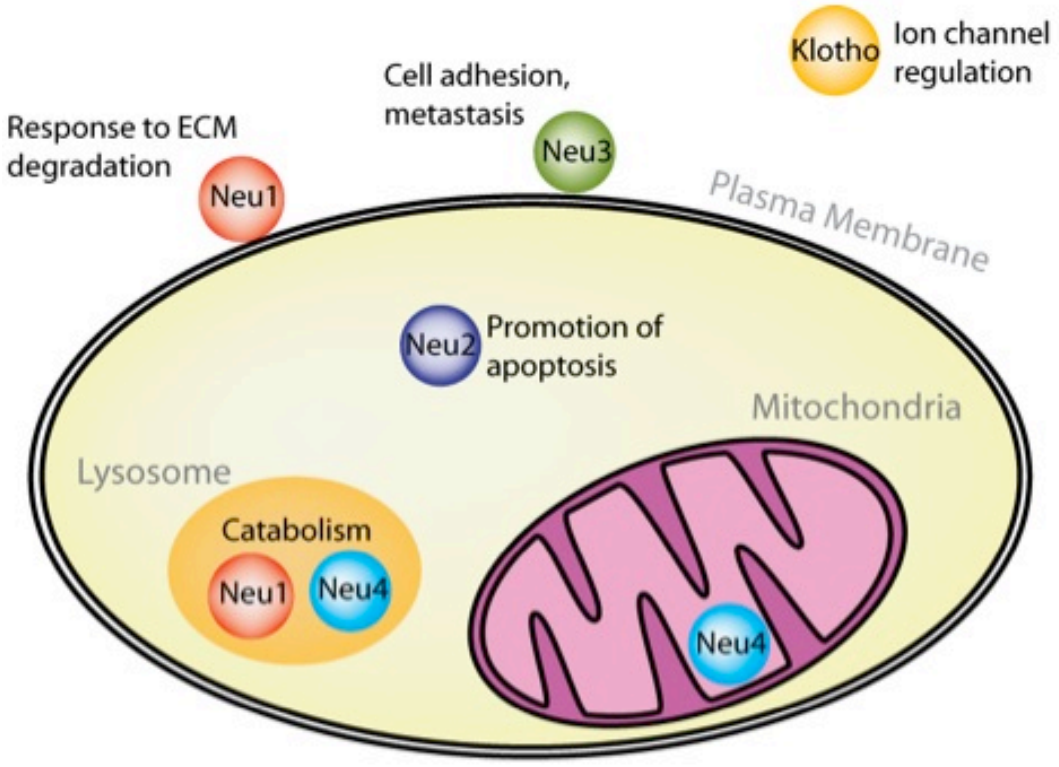

Figure 1.4. Localization and function of mammalian sialidases. Sialidases are found throughout the cell and extracellular space. Sialidase expression is implicated in numerous cellular processes. ECM = extracellular matrix

Purpose

Prior work has implicated sialidases in a numerous important biological processes. Most of these studies relied on overexpression or gene silencing of sialidases. Thus, mechanistic data on how sialidases exert these effects has generally remained lacking. This is because, in large part, the substrates of these enzymes have remained elusive, thus preventing a cohesive mechanism from being formed. Clearly, tools to better identify sialidase substrates would shine light on these interesting pathways. The major focus of my research has been to develop tools that allow for the identification and characterization of sialic acid mediated interactions including those between sialidases and their substrates. Chapter 2 discusses the design of a sialidase substrate specificity assay that allows for the rapid and high throughput characterization of sialidase reactivity towards a variety of complex glycans. Chapter 3 describes efforts to use photocrosslinking analogs of sialic acid to enable the discovery of sialidase substrates in a cellular context. Chapter 4 describes work to develop photocrosslinking probes to identify novel sialic acid mediated interactions.

Chapter 2. Development and implementation of a sialidase substrate specificity assay for complex glycans

Introduction

Sialic acid refers to a family of nine-carbon α-keto acids typically located at the non-reducing termini of many mammalian glycoproteins and glycolipids. Various sialic acids occur in nature, (*46*) including *N*-acetylneuraminic acid (Neu5Ac), the most common sialic acid in humans; *N*-glycolylneuraminic acid (Neu5Gc), produced by many nonhuman mammals; and deaminated sialic acid (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; KDN), produced by lower vertebrates and bacteria. In mammals, sialylated glycoconjugates, also called sialosides, play roles in physiological processes including cell differentiation, proliferation, apoptosis, and the immune response. Additionally, sialosides facilitate non-host interactions by serving as receptors for pathogens and secreted toxins. (*47*) The extent of glycoconjugate sialylation is regulated by sialyltransferases, which add sialic acid to glycoconjugates, and by sialidases, also called neuraminidases, which remove sialic acid from glycoconjugates. Sialidases hydrolytically cleave the ketosidic bond between sialic acid and an underlying glycan. These enzymes can regulate cell signaling through their ability to rapidly and dramatically change glycosylation of cell surface adhesion molecules, a transformation that can impact responses to extracellular stimuli.

Genes encoding sialidases are found in viral, bacterial, and eukaryotic genomes. Viral and bacterial sialidases play roles in pathogen infection of human hosts. In mammals, sialidase activities are critical to normal physiology, and changes in sialidase expression levels are associated with cancer, both in primary tumor samples and in cancer cell lines (as discussed above). (*24*) However, mechanistic link(s) between sialidase activity and cellular responses remain obscure. Improved knowledge of the substrate specificity of sialidases could reveal clues as to the regulatory processes that are normally controlled by sialidases and those that are disrupted by sialidase activity in infection and in cancer.

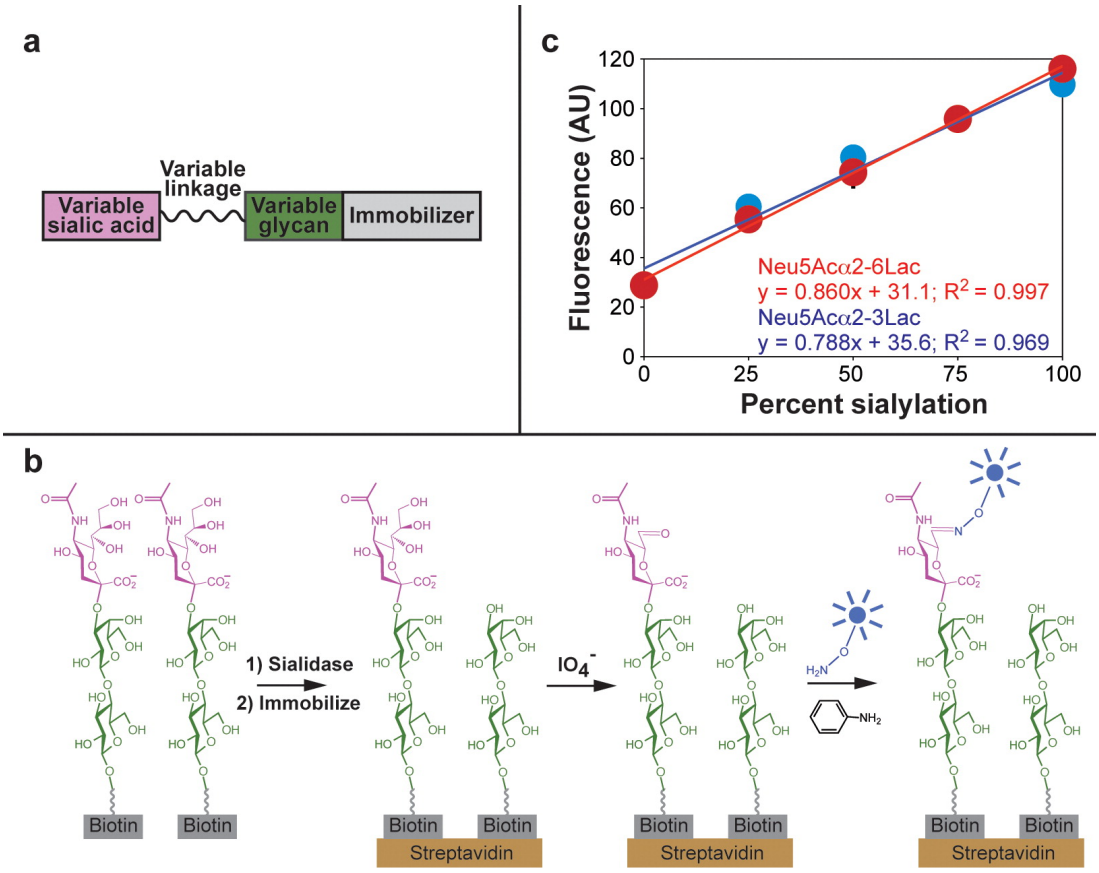

Figure 2.1. PAL method adapted to 96-well plate format. (a) The sialidase specificity assay accommodates a variety of sialosides. The form of sialic acid, the nature of the linkage, and structure of the underlying glycan can be varied. (b) Glycans are incubated with sialidase and then immobilized. Sialic acid containing glycoconjugates are fluorescently labeled by the periodate oxidation and aniline catalyzed ligation (PAL) method and detected. (c) The PAL method quantitatively detects sialylation. Biotinylated sialylated and unsialylated glycans were mixed in ratios indicated and immobilized. Sialylated glycans were labeled by periodate oxidation followed by aniline-catalyzed oxime ligation to Alexa Fluor 488. Error bars represent standard deviation of three trials. In some cases data points conceal error bars

Routine measurements of sialidase activity are conducted using methylumbelliferyl-*N*-acetyl-α-*D*-neuraminic acid (4-MU-NANA), an artificial substrate consisting of a proto-fluorophore linked to Neu5Ac. Hydrolysis of the ketosidic bond by a sialidase liberates the fluorophore, enabling facile detection of sialidase activity by continuous fluorescence measurement. However, the 4-

MU-NANA reagent cannot provide information about the sialidase's specificity for sialic acid linkage or the underlying glycan to which the sialic acid is attached. Specificity studies require examination of sialidase activity on a variety of sialoside substrates. One approach is to quantify the amount of sialic acid released, either by direct measurement (*48*) or by coupled enzyme assays, (*49*) but the availability of defined substrates and the quantity of material required limit the scope of these approaches. Another strategy for studying sialidase specificity involves the use of lectin binding to detect changes in the sialylation state of immobilized glycans. (*50, 51*) However, low specificity and variable affinity of lectins limits their utility. As an alternative, two groups reported disaccharide substrates that are tagged with *p*-nitrophenyl or methylumbelliferone groups at the reducing terminus. Removal of sialic acid by a sialidase frees a tagged monosaccharide that can be hydrolyzed by excess glycosidase, and the liberated tag produces a quantitative UV or fluorescent signal. (*52, 53*) This approach yielded information about the substrate specificity of both bacterial and mammalian sialidases (*54, 55*) but remains limited to disaccharide substrates.

In this chapter I describe a simple-to-use sialidase specificity assay that circumvents the limitations of existing assays. By employing a chemoselective reaction that labels any sialoside, this assay enables analysis of sialidase activity on a wide variety of sialosides, including relatively complex glycans. Implementing this assay, I was able demonstrate that the activity of the human cytosolic sialidase NEU2 is influenced by multiple structural elements contained within the sialic acid and the underlying glycan.

Results

I wanted a sialidase substrate specificity assay to be applicable to a wide array of complex glycan substrates. Because the primary limitation of existing sialidase specificity assays is access to suitable substrates, the ideal method should be readily applicable to existing glycans that have been prepared to study the

specificity of glycan-binding proteins. (*56*) Immobilized glycan arrays are widely used to rapidly assess the specificity of carbohydrate binding proteins. (*57-59*) Potential hits are then validated by solution-based binding measurements. I envisioned an analogous approach to studying sialidase specificity *i.e.* immobilized arrays of sialylated glycans could be subjected to sialidase treatment and the relative desialylation of potential substrates measured. Interesting trends in substrate specificity could be confirmed by further solution-based kinetics measurements. To measure the extent of desialylation, I turned to the periodate oxidation and aniline catalyzed ligation (PAL) method, which capitalizes on the unique reactivity of the sialic acid polyhydroxyl side chain. (*60*) Mild oxidation conditions selectively oxidize the C7 position of sialic acid to an aldehyde. Subsequent oxime formation by reaction with an aminooxy reagent is catalyzed by aniline, yielding a labeled sialic acid. This detection method is ideally suited to

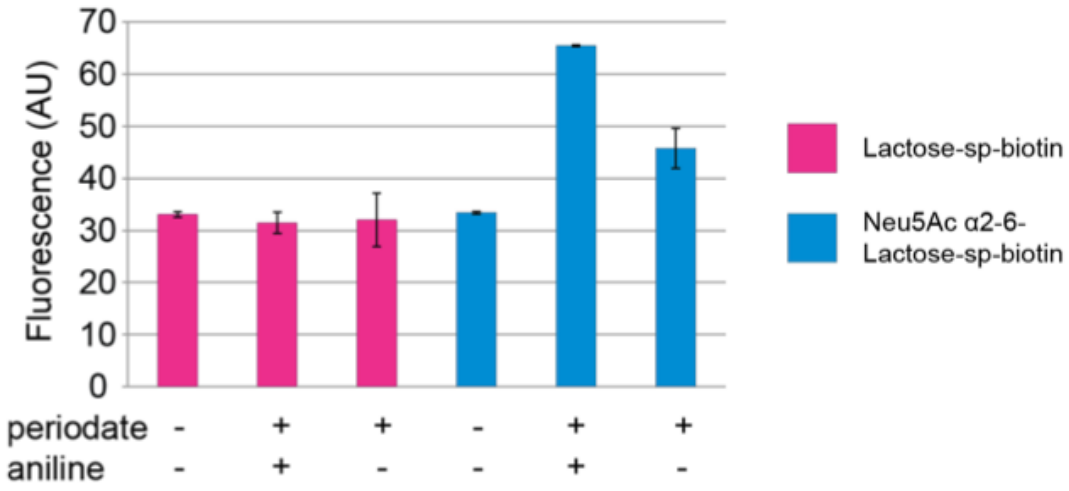

Figure 2.2. PAL signal is dependent on sialylation, aniline, and periodate. The indicated glycans were immobilized, oxidized, and functionalized with aminooxy-Alexa Fluor 488. Periodate or aniline was omitted from some reactions, as indicated. The increase in fluorescence after PAL is dependent on the presence of sialylated glycans. The increase in fluorescent signal is completely lost without periodate oxidation and significantly decreased if aniline is not present during oxime formation. Error bars represent standard deviation of three independent measurements.

specificity studies because it is independent of the glycan to which sialic acid is attached and of most modifications to the sialic acid itself, although modifications to the C7 and C8 positions of sialic acid preclude its use. I reasoned that I could employ the PAL method to detect the ability of sialidases to desialylate a variety of biotinylated sialosides in which the sialic acid structure, sialic acid linkage, and structure of the underlying glycan were varied (Figure 2.1, panel a).

First, I adapted the PAL reaction (*60*) for use in a 96-well format (Figure 2.1, panel b). I employed glycans conjugated to biotin at the reducing end *via* a spacer unit (Sp) (linker described in methods section). I immobilized each glycan in a single well of a streptavidin-coated plate and then oxidized the glycan by sodium periodate treatment. Oxidized glycans were reacted with an aminooxy detection reagent in the presence of aniline catalyst, using acidic conditions (pH 4.5) to promote the reaction. Sialic acids are known to be acid sensitive. Acidic conditions (pH ~2) and high temperatures (80 °C) are frequently used to hydrolyze sialic acids. (*61*) In order to minimize the chances of hydrolysis I used only mildly acidic conditions and kept the reaction on ice for the duration of the acid exposure. (*62*) This reaction yielded a 3-4 fold increase in fluorescence for sialylated glycans relative to that of unsialylated glycans (Figure 2.2 and 2.3). In the absence of periodate oxidation, there was no difference in fluorescence signal between sialylated and unsialylated glycans, and in the absence of aniline, the difference was greatly reduced (Figure 2.2). It is worth noting that even in the lactose sample not treated with periodate, some background fluorescence is still observed. This background fluorescence does not change when the lactose sample is treated with periodate. This suggests that the vicinal diols in lactose are not periodate sensitive and that the background fluorescence is from another source. I would speculate that this background fluorescence is due to binding of the fluorophores to the plastic and will not affect the assay. Taken together, these results indicate that the detection method reliably discriminates sialylated and unsialylated glycans.

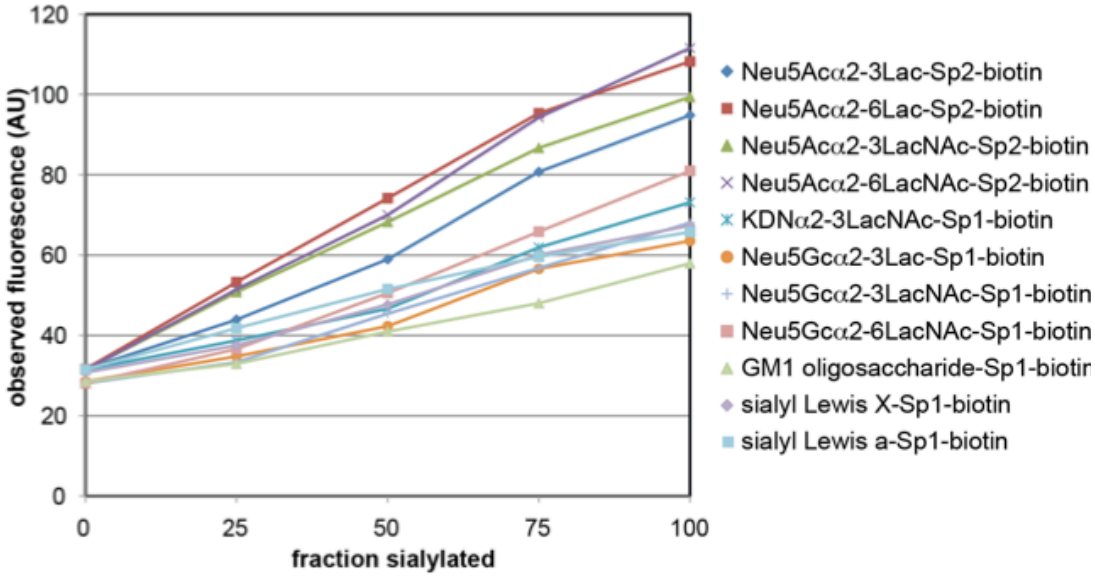

Figure 2.3. Fluorescence signal from PAL is proportional to percent sialylation. The indicated glycans were each mixed with their unsialylated counterpart in varying ratios. Whenever possible, I used an unsialylated reference compound that was identical to the sialylated molecule, except for the lack of sialic acid. However, in some cases the reference molecule had a different spacer length than the sialylated molecule and in one instance (GM1) I used a different but related unsialylated glycan as a reference. Glycan mixtures were immobilized, oxidized, and functionalized with aminooxy-Alexa Fluor 488. In all cases, the fluorescence signal was proportional to fraction of sialylated glycan.

Next, I tested conditions where the fluorescence signal accurately reflected the amount of sialylated glycan. I prepared defined mixtures of Neu5Acα2-3Lac-Sp-biotin and Lac-Sp-biotin. I immobilized the mixtures in individual wells of a 96-well streptavidin-coated plate and detected sialylated molecules using the PAL method. I observed fluorescence signal related linearly to the fraction of sialylated glycan present (Figure 2.1, panel c). Importantly, sialic acid linkage did not affect the fluorescence signal, and the signal was proportional to the fraction of glycan that was sialylated. The y-intercept is not zero due to the background fluorescence described above, however this does not appear to affect the assay as I still see a linear relationship between fluorescence and sialylation.

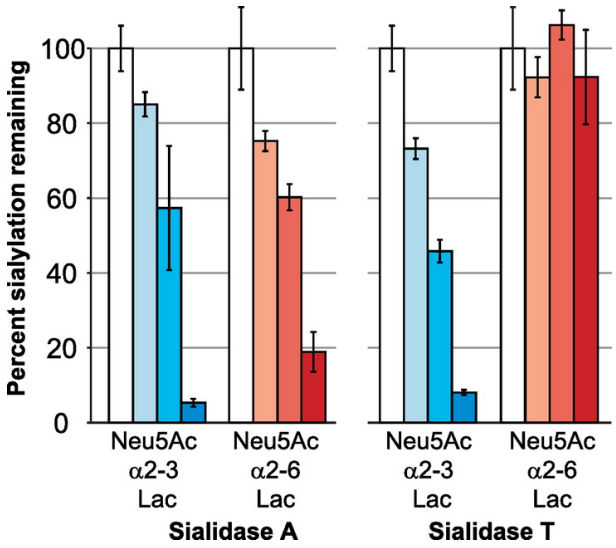

Figure 2.4. Sialidase assay accurately characterizes specificity of bacterial sialidases. Biotinylated Neu5Acα2-3Lac and Neu5Acα2-6Lac were incubated with *Arthrobacter ureafaciens* sialidase A (0, 0.55, 1.66, or 5 mU, bars left to right) or *Salmonella typhimurium* sialidase T (0, 6.4, 19.3, or 58 μU, bars left to right) for 2 h at 37 °C. The PAL method was used to determine the fraction of sialylated glycan remaining after sialidase treatment. Error bars represent standard deviation of three trials.

Subsequent data are reported as "percent sialylation," calculated for each experimental observation based on fluorescence signals of sialylated and unsialylated glycans (Figure 2.3).

I investigated whether I could accurately detect sialidase specificity by examining two well-characterized bacterial sialidases. Sialidase A from *Arthrobacter ureafaciens* hydrolytically cleaves both α2-3- and α2-6-linked sialic acids. (*63*) Sialidase T from *Salmonella typhimurium* exhibits a strong preference for sialic acids in the α2-3-linkage. (*64*) Using Neu5Acα2-3Lac-Sp-biotin and Neu5Acα2-6Lac-Sp-biotin, I observed desialylation of both α2-3- and α2-6-linked sialosides by sialidase A, while sialidase T displayed a strong preference for α2-3-linked sialic acid (Figure 2.4).

Next, I examined the human sialidase NEU2, also known as the cytosolic sialidase or the soluble sialidase. (*65*) Mammalian NEU2 is highly expressed in

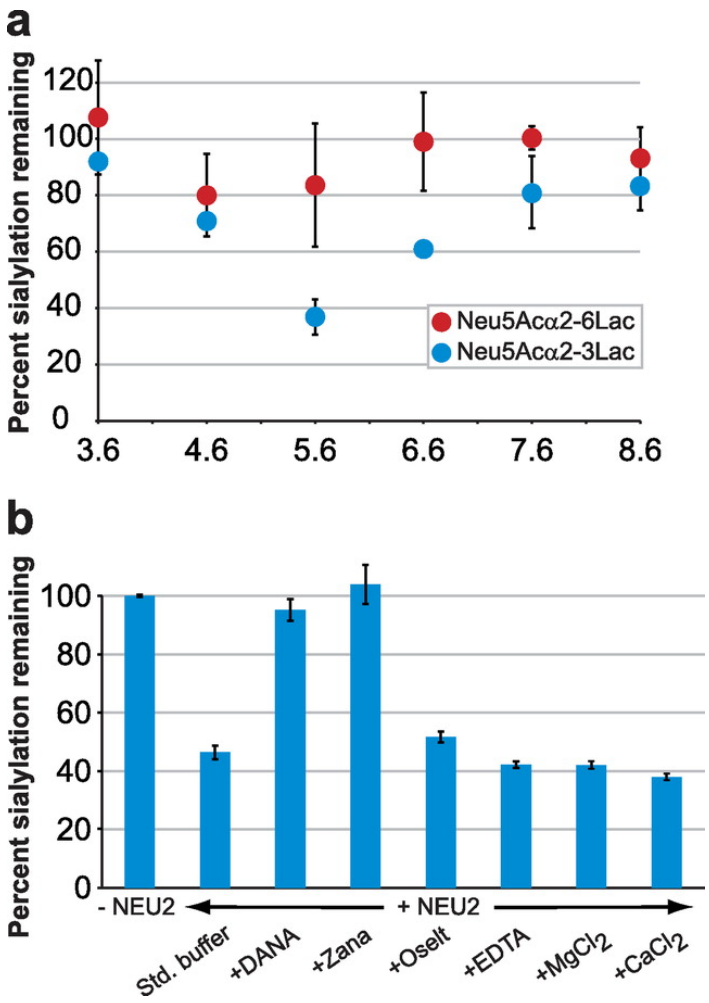

Figure 2.5. Activity of GST-NEU2 depends on buffer composition. (a) GST-NEU2 (1.13 mU) was incubated with Neu5Acα2-3Lac in buffer containing 200 mM sodium phosphate, 100 mM sodium acetate, and 100 mM NaCl at the indicated pH, for 1 h at 37 °C. Error bars represent standard deviation of three trials. (b) GST-NEU2 activity (1.13 mU) was measured in 100 mM NaOAc, pH 5.6, and 100 mM NaCl at 37 °C for 1 h. Buffer was varied by presence of 1 mM $CaCl_2$, $MgCl_2$, EDTA, zanamivir, DANA, or oseltamivir, as indicated. Error bars represent standard deviation of three trials.

skeletal muscle, where it is implicated in myoblast differentiation, (*66*) and also is present in other tissues. *In vitro* assays have demonstrated that NEU2 accepts

glycoprotein, glycolipid, and oligosaccharide substrates, (*67, 68*) but its physiological substrates remain unknown. I expressed NEU2 with a C-terminal glutathione *S*-transferase tag (GST-NEU2). I measured the pH dependence of GST-NEU2 activity toward Neu5Acα2-3Lac-Sp-biotin (Figure 2.5, panel a). Consistent with prior reports, GST-NEU2 exhibits optimal activity at pH 5.6. (*54, 68*) I also tested the effects of varying the buffer composition (Figure 2.5, panel b). GST-NEU2's activity toward Neu5Acα2-3Lac-Sp-biotin did not depend on divalent ions. On the other hand, GST-NEU2 was inhibited by sialidase inhibitors 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA) and zanamivir (Relenza) but was not affected significantly by oseltamivir (Tamiflu), consistent with previous findings. (*69*)

I examined the sialoside specificity of NEU2 using a small panel of potential substrates, with variable sialic acid linkage, sialic acid form, and underlying glycan structure (Figure 2.6, panel a). An early report describing the purification of cytosolic sialidase activity indicated that it prefers the α2-3-linkage (*70*) and subsequent studies using recombinant NEU2 support that finding. (*54, 68*) However, using lysates from cells overexpressing mouse NEU2, Koda *et al.* observed activity toward both α2-3- and α2-6-linked sialosides. (*71*) My data are consistent with the prevailing view that NEU2 exhibits a strong preference for α2-3-linked sialic acids as I observed only minor activity toward the α2-6-linked sialosides (Figure 2.6, panel b). I examined NEU2's tolerance of different forms of sialic acid. Consistent with the findings of others, (*54*) I observed that GST-NEU2 readily hydrolyzes both Neu5Ac and Neu5Gc, but not the deaminated sialic acid, KDN. I examined how sialidase activity depends on the structure of the underlying glycoconjugate and found that GST-NEU2 did not strongly discriminate between Lac- or LacNAc-based glycans. Although GST-NEU2 readily recognized Neu5Acα2-3LacNAc-Sp-biotin, it showed diminished activity toward the related glycan sialyl Lewis X, in which fucose is attached to the *N*-acetylglucosamine (GlcNAc) residue. Furthermore, sialyl Lewis a and the GM1

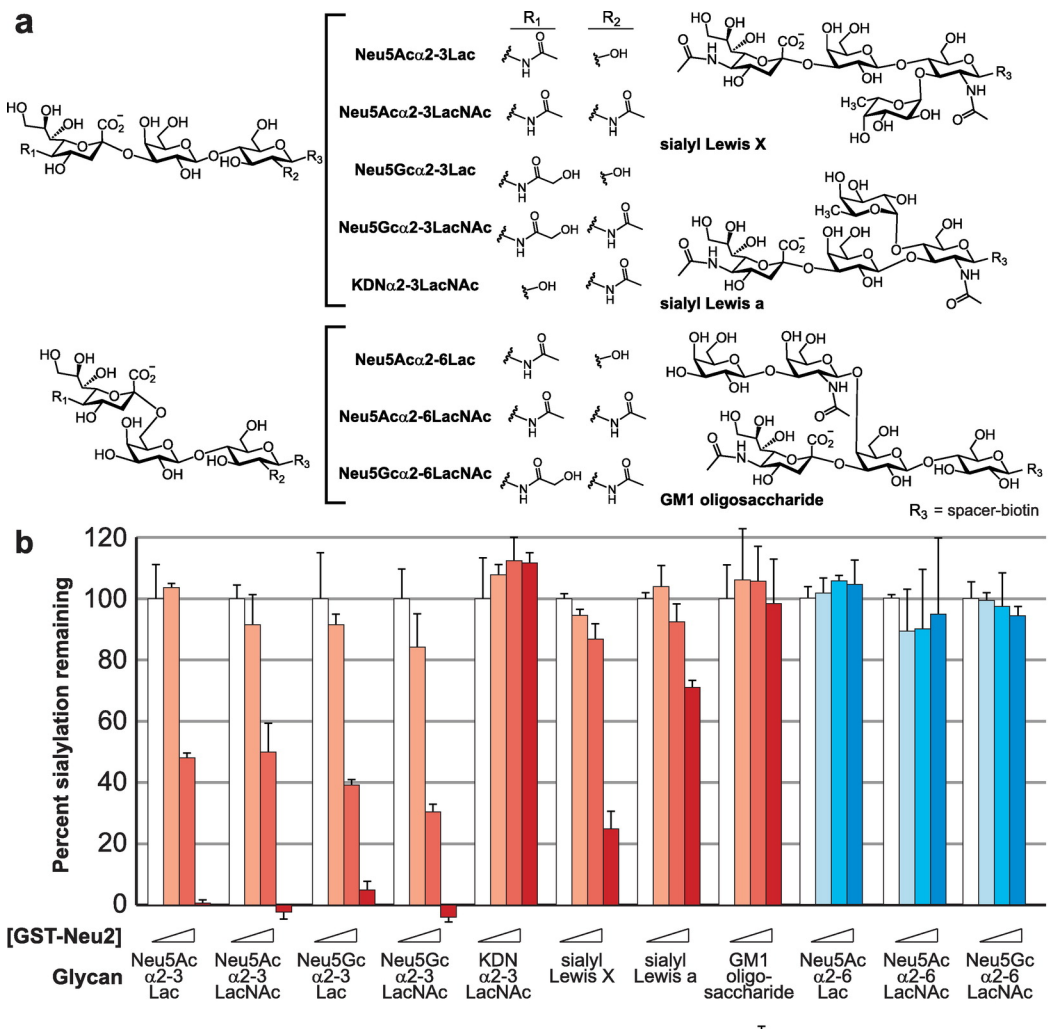

Figure 2.6. **Human cytosolic sialidase NEU2 exhibits sialoside specificity dependent on sialic acid linkage, sialic acid form, and underlying glycan structure.** (a) Sialylated glycans examined. (b) GST-NEU2 activity detected by PAL method. GST-NEU2 (0, 0.76, 2.3, or 6.9 mU, bars left to right) was incubated with 1 μM of glycan in 100 mM NaOAc, pH 5.6, and 100 mM NaCl for 2 h at 37 °C, followed by immobilization and PAL labeling. Error bars represent standard deviation of three trials.

oligosaccharide, both of which contain Neu5Acα2-3Gal, were also poor substrates, suggesting that the enzyme is sensitive to the glycan structure underlying the sialic acid residue. The lack of NEU2 activity on the GM1 oligosaccharide is consistent with other studies, (*68*) but sialyl Lewis X and sialyl Lewis a had not been examined previously. I validated these finding by

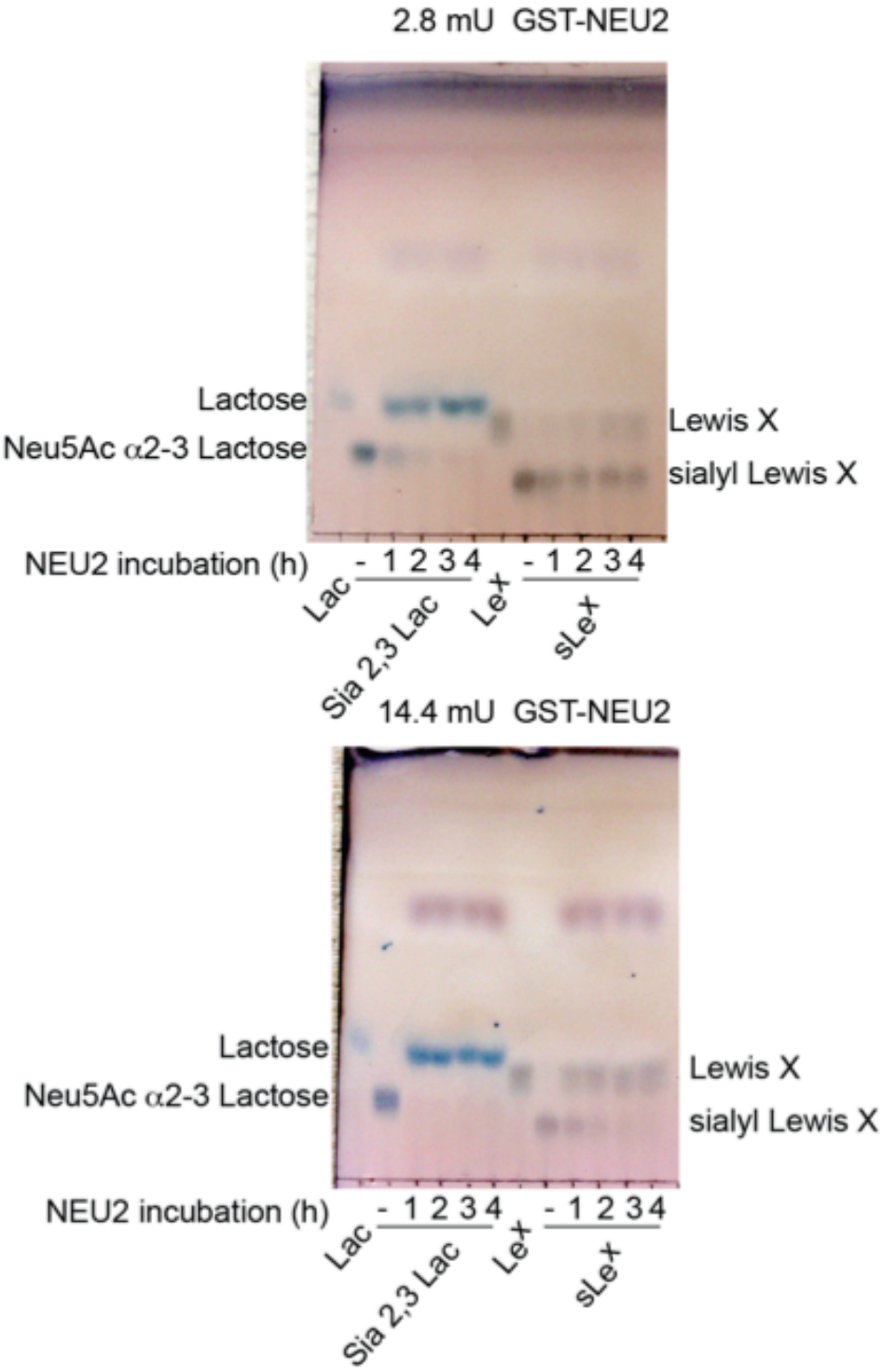

Figure 2.7. GST-NEU2 prefers Neu5Acα2-3Lactose over sialyl Lewis X. GST-NEU2 (2.8 or 14.4 mU) was added to 1 mM of the indicated glycan in buffer containing 100 mM sodium acetate, pH 5.6, and 100 mM NaCl for the indicated time. Reaction progress was analyzed by thin layer chromatography with the eluent 4:2:1:0.1 EtOAc:MeOH:H_2O:AcOH and visualized with *p*-anisaldehyde stain.

monitoring glycan desialylation by thin layer chromatography (Figure 2.7). GST-NEU2 hydrolyzes sialyl Lewis X more slowly than Neu5Acα2-3Lac, consistent with the specificity assay data.

Discussion

In summary, I report a method for evaluating sialidase specificity. I adapted the PAL method to monitor desialylation of immobilized glycoconjugates. Then, examined how the activities of mammalian and bacterial sialidases depend on multiple substrate features, including sialic acid linkage, sialic acid identity, and structure of the underlying glycan. In the future, immobilized glycoproteins and glycolipids could be examined, or the method could be adapted to interrogate larger arrays of robotically printed glycans. (*72*) Knowledge of sialidase specificity will provide insight into sialidase roles in normal physiology and in disease.

Existing sialidase assays rely on small molecule substrates that limit the degree to which complex substrates can be probed for substrate specificity. The current default substrate for sialidases is 4-MU-NANA which is composed of a sialic acid glycosidically linked to the protoflorophore. When enzymatically cleaved the extinction coefficient rises dramatically allowing for continuous fluorescence measurement. This assay is ideal for measuring total sialidase activity and I used it routinely to calculate specific activity of GST-NEU2. This assay remains the fastest and best approach for rapid determination of activity, but it provides no information about substrate specificity.

Currently the most complex glycans that can be evaluated in a high throughput fashion are disaccharides. These substrates are useful for determining linkage specificity but are of little utility for evaluating the influence on substrate recognition of structural elements below the adjacent carbohydrate.

Another approach to evaluate complex substrates relies on lectin binding. However lectins have variable binding affinities that are highly dependant on the

structure of the glycan being probed and therefore are of little use in a comparative study. For example, one could not directly compare the substrate preference of α2,3 and α2,6 linked glycan directly because they require the use of two distinct lectins. The approach described here circumvents these problems by utilizing a chemical detection reaction that is independent of the structure of the underlying glycan.

One potential drawback to my approach is the availability of biotinylated glycans. There are several sources, both from government-funded agencies (The Consortium for Functional Glycomics) as well as private companies (i.e. Glycotech) from which to get such glycans.

The limitations in availability of these glycans could be circumvented by adapting the assay described here to a glycan array, work that is currently underway in the lab. Glycan arrays are assembled from naturally occurring glycans that are derivitized with immobilization chemistry. The glycans are derivitized with a bifunctional amine containing linker in which one amine reacts with the free reducing end of the glycan and the other amine reacts with an NHS-ester immobilized on a glass slide, thus coupling the glycan to the slide via its reducing end. In principle any glycan, either naturally occurring or synthetic, can be used in this manner. Indeed, the Cummings group has demonstrated that essentially any glycan from a natural source can be derivitized, then purified by multi-dimensinal HPLC and spotted on an array. Thousands of such glycans have been purified and probed for glycan binding specificity. (*73*) It is easy to imagine my sialidase assay described here being applied to such a microarray. In fact, a post-doc in the lab is currently collaborating with the Cummings lab to do just that. We expect to learn quite a bit about substrate specificity from these studies.

Another potential drawback is that modifications to the glycerol side-chain cannot be probed with this method. For example acetylation of the 9- position of sialic acid has important roles in CD22 signaling (discussed above) but this

modification could not be probed by this assay due to the dependence on oxidation of the 7-position of sialic acid. Despite these drawbacks I expect this assay to be quite useful in determining the substrate specificity of sialidases from numerous sources.

This assay has already been used by others in our group to characterize bacterial sialidases implicated in disease. The pathogenic bacteria *Streptococcus pneumoniae* is the primary cause of bacterial meningitis in children. (*74*) The infection process requires the bacterium to attach to and invade the blood brain barrier (BBB). This bacterium expresses three sialidases (NanA, NanB and NanC) and one of these sialidases, NanA, is implicated in the invasion of the BBB, which leads to inflammation and the pathogenesis of the disease. What makes NanA different from the other two sialidases has remained unclear. Recently, using the assay that I describe here, a post-doctoral fellow in the Kohler lab probed the substrate specificity of these three sialidases. She found that NanB and NanC exhibited a strong preference for α2-3-linked sialic acids compared to α2-6-linked sialosides. However, NanA exhibited significant activity towards α2-6-linked sialosides making it unique among the sialidases expressed by this organism. Additionally, she noticed that all three of the sialidases had a strong preference for Neu5Ac compared to Neu5Gc. This is intriguing because Neu5Ac is enriched in glycans produced in the brain. It may be that substrate specificity rather than total sialidase activity is the key difference between the sialidases and that desialylation of an α2-6-linked, Neu5Ac-containing sialoside is an important step in invasion of the BBB. If so, this might provide new avenues for therapeutic intervention.

This assay could also be useful in discovering changes in sialidase substrate specificity of disease causing organisms. Host specificity of influenza strains is dependent in part on the glycan structures recognized by the influenza sialidase (also known as neuraminidase). (*18*) Routine screening of influenza sialidases

with this assay could alert us to novel mutations that could allow a flu strain to cross the species barrier.

Materials and methods

General.

Sialidase A and T were purchased from Prozyme (gk80040) and New England Biolabs (P0728), respectively. Sialidase inhibitors were purchased from Toronto Research Chemicals: oseltamivir (0700980), zanamivir (Z148000), and *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid (DANA) (A172200).

Description of biotinylated glycans.

Biotinylated glycans were acquired from the Consortium for Functional Glycomics (CFG), Glycotech, or were synthesized using established protocols. Glycans acquired from the CFG were: Neu5Gcα2-3Galβ1-4Glc-Sp1-biotin, Neu5Gcα2-3Galβ1- 4GlcNAc-Sp1-biotin, Neu5Gcα2-6Galβ1-4GlcNAc-Sp1-biotin, KDNα2-3Galβ1-4GlcNAc-Sp1- biotin, Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ-Sp1-biotin, Galβ1-4[Fucα1-3]GlcNAcβ-Sp1- biotin, Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAcβ-Sp1-biotin, Galβ1-3[Fucα1-4]GlcNAcβ-Sp1- biotin, Neu5Acα2-3[Galβ1-3GalNAcβ1-4]Galβ1-4Glcβ-Sp1-biotin, where Sp1 = $(CH_2)_2NHCO((CH_2)_5NH)_2$. Glycans acquired from Glycotech were: Neu5Acα2-3Galβ1-4Glc- Sp2-biotin, Neu5Acα2-6Galβ1-4Glc-Sp2-biotin, Neu5Acα2-3Galβ1-4GlcNAc-Sp2-biotin, Galβ1-4Glc-Sp2-biotin, Galβ1-4GlcNAc-Sp2-biotin where Sp2 = $(CH_2)_3NHCO(CH_2)_5NH$. Neu5Acα2-6Galβ1-4GlcNAc-Sp2-biotin was prepared chemoenzymatically from Galβ1- 4GlcNAc-Sp2-biotin as described by others. (*75*) Glycan concentration was determined using FluoReporter Biotin Quantitation Kit (Invitrogen F30755) according to the manufacturer's instructions.

Relationship between PAL signal and sialoside concentration.

Standard curves to convert observed fluorescence signal to percent sialylation

were obtained by mixing together known ratios of sialylated and unsialylated glycans to achieve a final concentration of 1 μM total glycan in PBS prior to immobilization. For standard curves, Neu5Acα2-3[Galβ1-3GlcNAcβ1-4]Galβ1-4Glcβ-Sp1-biotin, Neu5Gcα2-3Galβ1-4Glc-Sp1-biotin, Neu5Acα2-3Galβ1-4Glc-Sp2-biotin, and Neu5Acα2-6Galβ1-4Glc-Sp2-biotin were compared to Galβ1-4Glc-Sp2-biotin. Neu5Gcα2- 3Galβ1-4GlcNAc-Sp1-biotin, Neu5Gcα2-6Galβ1-4GlcNAc-Sp1-biotin, Neu5Acα2-6Galβ1- 4GlcNAc-Sp2-biotin, Neu5Acα2-3Galβ1-4GlcNAc-Sp2-biotin, and KDNα2-3Galβ1-4GlcNAc- Sp1-biotin were compared to Galβ1-4GlcNAc-Sp2-biotin. Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ-Sp1-biotin was compared to Galβ1-4[Fucα1-3]GlcNAcβ-Sp1-biotin. Neu5Acα2- 3Galβ1-3[Fucα1-4]GlcNAcβ-Sp1-biotin was compared to Galβ1-3[Fucα1-4]GlcNAcβ-Sp1- biotin.

For each mixture, a 100 μL aliquot was incubated in a single well of a 96-well streptavidin- coated plate for 1 h at room temperature. After incubation, the wells were washed three times with 100μL of PBST. Next, 100 μL of 1 mM or 0.1 mM $NaIO_4$ in PBS pH 7.4 was added to each well and the plate was incubated at 4 °C for 30 min. The oxidation reaction was quenched with 100 μL of 1 M glycerol in PBS pH 7.4 for 5 min followed by washing three times with 100 μL of PBST. To each well was added 100 μL of buffer containing 100 mM NaOAc pH 4.5, 10 mM aniline and 10 μM aminooxy-Alexa Fluor 488. The plate was incubated for 2 h at 4 °C. The wells were washed three times with 100 μL of PBST and the fluorescence was measured on a Spectramax M5 plate reader (excitation: 488 nm, emission: 515 nm).

Production of recombinant NEU2.

Recombinant GST-NEU2 was prepared essentially as described. (*65*) Plasmid DNA encoding the human *NEU2* ORF was purchased from OriGene (RC219858). *NEU2* was cloned into the pGEX-4T-1 vector in-frame with GST using BamHI and XhoI restriction enzymes. The pGEX-NEU2 plasmid was transformed into *E. coli* BL21(DE3). A single colony was selected and grown in

ampicillin-containing LB to OD_{600} = 0.6 prior to induction with 0.1 mM IPTG. Induction was carried out for 16 h at 30 °C. Cells were harvested by centrifugation and lysed in 50 mL phosphate buffered saline (PBS), pH 7.4 containing 2 mg mL^{-1} lysozyme (Sigma) and one Complete Protease Inhibitor Cocktail Tablet (Santa Cruz) for 30 min followed by sonication. Lysates were centrifuged at 47,800 *g* for 1 h and insoluble material was discarded. The supernatant was applied to glutathione sepharose 4B column (27-4574-01, GE Healthcare), washed three times with 50 mM Tris-HCl, pH 8.0, and eluted with buffer containing 10 mM reduced glutathione. Protein content was analyzed by SDS-PAGE followed by Coomassie stain or immunoblot using antibodies against GST (GST(B14):SC-138, Santa Cruz) or NEU2 (ab55568, Abcam). 1 L of bacterial culture yielded 1 mg of protein with a specific activity of 4.06 μmol min^{-1} mg^{-1} or 4.06 units mg^{-1} (a unit is defined as the amount of enzyme required to release 1 μmol of methylumbelliferone per minute).

Determination of GST-NEU2 specific activity.

GST-NEU2 (17 ng) was incubated with 1 mM methylumbelliferyl-*N*-acetyl-α-*D*-neuraminic acid (4-MU-NANA) in buffer containing 100 mM NaOAc, pH 5.6, and 100 mM NaCl for 1 h at 37 °C in a final volume of 100 μL. The reaction was quenched with 50 μL of 500 mM $NaHCO_3$ at a pH of 10.5 and the fluorescence was measured on a Spectramax M5 plate reader (excitation: 325 nm, emission: 420 nm). The fluorescence of methylumbelliferone released by sialidase activity was compared to that of methylumbelliferone standards to calculate the amount of sialic acid released, which was used to calculate specific activity.

Verification of GST-NEU2's preference for nonfucosylated glycans.

GST-NEU2 (2.8 or 14.4 mU) was added to 100 μL of 1 mM sialyl Lewis X (OS04058, Carbosynth) or Neu5Acα2- 3Lactose (OS04397 , Carbosynth) in 100 mM NaOAc, pH 5.6, 100 mM NaCl and incubated for the indicated time at 37 °C. The reaction was analyzed by thin layer chromatography with the eluent 4:2:1:0.1 EtOAc:MeOH:H_2O:AcOH and visualized with *p*-anisaldehyde stain (0.2 M *p*-

anisaldehyde, 0.6 M sulfuric acid, 0.2 M acetic acid in ethanol) by heating at 300 °C.

Chapter 3 Development of tools to identify sialidase substrates in cellular contexts

Introduction

Sialic acid refers to a family of nine-carbon α-keto acids typically located at the non-reducing termini of many mammalian glycoproteins and glycolipids. The presence of sialic acid on these biomolecules can have several effects. Sialic acid can alter the intrinsic structure of the protein or lipid. Sialic acid can also recruit specific sialic acid binding partners that alter the localization or activity of the protein. Alternatively, sialic acid can mask underlying antigens from recognition by binding partners. For example, the Ashwell receptor in hepatocytes binds to terminal galactose residues on circulating glycoproteins and mediates uptake and destruction of these proteins. Sialic acid linked to the terminal galactose residue prevents this binding and clearance by this receptor. (*76*) Sialidases, with their ability to dramatically alter the sialylation state of the cell, represent a means to rapidly desialylate and thereby alter the function of the sialylated protein. Mammalian sialidases are implicated in numerous physiological and pathological processes from development, immunity, and cancer. (*14*) However most of the experiments implicating sialidases in these processes are based on overexpression or gene silencing studies and therefore

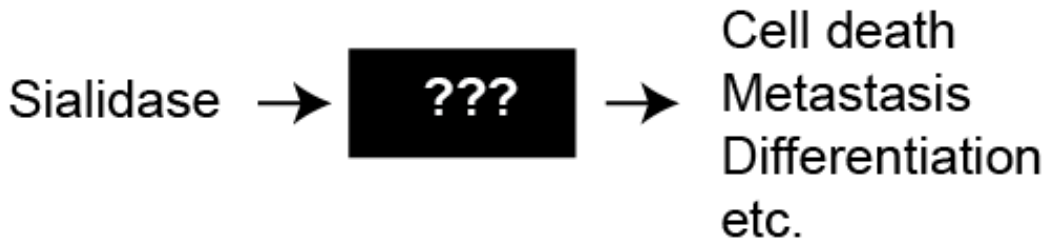

Figure 3.1. Sialidase substrates: the missing link. Sialidases are implicated in numerous physiological and pathological processe. However, the mechanism of action is unknown as we lack information about sialidase substrates.

little mechanistic information is known about how these enzymes cause such diverse outcomes. Presumably sialidases are removing sialic acid from a protein or lipid. This desialylation then alters the localization or activity of some unknown downstream component of a signaling pathway. This information is then converted to the diverse outcomes, whether it is immune evasion or cell death (Figure 3.1).

We lack better mechanistic understanding of sialidases' effects because, in most cases, the direct target of the sialidase is unknown. Knowledge of the substrates' identities in a given context would allow investigation into how desialylation of individual targets leads to diverse cellular outcomes. The best-studied mammalian sialidase in terms of regulating signaling is NEU1. It is known that NEU1 hydrolyses sialic acid residues from TLR4 and that this permits TLR4 dimerization and activation. (*77*) This information is based largely on immunoprecipitation data. It appears NEU1 is unusual amongst sialidases in that it forms stable protein-protein complexes with its substrates. This is perhaps not surprising given that NEU1 must form complexes with other proteins in order to have full activity. NEU1 exists as an obligate protein complex with PPCA (Protective protein/Cathepsin-A) and beta-galactosidase both in the lysosome and on the cell surface, though on the cell surface beta-galactosidase can be replaced by elastin binding protein (EBP). (*78*) However there is no data to suggest that other sialidases form such stable complexes with their substrates.

Currently there exist no discovery assays to identify the substrates of sialidases. The few substrates that have been identified were found by their association with protein complexes, and this is only true for NEU1. Clearly a broadly applicable discovery method for sialidase substrates is needed.

Purpose

Given the difficulty of identifying sialidase substrates I sought to develop a general approach to identify the protein or lipid component of sialidase

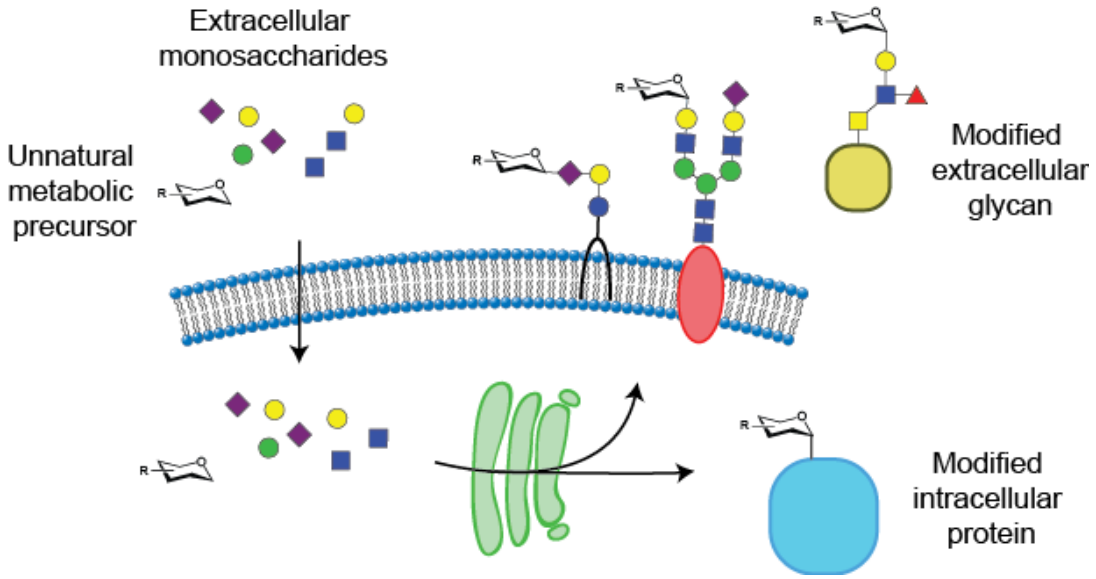

Figure 3.2. Metabolic oligosaccharide engineering introduces novel functionalities to glycans. Metabolic oligosaccharide engineering relies on modified monosaccharide analogs that are incorporated by the cell and processed much like their natural counterparts. These are installed into glycans by the cells endogenous machinery where they can be used as tools to probe various aspects of glycosylation.

substrates. This approach should be generalizable to any sialidase and any context. This approach should also directly demonstrate interactions between sialidases and their substrates. With this in mind I turned to metabolic incorporation of photocrosslinking sialic acids. Metabolic oligosaccharide engineering has been employed to place orthogonal functional groups onto glycans in cellular contexts. (*79, 80*)

ManNAc (*N*-acetylmannosamine) is the biological precursor to sialic acids and is frequently used as a platform to install orthogonal functional groups onto sialic acids. This approach was pioneered by Reutter and colleagues who demonstrated that ManNAc analogs bearing additional methylene units could be delivered to biological systems and converted to their corresponding sialic acids and presented on the cell surface. (*79*) This work was then expanded on by

Bertozzi and coworkers who utilized ManNAc analogs containing azido and keto functional groups. (*81*) Since then numerous chemical handles have been attached to ManNAc analogs including alkynes, thiols, and diazirines among others and were successfully converted to their sialic acid analogs. (*81*)

In order to make the ManNAc analogs cell-permeable, the hydroxyl groups are acetylated to form Ac_4ManNAc analogs. Cells are cultured with Ac_4ManNAc analogs, which then passively diffuse across the plasma membrane into the cytosol where the acetyl groups are hydrolyzed by non-specific esterases. Little is known about the nature of these esterases. The liberated ManNAc analog can then enter the sialic acid biosynthetic pathway to become a sialic acid analog (Figure 3.2).

Metabolic incorporation of photocrosslinking sialic acids sheds light on molecular interactions

Photocrosslinkers have been gaining increasing popularity to identify novel interactions among biomolecules and to gain insight into detailed molecular motion that would otherwise be difficult to discern. (*82*) Photocrosslinkers have been installed into proteins, lipids, and carbohydrates. I desired to use a sialic acid analog containing a photocrosslinker that would allow me to identify sialic acid binding partners. (*83*) The Kohler group pioneered the use of sialic acid analogs incorporating a diazirine functional group for use as a photocrosslinker. When exposed to ultraviolet light at 365 nm diazirines dissociate to form a highly reactive carbene. Within a matter of microseconds this carbene can form a covalent bond to any nearby heteroatom bond, thus converting a transient interaction between macromolecules into a covalently bound complex that can be isolated and the components identified. This approach of incorporating diazirines into sialic acid analogs has successfully been used to crosslink sialic acid dependent protein-protein as well as protein-lipid interactions. (*83, 84*) CD22 is

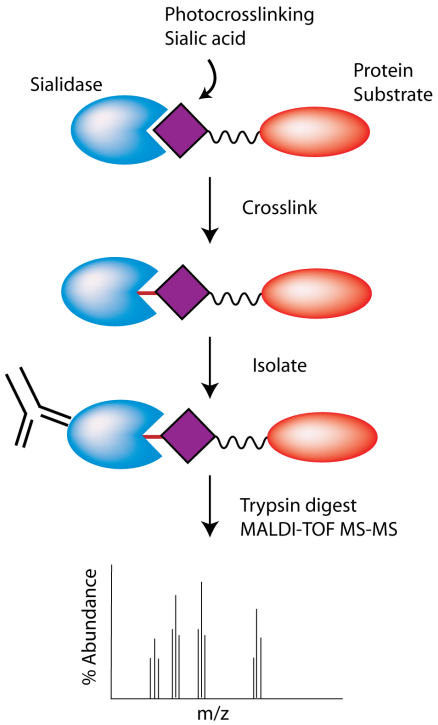

Figure 3.3. Schematic of photocrosslinking sialidase substrate identification approach. Cells expressing a catalytically inactive sialidase of interest are incubated with photocrosslinking sialic acid precursors then UV irradiated to crosslink sialidase with substrate. Purification of this sialidase-substrate complex allows identification of substrates by MS sequencing

an inhibitor of B-cell signaling that both presents and binds to sialic acid, (*85*) thereby creating sialic acid dependent oligomers on the cell surface. The Kohler lab has successfully trapped these sialic acid mediated interactions between CD22 monomers in live cells using metabolic incorporation of photocrosslinking sialic acids. Cholera toxin subunit B (CTxB) binds to the ganglioside (sialic acid containing glycosphingolipid) known as GM1 on host cells as the first step of intoxication. (*86*) Metabolic incorporation of photocrosslinking sialic acids has enabled the covalent entrapment of this protein-lipid complex.

Given the success of photocrosslinkers at capturing sialic acid mediated interactions, I decided to use this approach to trap sialidase-substrate

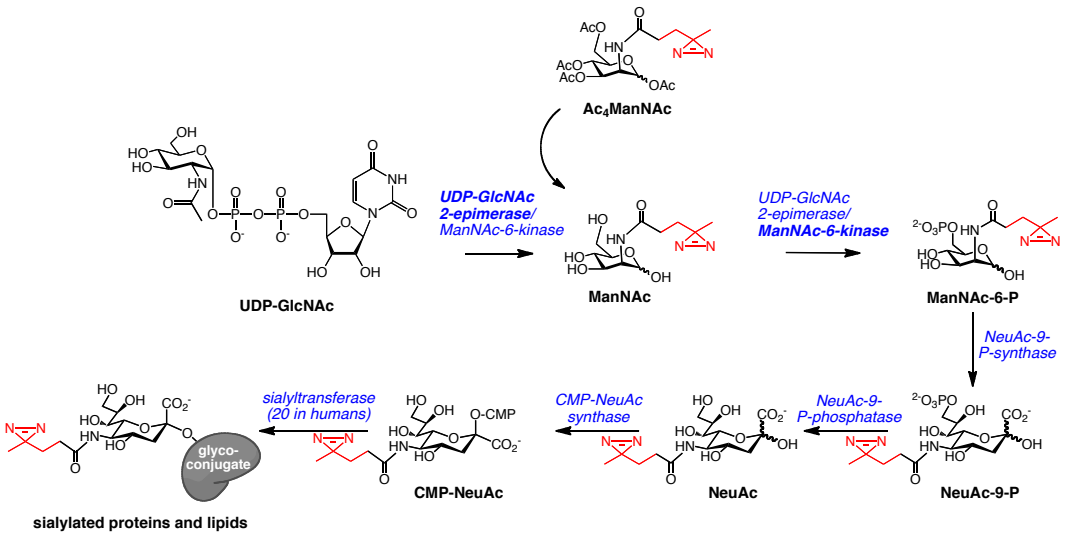

Figure 3.4. Schematic of metabolic incorporation of sialic acid analogs. Cells are fed Ac_4ManNDAz-2me which passively diffuses across the plasma membrane where it is deacetylated to release ManNDAz-2me. This compound is converted by the cells endogenous sialic acid biosynthetic pathway to SiaDAz-2me, which is then activated to form CMP-SiaDAz-2me. SiaDAz-2me is then transferred by sialyltransferases to its final destination on the glycan

interactions (Figure 3.3). This requires the use of diazirine containing ManNAc analogs (ManNDAz-2me) to generate photocrosslinking sialic acids in cells. Figure 3.4 shows the biosynthetic pathway for sialic acids as well as how we go about hijacking this pathway to install diazirines onto sialic acids. Cells are supplemented with Ac_4ManNDAz-2me, which can passively diffuse across the membrane due to acetylation of the hydroxyl groups. Once in the cytosol, the acetyl groups are cleaved by non-specific esterases to reveal ManNDAz-2me, which is then condensed with pyruvate to form SiaDAz-2me. SiaDAz-2me must then be activated by coupling with CTP to form CMP-SiaDAz-2me, which is the substrate for sialyltransferases that transfer SiaDAz-2me onto the glycan chain.

The chronic myelogenous leukemia cell line K562 is normally resistant to apoptotic stimuli due to the expression of anti-apoptotic proteins. (*87*) However, in 2008, Tringali *et al.* found that overexpression of the cytosolic sialidase NEU2 could restore sensitivity to apoptotic stimuli. Some mechanistic insight was gained as they demonstrated that the antiapoptotic protein Bcl-xl was

downregulated at the transcriptional level. (*34*) Yet, the question remains how this sialidase activity leads to this transcriptional change. Identification of the glycoprotein or lipid acted on by NEU2 in this context would provide direct mechanistic insight. Therefore I decided to use this system as a platform to develop a photocrosslinking sialidase substrate identification strategy. In brief, the plan consisted of incorporating our photocrosslinking sialic acids into K562. In principle, this would incorporate the photocrosslinking analogs into any sialic acid containing molecule, including NEU2's substrate(s). These cells would be expressing affinity tagged NEU2 or a catalytically inactive mutant of NEU2. This point mutant of NEU2 contains an alanine substitution to one of the residues in the catalytic triad. I predicted that the catalytically inactive NEU2 mutant would bind substrates without hydrolytically releasing sialic acid (Figure 3.5). Upon UV irradiation, I envisioned that the photocrosslinker would form a covalent bond between NEU2 and its substrate. Purification of NEU2 by affinity purification would co-purify this NEU2-substrate complex, allowing me to identify the

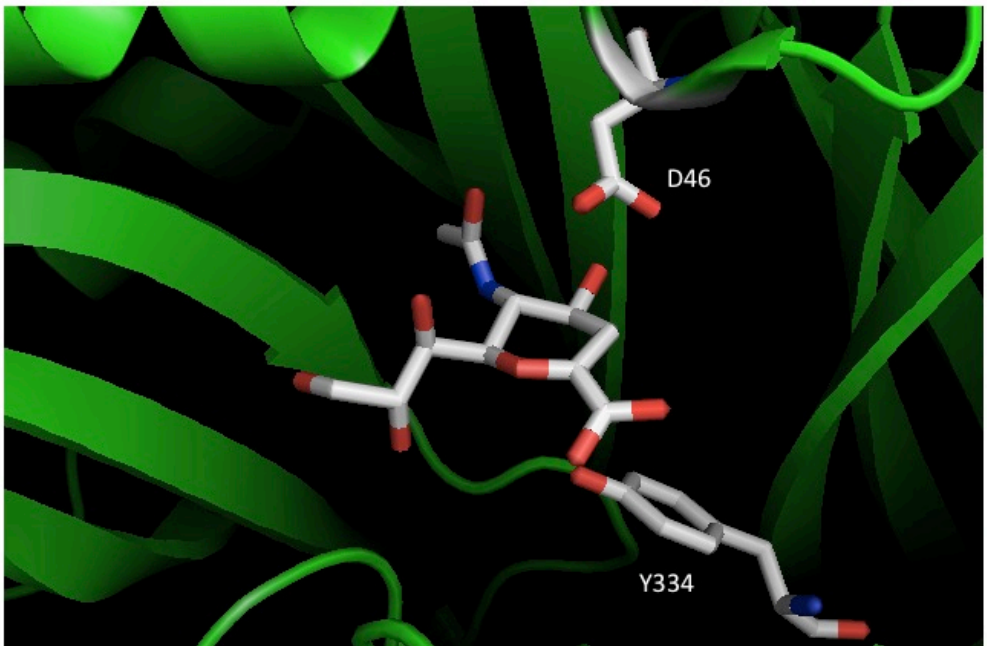

Figure 3.5. NEU2 active site. NEU2 active site highlighting catalytic residues D46 and Y334 shown as sticks around the sialic acid transition state inhibitor DANA (2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid)

substrate by mass spectrometry. This approach would also be generalizable to any sialidase/substrate combination making it a valuable tool in the glycobiologist's toolkit.

Results

Photocrosslinking of sialidases to their substrates

1) Crosslinking NEU2 to its substrates in K562 cells

In order to identify the substrates of NEU2 in K562 cells I first stably overexpressed a myc-tagged version of NEU2 or the catalytically inactive point mutant NEU2(D46A) (Figure 3.5). I verified the expression of the proteins by immunoblot against the myc tag**.** I also tested the lysates for sialidase activity and found that NEU2(D46A) expressing cells showed only basal levels of sialidase activity (data not shown). I then incubated these cells with 100 μM Ac_4ManNDAz-2me for 72 h. These cells were then exposed to 365 nm light for 45 min and then lysed and analyzed for high molecular weight bands by immunoblot against myc. In principle, bands should appear at a higher molecular weight that are reactive towards the myc antibody. These higher molecular weight species would represent the combined mass of NEU2 and its substrate. No crosslinked products could be detected as evidenced by a lack of high molecular weight species appearing in the +UV lanes (Figure 3.6).

a) Determining the fate of photocrosslinkers in K562 cells

To understand why this crosslinking was unsuccessful I looked at the fate of the diazirine containing ManNAc analogs that were fed to K562 cells. To study this, I relied on a well established approach for analyzing sialic acids from cells known as DMB derivitization. (*88*) In brief, this approach relies on the reactivity of the fluorophore DMB (1,2-diamino-4,5-methylene-dioxybenzene) with α-keto acids (such as sialic acids or its analogs) to produce a fluorescent compound that can

be analyzed by HPLC. This approach has been employed to evaluate sialic acids with various structural variations such as acetylation and hydroxylation thus giving me the confidence that this approach would be useful for analyzing our diazirine-containing sialic acid analogs. When K562 cells were supplemented with Ac_4ManNDAz-2me, SiaDAz-2me could be detected in the cytosolic fraction after derivitization. This indicated that K562 cells are capable of producing SiaDAz-2me from ManNDAz-2me. The first step of DMB derivitization is hydrolysis of the ketosidic bond between sialic acid and either CMP or an underlying glycan. This means that all of the sialic acid in the cell, whether activated or not, appears the same by this assay. One possible explanation for the lack of crosslinking that I observed is that the SiaDAz-2me is produced in these cells but not activated to CMP-SiaDAz-2me, thus preventing our photocrosslining sialic acid analogs from being incorporated into NEU2's substrates. To distinguish CMP-SiaDAz-2me and glycoconjugate-bound SiaDAz-2me from free SiaDAz-2me, I reduced the α-keto group of the free sialic acid with sodium borohydride prior to reacting with DMB. This process reduces the ketone of free sialic acids, but not CMP- or glycoconjugate-bound sialic acids thus

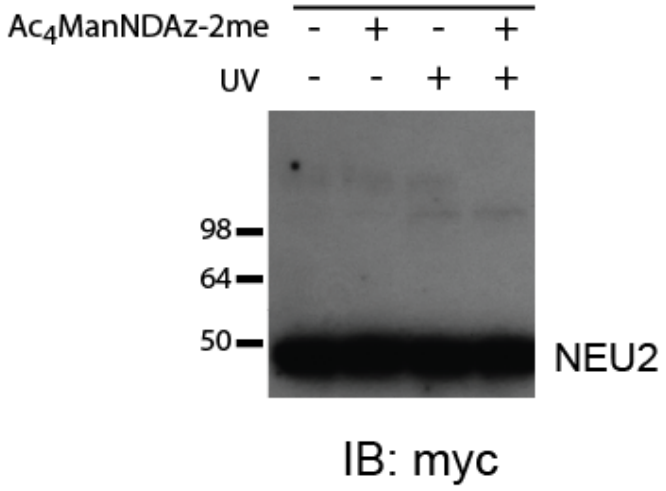

Figure 3.6. No crosslinking of NEU2 is detected in K562 cells. K562 cells expressing myc-NEU2(D46A) were grown in the presence of Ac_4ManNDAz-2me for 72 h followed by UV irradiation to initiate crosslinking. Cells were lysed and subjected to immunoblot for myc.

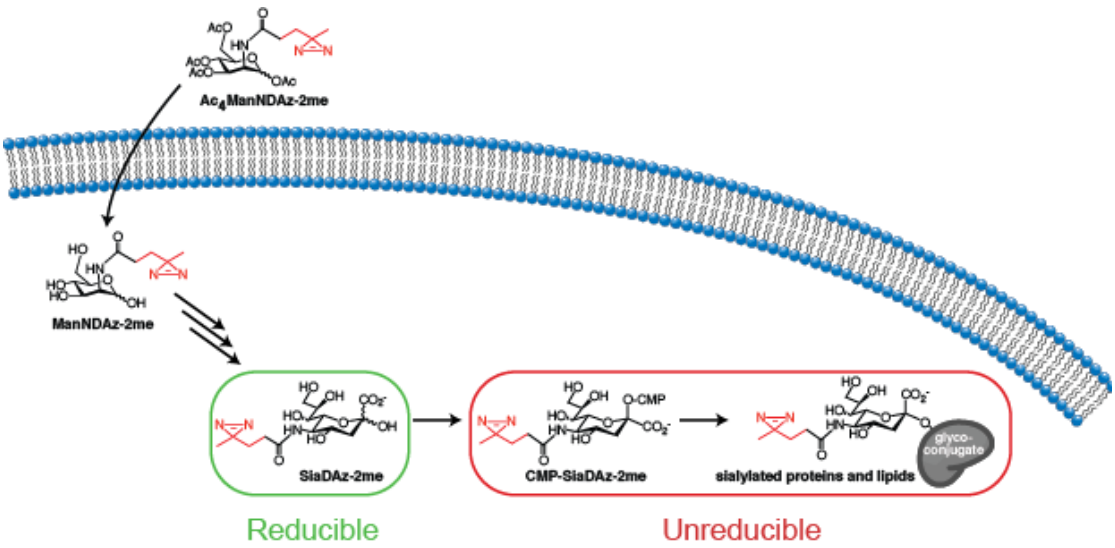

Figure 3.7. Schematic of the possible metabolic intermediates of SiaDAz-2me. Sialic acid analogs that are not conjugated to CMP or glycans can be reduced by borohydride prior to detection by DMB derivitization. CMP or glycan bound sialic acid analogs are insensitive to this reduction. This method can be used to distinguish the fate of sialic acid analogs in the cell.

preventing them from reacting with DMB (Figure 3.7) (*89*). Figure 3.8 shows that K562 cells supplemented with Ac_4ManNDAz-2me produce a peak corresponding to SiaDAz-2me but that this peak disappears completely upon reduction with borohydride. This is in contrast to results from Jurkat, a cell line that is known to produce cell surface SiaDAz-2me and has been used as a system to successfully crosslink sialic acid mediated interaction previously. (*84*) In Jurkat cells, a peak is still observed after borohydride reduction, indicating that CMP-SiaDAz-2me is being produced in Jurkat cells but not in K562 cells. These results indicate that K562 cells are not a useful cell line in which to attempt sialidase crosslinking. Further work will be needed to characterize what factors make K562 unable to incorporate these analogs.

2) Crosslinking GST-NEU2 to substrates in BJAB K20 cells

Next I tried using a cell line that has previously been demonstrated to incorporate our photosugars. (*83*) BJAB K20 are deficient in GNE which is the enzyme that catalyzes the first committed step of sialic acid biosynthesis. BJAB K20 are

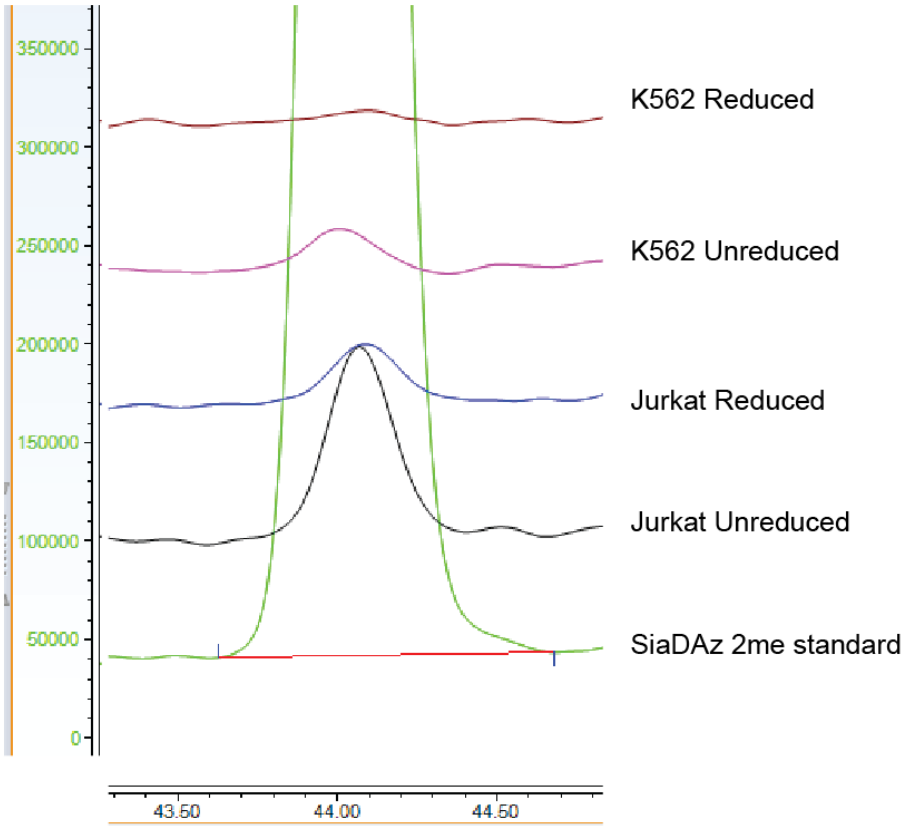

Figure 3.8. SiaDAz-2me remains unconjugated in K562 cells. K562 or Jurkat cells were incubated with 100 μM Ac_4ManNDAz-2me for 72 h. Cells were lysed and half the sample was treated with $NaBH_4$ to reduce unconjugated sialic acids. The samples were then DMB derivitized and analyzed by HPLC

dependent on exogenous sources of sialic acid or its precursors to generate sialic acid. (*90*) Upon serum starvation these cells show a dramatic reduction in their cell surface sialic acids as detected by flow cytometry analysis using sialic acid binding lectins. Upon supplementation with the cell permeable Ac_4ManNAc or Ac_4ManNDAz-2me, a restoration of cell surface sialic acid is observed. (*83*) I first tested that there were NEU2 substrates on the surface of K20 cells by demonstrating a reduction in cell surface staining for sialic acid upon treatment with WT GST-NEU2. (Figure 3.9 panel a). To do this I first serum starved the cells to deplete the cell surface of endogenous sialic acids. I then supplemented the cell with 100 μM of either Ac_4ManNAc or Ac_4ManNDAz-2me. I then treated

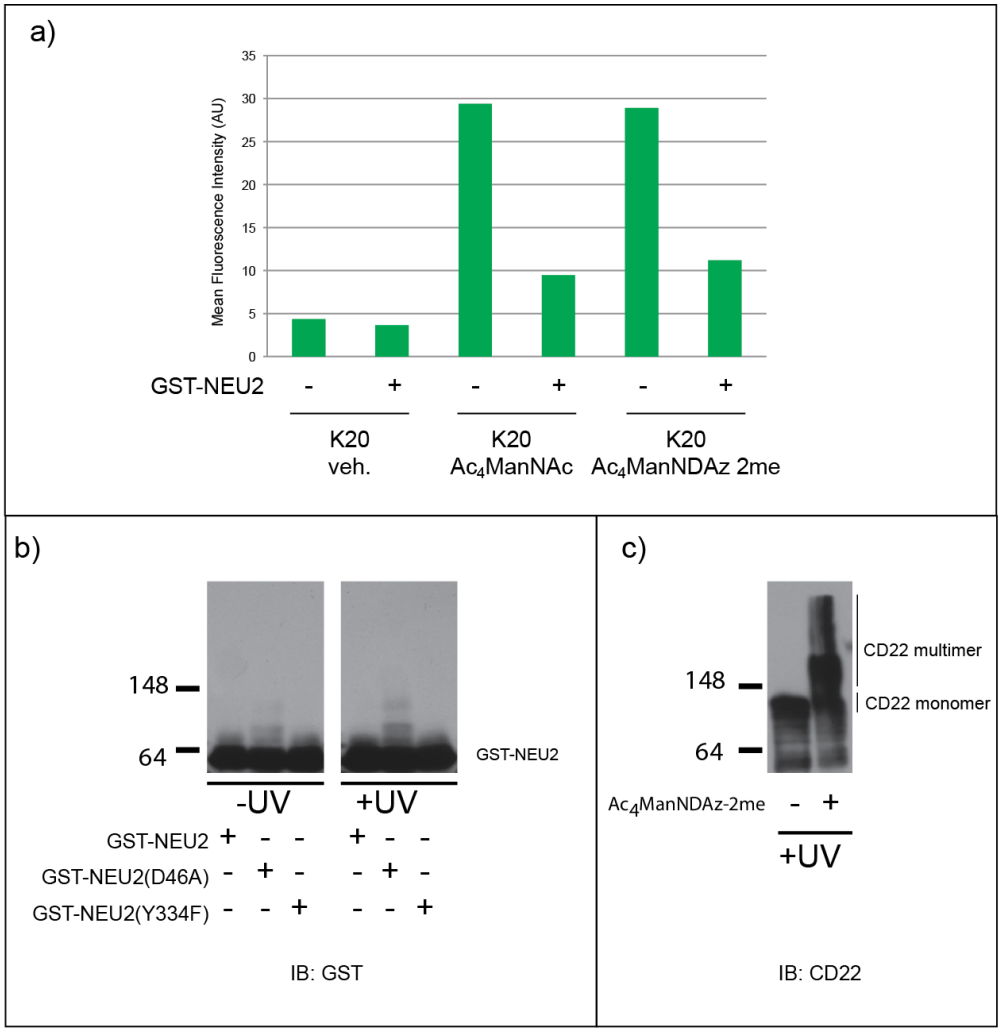

Figure 3.9. K20 cells contain NEU2 substrates yet crosslinking NEU2 to substrates does not occur. a) K20 cells were serum starved and supplemented either Ac_4ManNAc or Ac_4ManNDAz-2me then treated with GST-NEU2. Sialic acid content was analyzed by flow cytometry using MAL-biotin/Streptavidin-HRP b) K20 cells were serum starved and supplemented with Ac_4ManNDAz-2me. Cells were lysed and incubated with the indicated protein followed by UV irradiation and analysis by immunoblot against GST c) K20 cells were serum starved and supplemented with Ac_4ManNDAz-2me. Cells were UV irradiated and analyzed by immunoblot against CD22.

the cells with GST-NEU2 to hydrolyze cell surface sialic acids. I then probed for sialic acid on the cell surface with a biotinylated lectin MAL-II, which binds specifically to α2,3 linked sialic acids (*91*) followed by streptavidin-DTAF. I then measured the fluorescence by flow cytometry (Figure 3.9 panel a). I see a 6-fold increase in lectin binding upon supplementation with Ac_4ManNAc or Ac_4ManNDAz-2me after serum starvation. I also see a 3-fold decrease in sialylation upon GST-NEU2 treatment. These data suggest two things. First this indicates that there are substrates for NEU2 in these cells. Second, it demonstrates that NEU2 is able to hydrolyze SiaDAz-2me.

With the knowledge that there are indeed substrates for NEU2 on the surface of K20 cells I tested if I could crosslink GST-NEU2 to K20 cells. K20 cells were serum starved and then supplemented with Ac_4ManNDAz-2me for 72 h. The cells were then lysed and incubated with GST-NEU2 or one of two catalytically inactive mutants (D46A or Y334F). After 1 h the samples were exposed to 365 nm light to activate the diazirine and initiate crosslinking. After this the samples were separated by SDS-PAGE and analyzed by immunoblot using an antibody against GST. If crosslinking were successful I would expect to see high molecular weight bands or smears representing the combined mass of GST-NEU2 and its substrate(s). However, as shown in Figure 3.9 panel b, no such smearing appears in the lanes that were exposed to UV that is not also present in the samples protected from UV. Contrast these negative results with the positive control shown in Figure 3.9 panel c). In this case CD22 crosslinking is clearly evident in cells treated similarly as before. Note the high molecular weight bands and smear that appear in the Ac_4ManNDAz-2me and UV treated lanes. These results indicate that even though K20 contain substrates for NEU2 and can incorporate our photocrosslinking sialic acid analogs, they are unable to crosslink this sialidase with this substrate. This indicated that diazirine-mediated crosslinking was not a viable approach to identify NEU2 substrates in BJAB K20.

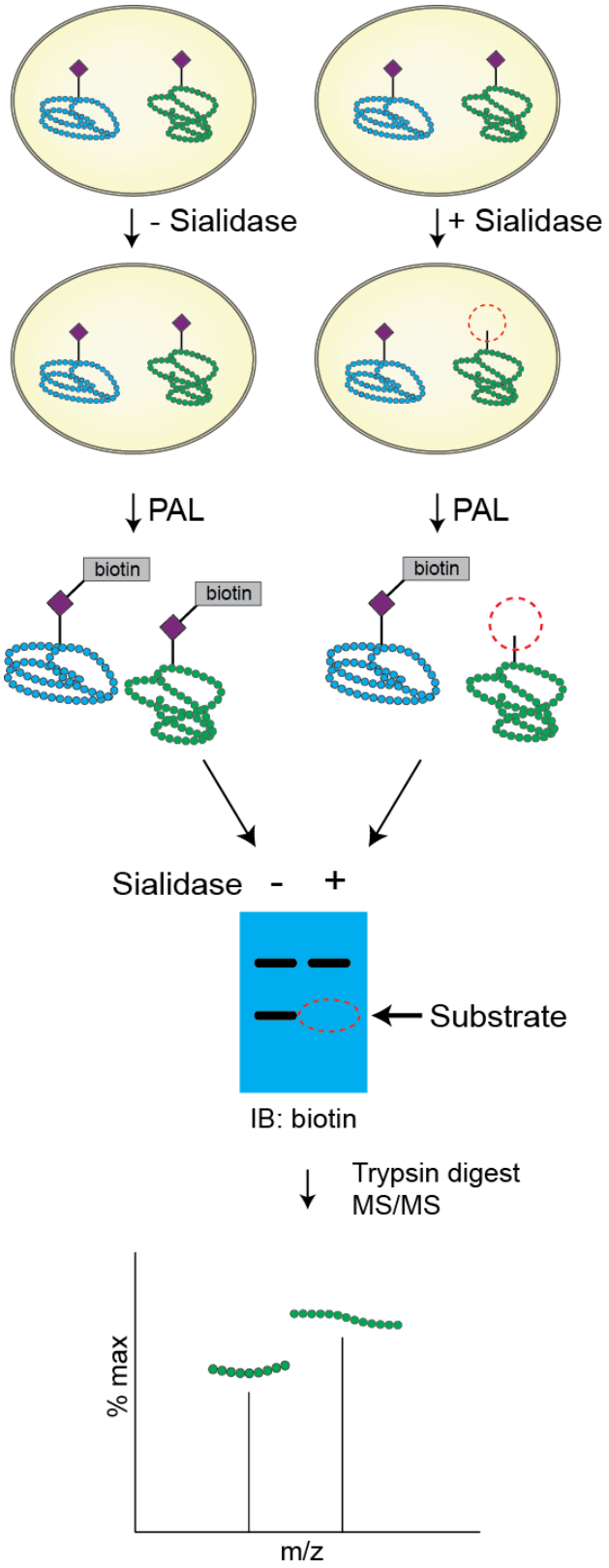

Figure 3.10 PAL labeling scheme. Cells expressing a sialidase or a catalytically inactive mutant are subjected to PAL analysis to label sialic acids with biotin. Differences in biotinylation can be detected by immunoblot and hits can be sequenced by MS/MS

PAL labeling of sialidase substrates

I next sought out an approach to discover sialidase substrates that did not rely on cellular incorporation of sialic acid analogs. I turned my attention to PAL (*P*eriodate oxidation and *A*niline catalyzed *L*igation) labeling of cells described by Paulson *et al.* as an approach to affinity label sialic acids of the plasma membrane in live cells. (*60*) PAL relies on mild periodate oxidation of the 7-position of sialic acids to an aldehyde. This aldehyde is then reacted with a hydroxylamine containing an affinity tag, in this case a biotin moiety. I envisioned an approach in which parallel cell lines, one expressing a sialidase and one not, were subjected to PAL to biotinylate the sialic acids of the cell. I would then probe for differences in sialic acid content of glycoproteins by immunoblot for the biotinylated sialic acids between the cells exposed to sialidase and those that were not. This could then be followed by label free mass spectrometry protein sequencing to determine the identity of the proteins desialylated by these sialidases (Figure 3.10).

To test this approach for feasibility, I once again turned to K562 cells. I stably overexpressed NEU2 or NEU2(D46A), a catalytically inactive mutant of NEU2 in these cells. These cells were lysed by sonication and the lysates were filtered through a 10 kDa molecular weight cut-off filter to remove small molecules that might inhibit the PAL labeling. PAL was then performed on the cell lysate to biotinylate the cells' sialic acid. As shown in Figure 3.11, I see biotin labeling of glycoproteins in samples treated with PAL but not in samples that were not treated with PAL. Unfortunately, I saw no differences between the lanes representing NEU2 expressing cells and NEU2(D46A) expressing cells. To test if this was simply a matter of expression level of the enzymes, I incubated the lysate expressing NEU2(D46A) with 40 μg (representing 100 mU of enzyme) of recombinant GST-NEU2 to attempt to see any desialylation could be detected. Even under these extremely high levels of active sialidase, no differences in sialylation could be detected. Attempts to enrich these samples using

streptavidin-coated beads yielded the same results: showing no difference between the NEU2 and NEU2(D46A) samples (data not shown). This indicates that PAL labeling is not a useful option for sialidase substrate discovery at least in the cytosol.

Discussion

Despite their importance relatively little is known about the mechanism through which sialidases influence signaling pathways. This is due in large part to the fact that the protein components of sialidase substrates remain unidentified. Currently there is no widely applicable approach available to identify the protein components of sialidase substrates.

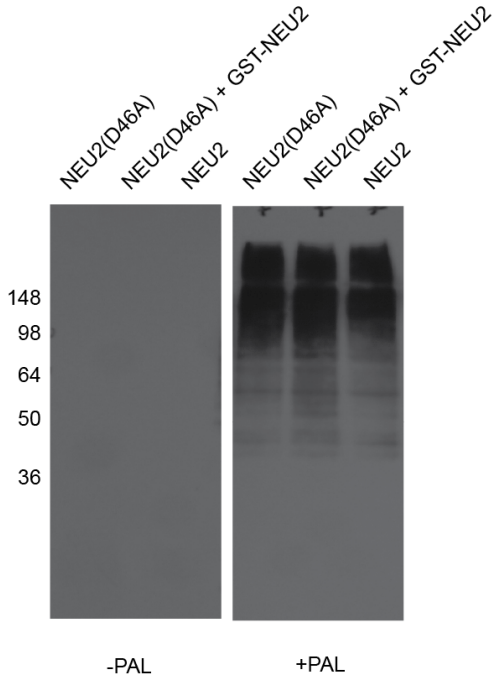

Figure 3.11 PAL labeling detects no changes in cellular sialic acid upon sialidase expression. K562 cells expressing NEU2 or NEU2(D46A) were lysed and PAL labeled as described in the methods. Samples were analyzed by immunoblot for biotin. Samples in lanes 2 and 5 were additionally incubated with 100 mU of GST-NEU2 to further hydrolyze sialic acids

The aim of this research was to generate tools that could be useful for identifying sialidase substrates. Towards this end I attempted two distinct approaches i) metabolic oligosaccharide engineering of photocrosslinking sialic acid analogs to covalently capture sialidase-substrate complexes and ii) PAL labeling of sialic acids with affinity handles for the identification of sialidase substrates.

i) Crosslinking

I attempted to incorporate our photocrosslinking sialic acid analogs into K562 cells because NEU2 restores sensitivity to apoptosis in these cells. Identification of the substrate of NEU2 in this context would have proven quite valuable. However, I observed that K562 are unable to incorporate our analogs. This was quite surprising because up to this point, all of the cell lines tested have been able to incorporate our analogs. Some cell lines, such as Jurkat and BJAB K20 cells, incorporate our analogs quite well. Yet my data indicate that K562 are unable to incorporate these analogs into glycoconjugates. Further work with cell lines in the lab demonstrated that some cell lines are able to incorporate our analogs while others are not. The cell lines that are able to incorporate our analogs are: Jurkat, BJAB K20, Daudi, HEK293, Caco-2, and T84. While those that do not incorporate our analogs include: K562, MDA-MB-231, HeLA and SW48. Why there is a discrepancy in the ability of different cell lines to incorporate our analogs remains a mystery. One possibility is that there is a mutation in K562 cells that causes the sialic acid metabolizing enzymes to no longer accept our analogs. Sequencing the genes known to be in the pathway would answer this question. Further work will be required to determine exactly why this discrepancy exists.

Metabolic incorporation of photocrosslinking sialic acids has proven useful for trapping known sialic acid mediated interactions. (*83, 84*) In principle, this should have worked for trapping sialidases with their substrates as well. There are several possibilities why this might not have worked. Despite several attempts I was never able to conclusively demonstrate that NEU2(D46A) is able to bind

sialic acids (data not shown). It is possible that this active site mutation abolishes the ability of the enzyme to bind its substrates. Another possibility is that the affinity of sialidases for their substrates is simply to low for this technique to trap these interactions. Such ephemeral interactions should be applicable to this photocrosslinking technology but it may be that I have found the lower limit of detection for this technique.

It is possible to estimate the lower limit of detection for crosslinking by comparing the affinities of the molecules that we know to be crosslinkable with those that are not crosslinkable. For example, it is known that CD22 crosslinks to itself readily in both prior experiments as well as in my hands (see Figure 3.9c). The affinity of CD22 for sialic acid is approximately 100μM (*92*) NEU2 meanwhile, does not appear to crosslink at all. There is no information about the affinity of human sialidases for their sialic acid. The affinity of bacterial sialidases for sialic acid ranges from the high-micromolar to millimolar K_d range. (*93*) It is reasonable to assume that human sialidases will share a similar millimolar affinity range. If human sialidases have a similarly weak affinity it is reasonable to assume that the lower limit of detection for crosslinking falls within this range *i.e*. millimolar affinities.

After the failures of metabolically incorporated SiaDAz to crosslink to NEU2 I wondered if I could generate a positive control that did not rely on cellular incorporation. I envisioned a small molecule or protein that contains our photocrosslinking sialic acids that could be generated *in vitro*. This compound could then be crosslinked to sialidases. This compound would eliminate the variables of the cell such as incomplete incorporation of crosslinker and allow me to test if sialidase mediated crosslinking is at all feasible. The development of these compounds is described in Chapter 4.

ii) PAL

PAL has proven useful for labeling sialic acids on the plasma membrane. (*60*) I was interested using this labeling approach to identify NEU2 substrates. Since NEU2 is a cytosolic protein, I attempted to adapt this procedure for use in whole cell lysates. There are several possibilities why this approach might not have worked. For one, it may be that the change in sialylation due to NEU2 expression is simply too low to be detected by this method. This explanation is somewhat belied by the dramatic decrease in cell surface sialylation that I see when I treat the cell surface with GST-NEU2 (Figure 3.9).

To test if PAL could detect sialidase activity against a model glycoprotein, the heavily sialylated glycoprotein fetuin was treated with the sialidase from *Arthrobacter ureafaciens*. In this experiment the band representing sialylated fetuin disappeared completely indicating that against a model glycoprotein and using a highly active sialidase desialylation could be detected by this method (data not shown).

Another possible reason why the PAL detection method did not prove useful in identifying sialidase substrates is the fact that I was looking for substrates in the cytosol. Initial experiments focused on cytosolic proteins as that is likely where NEU2 substrates will be found. Later proof of concept experiments looked at whole cell lysates in an effort to determine if the approach was feasible at all. Prior work in the lab has shown this labeling approach be useful in identifying sialidase substrates of bacterial sialidases on the surface of mammalian cells (Janet McCombs, personal communication). In these experiments she treated live cells with bacterial sialidases then performed PAL on intact cells then isolated the biotinylated sialosides for MS/MS analysis. This is in contrast to my experiments in which I attempted to probe whole cell lysates by PAL. My approach naturally includes components of the cytosol. This raises the possibility that there may be some unknown compound in the cytosol that inhibits the PAL reaction. I anticipated that the high concentration of free carbohydrates and DNA in the lysate might compromise the ligation so I sonicated and filtered the

samples prior to performing PAL. Yet, this had no effect on the detection reaction (data not shown). If there is indeed some inhibitory compound it is maintained by filtration suggesting that it is above 10 kDa in molecular weight as this is the cutoff of the filter that I used. Further work may be needed to identify this inhibitory factor of PAL labeling in the cytosol.

Given the important cellular processes that sialidases are involved in, identification of sialidase substrates remains an important and worthwhile field of research. Sialidases other than NEU2 may be applicable to either crosslinking or PAL labeling described in this chapter. NEU1 and NEU3, with their plasma membrane localization, should be especially applicable to PAL labeling on the cell surface.

Materials and Methods

Cell culture

K562 and BJAB K20 cells were cultivated in RPMI 1640 media containing 2 mM glutamine and supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, and 100 µg ml^{-1} streptomycin. Unless otherwise noted, cells were maintained in a water-saturated atmosphere at 37 °C and 5% CO_2. Typically, cell densities were maintained between 2.5 x 10^5 and 2.0 x 10^6. To generate BJAB cells in serum free conditions, the cells were grown in RPMI 1640 with 2 mM L-glutamine containing 1X Nutridoma SP, 50 U ml^{-1} penicillin, and 50 µg ml^{-1} streptomycin. Cells were cultured for two passages at 2.5 x 10^5 cells mL^{-1} in media for 72 h prior to supplementation with monosaccharides. Prior to the addition of cells to the tissue culture plate, Ac_4ManNAc, Ac_4ManNDAz-2me (10 mM stock in EtOH) was added to a final concentration of 100 µM and the solvent was allowed to evaporate in the dark prior to the addition of cells. Cells were incubated with the indicated sugar for 72 h.

NEU2 cloning and mutagenesis

Recombinant GST-NEU2 was prepared essentially as described. (*65*) Plasmid DNA encoding the human *NEU2* ORF was purchased from OriGene (RC219858). *NEU2* was cloned into the pGEX-4T-1 vector in-frame with GST using BamHI and XhoI restriction enzymes. Mutagenasis was carried out according to the Quikchange protocol. The pGEX-NEU2 plasmid was transformed into *E. coli* BL21(DE3). A single colony was selected and grown in ampicillin-containing LB to OD_{600} = 0.6 prior to induction with 0.1 mM IPTG. Induction was carried out for 16 h at 30 °C. Cells were harvested by centrifugation and lysed in 50 mL phosphate buffered saline (PBS), pH 7.4 containing 2 mg mL^{-1} lysozyme (Sigma) and one Complete Protease Inhibitor Cocktail Tablet (Santa Cruz) for 30 min followed by sonication. Lysates were centrifuged at 47,800 *g* for 1 h and insoluble material was discarded. The supernatant was applied to glutathione sepharose 4B column (27-4574-01, GE Healthcare), washed three times with 50 mM Tris-HCl, pH 8.0, and eluted with buffer containing 10 mM reduced glutathione. Protein content was analyzed by SDS-PAGE followed by Coomassie stain or immunoblot using antibodies against GST (GST(B14):SC-138, Santa Cruz) or NEU2 (ab55568, Abcam). 1 L of bacterial culture yielded 1 mg of protein with a specific activity of 4.06 μmol min^{-1} mg^{-1} or 4.06 units mg^{-1} (a unit is defined as the amount of enzyme required to release 1 μmol of methylumbelliferone per minute).

For stable expression of NEU2 in K562 cells the myc-*NEU2* ORF was cloned into a custom vector courtesy of Matthew Porteus (Stanford) for insertion into the CCR5 locus. This plasmid along with two Zinc finger nucleases was nucleofected into K562 cells according to manufacturers instructions. Generation of these vectors is described in (*94*). Cells were selected with 0.5 μg mL^{-1} puromycin and counterselected with 5 μM ganciclovir. Expression of NEU2 and its variants was verified by blotting against the myc tag with an anti-myc antibody (Cell Signaling 9B11)

Determination of GST-NEU2 specific activity.

GST-NEU2 (17 ng) was incubated with 1 mM methylumbelliferyl-*N*-acetyl-α-*D*-neuraminic acid (4-MU-NANA) in buffer containing 100 mM NaOAc, pH 5.6, and 100 mM NaCl for 1 h at 37 °C in a final volume of 100 µL. The reaction was quenched with 50 µL of 500 mM $NaHCO_3$ at a pH of 10.5 and the fluorescence was measured on a Spectramax M5 plate reader (excitation: 325 nm, emission: 420 nm). The fluorescence of methylumbelliferone released by sialidase activity was compared to that of methylumbelliferone standards to calculate the amount of sialic acid released, which was used to calculate specific activity.

Crosslinking NEU2 in K562 cells

K562 cells stably expressing myc tagged NEU2 or NEU2(D46A) were incubated with 100 μM Ac_4ManNDAz-2me for 72 h followed by UV irradiation for 20 min (Black Ray Lamp, XX- 20BLB, VWR cat #21474-676). The cells were lysed in RIPA buffer containing 1x Complete protease inhibitor cocktail (Santa Cruz). Samples were immunoblotted for myc (Cell Signaling 9B11)

DMB derivitization

Cells were harvested and lysed in hypotonic lysis buffer by passage through a 25.5 gauge needle 30 times. Borohydride reduction was carried out by adding sodium borohydride to a final concentration of 0.4 M and incubating samples at 0° C for 16 h. Acetic acid was added to a final concentration of 2 M and the samples were heated at 80° C for 2 h. The samples were cooled and 80 μL of DMB reaction solution (7.0 mM DMB, 0.75 M 2-mercaptoethanol, 18 mM $Na_2S_2O_4$, 1.4 M acetic acid) was added. These samples were heated at 50° C for 2 h. The samples were cooled and filtered prior to HPLC analysis (Dionex Ultimate 3000 system. Column: Dionex polaradvantage (061321). eluent: acetonitrile/water)

Sialidase treatment of cells

BJAB K20 cells were serum starved and supplemented with 100 μM of the indicated sugar for 72 h. Cells were harvested and 0.1 x 10^6 cells were incubated in 100 mM NaOAc/AcOH buffer pH 5.6 with 100 mM NaCl containing 100 mU of GST-NEU2 for 1 h. the cells were pelleted at 900 x *g* and washed in PBS pH 7.4 + 0.1% BSA. Cells were incubated with 10 μg mL^{-1} of MAL-II-biotin for 1 h on ice. Cells were washed twice in PBS + 0.1% BSA and incubated in 10 μg mL^{-1} Streptavidin-DTAF for 1h followed by washing twice with PBS + 0.1% BSA. Cells were analyzed on FACSCaliber flow cytometer. DTAF was measured in the FL1 channel. Data was analyzed on FlowJo (Tree Star Inc.)

Crosslinking GST-NEU2 to K20 lysates

BJAB K20 cells were serum starved and supplemented with 100 μM of the indicated sugar for 72 h. These cells were lysed in hypotonic lysis buffer and GST-NEU2 or its mutants were added to a final concentration of 1 μg mL^{-1}. Samples were incubated 16 h at 4° C followed by UV irradiation at 365 nm light (Black Ray Lamp, XX- 20BLB, VWR cat #21474-676) for 45 min on ice. Samples were then analyzed by immunoblot for GST using anti-GST antibody (Santa Cruz SC-138)

PAL labeling K562 cells

K562 cells stably expressing NEU2 or NEU2(D46A) were lysed in hypotonic lysis buffer by sonication on Fisher scientific 500W probe sonicator at 20% amplitude for 3 x 5 s. This lysate was applied to Amicon ultra 10 kDa molecular weight cutoff filters and centrifuged at 17,000 x *g* for 5 min. The concentrated sample was diluted in PBS pH 7.4 ten-fold and the filtration was repeated a total of three times. $NaIO_4$ was added to a final concentration of 1 mM and the lysate was incubated on ice for 30 min. An excess of glycerol was added to quench the oxidation and the lysates were filtered as described above three times.

Aminooxy-biotin was added to a final concentration of 10 μM and aniline was added to a final concentration of 10 mM. The samples were incubated on ice for 90 min. The lysates were then immunoblotted for biotin using anti-biotin antibody (Jackson research 200-032-211).

Chapter 4: Generation of photocrosslinking probes *in vitro*

Introduction

Sialic acids are an important class of carbohydrate responsible for mediating numerous interactions in the cell. These carbohydrates are positioned at the terminal position of the glycan chain putting them in prime position to interact with the environment. Indeed, many cell-cell and protein-protein interactions are mediated by sialic acids. Leukocyte homing and rolling along the endothelium is mediated by sialic acid-protein interactions. (*7*) Glycan-protein interactions are typically low affinity and have rapid off-rates making discovery of novel interactions difficult to perform by traditional biochemical approaches. (*15*) Therefore, new tools would be useful to aid in discovering novel sialic acid mediated interactions.

An *in vitro* reaction for generating photocrosslinking sialic acid analogs

Photocrosslinkers have been gaining increasing popularity to identify novel interactions between biomolecules. (*82*) Photocrosslinkers have been installed into proteins, lipids, and carbohydrates. I desired to use a sialic acid analog containing a photocrosslinker that would allow me to identify sialic acid binding partners. (*83*) The Kohler group pioneered the use of sialic acid analogs incorporating a diazirine functional group for use as a photocrosslinker. When exposed to ultraviolet light at 365 nm, diazirines dissociate to form a highly reactive carbene. This carbene can then form a covalent bond to any nearby heteroatom bond. This approach of incorporating diazirines into sialic acid analogs has successfully been used to crosslink sialic acid dependent protein-protein as well as protein-lipid interactions. (*83, 84*)

Having shown in Chapter 3 that our photocrosslinking sialic acid analogs are unable to be incorporated into certain cell types I next wanted to evaluate whether I could generate these photocrosslinking sialic acids in vitro, outside the

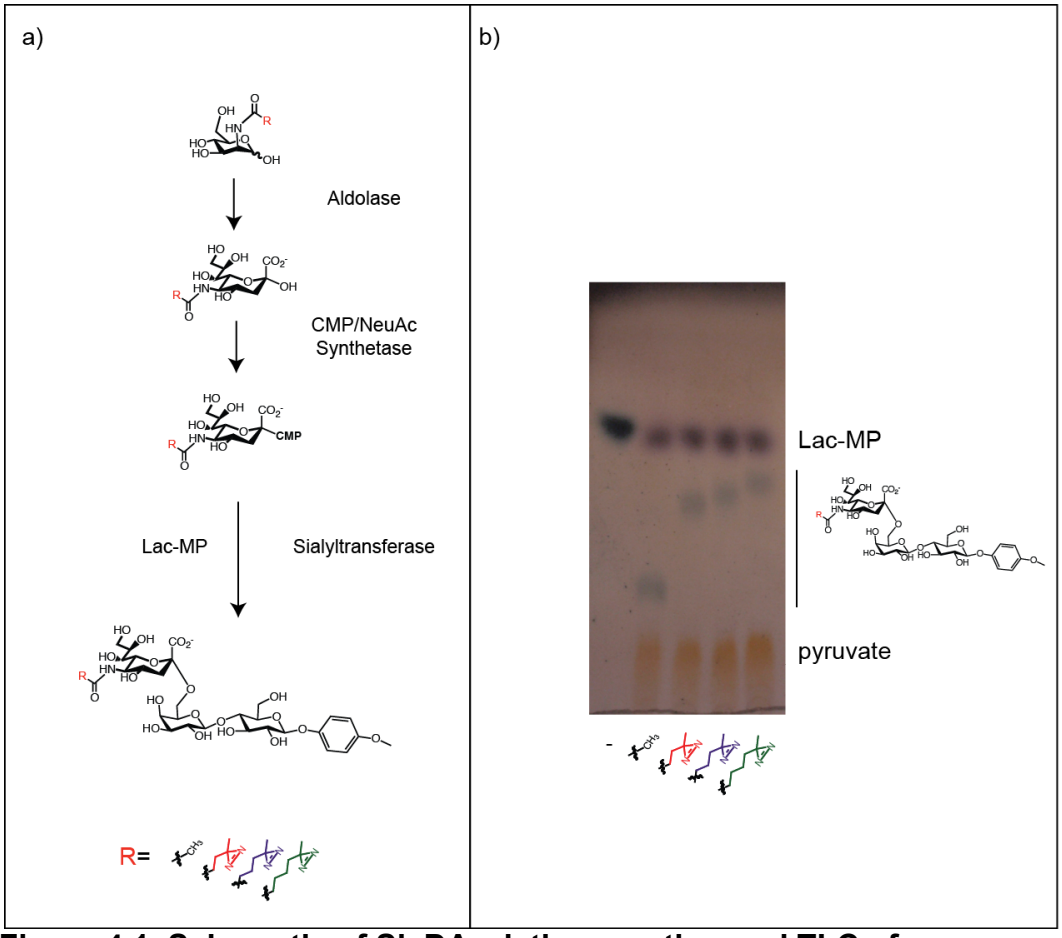

Figure 4.1. Schematic of SiaDAzylation reaction and TLC of SiaDAzylation of Lac-MP. a) Schematic of SiaDAzylation reaction b) Lac-MP was SiaDAzylated with the indicated sugars and the reaction was analyzed by TLC

confines of the cells metabolic machinery. In principle these sialic acid analog probes would be attached in place of endogenous sialic acid to oligosaccharides on glycoproteins. I was inspired by the work of Xi Chen who has shown that bacterial enzymes can be used to generate non-natural sialic acid analogs. (*52, 75*)

The first step of the reaction involves a NeuAc aldolase from E. coli which catalyses the condensation of ManNAc with pyruvate to form the sialic acid analog. Then the CMP-sialic acid synthetase couples this sialic acid analog to

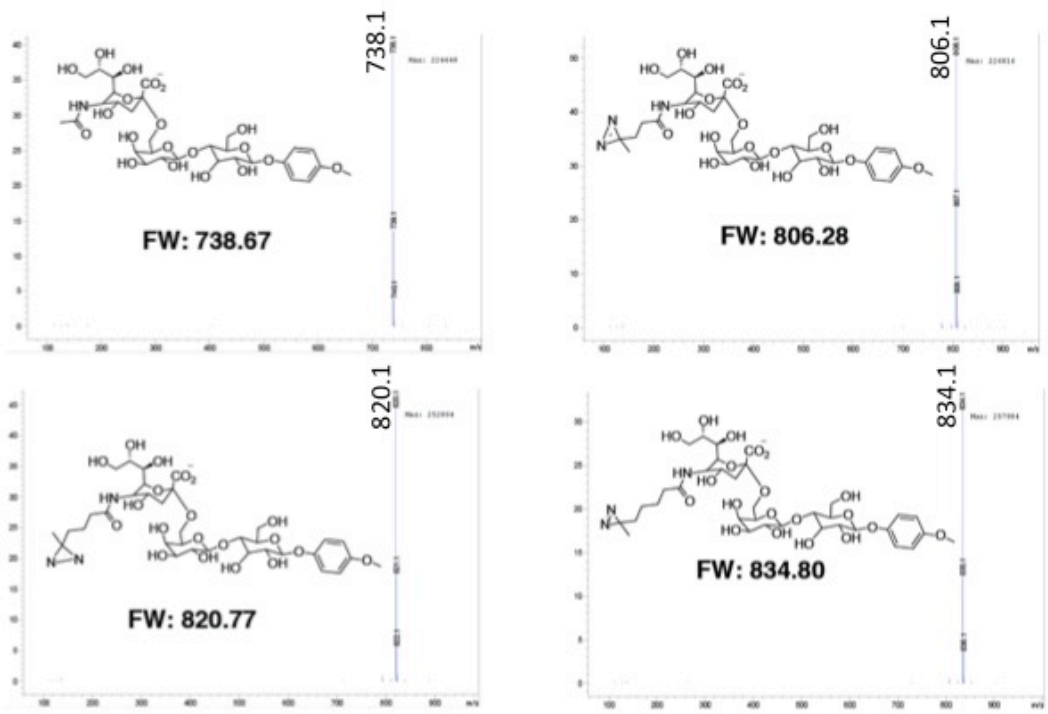

Figure 4.2. LC/MS confirms the identity of SiaDAz-ylated compounds.

CTP to produce CMP-sialic acid analog. Finally, a sialyltransferase transfers the sialic acid analog to the acceptor sugar (Figure 4.1).

Using this reaction Xi Chen was able to make numerous sialic acid analogs with modifications at the *N*-acyl position of sialic acid. (*75*) I was inspired by this to see if I could use this reaction to generate diazirine containing sialic acid analogs in vitro rather than in cells.

Prior experiments in the lab have shown that the length of the linker between the N-acyl and diazirine groups can affect the crosslinking efficiency of these analogs. The distance between the diazirine and carbonyl group is measured in methylene units (2me, 3me 4me). The longer linker lengths (3me, 4me) have shown good crosslinking of sialic acid mediated interactions even though the incorporation of these molecules into the cell surface was quite poor. Therefore, I

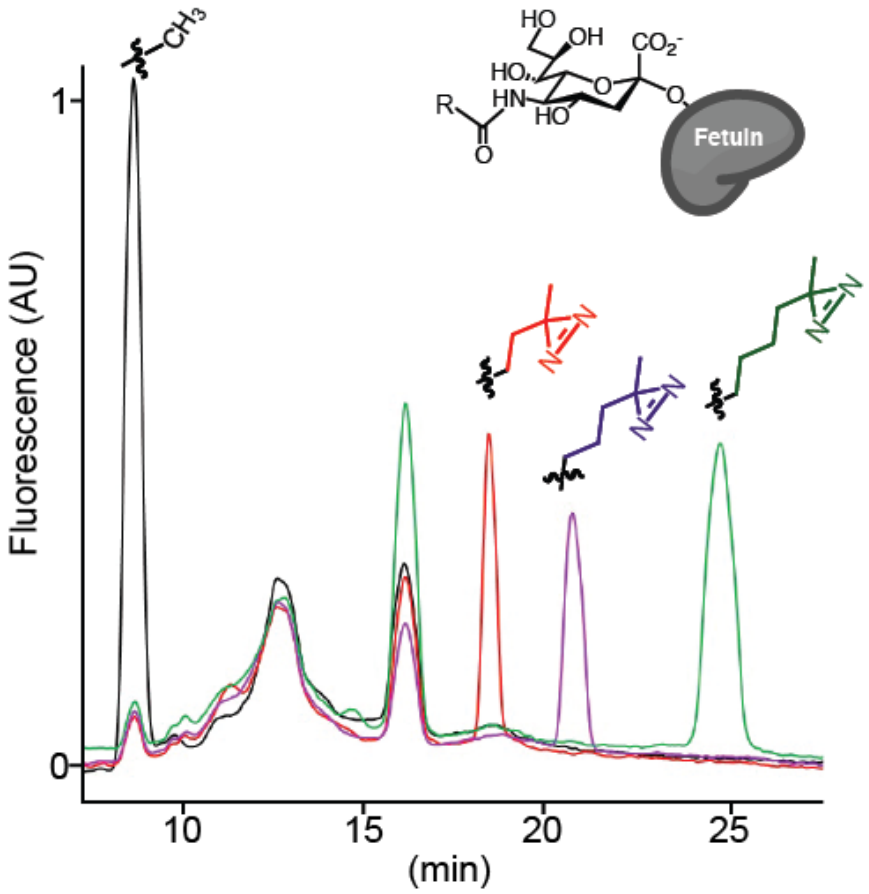

Figure 4.3. Photocrosslinking analogs are incorporated into asialofetuin. Asialofetuin was SiaDAz-ylated as described and after extensive washing the sialic acid analogs were hydrolyzed and DMB derivitized prior to HPLC analysis. Peak labels identify each analog by its side chain modification.

decided to test if I could standardize the incorporation of these analogs using this *in vitro* SiaDAz-ylation approach.

Results

Incorporation of photocrosslinking sialic acids into a small molecule

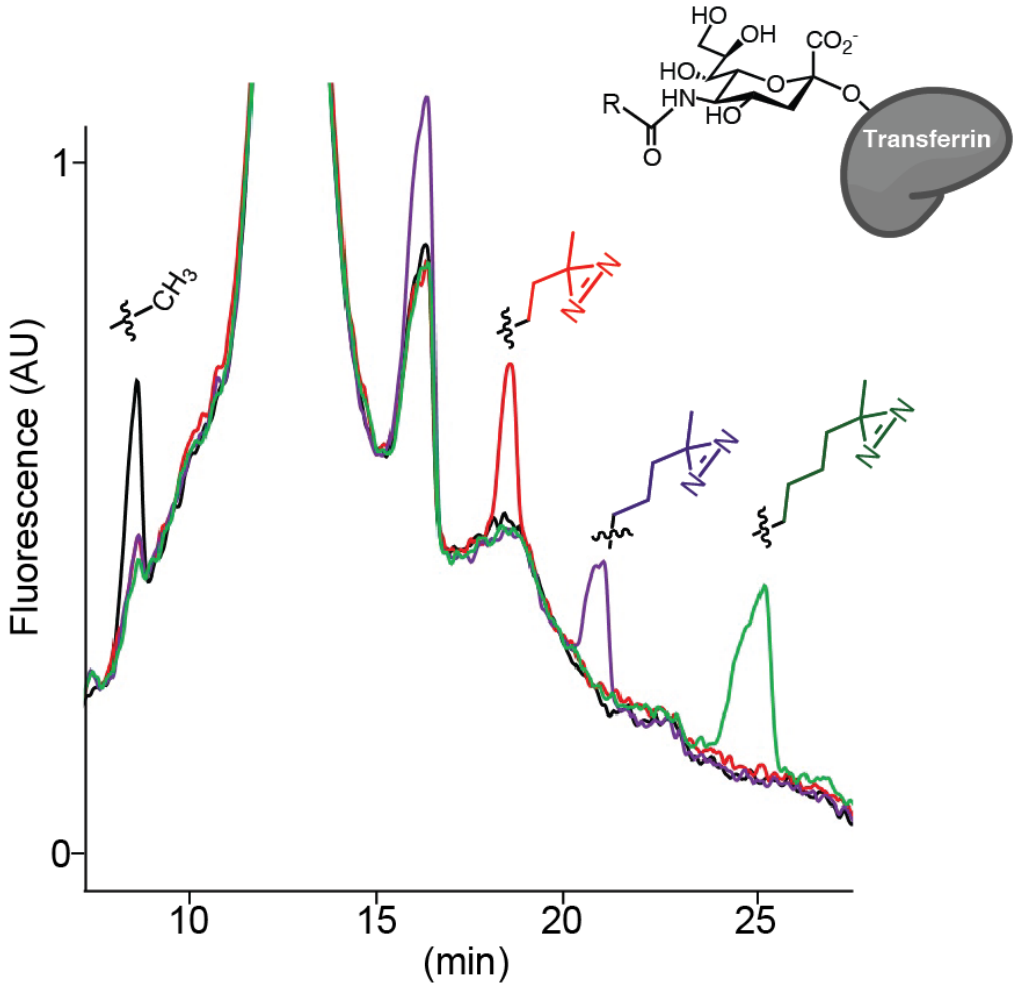

Figure 4.4. Photocrosslinking analogs are incorporated into transferrin. Transferrin was sialidase treated and SiaDAz-ylated as described and after extensive washing the sialic acid analogs were hydrolyzed and DMB derivitized prior to HPLC analysis. Peak labels identify each analog by its side chain modification.

I first tested whether this reaction could tolerate our photocrosslinking ManNAc analogs using a small molecule acceptor. Lactose-methoxyphenyl (Lac-MP) was used as an acceptor because it is easily detectable by TLC and HPLC due to the UV absorbing methoxyphenyl group. When the SiaDAz-ylation reaction was performed with either ManNAc or ManNDAz-2me, ManNDAz-3me or ManNDAz-4me new spots appeared by TLC that correspond to the decreasing polarity of these analogs (Figure 4.1). LC/MS of the reaction confirmed the presence of SiaDAz-Lac-MP as a product (Figure 4.2).

Incorporation of photocrosslinkers into proteins

Having shown that the SiaDAz-ylation reaction works on small molecule acceptors I next looked to see if I could incorporate our analogs into glycoproteins. Fetuin is a well-studied model glycoprotein that is heavily sialylated. There are 13 residues of sialic acid per molecule of protein. (*95*) Both fetuin and asialofetuin (chemically desialylated fetuin) are commercially available making them attractive targets for testing this SiaDAz-ylation reaction. The SiaDAz-ylation reaction was performed on asialofetuin to ensure that there were vacant acceptor sites available for the sialic acid analogs. After the reaction, the protein was washed exhaustively by filtration to remove any unreacted sialic acid analog. The protein was then subjected to DMB derivitization. In brief: sialic acids were hydrolyzed by incubation with 2M acetic acid then the freed sialic aids were reacted with the fluorophore DMB to produce DMB-sialic acid or its analogs. These could then be resolved by HPLC with fluorescent detection. Figure 4.3 shows that all the analogs tested (2me 3me and 4me) were incorporated equally well by these enzymes into asialofetuin.

Most of the proteins that one would want to SiaDAz-ylate will already be sialylated, which would prevent the addition of our photocrosslinking analogs. I wanted to develop a protocol to desialylate these proteins and subsequently SiaDAz-ylate them with our analogs. To test this I chose the sialylated protein transferrin. First I desialylated it using the broad specificity sialidase from *Arthrobacter ureacfaciens*, I then washed the protein to remove the liberated sialic acid and then SiaDAz-ylated it as above. As shown in Figure 4.4 each of the analogs is incorporated into transferrin.

Characterization of the glycans that are SiaDAz-ylated

Fetuin contains both *N*- and *O*-linked glycans. Some of the anti-inflammatory properties of fetuin are dependent on the presence of sialic acids contained in *N*-linked glycans. (*96*) In order to use this SiaDAz-ylation approach to capture sialic

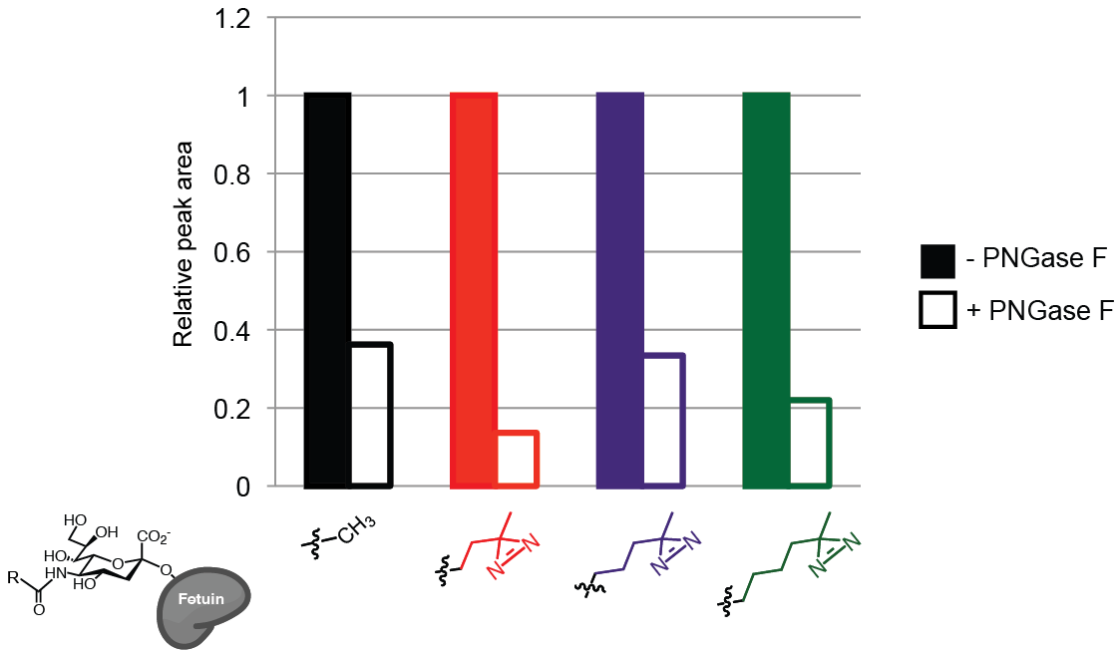

Figure 4.5. Sialic acid analogs are incorporated into *N*-linked glycans. Asialofetuin was SiaDAz-ylated with the indicated analog and then the *N*-linked glycans were hydrolyzed with PNGase F. After washing, the remaining glycans were hydrolyzed and DMB derivitized and analyzed by HPLC.

acid mediated interactions it will be important to fully characterize the types of glycans that SiaDAz is incorporated into. Therefore, I next wanted to determine whether our sialic aid analogs are incorporated to one or both of these types of glycans.

To determine if SiaDAz is incorporated into *N*-linked glycans I used an enzymatic method to hydrolyze the *N*-linked glycans and then looked to see if there was a change in the levels of SiaDAz on the protein. PNGase F is an enzyme that is widely used in the glycobiology field because it specifically cleaves the bond between *N*-linked glycans and the asparagine to which they are attached. (*97*) Therefore, I first SiaDAz-ylated fetuin as before, then treated the sample with PNGase F to remove *N*-linked glycans. After washing away the liberated glycans I then performed DMB derivitization as before to evaluate the amount of SiaDAz remaining. I saw a dramatic decrease in the peak area of the SiaDAz analogs

after PNGase F treatment (Figure 4.4, hollow bars) compared to the untreated samples (Figure 4.4, solid bars) indicating that our SiaDAz analogs are incorporated into *N*-linked glycans. The SiaDAz remaining after PNGase F treatment could be due to SiaDAz contained in *O*-linked glycans or it could be due to incomplete removal of *N*-linked glycans by the enzyme. Further work will be needed to distinguish these two possibilities.

Discussion

The difficulties that I had incorporating photocrosslinking sialic acid analogs into cells (discussed in Chapter 3) led me to explore alternative approaches to generate these analogs. I decided to create probes *in vitro* that contain our sialic acid analogs. Performing the SiaDAz-ylation reaction *in vitro* would allow me to alter the reaction conditions to maximize products in such a way that would not be possible when doing this in cells.

There are a number of shortcomings with metabolic incorporation of photocrosslinking sialic acid analogs into cells that are addressed by these *in vitro* generated probes. For one, they eliminate the need for cellular metabolism of the analogs. As I demonstrated in Chapter 3, this can be a significant drawback, as some cell lines simply cannot process our analogs. Additionally, with the *in vitro* reaction, I was able to show equal incorporation of our different linker lengths (SiaDAz-2me, SiaDAz-3me, and SiaDAz-4me). This was quite a pleasant surprise since cellular experiments using the longer linker lengths showed little to no incorporation of these analogs onto the cell surface. Despite this low incorporation, SiaDAz-3me and SiaDAz-4me still showed detectable crosslinking of proteins. (*83*) This suggests that the longer linker lengths make more efficient crosslinkers. With these photocrosllinking probes in hand we can directly test whether this is the case or not. Another drawback to using cells to generate photocrosslinkers is that the amount of crosslinked material generated

is usually quite low, making analysis by immunoblot difficult. These photocrosslinking probes described here should circumvent both of these problems.

I have successfully demonstrated incorporation of SiaDAz into small molecules. I have demonstrated that SiaDAz-2me, SiaDAz-3me, and SiaDAz-4me are all incorporated into Lac-MP at comparable levels. Incorporation of SiaDAz into small molecules could easily be adapted to generate biotinylated small molecules with photocrosslinking sialic acid analogs. This could be applied to cells or tissues and irradiated to crosslink sialic acid binding partners that could then be purified by the biotin affinity handle. This approach could be useful for discovering novel sialic acid binding proteins.

SiaDAz-ylation of proteins *in vitro* could be useful for discovery of sialic acid binding partners when one component is already known. For example, fetuin has anti-inflammatory effects that are dependent on sialic acids. Administration of fetuin, but not asialofetuin, to mice or isolated macrophages leads to a decrease in the secretion of the inflammatory cytokine HMGB-1. (*96*) Clearly, sialic acid is important for this process, but the receptor for sialylated fetuin in this context remains unidentified. SiaDAzylated fetuin could be applied to isolated macrophages and crosslinked to its receptor(s). Mass spectrometry sequencing could then be used to identify the receptor(s).

There are a number of examples in the literature where sialylation of a protein is important for mediating interactions. Discovering the identity of the interacting partner is often is difficult due to the low affinity nature of these interactions. One example is the Immunoglobulin G (IgG) antibodies. IgG antibodies consist of highly variable FAb domains, which bind antigens, and relatively constant Fc domains, which bind to Fc receptors (FcgR) on leukocytes. However, recent data suggests that the Fc domains are not as constant as previously thought and that the source of this variability is due to an *N*-linked glycan. (*98*) Even monoclonal

antibodies, which are supposedly identical, have a distribution of glycans. Importantly, differences in sialylation on the Fc domain affect the biological activity of IgG, switching it from being pro- to anti-inflammatory. (*99*) How the presence of sialic acid does this is currently a mystery as the sialic acid resides far from the Fc receptor binding site and doesn't significantly alter the structure of the Fc receptor binding interface. (*100*) This raises the intriguing possibility that there is an alternate receptor for sialylated IgG that specifically recognizes the sialylated form of this protein. Use of my in vitro strategy to incorporate photocrosslinking sialic acids could reveal just such an alternate receptor.

One potential drawback to this approach is that the diazirine might disrupt binding to sialic acid binding partners. If this proves to be the case the diazirine can be moved to the 9-position of sialic acid to accommodate tight binding pockets around the *N*-acyl side chain of sialic acid.

Another potential pitfall is that the chosen sialyltransferase might have substrate specificity that precludes certain glycan types. Human sialyltransferases are known to have preferences for certain substitutions at varying positions in their acceptors. (*101*) It is possible that the bacterial sialyltransferase that I am using will run in to this problem as well. I have already tested that human ST6Gal1 is active under these enzyme conditions and is capable of transferring all of our photosugars to fetuin (data not shown). Future work should include testing a panel of sialyltransferases for activity in this assay and with our photosugars.

So far I have only tested sialyltransferases that catalyze the sialic acid in an α2,6 linkage. It will certainly be desirable to be able to add photosugars in an α2,3 linkage as well as an α2,8 linkage. Future work should include attempts to use α2,3 and α2,8 specific sialyltransferases in this reaction.

Materials and Methods

General

Fetuin, asialofetuin, *Pasteurella damsela* sialyltransferase (PdST), *Neisseria meningititis* CMP-Sialic acid synthetase (NmCSS), Cytidine triphosphate disodium salt, sodium pyruvate, and *N*-acetylmannosamine (ManNAc) were purchased from Sigma-Aldrich. NeuAc aldolase was purchased from Toyobo. ManNAc analogs were a gift from Dr. Yibing Wang synthesized essentially as in (*83*). Lac-MP (4-methoxyphenyl-β-D-lactopyranoside) was purchased from TCI (M-1805).

SiaDAz-ylation reaction

The SiaDAz-ylation of Lac-MP was carried out in 100mM Tris-HCl pH 8.8 containing 20 mM $MgCl_2$, 7.5 mM ManNAc (or analog), 7.5 mM CTP, 40 mM sodium pyruvate, 2.5 mM Lac-MP, 2.3 mg mL^{-1} NeuAc Aldolase, 0.4 mU NmCSS, 0.2 mU Pd2,6ST, and 0.05% NaN_3 in a final volume of 50 μL. This reaction was incubated for 16 h at 37°C. The reaction was analyzed by TLC using 4:2:1:0.1 Ethylacetate:methanol:water:acetic acid as eluent and detected by *p*-anisaldehyde stain

The sialylation of fetuin and transferrin was carried out in 100 mM Tris-HCl pH 8.8 containing 20 mM $MgCl_2$, 7.5 mM ManNAc (or analog), 7.5 mM CTP, 40 mM sodium pyruvate, 0.2 mg mL^{-1} asialofetuin, 2.3 mg mL^{-1} NeuAc Aldolase, 0.4 mU NmCSS, 0.2 mU Pd2,6ST and 0.05% NaN_3 in a final volume of 50 μL. This reaction was incubated for 16 h at 37° C. The samples were loaded onto an Amicon Ultra 10 kDa MWCO filter and centrifuged at 17,000 x *g* for 5 min. The sample was diluted ten fold in PBS pH 7.4 and centrifuged again. This process was repeated a total of 8 times. Acetic acid was added to a final concentration of 2 M in 100 μL and this reaction was heated at 80° C for 2 h. This reaction was cooled and 80 μL of DMB reaction solution (7.0 mM DMB, 0.75 M 2-mercaptoethanol, 18 mM Na_2S_2O4, 1.4 M acetic acid) was added and the

reaction was heated at 50° C for 2 h. The samples were cooled and filtered prior to HPLC analysis (Dionex Ultimate 3000 system. Column: Dionex polaradvantage (061321). Eluent: acetonitrile/water)

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