University of Alberta

Exploring the role of sialic acid in the glycoprotein LFA-1 using bioconjugate chemistry

by

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Dedicated to My Parents and Sister

ABSTRACT

The lymphocyte function associated antigen-I (LFA-1) is important to a variety of immune cell processes including immune synapse formation and lymphocyte homing. Studies have shown that lymphocytes exhibit increased adhesion upon sialic acid removal by neuraminidase. Recent studies of β1 integrin function have shown a role for sialic acid. We hypothesized that modifications to sialic acids on LFA-1 may influence ligand binding, as a potential mechanism responsible for the neuraminidase effect. Herein, we describe progress towards studying LFA-1 sialic acids and their role in LFA-1 mediated adhesion. We describe applications of metabolic labeling strategies. We demonstrate bioconjugation of alkynyl drag-tags for resolution of sialoforms of metabolically-labeled murine IgG. We demonstrate bioconjugation of synthetic carbohydrate epitopes to an azide-containing model protein by Staudinger ligation. Finally, we report the production of stable HeLa cell lines expressing GFP-fused NEU3, as a model system for *in vivo* studies of NEU3.

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ADMIDAS	Adjacent to Metal Ion-Dependent Adhesion Site
CMP	Cytidine monophosphate
EC	Endothelial cell
ECM	Extracellular matrix
EM	Electron microscopy
ESI	Electrospray ionization
FACS	Fluorescence activated cell sorting
FRET	Förster resonance energy transfer
Gal	D-Galactose
GalNAc	*N*-Acetylgalactosamine
GlcNAc	*N*-Acetylglucosamine
GFP	Green fluorescent protein
GnT	*N*-Acetylglucosaminyltransferase
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICAM	Intercellular Adhesion Molecule
IEF	Isoelectric focusing
Ig	Immunoglobulin
Lac	Lactose
LacNAc	*N*-Acetyllactosamine
LAD	Leukocyte Adhesion Deficiency
LC	Liquid Chromatography

LFA-1	Lymphocyte Function-Associated Antigen-1
LIMBS	Ligand induced metal ion binding site
mAb	Monoclonal Antibody
MAL-1	*Maackia amurensis* lectin-1
Man	D-Mannose
ManNAc	*N*-Acetylmannosamine
ManNAz	*N*-Azidoacetylmannosamine
MIDAS	Metal ion-dependent adhesion site
MS	Mass spectrometry
NEU	Neuraminidase
Neu5Ac	5*N*-Acetylneuraminic acid
PEG	Polyethylene glycol
PMA	Phorbol 12-myristate 13-acetate
PNGase F	Peptide N-glycosidase F
PTM	Post-translational modification
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNA	*Sambucus nigra* agglutinin
SPR	Surface plasmon resonance
ST6Gal I	β-galactoside- α-(2-6)-sialyltransferase

Chapter 1: Integrin structure, function, and glycosylation

1.1 Introduction to integrins

Cell adhesion molecules mediate adhesive interactions between cells, and with proteins in the extracellular matrix (ECM). These cell surface receptors are essential to life in multicellular organisms, allowing for growth of tissues and organs, cellular differentiation, and cell migration. Immune cells in circulation are especially dependent on adhesion receptors, which are vital for the trafficking of circulating immune cells, their migration through tissues, and for immune synapse formation.[1] Cell adhesion molecules are generally divided into four subgroups: integrins, selectins, cadherins, and the immunoglobulin superfamily.[2,3]

A classic example of the importance of cell adhesion molecules is provided by the inflammatory response, where circulating white blood cells must reach the peripheral tissue by interacting with endothelial surfaces of blood vessels. As shown in **Figure 1.1**, initial contact of a leukocyte with the endothelial surface is mediated by the selectins, which form weak, transient interactions with carbohydrate epitopes (such as the sialyl-Lewis^X tetrasaccharide)[4] on the leukocyte cell surface.[4] This interaction induces rolling of the leukocyte along the endothelial surface. The rolling phase allows for stronger adhesive interactions between leukocyte integrins and their ligands on the endothelial cell surface, leading to integrin-mediated firm adhesion, and subsequent extravasation of the leukocyte to the tissues.[5,6,7]

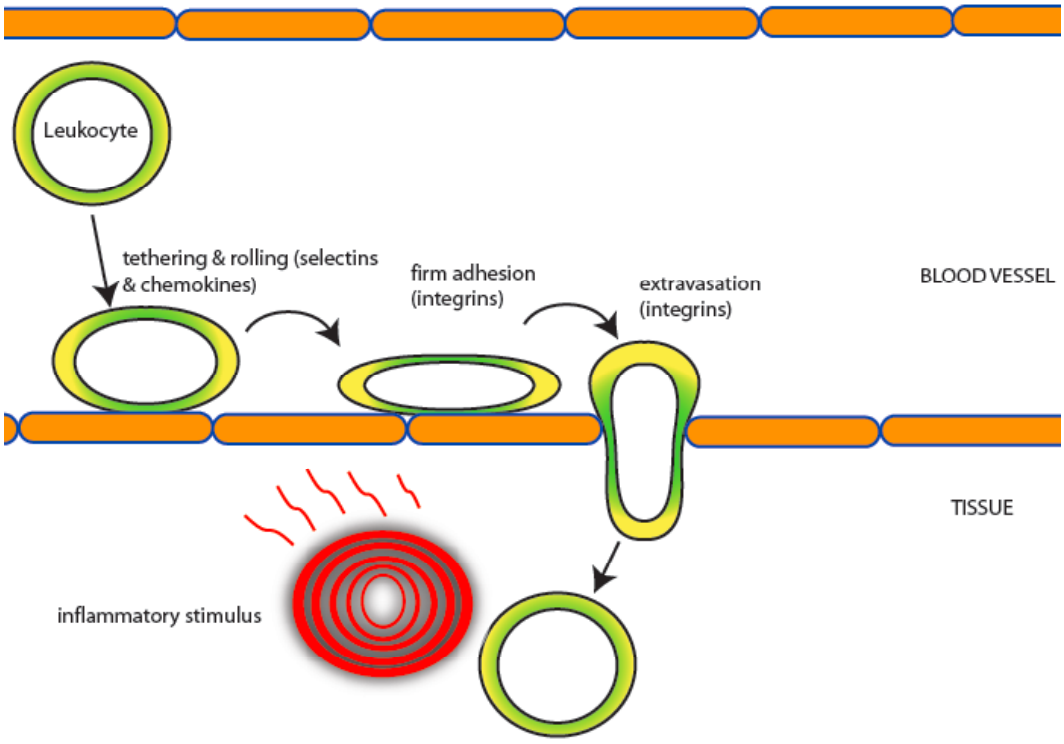

Figure 1.1 Leukocyte interaction with endothelial surfaces. Weak interactions between selectins and carbohydrate epitopes on the white blood cell, initiate the rolling phase, which is followed by integrin-mediated firm adhesion, and extravasation to the tissues.

Integrins are a family of cell surface receptors consisting of two non-covalently associated, α and β transmembrane glycoproteins, forming a heterodimer complex.[2] Integrins bind to protein ligands within the ECM or on the surface of other cells (e.g., fibronectin, collagen, and laminin). Proper integrin activity is essential for normal cellular function, growth, and survival. Integrin receptors have been implicated in a variety of physiological and pathological processes, including embryogenesis, immune synapse formation, wound healing, and tumour metastasis.[3,8] There are two general modes by which the cell can

regulate integrin binding; by modulating avidity, through integrin clustering, for example, or by modifying affinity, through integrin conformational change.[2,8,9] Integrins are unique cell adhesion molecules because their adhesiveness is dynamically regulated; either through inside-out signaling mechanisms, initiated by interactions with signaling molecules and cytoskeletal components inside the cell; or outside-in signaling, where ligand binding events outside the cell transmit signals to the cytoplasm. This ability to *integrate* intracellular and extracellular cues is the reason these receptors are called integrins.[1,2,10,11,12]

In mammals, 18 different α subunits and eight β subunits have been found, forming 24 unique integrin αβ heterodimers (**Figure 1.2**).[2,10] Different heterodimers give rise to different ligand specificities, and many integrins are unique to specific cell types.[2,10] The β2 integrins, for example, are only expressed on leukocytes. The variety of integrins (and other cellular adhesion molecules) expressed on a cell will largely govern cellular interactions with the surrounding environment. Because integrins are crucial to many immunological processes, defects in integrin expression or function have been implicated in an ever-growing list of pathologies. This list includes genetic diseases (such as Leukocyte Adhesion Deficiency (LAD)), autoimmune diseases (e.g. multiple sclerosis), bacterial infections (such as *Actinobacillus actinomycetemcomitans*, the main causative agent of periodontitis), viral infections (including HIV), and cancer metastasis.[2,3,10,11]

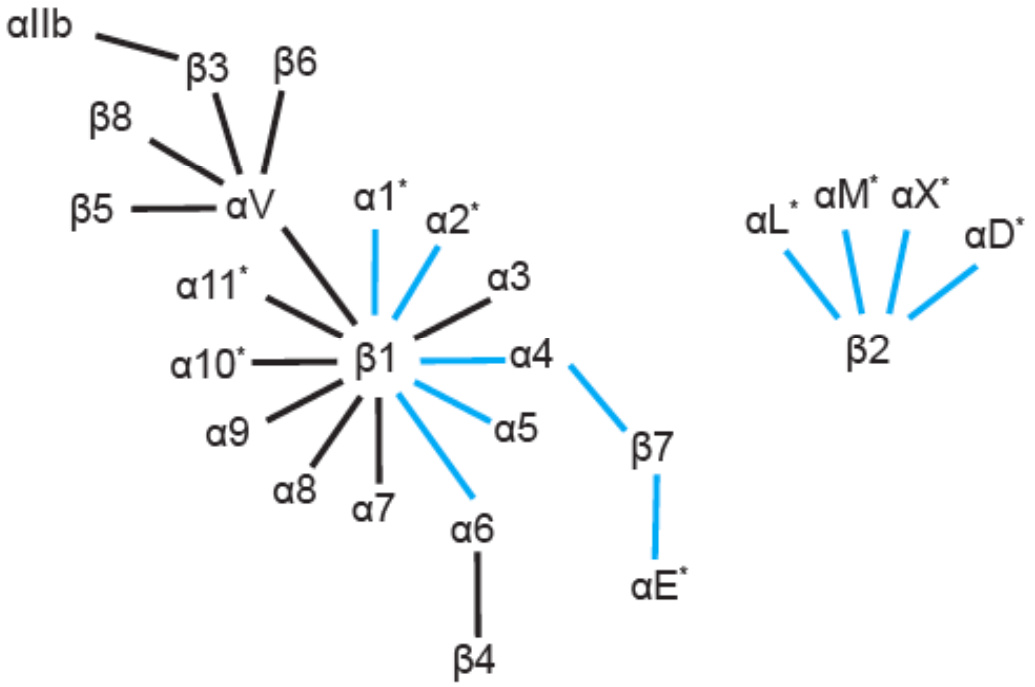

Figure 1.2 Integrin heterodimers. Integrin heterodimers that are unique to immune cells are connected by light-coloured lines. The α subunits containing I domains are indicated with an asterisk.[2,10]

1.1.1 Integrin structure

Each integrin is composed of a non-covalently associated heterodimer of α and β subunits. Both integrin subunits are type I transmembrane glycoproteins with short *C*-terminal cytoplasmic regions, single-spanning transmembrane domains, and large extracellular domains possessing ligand-binding headpieces at the *N*-termini (**Figure 1.3**).[2] The *C*-terminal segments mediate interactions with the cytoskeleton and with signaling molecules, while the *N*-terminal domains of each subunit associate to form the ligand-binding headpiece. The *N*-terminal head region of integrin α subunits contains a region of seven, 60-amino acid segments,

which collectively form the β-propeller region of the α subunit. Approximately half of the α subunits contain an inserted domain (I domain) in the β propeller region, which is directly involved in ligand binding. The β chain may also contain a similar I domain, with a specificity-determining loop involved in ligand binding.[2,13,14] The I domain of the β chain has a segment that directly binds to the β-propeller of the α subunit. Mutations that disrupt this interaction between the β I domain and the β-propeller region at the head of the α subunit are known to cause LAD by disrupting $\alpha_L\beta_2$ activity.[14] While both the α and β I domains contain a metal ion-dependent adhesion site (MIDAS) which binds Mg^{2+}, only the β I domain contains adjacent metal binding sites. The β I domain contains the ligand-induced metal ion-binding site (LIMBS), and the adjacent to metal ion-dependent adhesion site (ADMIDAS), which both bind Ca^{2+}.[2] When the α I domain is present, it is directly involved in ligand binding, and is allosterically regulated by the conformation of the β subunit.[2,8,15] However, when the α I domain is absent, it is believed that the β I-like domain plays a greater role in ligand binding. This difference in ligand binding adds further complexity to the study of integrins.[2,8,9]

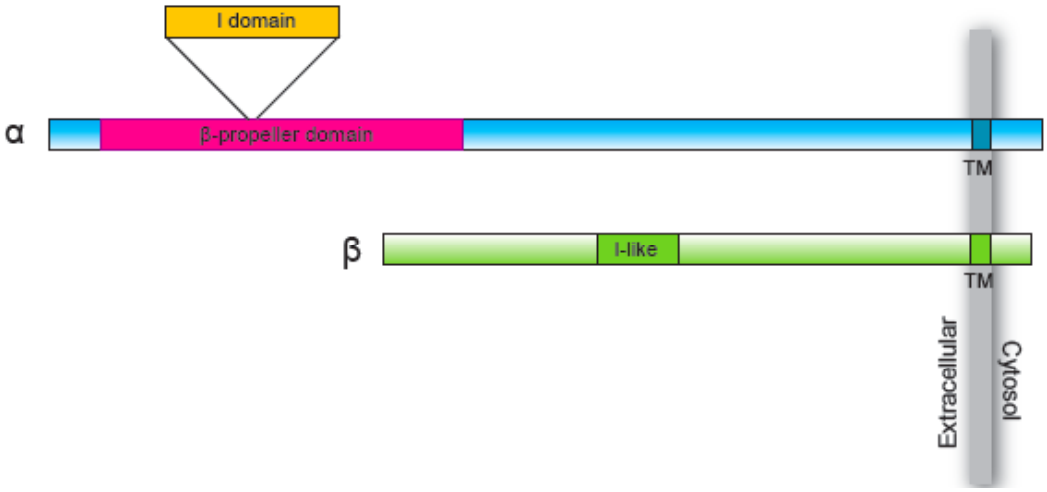

Figure 1.3 General integrin structure. Both integrin subunits are type-I transmembrane proteins, containing short cytoplasmic domains, a single-pass transmembrane domain, and large extracellular domains. All β subunits possess some form of an I, or I-like domain, while only half of the α subunits contain I domains.[2,8]

1.1.2 Integrin conformational change

The general structure of integrins has been well known for over twenty years. However, the importance of conformational change in integrin function was not fully appreciated until 2001, when X-ray crystal structures of the $\alpha_V\beta_3$ integrin showed that it existed in a bent conformation.[8,16] In this structure, the ligand binding site at the extracellular head was bent towards the membrane, and the extracellular stalks of both subunits were closely-associated (**Figure 1.4**).[8,16] As will be described below, it has since been found that this is one of at least three major integrin conformational states.[2,8,15,17,18,19,20,21,22,23] The bent conformation represents the low-affinity state for integrin ligands.[24,25] A switch-blade like

model was proposed by Springer and colleagues, in which the extracellular stalks extend.[25,26] In the intermediate affinity state, the stalks are extended near the head region, although the head remains closed and the stalks are still closely associated near the membrane.[27] Full extension of the legs, occurring at the thigh/genu interface results in the high affinity state, where the stalks of each subunit are completely separated and the headpiece is open for ligand binding.[2,8,15,19,26,27,28]

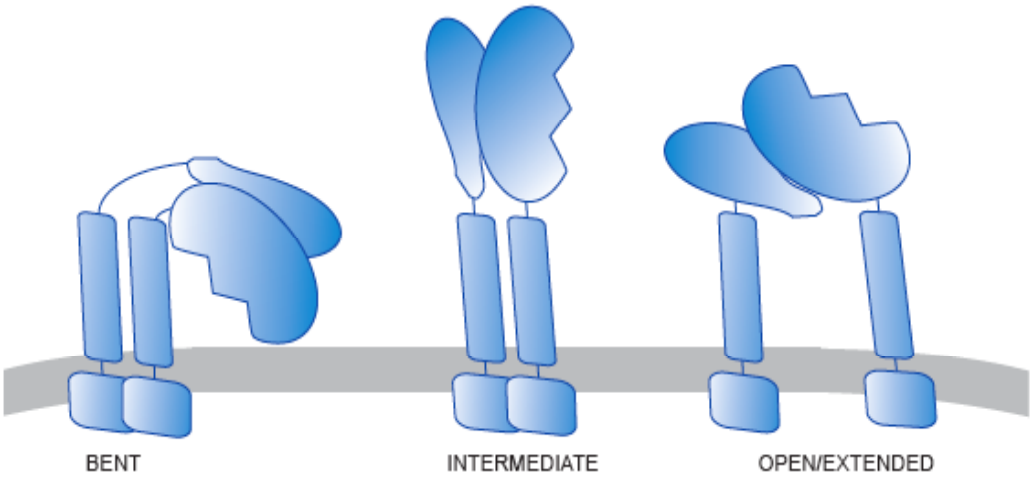

Figure 1.4 Switch-blade model of integrin conformational change. The switch-blade model proposes that integrins exist in at least three conformations (closed, intermediate, and open), each with a different affinity for integrin ligands. As the stalks extend, the integrin goes from having a low ligand affinity to a moderate, then high affinity. When present, the α I domain is directly involved in ligand binding. If the α I domain is absent, the β I domain is more directly involved.[2,8,26]

There has been much debate regarding conformational models for integrin activation.[2,26,29] The switch-blade (extension) model, described above, was pioneered by Springer and co-workers, and was widely supported in the

literature.[19,24,28,30,31,32] Springer's group used FRET (Forster resonance energy transfer) to demonstrate that inside-out activation, through phorbel-ester-induced stimulation, results in an activated integrin, where the stalks of each subunit are separated.[25] Springer and coworkers also used electron microscopy (EM) studies to demonstrate extension, due to outside-in activation induced by binding of soluble ligands.[25] Moreover, mapping studies using activation-dependent mAbs point to a region of the β I-hybrid domain that is only exposed in the open, extended conformation.[28] Despite the wealth of support for integrin extension, an EM study of the $\alpha_V\beta_3$ integrin by Adair et al. in 2005 led to the proposition of an alternative mechanism for integrin activation, known as the deadbolt model.[29] This model proposed that integrins could bind ligands while still in the bent conformation, without extension of the stalks. Springer and co-workers tested these alternative models by mutations to introduce disulfide bonds locking the integrin in the bent conformation. Using their system, they demonstrated that integrins that were incapable of extension were resistant to inside-out activation. These results are more consistent with the switch-blade, rather than the deadbolt model, for integrin conformational change.[26]

Integrins show a conformational, and therefore, functional dependence on divalent metal cations. All integrins require divalent cations for ligand binding. In many cases, the divalent cation is directly involved in ligand binding. For example, many integrin ligands, such as fibronectin, contain an RGD (Arg-Gly-Asp) sequence.[9,33,34] The interaction between the RGD sequence and the metal cation involves the coordination of an aspartic acid residue to Mg^{2+} in the β I-

domain, while the hydrogens of the arginine of the RGD sequence also coordinate to aspartic acid residues on the β propeller of the α subunit. Divalent cations can have both positive and negative regulatory effects on integrin binding.[35] Addition of Mg^{2+} or Mn^{2+}, or removal of Ca^{2+} will increase integrin adhesiveness.[2,8] Binding of metals to the LIMBS will increase integrin adhesiveness, by stabilizing the open conformation,[35,36] while binding to the ADMIDAS will have a negative regulatory effect, stabilizing the bent conformation.[37] The effects of Ca^{2+} are an example of the precise dynamic regulation of integrin adhesiveness. High concentrations of Ca^{2+} will inhibit adhesion through the ADMIDAS, while low Ca^{2+} levels will support adhesion through interactions with the LIMBS.[2,8,35,36,37]

1.2 Integrin glycosylation

Glycosylation is among the most common post-translational modifications (PTMs) of proteins, with approximately half of all eukaryotic proteins being glycosylated, including most cell surface receptors.[4,38,39] The biosynthesis of oligosaccharides attached to proteins (glycans) is achieved by the activity of glycosyltransferases, enzymes that add sugar residues from a nucleotide-sugar donor to an acceptor, in the formation of a glycosidic linkage.[4] Glycans are classified based on their linkage to the protein, as N-linked (involving a linkage between an asparagine residue and *N*-acetylglucsoamine, GlcNAc), or O-linked (involving the hydroxyl group of serine or threonine residues with *N*-acetylgalactosamine, GalNAc). All N-linked glycans contain a common core

structure (Man-α(1→6)(Man-α(1→3))Man-β(1→4)GlcNAc-β(1→4)GlcNAc).[4] After the addition of a chitobiose core, N-glycans are processed in the Golgi, where additional residues are added by glycosyltransferases, and others are removed by the action of glycosidases (enzymes which cleave glycosidic linkages). Depending on the modifications made to this core, the N-linked glycans are classified into three general categories: high-mannose, complex, and hybrid glycans (**Figure 1.5)**.[4,39] Two relevant modifications, branching, and sialylation, are discussed in more detail below.

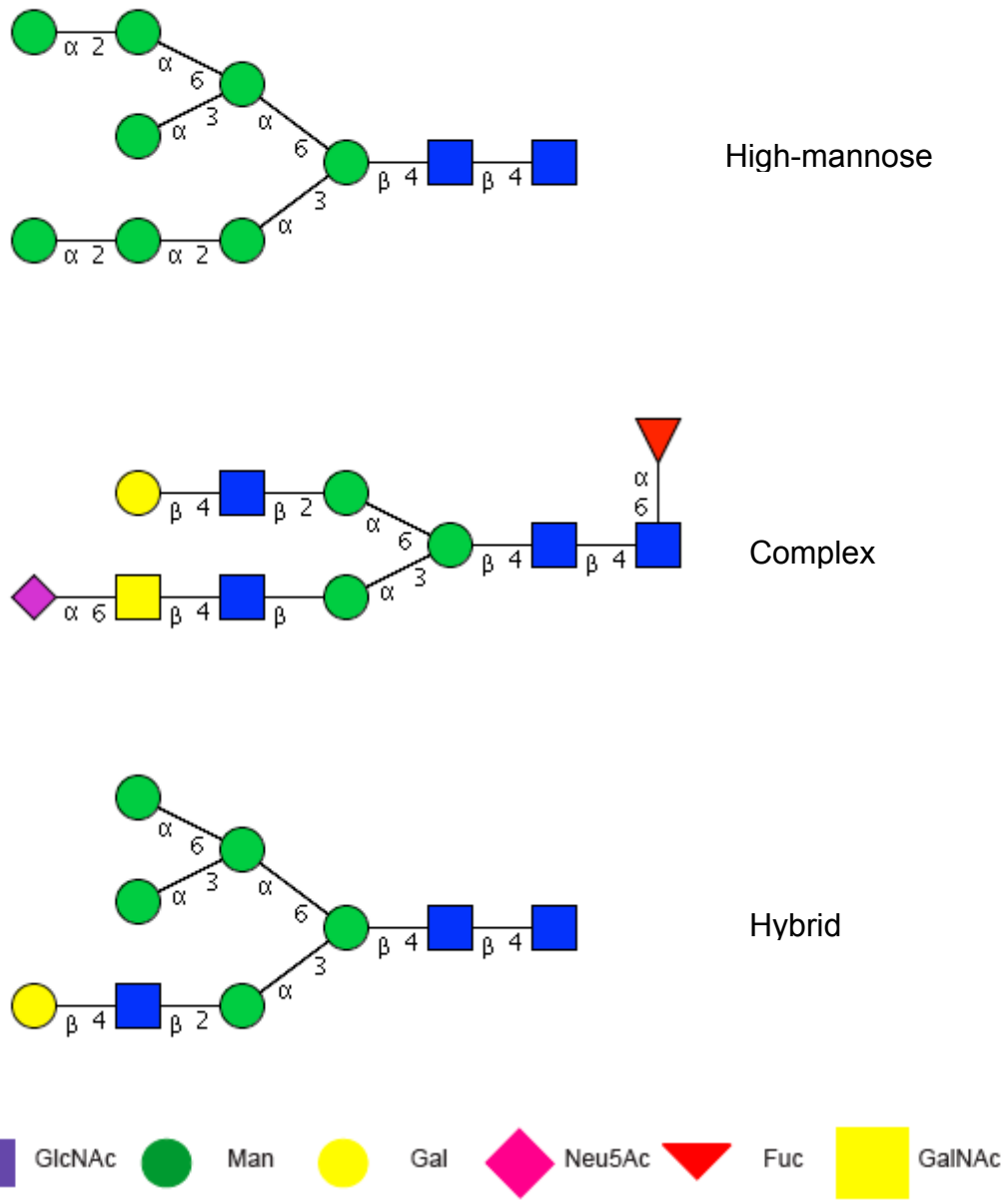

Figure 1.5 Types of N-glycans. N-Linked glycans are classified as either high-mannose (A), complex (B), or hybrid type (C). All N-glycans contain a common pentasaccharide core, Man-α(1→6)(Man-α(1→3))Man-β(1→4)GlcNAc-β(1→4)GlcNAc. Linkage to asparagine is via the core GlcNAc.[4]

1.2.1 Branched N-glycans

Branched glycans, such as bisected GlcNAc or β1→6GlcNAc, are associated with processes such as cell adhesion, migration, and cancer metastasis.[9,39] These structures are attributed to the activity of *N*-acetylglucosaminyltransferases III and V (GnT-III, GnT-V).[9,39] GnT-III adds a bisecting β-(1→4) GlcNAc residue to the branching mannose residue of the N-glycan core. Changes in β-(1→6) branching are due to the action of a Gogli enzyme known as GnT-V (β-(1→6) *N*-acetylglucosaminyltransferase V), which adds GlcNAc residues to mannose in a β-(1→6) linkage. This often results in the formation of poly *N*-acetyllactosamine (LacNAc) chains, which are can be capped with terminal sialic acid residues.[9,39]

1.2.2 Sialic acids

Sialic acids, or *N*-acetylneuraminic acids (Neu5Ac), are negatively-charged, nine-carbon sugars added to terminal residues of N-glycans, via linkage to galactose (α-(2→3) or α-(2→6)) or sialic acid residues (α-(2→3), α-(2→6), or α-(2→8) linkages).[9] These residues are transferred by enzymes collectively known as sialyltransferases, which transfer sialic acid from CMP-Neu5Ac to glycans on glycoproteins and glycolipids (**Figure 1.6**). The most common linkage, α2→3, is catalyzed by several sialyltransferases, while ST6Gal I (β-galactoside, α2→6-sialyltransferase) directs most α2→6 linkages.[9,40] Because sialic acids bear a net negative charge at physiological pH and are located terminally, they have the potential to affect cellular adhesion interactions.

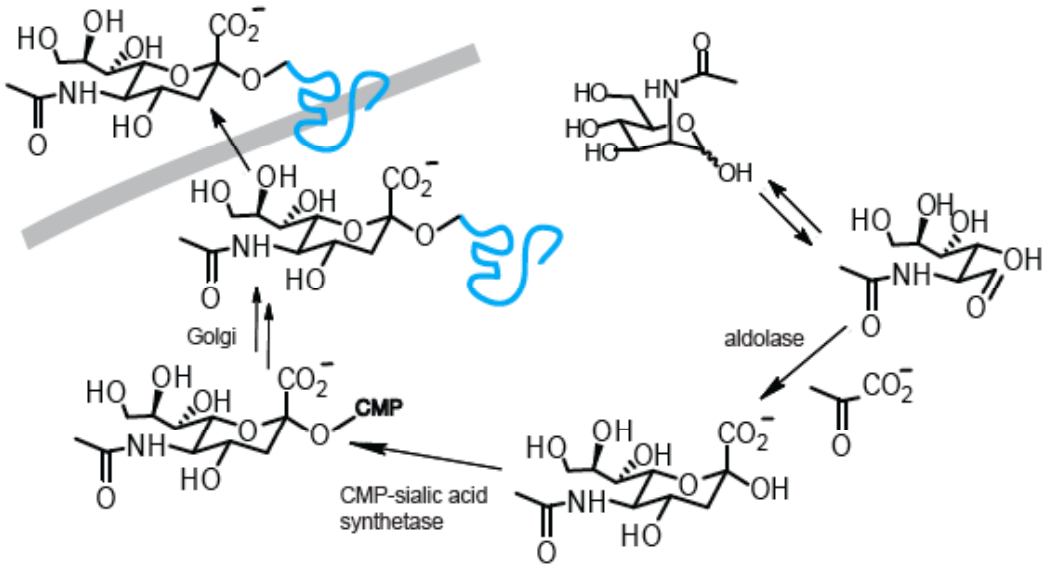

Figure 1.6 Biosynthesis of sialic acids. *N*-acetylmannosamine (ManNAc) is combined with an enolized pyruvate, yielding sialic acid. After synthesis of CMP-sialic acid, sialyltransferases in the Golgi transfer sialic acids onto glycoproteins which could be shuttled to the cell surface.[4]

1.2.3 Potential roles for glycans

Carbohydrates can affect protein function, trafficking, and turnover.[9,39,41] As discussed below, proteins with variable glycosylation are often localized to different cellular compartments. Glycans can also affect protein turnover, as variations in glycan structure can affect whether or not a protein is targeted for degradation.[39,41] Similarly, glycans can mask parts of the protein backbone, protecting it from degradation by nonspecific proteases, and improving protein stability.[39] Glycans can also affect the shape of proteins, by influencing protein folding, or conformation.[23] As discussed above, integrin conformational changes are directly linked to the regulation of their ligand affinity. An example of how

glycosylation can affect integrin conformation is discussed below. Because of their predominance in the extracellular space, glycans play important roles in cell-surface recognition events. For example, selectins are cell surface receptors that recognize and bind to specific carbohydrate epitopes, and are responsible for the rolling phase of leukocyte migration to the tissues (**Figure 1.1)**.[4] Although this example illustrates the role of carbohydrates in selectin-mediated adhesion events, there is increasing interest in the structure, regulation, and functional significance of integrin glycosylation.

1.2.4 Integrin glycoforms: known structural variation in integrin glycans

Although glycosylation is a common post-translational modification, the glycan structures of most integrins remain to be characterized. This is especially surprising considering that, as discussed below, evidence for variable integrin glycoforms has existed for over 20 years.[9,42] However, several general features of integrins and their glycosylation have been established. These large (>250 kDa heterodimers) glycoprotein receptors are typically heavily glycosylated, with several glycans found on the β subunits, and even more on the larger, α subunits. The extent of glycosylation is quite significant, and can account for more than 20% of the integrin mass. Most of these glycans appear to be N-linked.[9,38,42] This was established in a variety of studies where differences in the electrophoretic mobility of purified integrins could be eliminated upon treatment with PNGase F. The $\alpha_5\beta_1$ integrin, for example, contains 26 potential N-glycosylation sites, including 14 on the α subunit. [9,40,43] Considering the role glycan structure often

plays in protein conformation, it is perhaps not surprising that these N-glycans are functionally significant, as will be discussed below.

Limited information is available about the structure of integrin N-glycans. Only the glycan structures of the $\alpha_V\beta_3$, $\alpha_3\beta_1$, $\alpha_L\beta_2$ and $\alpha_5\beta_1$ integrins have been studied in detail.[39,44,45,46,47] Integrins possess various N-linked glycan structures, including high mannose, hybrid type, as well as complex bi-, tri-, and tetra-antennary structures. Structural studies of the $\alpha_3\beta_1$ integrin by mass spectrometry by Litynska et al., and HPLC mapping studies of the $\alpha_5\beta_1$ integrin by Nakagawa et al., have both revealed that β1 integrins possess a large number of LacNAc type multi-antennary N-glycans.[46,47] Sequential glycosidase digestion and methylation analysis of LFA-1 N-glycans released by hydrazinolysis allowed Asada and co-workers to propose that LFA-1 possesses mainly high mannose and bi-, tri-, and tetra-antennary complex structures. Although, some amount of monoantennary and hybrid structures were also found.[44] These studies also revealed significant heterogeneity in integrin N-glycans (varying glycan structures attached to the same site). Specific changes associated with variable degrees of branching, and sialylation of integrins are discussed further below. This variability in glycan structure is consistent with previous lectin, and electrophoresis-based analyses performed by Pardi et al. ($\alpha_L\beta_2$), Jasiulionis et al. ($\alpha_6\beta_1$), and Prokopishyn et al ($\alpha_3\beta_1$).[42,48,49]

Although the biological significance of glycan structural variability has yet to be fully established, researchers have observed that different cell types express variant β1 glycoforms. Kim et al. observed altered glycosylation and cell surface

expression of β1 integrins during keratinocyte activation, while Symington and colleagues have found that activation with phorbol esters can induce both altered glycosylation and expression of β1 integrins.[50,51] Furthermore, like many other cell surface proteins, integrins exhibit differential glycosylation during tumour development and metastasis.[43,52] Collectively, these findings support a role for different glycan structures in integrin function.

1.2.5 Regulation and functional significance of integrin glycosylation

It is well known that tumour cells often exhibit altered glycosylation profiles.[9,39,43] There is strong evidence that both GnT-V and ST6Gal I contribute to this observation, as elevated expression of these enzymes have been observed in tumour cells.[9] Despite the evidence, there is little information about the protein targets of these enzymes and their role in cancer progression. Several studies suggest that β1 integrins are likely targets of both GnT-V and ST6Gal I. Bellis and coworkers have shown that increased expression of the ras oncogene results in increased α-(2→6) sialylation of β1, and not β3 or β5 integrins, due to induction of ST6Gal I expression in colon epithelial cells.[43] These cells have increased adhesiveness to β1 ligands, such as collagen and fibronectin, but not β3 or β5 ligands, such as vitronectin.

The regulation of β1 sialylation by ST6Gal I appears to be even more crucial in immune cells. In myeloid differentiation of U937 cells, PMA-stimulation of cell differentiation resulted in expression of a hyposialylated β1 integrin, as a result of decreased expression of ST6Gal I. The appearance of

hyposialylated β1 integrin was temporally correlated to enhanced binding to fibronectin.[40] Bellis provided further support for this model by demonstrating that a U937 cell variant which does not exhibit PMA-induced binding to fibronectin, also does not down-regulate ST6Gal I expression. Bellis' model for increased adhesiveness due to hyposialylation of β1 integrins is supported by adhesion data in live cells, and with purified $\alpha_5\beta_1$ integrins.[9] Removal of sialic acid upon treatment with neuraminidase enzymes also resulted in increased cell binding to fibronectin in myeloid, epithelial, and tumour cell lines.[9] The same result was observed in β1 integrins from myeloid cells expressing non-natural sialic acids.[9] These findings are consistent with animal studies where complete knockout of ST6Gal I resulted in immunocompromised mice. However, mice with only reduced expression in ST6Gal I show increased inflammatory responses.[53,54]

Integrin glycoforms arising from differences in the degree of β-(1→6) branching also appear to be functionally significant. Integrins, with varied degrees of β-(1→6) branching due to GnT-V have shown altered subcellular localization. Studies by Yammamoto et al. involving the $\alpha_3\beta_1$ integrin have shown that increased expression of GnT-V resulted in an increase in $\alpha_3\beta_1$ targeting to the leading edge of polarized cells.[55,56] Moreover Guo and coworkers showed that increased expression of GnT-V in fibrosarcoma cells resulted in increased β-(1→6) branching on the β1 subunit, and was associated with reduced $\alpha_5\beta_1$ clustering. They also found that the β1 subunit, but not the α5, was a substrate for GnT-V.[57]

Although most studies of integrin glycosylation have focused on biosynthetic mechanisms, alternative pathways could also regulate their structures. Several glycosyltransferases and glycosidases are known to act at the cell surface.[58] Several more are known to act inside the cell.[9,59] These changes could be further varied by time or localization of the integrin or enzyme, giving rise to many possible mechanisms for integrin glycan regulation.

There is evidence for differences in how cells regulate the expression of specific integrin glycoforms at the cell surface. Early studies of integrin glycosylation, using tunicamycin (an inhibitor of N-glycan biosynthesis) or swainsonine (mannosidase I/II inhibitor) also found that N-glycosylation was required for normal integrin αβ subunit pairing.[39,60] This finding has since been supported through site-directed mutagenesis studies that removed specific N-glycosylation sites on the α5 subunit. Without N-glycans at specific sites, heterodimerization and cell surface expression of the $\alpha_5\beta_1$ integrin was blocked.[61] Some integrin subunits, such as the β1 subunit, are stored in pools in the endoplasmic reticulum, allowing the cell to direct varying integrin glycoforms to the cell surface without the need for *de novo* protein synthesis. While this is true for the β1 subunits, it is not true for the α subunits. Moreover, Heino et al. have demonstrated that β1 chains are expressed in excess over α subunits in a variety of cell types.[9,62]

An alternative mechanism, studied intensely by Bellis and coworkers, involves the possibility that sialic acids, with their negative charge at physiological pH, are used as masking groups for galactose residues. This is

widely believed to be a major role for sialic acids in cell biology.[9,63] However, the relationship between sialic acids and β1 integrin function appears to be complex. As discussed above, the Bellis lab has observed that hyposialylation of β1 integrins, due to either enzymatic removal by sialidases *in vitro*, or downregulation of sialyltransferases *in vivo*, results in increased $\alpha_5\beta_1$ binding to fibronectin.[39,40,43] However, Bellis has also found that the same treatments inhibit $\alpha_1\beta_1$ integrin binding to collagen. Furthermore, Nadanaka et al. have observed that oligosialic acid on the α5 subunit was required for binding to fibronectin.[64] Bellis et al. have proposed that differences in β1 binding could be due to the presence or absence, respectively, of an α I domain (present in α1, but not α3 or α5 subunits). As discussed earlier, regulation of integrin binding is heterodimer-specific, and dependent on the presence of an α I domain. Whether or not the simple presence or absence of an α I domain can help explain these differences in glycosylation-dependent integrin regulation has yet to be determined. Clearly, future studies of integrin glycosylation will be required to explore the structures and functional roles of integrin glycans.

It is possible that modification of integrin glycans is a general mechanism for conformational, and therefore functional, regulation of integrin binding. Springer and colleagues have demonstrated that the open conformation of β1 and β3 integrins can be stabilized by introducing an N-glycosylation site into their I domains.[23] Correspondingly, this glycan wedge resulted in an increased ligand binding affinity. Introduction of a bisecting GlcNAc into the α5 subunit, however, resulted in significantly reduced binding to fibronectin.[39] Liu et al. have presented

a model of these effects with molecular dynamics studies, in which changes in α2→6 linked sialic acids on N-glycans in the β1 I domain resulted in significant conformational changes in regions of the β1 integrin required for binding.[39,65] Thus, there is increasing evidence for a role for N-glycosylation in integrin conformational change.

It is not yet possible to rule out indirect effects of integrin glycosylation, perhaps through associations with glycosphingolipids.[9] Glycolipids have been implicated in cell adhesion events, including work by Wang et al. who demonstrated that interactions with GT1b affect $\alpha_5\beta_1$ integrin binding to fibronectin.[66] As these studies show, there are still many unanswered questions about the structure of integrin glycans, the role of these glycans in integrin function, and the regulation of these glycans by neighbouring enzymes. A better understanding of integrin glycan structure and the mechanisms that regulate them, should allow for an improved understanding of how glycans affect cell adhesion. As aberrant glycosylation is a hallmark of cancer development and autoimmune disorders, perhaps these studies will help clarify the molecular basis of these important diseases.

1.3 LFA-1 glycosylation

Integrin N-glycosylation plays an important role in the function of this receptor family. Integrins are well known to undergo significant conformational changes at the cell surface, leading to changes in ligand binding affinity. While we have presented evidence for a role for glycosylation in this process, most

studies of integrin glycosylation to date have focused on β1 integrins. However, there is growing evidence that β2 integrins, such as the lymphocyte function associated antigen-I (LFA-1), are also subject to conformational regulation by N-glycans. β2 integrins are vital to immune processes, and are only expressed by immune cells.[10] LFA-1 ligands are typically members of the immunoglobulin superfamily, including intercellular adhesion molecule-1 (ICAM-1), ICAM-2, and ICAM-3.[67] These interactions play an important role in several key immunological functions including lymphocyte activation, and immune synapse formation.[3,68] As a testament to its importance to immune function, LFA-1 has been implicated in a wide variety of diseases including leukocyte adhesion deficiency (LAD), HIV infection, tumour metastasis, asthma, multiple sclerosis, and other autoimmune diseases.[3,12]

While LFA-1 is among the best-studied integrins, little attention has been devoted to the role of glycosylation in LFA-1 function. LFA-1 was first identified by Springer and coworkers in 1981,[69,70,71] and, in 1982, they were the first group to purify LFA-1.[72] As discussed in Section 1.2, Asada et al. studied LFA-1 glycosylation in 1991.[44] They proposed that LFA-1 possessed high mannose, and bi-, tri-, and tetra-antennary complex N-glycans. Another major study of LFA-1 N-glycosylation was performed in 1989 by Pardi and coworkers.[42] Using isolectric focusing, they showed that purified LFA-1 existed as several isoforms with varying electrophoretic mobility. However, upon treatment with neuraminidase, the same samples yielded only a single band, implying that these isoforms were sialoforms. This study also demonstrated that T cells treated with

neuraminidase exhibited increased adhesion to endothelial cells. This phenomenon has since been coined "the neuraminidase effect," and Bagriacik et al. have suggested that this observation is due to interactions between LFA-1 and ICAM-1.[42,73] While no study has directly tested the role of LFA-1 in the neuraminidase effect, there is strong evidence linking LFA-1 to this phenomenon. In Pardi's adhesion experiments (**Figure 1.7**), treatment with monoclonal antibodies that block LFA-1 binding resulted in a significant decrease in adhesion in all lymphocyte subsets (with or without neuraminidase). Moreover, Pardi found that the increased adhesion due to sialic acid removal was proportional to amount of LFA-1 expressed on the surface of each lymphocyte subset. Subsequent work by Quinn et al. using flow cytometry to support a link between the neuraminidase effect and LFA-1.[74] Quinn identified a monoclonal antibody specific for a carbohydrate epitope on β2 integrins, which showed increased expression upon neuraminidase treatment or cellular activation. This epitope was removed with PNGase F treatment, confirming that the epitope was part of an N-linked glycan.[74] Consistent with this finding, Josefsson et al. have identified a lectin domain on β2 integrins that appears to influence ligand binding, and functions independently of the α I domain.[75]

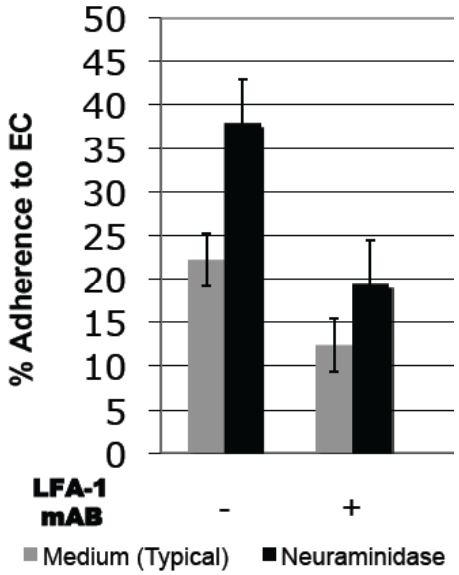

Figure 1.7 Endothelial cell (EC) adherence of neuraminidase-treated T cells. Representative data from the adhesion experiment performed by Pardi et al.[42] The effect of sialic acid removal by neuraminidase (from *Clostridium perfringens*) on the adhesion of purified lymphocyte subsets to ECs was compared. For antibody blocking, the TS1/22 mAb (anti-LFA-1, α subunit) was used.

Bellis has demonstrated a role for sialyltransferase activity in hyposiaylation of β1 integrins. However, sialyltransferase activity alone cannot explain how enzymatic removal of sialic acids on live cells results in increased adhesion. Therefore, sialyltransferase activity alone cannot explain the neuraminidase effect. As has been found with other mechanisms of integrin regulation, dynamic regulation is typical in these systems. If sialic acids are relevant to integrin function, neuraminidases may be involved. Four mammalian neuraminidase enzymes have been identified to date (NEU1, NEU2, NEU3, and NEU4).[76] Two of these, the plasma membrane-associated sialidase, NEU3, and

the lysosomal sialidase, NEU1, have demonstrated activity at the cell surface.[77] NEU3 is ganglioside-specific, however, minor activity on glycoproteins has also been observed.[78] Furthermore, Uemura et al. have recently linked the suppression of metastasis in human colon cancer cells to desialylation of β4 integrins by NEU1.[76] This is the first report of integrin desialylation by a mammalian neuraminidase.

1.4 Hypothesis and project objectives

We have presented evidence that suggests that sialic acids on LFA-1 N-glycans are involved in conformational regulation of LFA-1 binding to ICAM-1. Consistent with hyposialylation of β1 integrins, enzymatic removal of LFA-1 sialic acids by neuraminidase enzymes could result in an increased affinity for ICAM-1 (**Figure 1.8**).

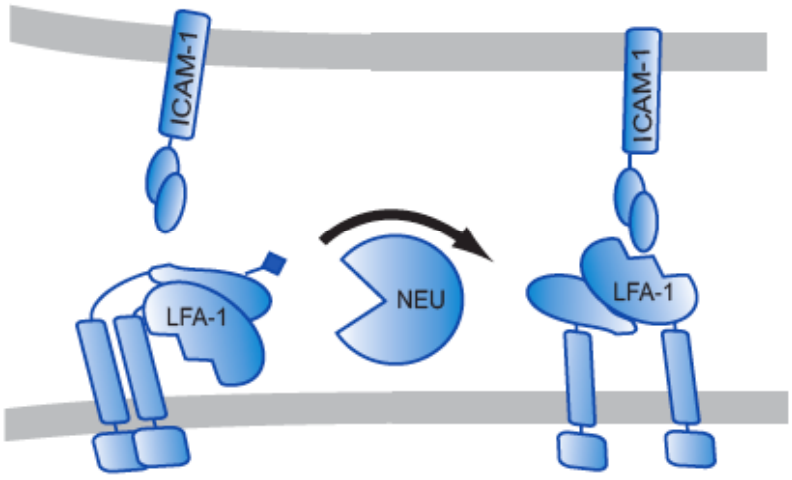

Figure 1.8 Proposed model of LFA-1 regulation. Sialic acid removal by neuraminidase enzymes at the cell surface could cause a conformational change in LFA-1, resulting in an increased affinity for ICAM-1.

Typical studies of integrins have involved the use of antibodies, which are relatively large, and could perturb normal protein function. Until recently, studies of glycosylation were heavily dependent on lectins, which show relatively weak binding to their ligands. Systematic digestion by glycosidases are limited as they must be done in vitro. Over the past ten years, new methods for studying glycans have been developed. Bertozzi and co-workers have pioneered bioorthogonal, small-molecule labeling strategies to study glycans.[79] Bioorthogonal chemical reporters are unnatural, non-perturbing chemical groups that can be selectively modified in living systems with exogenous probes.[80] As shown in **Figure 1.9**, non-natural monosaccharide precursors bearing bioorthogonal functional groups, such as the azide-bearing *N*-azidoacetylmannoasmine (ManNAz), can be incorporated into cellular biosynthetic machinery, resulting in glycoproteins bearing azide-labeled sialic acids at the cell surface.[80] Azides can be linked to small reporter groups using either alkynes, in the Cu(I)-catalyzed azide-alkyne click reaction developed by Meldal and Sharpless et al.,[81,82] or phosphines, in the Staudinger Ligation developed by Bertozzi's group.[80,83,84]

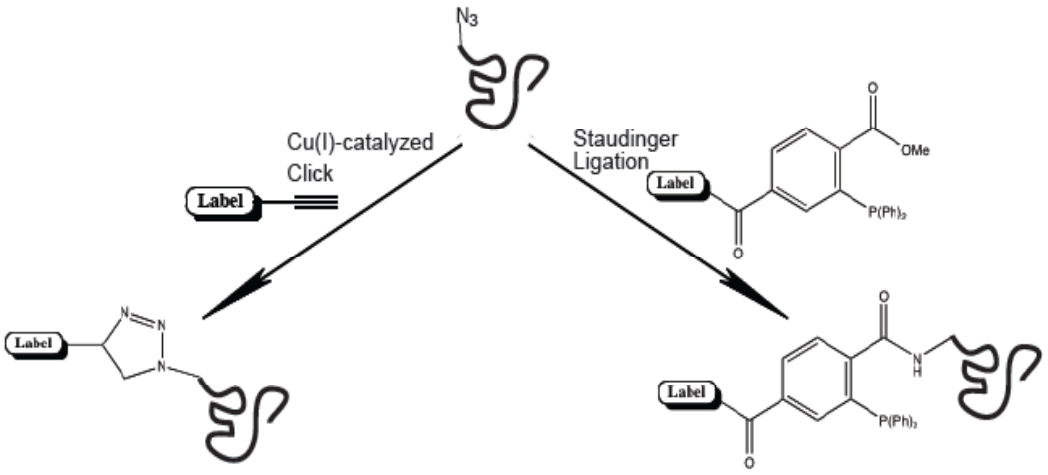

Figure 1.9 Derivitization of azides via the Cu(I)-catalyzed click reaction or the Staudinger Ligation. Azide-bearing biomolecules can react with labeled alkynes, in the Cu(I)-catalyzed click reaction, to attach labels via a triazole. Alternatively, labeled phosphine reagents can react with azides by Stadinger ligation.[79,83]

We hypothesized that metabolic labeling strategies could be used to test the role of LFA-1 sialic acid, and their role in LFA-1-mediated adhesion to ICAM-1. Furthermore, we considered that sensitive bioorthogonal methods could be used to test if LFA-1 sialic acid is modified by mammalian neuraminidases. If integrins are indeed susceptible to membrane-associated NEU activity, the neuraminidase effect may be evidence of this regulatory pathway. Should our methodology prove successful, this would be the first study of integrin conformational change using small molecules, and could serve as a general strategy for studying enzymatic modification of cell surface glycoconjugates.

In Chapter 2 we explore the use of bioorthogonal strategies including Bertozzi's ManNAz labeling strategy, to investigate the incorporation of sialic acid in LFA-1. In Chapter 3 we discuss alternative bioconjugation strategies for glycoprotein labeling.

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Chapter 2: Purification, labeling, and glycosylation of LFA-1[1,2]

[1] Compounds **2.1** and **2.2** were synthesized by Ravi S. Loka.
[2] MS analysis of LFA-1 was performed by Messela A. Fentabil.

2.1 Introduction

Glycosylation is a common post-translational modification of proteins.[1,2,3] Glycans play important roles in protein conformation, trafficking, and turnover. N-linked glycan structures have been implicated in the function of variety of proteins, including β1 integrins, as discussed in Chapter 1.[1,4] However, the role of N-linked glycans in the function of β2 integrins, such as the lymphocyte function-associated antigen I (LFA-1), remains to be determined. Although LFA-1 has been shown to be heavily decorated with N-linked glycans,[5,6] relatively little attention has been given to their role in the regulation of LFA-1 function. As discussed in Chapter 1, there is growing evidence that suggests a role for LFA-1 in the neuraminidase effect, an observed increase in adhesion of lymphocytes to endothelial cells upon sialic acid removal.[5,6,7] Herein, we describe our progress towards determining the role of sialic acids in LFA-1-mediated T cell adhesion. We used electrophoresis and blotting techniques, in combination with metabolic labeling and chemoselective ligation, to look for changes in LFA-1 sialic acid content upon cell activation. We also attempted structural characterization of LFA-1 glycans, and a functional assay to measure how sialic acid affects LFA-1-mediated T cell adhesion.

In 2007, Bonasio et al. reported a novel LFA-1 labeling strategy.[8] Their method involved the use of a recombinantly-expressed LFA-1 fused to a cutinase enzyme, which could be labeled with suicide inhibitors. While this strategy is elegant, it required significant control experiments to ensure that the cutinase did not alter LFA-1 activity. Moreover, this strategy is not suitable for the study of

mammalian glycans due to the significant differences in protein glycosylation between mammalian and bacterial cells.[2] Other labeling studies of LFA-1 have utilized monoclonal antibodies.[9] Although they exhibit excellent affinity for their targets, antibodies are relatively large (150 kDa), and, as demonstrated in Pardi's adhesion experiment (**Figure 1.7**),[6] antibodies can affect the function of their targets. Until recently, studies of glycan epitopes have relied on lectins, which have weak affinities for their targets.[10] Lectins, and antibodies which bind to glycans, can also have issues with cross-reactivity. Metabolic oligosaccharide engineering strategies, allowing the incorporation of bioorthogonal functional groups into specific carbohydrate residues, present an alternative for specific labeling and detection of glycans.[11] Bertozzi and co-workers were the first to specifically label sialic acids of cell surface glycoproteins using the Cu(I)-catalyzed Click reaction, or the Staudinger ligation. Unlike antibodies, this labeling strategy allows for introduction of small molecule labels, which are much less likely to perturb native protein function. Our goal was to utilize this sialic acid labeling strategy in our studies of LFA-1.

One of the potential disadvantages of metabolic oligosaccharide labeling is non-specific incorporation of the azide tag into all sialic acid residues of cell surface glycoproteins and glycolipids.[12,13] To overcome this challenge, we planned to combine the azide incorporation strategy with an immunoaffinity purification approach developed by Springer et al., to yield functional, azide-tagged LFA-1.[14,15]

The purification of integrins is challenging for a variety of reasons. First, because we want to study normal LFA-1 glycosylation in T cells, we cannot express recombinant LFA-1 in bacteria. We selected Jurkat cells (clone E6.1, ATCC) as a model lymphocyte line. Mammalian cells are more challenging to grow on larger scales. Unlike recombinant proteins expressed in bacteria, it is difficult to up-regulate expression of the protein of interest. Moreover, as transmembrane proteins, integrins are especially prone to aggregation upon purification. In addition to these challenges, Springer's group has shown that LFA-1 subunits will dissociate with prolonged exposure to the elution conditions.[15] Finally, as discussed below, the pH required for elution of LFA-1 is in conflict with typical protein immobilization chemistries utilized for immunoaffinity purification. Together, these challenges make preparative scale purification of native LFA-1 especially difficult.

Once we established protocols for the purification of LFA-1 and metabolic labeling of sialic acids, we aimed to employ the Staudinger ligation to specifically label LFA-1 sialic acids. This labeling strategy would allow us to directly study changes in LFA-1 sialic acid content, in vitro, using gel and blotting techniques, as well as *in vivo*, using established protocols for incorporation of purified integrins into artificial lipisomes.[16] To support gel-migration studies, we also aimed to utilize gel-migration studies, similar to those performed by Semel et al. in their studies of β1 integrins, to obtain evidence for changes to LFA-1 sialic acid content upon cellular activation with phorbol esters.[4] Alternatively, we attempted cellular adhesion assays similar to those described by Cherry et al., and others, to

determine if there is a role for sialic acid in LFA-1-mediated T cell adhesion to ICAM-1.[17]

2.2 Monoclonal antibodies for immunoaffinity purification

In order to purify LFA-1 by immunoaffinity chromatography, we required quantities of a monoclonal antibody (mAb) specific to the $\alpha_L\beta_2$ heterodimer. We prepared a stock of the TS1/18 mAb, which had been generated by Springer and coworkers in previously reported purifications of LFA-1.[18] The TS1/18 mAb is produced by the HB203 mouse hybridoma line (ATCC, Burlington, ONT).[18] The purified antibody could be used for immunoaffinity purification, immunoprecipitations, immunoblotting, ELISA-based adhesion studies, surface plasmon resonance (SPR) binding studies, and as controls to block integrin activity during adhesion studies. For these reasons, we chose to produce large quantities of antibody using commercial tissue culture chambers (NUNC Opticell MAX), which are designed for harvesting secreted antibody from hybridoma cells. It is worth noting that many antibodies to integrins are conformationally dependent. The TS1/18 mAb, for example, is specific for the β2 subunit, while the TS2/4 mAb, produced by the HB244 hybridoma cell line (ATCC)[15] binds the αL subunit. However, neither antibody will bind unless the subunits are associated.[15] Dustin et al. have reported that the TS2/4 mAb was best for purification of intact, functional LFA-1.[15] Therefore, this antibody was used for our experiments.

It is also worth noting that there are a variety of IgG subtypes (IgG_1, IgG_2, etc.), which exhibit small structural differences despite having over 95% amino

acid sequence homology.[19] While IgG_2 is a more commonly encountered subtype in research, the TS1/18 and TS2/4 mAbs used in this study were both IgG_1. Purification of this isotype required the use of a buffer system specialized for IgG_1 developed by Bio-Rad (see Methods). As shown in **Figure 2.1**, we were successful in producing multi-mg quantities of both the TS2/4 and TS1/18 mAbs. SDS-PAGE analysis revealed bands corresponding to both the IgG heavy- and light-chains, observed at the expected molecular weights (approximately 50 kDa and 25 kDa, respectively).[19]

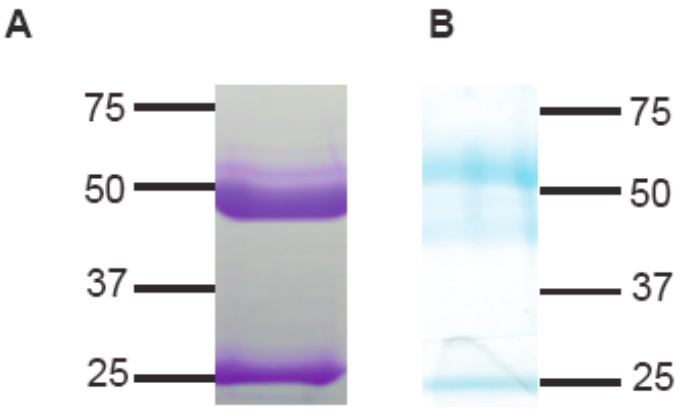


Figure 2.1 SDS-PAGE analysis of purified mAbs. TS2/4 mAb (A) and TS1/18 mAb (B) were purified using a Bio Rad MAPS II Protein A Purification Kit, then analyzed by SDS-PAGE under reducing conditions. Bands were visualized with Coomassie Brilliant Blue. The expected molecular weight of the heavy chain is 50 kDa, and the light chain is 25 kDa.

2.3 Purification of LFA-1

With sufficient quantities of TS2/4 available, the antibody was immobilized to Sepharose to generate an immunoaffinity column. Two standard

coupling chemistries were used for immobilization (**Figure 2.2**). Cyanogen bromide-activated Sepharose was used successfully in several reports by Springer et al., while protein immobilizations via *N*-hydroxysuccinimide (NHS) coupling have a slightly higher pH stability.[14] Interestingly, Springer's method involves elution of LFA-1 at pH 11.5, while protein immobilization is only reported to be stable to pH 11. Surprisingly, Springer does not report antibody leakage during elution of LFA-1. In our experiments, we observed significant release of antibody during purifications of LFA-1 at the reported pH, and this challenge led to the use of NHS-activated sepharose, which has improved stability at high pH. Despite the change in immobilization chemistry, we still observed release of small amounts of antibody during the elution step. One potential cause could be excess antibody used during the coupling step. However, antibody was observed to elute even after several wash steps, and the media was expected to have coupling capacities (up to 18 mg/mL) higher than we used (5 mg/mL).

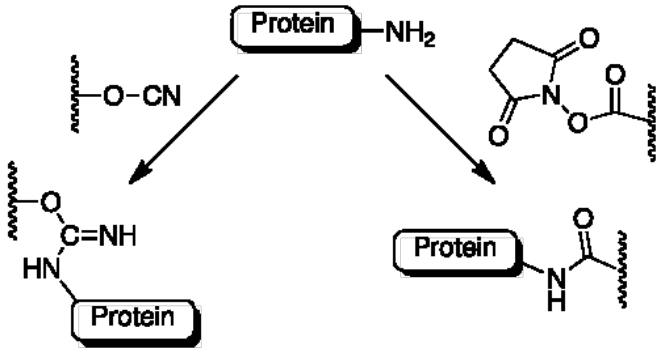


Figure 2.2 Antibody immobilization chemistries. Primary amines of lysine residues on antibodies can be used for immobilization to cyanogen bromide or NHS-activated Sepharose.

Typical immunoaffinity purifications of LFA-1 yielded between 500 μg and 1 mg of protein per litre of Jurkat culture. We required large quantities to overcome the significant glycan heterogeneity exhibited by integrins such as LFA-1. Despite the challenges associated with preparative-scale purification, LFA-1 was successfully purified from Jurkat (E6.1) cells, as shown in **Figure 2.3**. As shown by native immunoblotting, LFA-1 was purified as an intact heterodimer (later demonstrated to be functional, **Figure 2.10**). Under standard, reducing, and denaturing SDS-PAGE conditions. Two major bands were observed for purified LFA-1. One band corresponded to the αL subunit (177 kDa), while the second corresponded to the β2 subunit (95 kDa). The migration of both bands was consistent with reported results. [6,14,18]

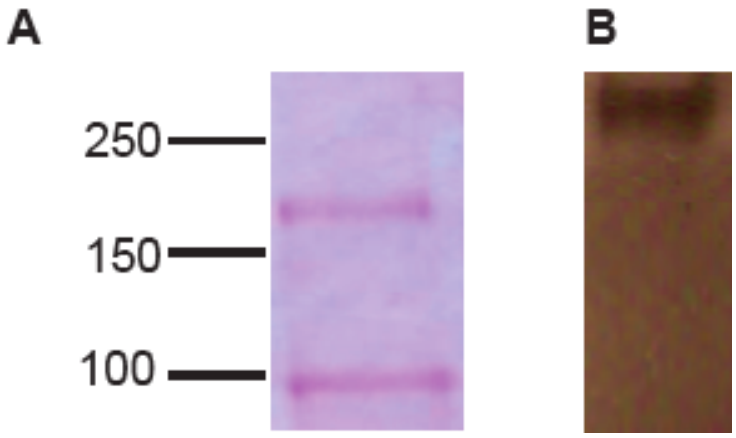


Figure 2.3 Analysis of purified LFA-1. Immunoaffinity purified LFA-1 was analyzed by SDS-PAGE (A) and Western blot (B). For SDS-PAGE, bands corresponding to LFA-1 αL and β2 subunits were visualized by Coomassie Brilliant Blue. For Western blot, LFA-1 was separated under native conditions. Intact LFA-1 was detected using the TS2/4 mAb (primary) followed by a goat-anti mouse IgG-HRP conjugate as the secondary antibody. The expected molecular weights are 177 kDa for the αL subunit, 95 kDa for the β2 subunit. and 270 kDa for the intact heterodimer.

2.4 Lectin Analysis

We also used gel and blotting-based approaches to gain insight into LFA-1 glycosylation. As discussed in Chapter 1, Bellis and co-workers demonstrated hyposialylation of β1 integrins upon activation of myeloid cells with phorbol esters. In a study by Semel et al., β1 integrins immunoprecipitated from PMA-treated myeloid cells were compared to those immunoprecipitated from untreated cells.[4] Using SNA (*Sambucus nigra* agglutinin), a lectin that binds α2→6 linked sialic acids, Semel et al. showed that activated myeloid cells express β1 integrins

lacking α-(2→6) sialylation. We performed a similar analysis of LFA-1 immunoprecipitated from Jurkat cells (**Figure 2.4**). We compared LFA-1 from PMA-treated Jurkat cells to that obtained from untreated controls, at different time points. Despite the low signal, this experiment was successful, yielding several interesting results. In all samples, there was more staining of the α subunit, which is consistent with the increased number of N-glycans relative to the β2 subunit.[5] At 1 h, there is only background staining, and at 6 h there is more staining of LFA-1 from control cells. Both observations are consistent with Semel et al.[4] However, at 12 h, there is more staining in LFA-1 subunits from PMA-treated cells. This implies that unlike the myeloid β1 integrins studied by Semel et al., PMA-treated Jurkat cells express LFA-1 bearing α-(2-6) linked sialic acids.

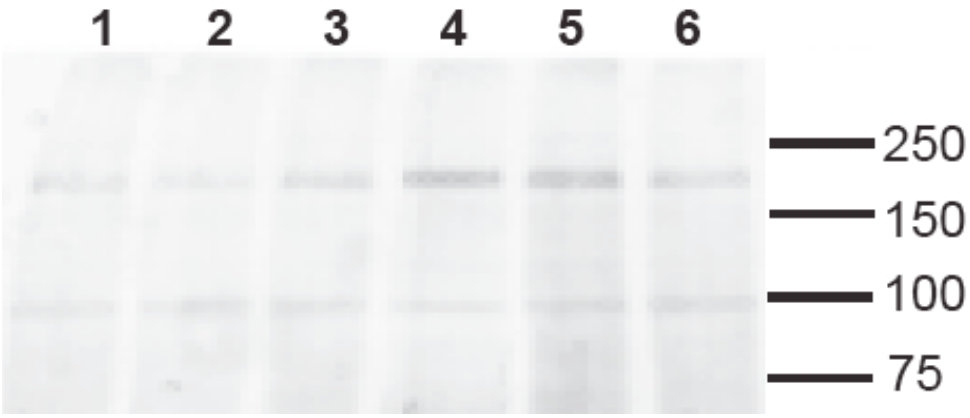
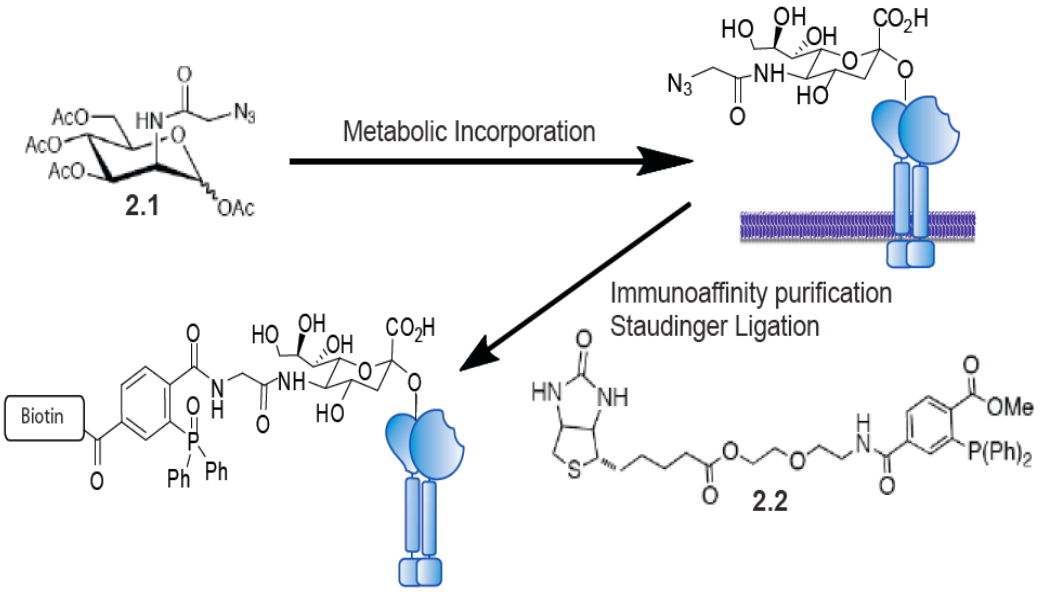


Figure 2.4 PMA-treated Jurkat cells express LFA-1 bearing α-(2-6) sialic acids. LFA-1 was immunoprecipitated from Jurkat cells at 1, 6, or 12 h following treatment of 50 ng/mL PMA or an equal volume of DMSO as a control. The samples were probed for α-(2-6) linked sialic acids using a biotinylated lectin, SNA followed by HRP-conjugated streptavidin. Bands were visualized by chemiluminescence. Lanes shown are as follows: 1-PMA-treated, 1 h; 2-Control, 1 h; 3-PMA-treated, 6 h; 4-Control, 6 h; 5-PMA-treated, 12 h; 6-Control, 12 h.

Additional trials of this experiment could provide more information about LFA-1 α2→6 sialic acid content. The lectin-based approach is well-established; however, the relatively weak affinity of lectins for their carbohydrate ligands gave rise to a low signal-to-noise ratio in **Figure 2.4**. As discussed in the next section, we explored chemical strategies to improve the signal, and obtain more conclusive information about LFA-1 sialic acids.

2.5 Metabolic labeling

To overcome the weak affinity of lectins, chemical strategies to label LFA-1 sialic acids were utilized. A chemical approach to studying LFA-1 glycosylation is available through metabolic oligosaccharide engineering, a method popularized by Bertozzi and coworkers.[13,20,21,22] Our strategy was to supplement Jurkat cell culture media with synthetic peracetylated *N*-azidoacetyl-mannosamine (ManNAz, compound **2.1**, **Scheme 2.1**), enabling the incorporation of azide tags into terminal sialic acids of Jurkat cell surface glycoproteins. We then utilized Springer's immunoaffinity purification strategy discussed above to purify LFA-1, many of which should contain azides in their sialic acids. Subsequent *in vitro* biotinylation of LFA-1 sialic acids was achieved using a biotinylated phosphine (compound **2.2, Scheme 2.1**).



Scheme 2.1 LFA-1 sialic acid biotinylation strategy. Compound **2.1** (peracetylated *N*-azidoacetyl-mannosamine) is incorporated into the cell's biosynthetic machinery, resulting in azide-bearing sialic acids on the cell surface. Immunoaffinity purification followed by the Staudinger ligation with a biotinylated phosphine (**2.2**) yields biotinylated LFA-1.

We tested this labeling strategy using Western blotting. A sample of LFA-1 bearing biotinylated sialic acids was split in two. One sample was treated with Peptide N-glycosidase F (PNGase F) to remove N-linked glycans. Both the PNGase F-treated, and the untreated samples were subjected to Western blotting with an HRP-conjugated streptavidin probe. As shown in **Figure 2.5**, our metabolic labeling strategy was successful. We were able to incorporate the azide, and subsequently biotinylate sialic acids in both the αL and β2 subunits of LFA-1. Not only do our results show metabolic incorporation in both LFA-1 subunits, PNGase-F treatment confirms that these sialic acids are on N-linked glycans in

both subunits. To date, this is only the second example of a purified protein that has been labeled this way. Bertozzi and coworkers were able to demonstrate this approach with dihydrofolate reductase (DHFR).[22]

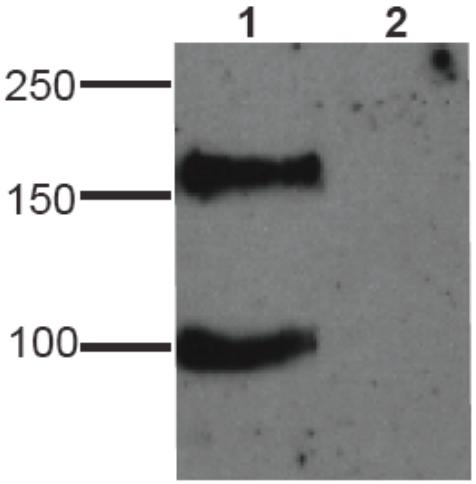


Figure 2.5 Metabolic labeling and biotinylation of LFA-1 sialic acids. Jurkat cells were cultured in media supplemented with ManNAz (**2.1**), and LFA-1 was purified by immunoaffinity chromatography. Azides were biotinylated by Staudinger ligation with the biotinylated phosphine **2.2**. The biotinylated proteins were separated by SDS-PAGE, blotted to nitrocellulose, and probed with streptavidin-HRP conjugate. Bands were detected by chemiluminescence. PNGase-F treatment was performed overnight at 37 °C on the native heterodimer. Lanes shown are as follows: 1-Untreated; 2-PNGase-F-treated.

The labeling of LFA-1 shown in **Figure 2.5** provides a bioorthogonal chemical handle that can be easily modified by established protocols. Alternative phosphines could also be used, bearing different labels (fluorescent dyes, for example), allowing for tailoring these labels for imaging. Furthermore, without

the limitations in binding specificity imposed by lectins, there is a significant improvement in signal to noise, relative to the lectin blot in **Figure 2.4**. One potential drawback to this approach is the time required. Incorporation of the azide into LFA-1 required at least 4 h. However, maximal incorporation is observed after 48 h, while immunoprecipitation requires an additional 5 h. The Staudinger ligation is typically performed overnight, followed by SDS-PAGE and immunoblotting. Therefore, the entire procedure takes several days, depending on incorporation time. The use of alkyne-linked labels would reduce the time required for derivitization of the azide, as click reactions are known to be significantly faster than the Staudinger ligation.[11] However, click reactions are typically performed with copper, which, due to potential cytotoxicity, might be undesirable for *in vivo* applications. Metabolic labeling provides information about total sialic acid, unlike lectins; which only recognize sialic acids of specific linkages.

2.6 Electrophoresis studies

In 2002, Semel et al. reported that β1 subunits in PMA-treated myeloid cells exhibited an increased electrophoretic migration relative to β1 subunits from untreated cells.[4] This difference in electrophoretic migration increased over time (up to 12 h). Bellis and coworkers have attributed this increased migration to hyposialylation of β1 integrins, due to reduced expression of the ST6Gal I sialyltransferase upon cell activation. We performed several electrophoresis-based

analyses to look for changes in LFA-1 sialylation upon PMA-stimulated cell activation.

2.6.1 Metabolic labeling of LFA-1 in band migration studies

We used the metabolic labeling and immunoaffinity purification strategies discussed above to look for changes in LFA-1 sialylation due to Jurkat cell activation. Metabolically labeled LFA-1, obtained from PMA-treated and untreated Jurkat cells at different time points, could be biotinylated by Staudinger ligation and analyzed by Western blot, using a streptavidin probe. This approach could yield information about changes in LFA-1 sialylation, not only by differences in band migration, but also by differences in band intensity.

As shown in **Figure 2.6**, this approach is a viable method to study changes in sialic acid content of LFA-1. Interestingly, we again observed more labeling of the αL subunit, consistent with our results using SNA (**Figure 2.4**) and with Asada's LFA-1 glycan analysis.[5] We also detected changes in the relative labeling of the αL subunit due to PMA treatment, which suggested that activation leads to increased sialylation between 1 and 12 h. This observation could be the result of increased sialyltransferase activity, as proposed by Bellis for the β1 integrin. Although this approach can certainly be used to study LFA-1 sialylation, it also suffers from the same signal variability and resolution problems, and as discussed above. The small scale of the immunoprecipitation makes it difficult to obtain quantitation by densitometry, as small variability can result in major differences in signal intensity. This variability is most evident in the analysis of the αL

subunit in **Figure 2.8**, where differences in band intensity are not expected. Although overcoming this variability will be challenging, the significant improvement in signal to noise makes this labeling strategy a legitimate alternative to lectin-based detection.

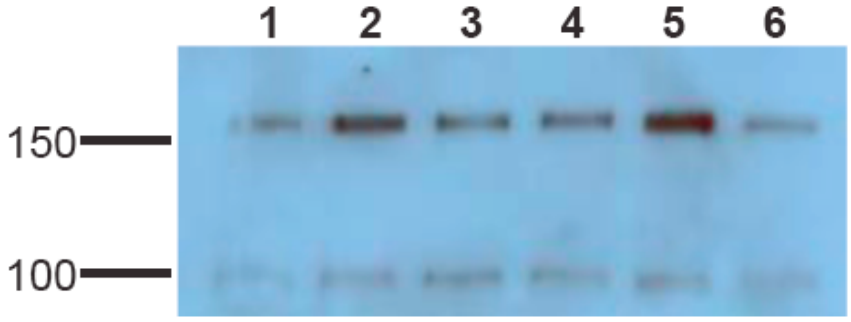


Figure 2.6 LFA-1 band migration study with biotinylated sialic acids. Jurkat cells were cultured in media supplemented with ManNAz (**2.1**) and LFA-1 was immunoprecipitated at 1, 6, or 12 h following treatment of PMA, or an equal volume of DMSO (control). Azide-bearing sialic acids were biotinylated by Staudinger ligation with compound **2.2**, and the samples were analyzed by immunoblotting using a streptavidin-HRP conjugate. Bands were visualized by chemiluminescence. Lanes shown are as follows: 1-PMA-treated, 1 h; 2-Control, 1 h; 3-PMA-treated, 6 h; 4-Control, 6 h; 5-PMA-treated, 12 h; 6-Control, 12 h.

An improved strategy for tracking changes in LFA-1 sialylation using metabolic labeling could involve the use of pulse-chase labeling. Tauber and coworkers used pulse-chase studies with radiolabeled monosaccharide precursors in classic studies of changes in protein glycosylation.[23] We hoped to perform similar pulse-chase labeling experiments, using ManNAz (**2.1**) as the pulse, and *N*-acetyl-mannosamine (ManNAc) as the chase. By again combining this

metabolic labeling with immunoprecipitation and biotinylation by Staudinger ligation, changes in LFA-1 sialic acid content could be detected by Western blot (with a streptavidin probe). We tested two pulse times, 0.5 h and 1 h, and three chase times, 1, 3.5 and 6 h. The times were selected based on previous studies.[23] As shown in **Figure 2.7**, this strategy for pulse-chase analysis of LFA-1 is viable. We observed incorporation of azide into LFA-1 sialic acids at 4 h after initial exposure to ManNAz. As expected, both longer pulse and longer chase times gave rise stronger signal in both subunits. We observed stronger signal in the αL subunit, consistent with **Figure 2.4** and **Figure 2.6** above, as well as reported studies of LFA-1.[5] It should be noted that the results in **Figure 2.7** are from the use of unsupplemented (ManNAc-free) media as the chase. Subsequent attempts using excess concentrations of ManNAc in the chase media gave rise to lower signal. This challenge could be overcome with the optimization of labeling concentrations and pulse-chase times. This pulse-chase labeling strategy could then be used to study changes in LFA-1 sialic acid due to cell activation, or other stimuli.

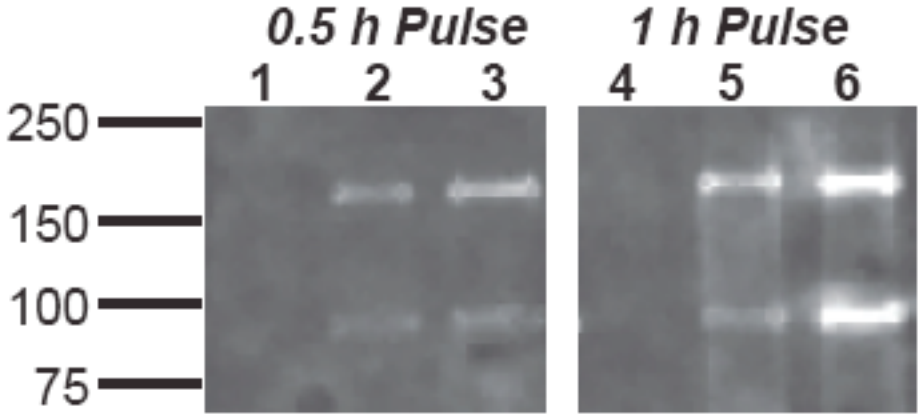


Figure 2.7 Pulse-chase labeling of LFA-1 sialic acids. Jurkat cells were cultured in media supplemented with ManNAz (**2.1**) for 0.5 h to 1 h (pulse). Cells were washed with unsupplemented media then incubated in that media for 1, 3.5, or 6 h (chase). LFA-1 bearing azide-labeled sialic (LFA-1-Az) was immunoprecipitated with TS2/4 mAb sepharose after 1, 3.5, or 6 hours. Before elution from the beads, azide-tagged sialic acids were biotinylated by Staudinger ligation with compound **2.2**. The samples were analyzed by immunoblotting using a streptavidin-HRP conjugate. Bands were visualized by chemiluminescence. Lanes shown are as follows: 1- 0.5 h pulse, 1 h chase; 2- 0.5 h pulse, 3.5 h chase; 3- 0.5 h pulse, 6 h chase; 4- 1 h pulse, 1 h chase; 5- 1 h pulse, 3.5 h chase; 6- 1 h pulse, 6 h chase.

2.6.2 LFA-1 analysis using subunit-specific antibodies

Our final attempts to observe changes in the electrophoretic migration of LFA-1 due to PMA treatment of Jurkat cells were performed identically to the study performed by Semel et al. in 2002.[4] Using separate antibodies in a Western blot, specific for either the αL (TS1/22) or β2 subunit (MEM-48), the effect of

PMA treatment on the electrophoretic migration of LFA-1 subunits was compared over time (**Figure 2.8**).

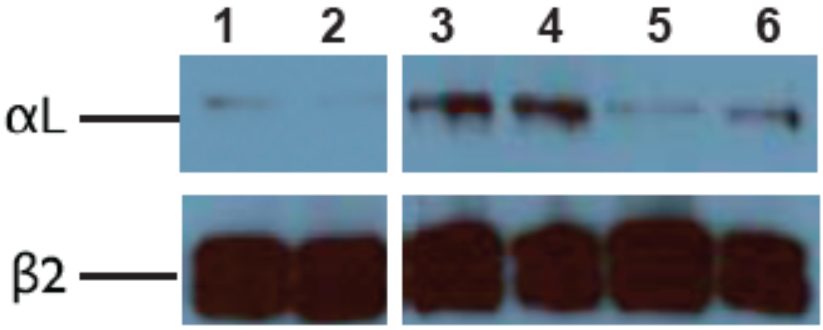


Figure 2.8 Band migration studies of LFA-1 using subunit-specific mAbs. LFA-1 was immunoprecipitated from Jurkat cells at 1, 6, or 12 h following treatment with PMA, or an equal volume of DMSO as a control. The samples were analyzed by Western Blot, using the αL-specific TS1/22 mAb (*Top*) or the β2-specific MEM-48 mAb (*Bottom*). Both blots utilized an HRP-conjugated goat anti-mouse IgG as the secondary antibody. Bands were detected by chemiluminescence. Lanes shown are as follows: 1- Control, 1 h; 2- PMA-treated, 1 h; 3- Control, 6 h; 4- PMA-treated, 6 h; 5-Control, 12 h; 6- PMA-treated, 12 h.

As shown in **Figure 2.8**, there appears to be a slight increase in the migration of the αL subunit 12 h after PMA treatment. This observation conflicts with the results obtained in **Figure 2.6**, where after 12 h, the αL subunit from PMA-treated cells migrated less than the αL subunit from untreated cells. As mentioned above, there is significant variability in the intensity of bands. In this experiment, the intensity of all bands should be the same in each lane, unless there are activation-dependent changes in antibody affinity for either subunit. The blot

for the β2 subunit shows consistent staining in each lane, although the bands are slightly overexposed. However, the blot for the αL subunit shows significant variability in signal intensity. Although these struggles are common to blotting techniques, they make these analyses more challenging. To obtain more conclusive evidence for changes in LFA-1 sialylation, additional resolution is required. We considered that this resolution could be provided by a second dimension of separation.

2.6.3 2D characterization of LFA-1

To improve the resolution of our gel-based analysis of LFA-1, two-dimensional gel electrophoresis strategies were explored. This involves separation of glycoprotein isoforms by isoelectric focusing [24] in the first dimension, followed by molecular weight separation by SDS-PAGE in the second dimension. Pardi et al. observed the presence of several LFA-1 sialoforms using IEF, and were able to visualize radiolabeled LFA-1 on a 2D gel.[6] Springer and coworkers have also used radiolabeling to characterize LFA-1 by 2D electrophoresis; however, separation in both dimensions was achieved by SDS-PAGE.[18]

Ideally, the metabolic labeling strategy discussed above could be used to perform two-dimensional analysis of LFA-1 bearing biotinylated sialic acids. We hoped that the improved sensitivity afforded by chemiluminescent detection in a Western blot would be sufficient to visualize LFA-1. However, we have yet to observe sufficient signal for any characterization of LFA-1 in this way. These signal-to-noise problems are attributed to a number of challenges. Several spots

are expected, due to LFA-1's significant glycan heterogeneity. It is also likely that biotinylation exacerbates the heterogeneity problem, increasing the number of potential spots on the 2D gel. It is also expected that the samples of purified LFA-1 are mixtures reflecting varying levels of ManNAz incorporation. This could range from no incorporation, to incorporation in several sialic acid residues. This variability certainly limited signal-to-noise, as it effectively dilutes the sample. We also observed significant aggregation of purified samples of LFA-1 prior to loading on the IEF strip. This aggregation resulted in loss of sample, and prevented detailed analysis by IEF. In our attempts to concentrate fractions of purified LFA-1, we observed that reconstitution after lyophilization required the use of detergents (such as Triton or SDS) at concentrations incompatible with IEF. Without the ability to concentrate the sample, it was difficult to detect LFA-1 glycoforms.

As an alternative to 2D detection of metabolically labeled LFA-1, we attempted 2D analysis of unlabeled LFA-1. However, instead of using radiolabeling, we used silver staining, which detects total protein. While less sensitive than detection of radiolabels, we were able to use this detection for both LFA-1 subunits. As shown in **Figure 2.9**, the results were consistent with those obtained by Pardi et al.[6] We attempted to compare purified samples of native LFA-1 to LFA-1 that had been subjected to neuraminidase treatment. Both the αL and β2 subunits were resolved by IEF, revealing that the αL chain was more negatively-charged than the β2 chain. This would be consistent with increased sialic acid content on the αL subunit; however, there are additional negatively-

charged groups (phosphates, sulfates, etc.) that could affect resolution by IEF. Each chain resolved over a range of electric potentials, supporting the expected heterogeneity of the protein.

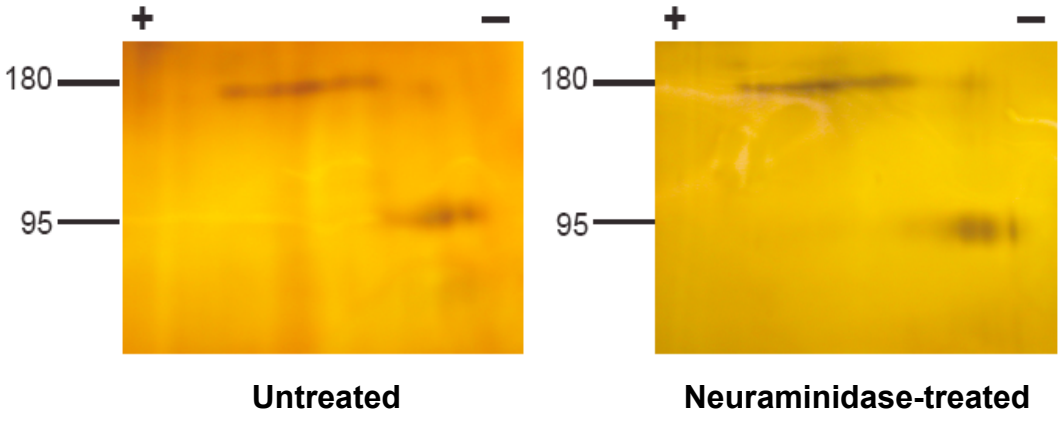


Figure 2.9 2D analysis of LFA-1. LFA-1 immunoprecipitated from Jurkat cells was subjected to isoelectric focusing (pH 4-7) followed by SDS-PAGE. Protein bands were detected by silver staining. Neuraminidase treatment was attempted prior to IEF, using neuraminidase from *C. perfringens* at 37 °C for 1 h at pH 5.5.

2.7 MS characterization of LFA-1 glycans

In their study of LFA-1, Asada and co-workers predicted 12 N-linked glycosylation sites on the αL subunit, and 6 sites on the β2 subunit. In addition to the gel-based analyses, we attempted to confirm those predicted glycosylation sites using mass spectrometry (MS) techniques. To date, the only reports of integrin glycan characterization by MS are studies of the $\alpha_3\beta_1$ integrin by Pochec et al. using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).[3,25,26,27]

Our initial attempts to characterize LFA-1 by MS involved the use of ESI-LC-MS-MS. We confirmed one N-linked glycosylation site (Asn 213 on the β2 chain). As shown in **Figure 2.10,** even this assignment is tenuous, as it relies on the assignment of low intensity signals.

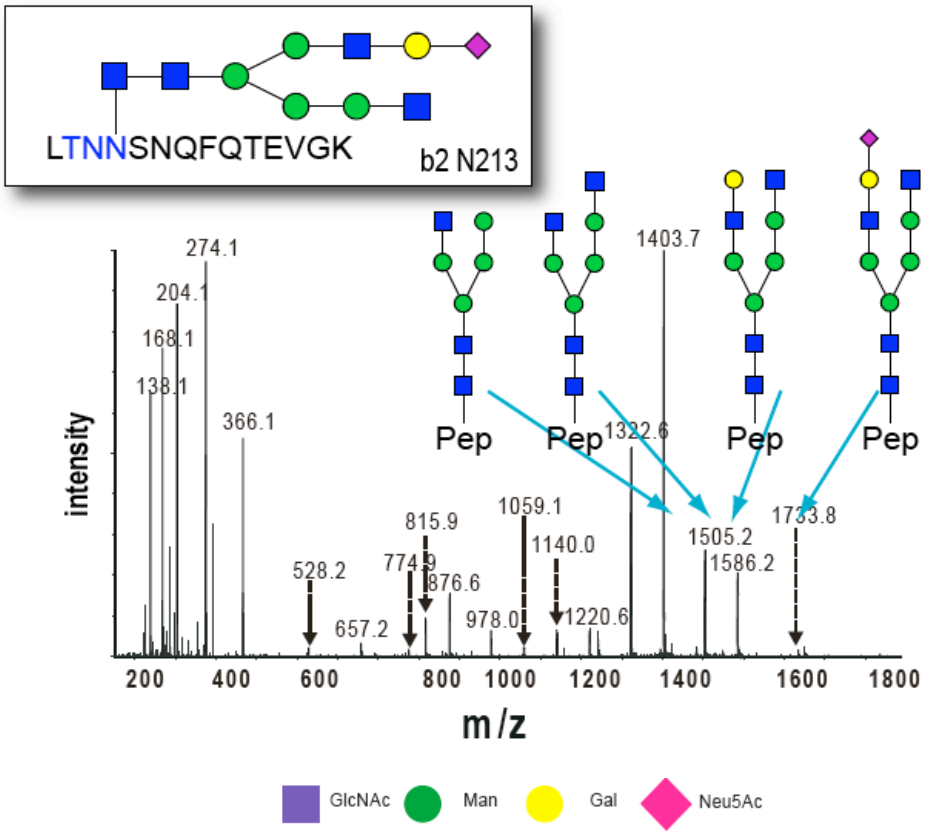


Figure 2.10. MS/MS of a glycopeptide ion from the β2 subunit of LFA-1. Positive-ion MS/MS spectrum with hypothetical structures of N-glycans of a doubly-charged glycopeptide ion obtained from trypsin digestion of LFA-1. Data provided by M. Fentabil (University of Alberta).

As discussed above, small amounts of antibody consistently leaked from the column during the immunoaffinity purification of LFA-1. This problem

further complicated attempts at glycan analysis of LFA-1 by mass spectrometry. N-glycans of mammalian IgG are less heavily glycosylated, and exhibit significantly less heterogeneity in their glycans as compared to integrins such as LFA-1.[19,28] Thus, even small contaminating amounts of antibody could significantly inhibit MS characterization of LFA-1 glycans. Despite separation of protein bands by SDS-PAGE, and direct excision of desired protein bands from the polyacrylamide gel, contaminating glycopeptides from TS2/4 were often found during attempts at characterizing either LFA-1 subunit. IgG heavy chains are typically about 50 kDa, while their light chains are over 25 kDa. It is not surprising then, that variable glycoforms of the antibody could co-migrate with either subunit during SDS-PAGE. When considering the size of the αL subunit (177 kDa), the breadth of the protein band, and the corresponding molecular weight range of the excised band, a heavily glycosylated form of the intact antibody, or some aggregate of antibody fragments, could migrate within that range. The β2 subunit (95 kDa) could also co-migrate with a combination of two IgG heavy chains, or a single heavy chain with two light chains. Due to the relatively limited heterogeneity of IgG N-glycans, these contaminants gave the strongest signals when using software-based filters to search for characteristic markers of N-glycans. Improved separation of LFA-1 from antibody was observed in SDS-PAGE when commercial gradient gels were used. Also, by running the gels at higher voltages, protein bands could be kept from broadening, allowing for better excision of the target band.

2.8 Adhesion of LFA-1

With a purification protocol for native LFA-1 available, we attempted to reproduce protocols for cellular adhesion assays with immobilized protein.[17,29] A reliable adhesion assay would allow us to study the role of glycosylation in LFA-1-mediated T cell adhesion to ICAM-1 directly. We first followed the protocol of Springer et al., who were the first to show that purified, native LFA-1 could be adhered to polystyrene surfaces by dilution of detergent concentrations.[30] After blocking the uncoated surface, the adhesion of cells to the purified LFA-1 was quantified visually by cell counting. An alternative approach, reported by Cherry et al.[17] involved measuring the fluorescence of dye-loaded cells after adherence to a surface. If either procedure were successful, we could use this assay to test the role of enzymatic modification of LFA-1 glycans.

Before testing the effect of glycan modifications, we attempted to reproduce the adhesion assay protocols reported in Springer and Klickstein's labs.[17] Plates were prepared by allowing immunoaffinity purified LFA-1 or commercial ICAM-1 (sICAM-1-Fc, R&D Sytems, Minneapolis, MN) to adhere to polystyrene dishes, which were then blocked with BSA. The adhesion of Jurkat cells treated with PMA was then compared to untreated cells. Detecting cell binding by cell counting confirmed that the purified LFA-1 was capable of adhesion, as shown in **Figure 2.11**. PMA stimulated cells show increased adhesion to areas coated with adhesion receptors (ICAM-1 or LFA-1). As expected, we observed relatively low background adhesion to surfaces lacking

LFA-1 or ICAM-1. However, in subsequent attempts, we found the assay to be inconsistent.

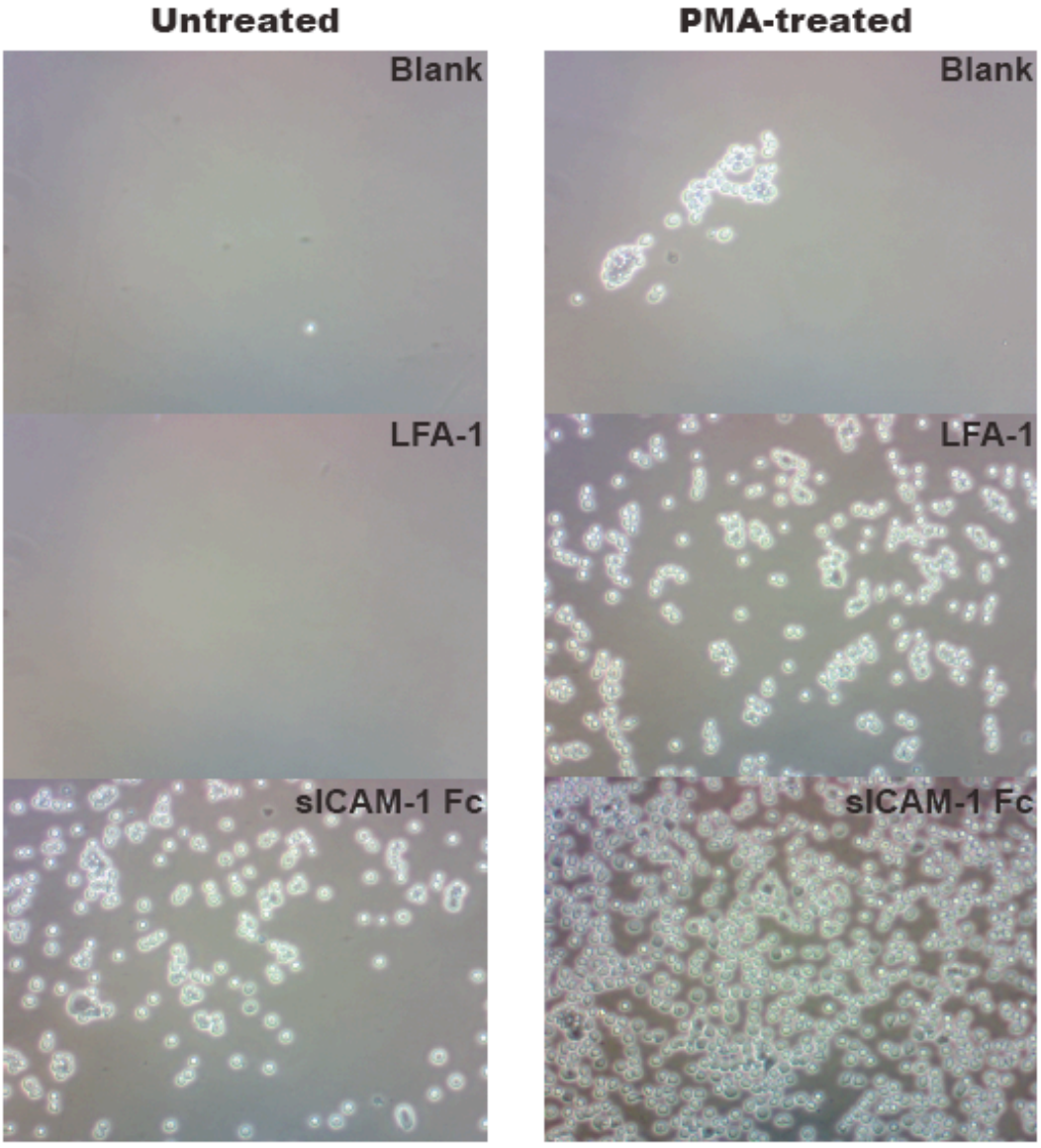


Figure 2.11 Jurkat cell adhesion assay. Adhesion of Jurkat PMA treated and untreated cells was compared using uncoated polystyrene plates coated with adhesion receptors (either immunoaffinity purified LFA-1, or commercial sICAM-1-Fc). Images were taken by attaching a digital camera to a light microscope. *Left:* Untreated Jurkat cells; *Right:* PMA treated Jurkat cells.

We also attempted to assay LFA-1 adhesion using a 96-well plate assay, involving Jurkat cells loaded with a fluorescent dye, 2',7'-Bis- 2-Carboxyethyl)-5-(And-6)- carboxyfluorescein (BCECF). Like Springer's assay, adhesion receptors were diluted to achieve low detergent concentrations necessary for immobilization. After immobilization of adhesion receptors, the plates were blocked with BSA. The adhesion of BCECF-loaded Jurkat cells treated with PMA was then compared to untreated cells. Results were quantified using a fluorescence plate reader. Once again, we found this assay to be inconsistent. Although the results in **Figure 2.12** show a higher percentage of bound cells after PMA treatment, the background adhesion to uncoated wells is high.

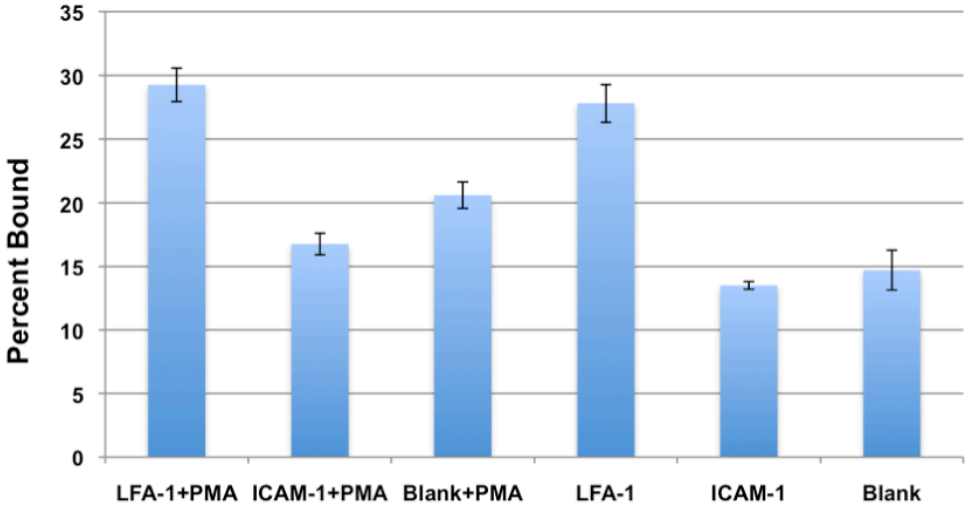


Figure 2.12 Jurkat cell adhesion assay. Adhesion of Jurkat cells to 96-well plates coated with adhesion receptors (LFA-1, sICAM-1-Fc). Bars indicate the percentage of cells remaining adherent to wells after washing. Data shown are mean values for a representative experiment of cells treated or untreated with PMA, performed in replicates of six. Error bars indicate standard error of the mean.

Lack of reproducibility in our cellular adhesion assays prevented a conclusive test of our hypothesis regarding the role of LFA-1 glycosylation in adhesion. These assays are notoriously difficult procedures to develop, as evidenced by the multitude of slight, but significant, variations in reported protocols.[17,30,31] These differences are most commonly observed in the washing steps, which are arguably the most critical part of cellular adhesion assays. We tried several different strategies, including passively flowing wash buffer over the area in a dish assay, flicking the 96-well plates, aspiration of wash buffer, or allowing cells to passively fall out of wells by inversion of plates into large volumes of wash buffer. Additionally, based on our attempts to study integrin structure, it is likely that post-purification aggregation of LFA-1 is also contributing to variability. Other members of our group have attempted alternative adhesion protocols, including a FACS-based approach.[31] However, these studies have also faced issues with reproducibility (Ebesoh and Cairo, personal communication). In the absence of a reproducible assay for cellular adhesion, the functional role of LFA-1 glycans remains an unresolved question.

2.9 Conclusions and future directions

In this chapter, we described the immunoaffinity purification of LFA-1 in its native form using the TS2/4 mAb. Although we observed no significant changes in band migration, we were able to obtain data consistent with an increase in sialic acid content, and $\alpha 2 \rightarrow 6$ linkages in the αL subunit of LFA-1. The metabolic incorporation of azides into cell surface sialic acids, and their

subsequent biotinylation by Staudinger ligation proved a successful strategy for detecting changes in sialic acid in purified LFA-1. We have also shown that this label is specifically incorporated into the N-linked glycans of the αL and β2 subunits, using PNGase F.

Although the key questions about LFA-1 glycan structure, function, and regulation still remain unanswered, most of the methodological steps required to answer those questions have been taken. Optimization of the immunoprecipitation, separation, and detection methods could reveal more subtle changes than those discussed here. It is also possible that sialic acids on LFA-1 are not modified upon activation, or do not affect LFA-1 function. As Bellis has noted, although hyposialylation is observed in some β1 integrins after PMA treatment in myeloid cells, the same treatment does not result in hyposialylation of several other β1 integrins.[1] Bellis proposed that this difference could be due the presence or absence of an α I domain.[1]

Despite significant effort, we have thus far been unable to address functional questions about LFA-1 glycans. This is due to the absence of a reliable adhesion assay. Errors in LFA-1 handling, storage, or errors in washing technique are the most likely sources of difficulty in this assay. Once these problems are resolved, the role of glycans in LFA-1 mediated adhesion can be measured.

With much of the preliminary work completed, the development of a reproducible adhesion assay, either by SPR,[32] ELISA,[25,27,32] or plate-based methods[17] could enable future researchers to quickly gain insight into the role of sialic acids in LFA-1 function. Additionally, a solution to the antibody leakage

problem, combined improved tools for glycan analysis, should make structural characterization of LFA-1 a less daunting challenge.

Our lab is also currently studying mammalian sialidases, and has already developed protocols to study NEU3 activity.[33,34] Moreover, there are protocols (developed by Dustin et al.) for reincorporation of purified integrins into artificial bilayers.[16] If these protocols can be reproduced successfully, the metabolic labeling and purification protocols described in this chapter could be used to test mammalian sialidases as potential regulators of LFA-1 sialic acids in vivo. This could serve as a general method for studying the glycobiology of transmembrane proteins, which could allow more sensitive detection of changes in the glycan structure.

In Chapter 3 we discuss initial progress towards in vivo studies of NEU3 using fluorescence microscopy, as well as progress towards the use of drag-tags for quantitation of sialoforms, as an application of azide metabolic labeling strategy discussed in Chapter 2. We will also demonstrate the use of the Staudinger ligation as a method for bioconjugation of synthetic carbohydrates, which are capable of recognition by lectins, as a way to probe the role of specific glycans in protein-carbohydrate recognition.

2.10 Materials and Methods

2.10.1 Antibodies

HB203 and HB244 hybridoma cells (which produce the TS1/18 and TS2/4 mAbs, respectively) were obtained from ATCC (Manassas, VA) and cultured with either ATCC 46-X Hybri-Care Media (HB203), or DMEM (HB244), supplemented with 10% IgG-depleted FBS and 100 μg/mL penicillin and streptomycin. For antibody production from HB244 cells, the NUNC OptiCell Max 2100 Starter Kit (NUNC, Rochester, NY) was used according to the manufacturer's instructions. OptiCell chambers were seeded with 30 mL of media containing 1 x 10^6 HB244 cells in log phase. Cells were cultured at 37 °C in a humidified, 5% CO_2 incubator. 20 mL harvests were performed and replaced at three-day intervals for one month per chamber. Antibodies were precipitated by ammonium sulfate precipitation, and then purified using a Bio-Rad MAPS II Protein A Purification Kit (Bio-Rad, Hercules, CA) according to the supplier's directions.

2.10.2 Purification of LFA-1[14,15]

All LFA-1 samples were obtained from Jurkat (E6.1) cells obtained from ATCC. Cells were cultured with RPMI 1640 supplemented with 10% FBS, and 100 μg/mL penicillin and streptomycin, and cultured in at 37 °C in a humidified, 5% CO_2 incubator. LFA-1 was immunoaffinity purified from Jurkat cells, according to the method by Springer et al., using the TS2/4 mAb immobilized on Sepharose.[14,15] TS2/4 mAb was immobilized using either cyanogen bromide-

activated Sepharose or *N*-hydroxy succinimide-activated Sepharose, according to the manufacturer's instructions. For preparative-scale purifications, Jurkat (E6.1) cells in log phase were harvested from 1 L spinner cultures (seeded at 100 000 cells/mL) and centrifuged at 500 x g for 10 min at 20 °C. The cells were washed three times with DPBS, then lysed at a density of 50 x 10^6 cells/mL in lysis buffer (20 mM Tris-HCl buffer pH 8.0, containing 0.5% Trition X-100, 1 mM PMSF, 5 mM iodoacetamide, 0.2 units/mL aprotinin, 2 mM $MgCl_2$, and 0.025% sodium azide). The sample was mixed at 4 °C overnight, then subjected to centrifugation at 3900 x g for 25 m at 4 °C. The supernatant was collected, and sodium deoxycholate was subsequently added to a concentration of 0.5% m/v. The lysate was then passed through a control BSA-Sepharose column to clear any particulates and non-specifically adsorbed material. Upon elution from the column, the lysate was passed through a TS2/4 mAb-Sepharose column at flow rate of 0.4 mL/minute. The column was washed with five column volumes of 1% Triton X-100 in TSA pH 8, followed by five column volumes of 1% octylglucoside in 20 mM Tris-HCl pH 8, containing 5 mM $MgCl_2$. LFA-1 was then eluted using 50 mM triethylamine, pH 11.5, 1% octylglucoside, 5 mM $MgCl_2$. 1.5 mL fractions were collected, each fraction containing 150 μl of neutralization buffer (1 M Tris pH 7.5, 1% octylglucoside, 5 mM $MgCl_2$). For band migration studies, LFA-1 was immunoprecipitated with TS2/4 mAb Sepharose using the same buffers used for the preparative-scale purification. Between wash steps, beads were centrifuged at 400 x g for 30 seconds. Each wash

step was performed 3X. Sepharose TS2/4-bound LFA-1 was boiled at 95 °C in Laemmli buffer prior to SDS-PAGE.

2.10.3 Lectin blotting[35]

Jurkat (E6.1) cells were treated with PMA (50 ng/mL) or an equal volume of DMSO (CTRL) and incubated in growth media at 37 ^{o}C, 5% CO_2 atmosphere. Cells were harvested after 1 h, 6 h, and 12 h. LFA-1 was immunoprecipitated from each sample using TS2/4 mAb Sepharose. Proteins were separated by 4/7% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk TBS-T, then incubated with biotinylated lectin, SNA (Vector Labs, Burlingame, CA), in 2.5% milk TBS-T. The membranes were then probed with horseradish peroxidase-conjugated streptavidin (Bio-Rad, Hercules, CA) for 1 h. Bands were visualized by chemiluminescence.

2.10.4 Metabolic labeling and detection by Western blot

Metabolic labeling of Jurkat sialic acids was achieved seeding cells (100 000 cells/mL) in growth media supplemented with 14 μM ManNAz (**2.1**) for variable time periods, depending on the assay. LFA-1 bearing azide-labeled sialic (LFA-1-Az) was immunoprecipitated with TS2/4 mAb sepharose as described above. However, before elution from the beads, azide-tagged sialic acids were biotinylated by Staudinger ligation by adding compound **2.2** (50 μg/mL) and mixing overnight at 4 °C in neutralized LFA-1 elution buffer.

Detection of biotinylated LFA-1 was achieved by Western blotting. Proteins were eluted by boiling at 95°C in Laemmli buffer, and separated by 4/7% SDS-PAGE. Proteins were then transferred to nitrocellulose, then the membranes were blocked with 5% milk in TBS-T. Membranes were probed with streptavidin-HRP (Bio-Rad, Hercules, CA) in 2.5% milk TBS-T. Bands were visualized by chemiluminescence. Removal of biotinylated sialic acids through removal of N-glycans, was achieved by treating immunoaffinity purified LFA-1-Az with PNGase F (New England Biolabs, Ipswich, MA). Native LFA-1 was incubated with PNGase F overnight at 37 °C.

2.10.5 Band migration studies

All band migration studies were performed as described above, with different detection strategies. First, Jurkat (E6.1) cells were treated with PMA (50 ng/mL) or an equal volume of DMSO (control) and incubated in growth media at 37 ^{o}C in a 5% CO_2 atmosphere. Cells were harvested after 1 h, 6 h, and 12 h. LFA-1 was immunoprecipitated from each sample using TS2/4 mAb sepharose, and eluted by boiling at 95 ^{o}C in Laemmli buffer. Proteins were separated by 4/7% SDS-PAGE and transferred to nitrocellulose membranes. Detection of metabolically labeled sialic acids was achieved using HRP-conjugated streptavidin. Separate, specific detection of αL and β2 subunits was achieved using the TS1/22 and MEM-48 mAbs (AbCam, Cambridge, UK), respectively. Membranes were blocked with 5% milk TBS-T, incubated with the antibody in 2.5% milk TBS-T for 1 hour, then probed with horseradish peroxidase-conjugated

goat anti-mouse IgG in 2.5% milk TBS-T for 1 h. Bands were visualized by chemiluminescence.

2.10.6 Pulse-chase labeling of LFA-1 sialic acids

Metabolic labeling was achieved by culturing Jurkat cells in growth media supplemented with 50 μM ManNAz (**2.1**) for 30 or 60 m (pulse). The cells were washed with unsupplemented media and then incubated in unsupplemented media for varying time periods (chase). LFA-1 bearing azide-labeled sialic (LFA-1-Az) was immunoprecipitated with TS2/4 mAb sepharose as described above after 1, 3.5, or 6 h. Before elution from the beads, azide-tagged sialic acids were biotinylated by Staudinger ligation by adding compound **2.2** (50 μg/mL) and mixing overnight at 4 °C. Detection of biotinylated LFA-1 was achieved by Western blot using HRP-conjugated streptavidin. Proteins were eluted by boiling at 95 °C in Laemmli buffer, and separated by 4/7% SDS-PAGE. Proteins were transferred to nitrocellulose, then the membranes were blocked with 5% milk in TBS-T. Membranes were probed with streptavidin-HRP (Bio-Rad, Hercules, CA) in 2.5% milk TBS-T. Bands were visualized by chemiluminescence. Compounds **2.1** and **2.2** were synthesized by R. Loka (University of Alberta).

2.10.7 2D electrophoresis

2D gels of LFA-1 were performed using a Bio-Rad 2D electrophoresis kit. Fractions of immunoaffinity purified LFA-1 were loaded onto IEF strips with a pH range of 4-7, according to the supplier's directions. Isoelectric focusing in the

first dimension was followed by 8% SDS-PAGE. Protein bands were visualized by silver staining (Bio-Rad, Hercules CA). Neuraminidase treatment of LFA-1 was attempted by incubation with a bacterial neuraminidase from *Clostridium perfringens* (Sigma, St. Louis, MO) at 37 ^{o}C for 1 h prior to loading samples on to IEF strips.

2.10.8 Preparation of samples for MS

LFA-1 was purified as described in section **2.10.2**, and separated by SDS-PAGE with 4%/7% gel. Protein bands were visualized with Coomassie Brilliant Blue. Bands corresponding to the αL subunit (177 kDa) and the β2 subunit (95 kDa) were excised, and digested with trypsin overnight at 37 °C. Samples were submitted to collaborators for analysis by ESI-LC-MS-MS.

2.10.9 Jurkat cell adhesion assays

Adhesion assays were performed by adhering immunoaffinity purified LFA-1 or commercial sICAM-1-Fc (R&D Sytems, Minneapolis, MN) onto polystyrene dishes. 5 μL of individual fractions of purified LFA-1 were diluted in 45 μL of TSM at the centre of polystyrene dishes. The plates were then blocked with 1% heat-denatured BSA and incubated with Jurkat cells, which were treated with 50 ng/mL PMA or an equal volume of DMSO, for 1 h) for 1 h. Non-adhering cells were removed by washing with assay buffer (HBSS-M). When performing the 96-well plate assay, Jurkat cells were loaded with the fluorescent dye, 2',7'-Bis- 2-Carboxyethyl)-5- (and-6)- carboxyfluorescein (BCECF, Invitrogen,

Carlsbad, CA), then adhesion was measured with a fluorescence plate reader (excitation 490 nm, emission 535 nm). [17,29]

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Chapter 3: Bioconjugate strategies for detecting glycoproteins[3,4]

[3] Portions of the work described in this chapter have been published in R.S. Loka, C.M. Sadek, N.A. Romaniuk, and C.W. Cairo, *Bioconjug Chem*, **2010**, 21(10), 1842-9.

[4] Bioconjugation of PEG drag-tags and subsequent SDS-PAGE analysis of labeled IgG was performed by N. A. Romaniuk (University of Alberta).

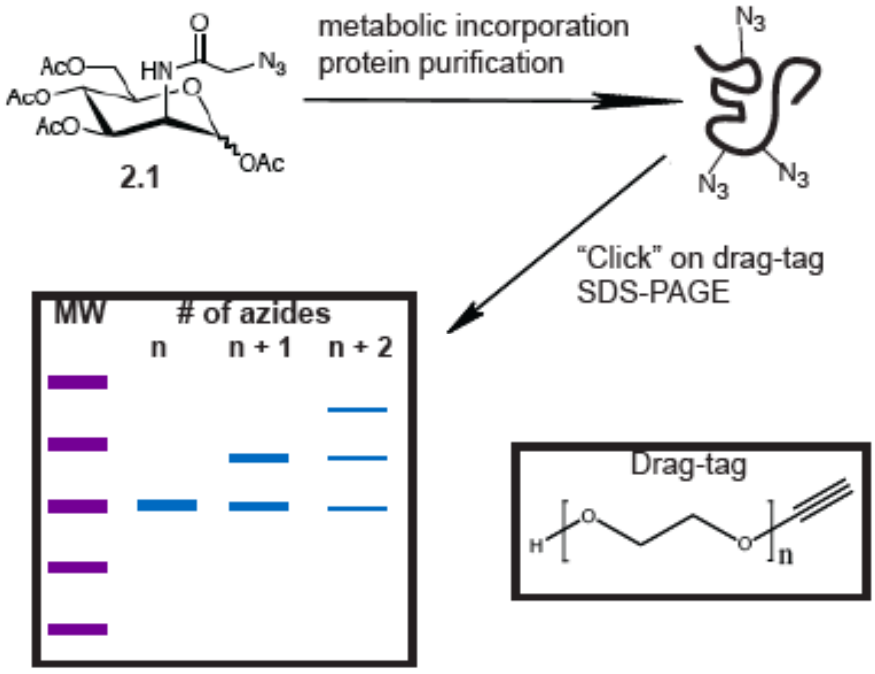
3.1 Introduction

Our group is interested in cell surface glycobiology, and the development of methods to study the role of carbohydrates in cell surface interactions. In Chapter 2, we attempted to determine a role for sialic acid in LFA-1 function. Although the role for sialic acid in LFA-1 function remains unresolved, the methods introduced in Chapter 2 have been utilized in other systems. In Chapter 3, we will discuss additional applications of metabolic labeling and Staudinger ligation methodologies,[1] as well as progress towards in vivo studies of the mammalian sialidase, NEU3. We demonstrate the bioconjugation of alkynyl drag-tags, by the Cu(I)-catalyzed click reaction, to resolve sialoforms of metabolically-labeled murine IgG. We also demonstrate chemoselective ligation, using synthetic carbohydrate epitopes and a model protein bearing synthetically-introduced azides, by Staudinger ligation.[1] We expect both of these strategies to be applicable to more complex systems. Finally, we report the production of stable HeLa cell lines expressing GFP-fused NEU3, as a model system for in vivo studies of NEU3 by fluorescence microscopy.

3.2 Determination of sialoforms using metabolic labeling and bioconjugation of drag tags

As discussed in Chapter 2, non-natural azidosugars can be metabolized by cells and incorporated into carbohydrate residues, incorporated into post-translational modifications of glycoproteins. The azide tag can serve as a chemical handle for the bioconjugation of detection reagents. Reported methods for

labeling include the Staudinger ligation, or the Cu(I)-catalyzed click reaction between alkynes and azides.[2,3,4] However, a shortcoming of this methodology is the non-specific incorporation of azidosugars into glycans. For example, as we observed in Chapter 2, low abundance glycoforms will dilute signal and non-specific labeling requires purification of the protein of interest. We envisioned that non-specifically incorporated azide tags could be used as a handle to detect the presence of sialoforms. Large molecular weight linkers of controllable size, known as drag-tags, have been used to study protein glycoforms.[5] With an azide-reactive drag-tag of sufficient size, glycoproteins with varying numbers of azides due to metabolic incorporation of azidosugars could be resolved electrophoretically (as shown in **Scheme 3.1**). Thus, sialoforms could then be discriminated by large differences in molecular weight or electrophoretic mobility. Like our labeling strategy in Chapter 2, this approach requires purification of the protein of interest after metabolic incorporation of the azidosugar. This method could also be used to identify and characterize protein glycoforms, and to study the expression of these glycoforms in response to specific stimuli.



Scheme 3.1 Bioconjugation of drag-tags to metabolically labeled sialic acids. Bioconjugation of alkyne-bearing drag-tags of defined, uniform size, could allow for the identification of sialoforms by SDS-PAGE.

In order to test the feasibility of the drag-tag approach, we employed murine IgG as a model glycoprotein. This glycoprotein is easy to purify in large quantities, and IgG glycosylation has been studied.[6,7,8,9,10] As discussed in Chapter 2, antibodies contain two heavy chains (50 kDa each), and two light chains (25 kDa each) each. The intact antibody is approximately 150 kDa. However, the heavy chains and light chains are held together by disulfide bonds. Under the reducing conditions typically used for SDS-PAGE, the heavy and light chains are resolvable.[9] IgG glycosylation varies in different species, however the Fc domains of IgG heavy chains are known to be N-glycosylated.[6,7,8,10] Sialylation of both Fc

domains gives rise to three different potential sialoforms, containing zero, one, or two sialic acids. The non- and mono-sialylated glycoforms predominate.[8,10]

HB244 hybridoma cells, which produce the TS2/4 mAb (IgG_1, mouse)[11] were cultured in media supplemented with ManNAz (**2.1**), following the protocols outlined in section **2.10**. The resulting azide-tagged antibody was purified. Labeling of the azidoprotein with alkynyl polyethylene glycol drag tags using the Cu(I)-catalyzed click reaction was performed as described in section 3.6.2. The drag-tag-conjugated antibody was then analyzed by SDS-PAGE.

As shown in **Figure 3.1**, separation by SDS-PAGE, and visualization using a fluorescent protein stain gave rise to single band corresponding to IgG light chain (25 kDa), but additional bands above the IgG heavy chain (50 kDa). This is consistent with the expected results.[8] Densitometric analysis of the gel revealed three distinct sialoforms, corresponding to the incorporation of zero, one, or two azide tags. This is consistent with published reports of IgG glycosylation.[6,7,8,10]

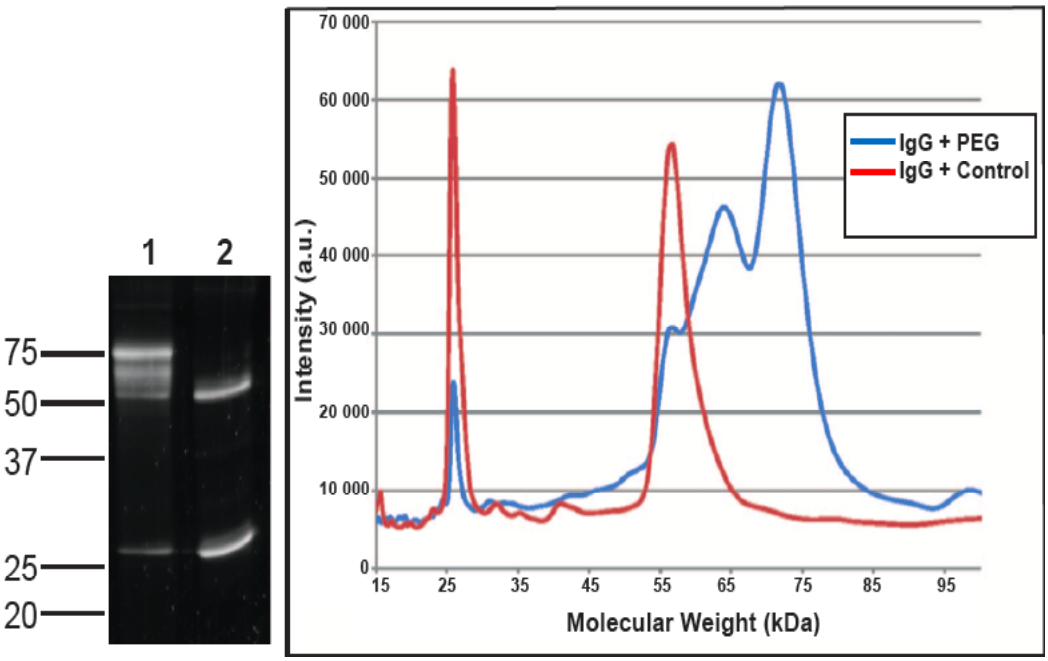


Figure 3.1 In gel fluorescence showing IgG sialoforms resolved by PEG drag-tag and their corresponding electrophoretic distribution. *Left:* TS2/4 mAb was purified from HB 244 cells cultured in media supplemented with ManNAz (**2.1**). The Cu(I)-catalyzed click reaction was used for bioconjugation of alkynyl polyethylene glycol drag-tags. Samples were analyzed by SDS-PAGE and visualized by fluorescence. Lanes shown are as follows: 1-IgG + drag-tag; 2-IgG + control. *Right:* Corresponding electrophoretic distribution of lanes 1 and 2.

As shown in **Figure 3.1** the metabolic incorporation of ManNAz (**2.1**) followed by the bioconjugation of polyethylene glycol drag tags can be used to determine the number of protein sialoforms. Subsequent attempts at using this method to analyze LFA-1 were not successful, likely due to issues of aggregation, higher molecular weight, and increased number of sialoforms. Any high

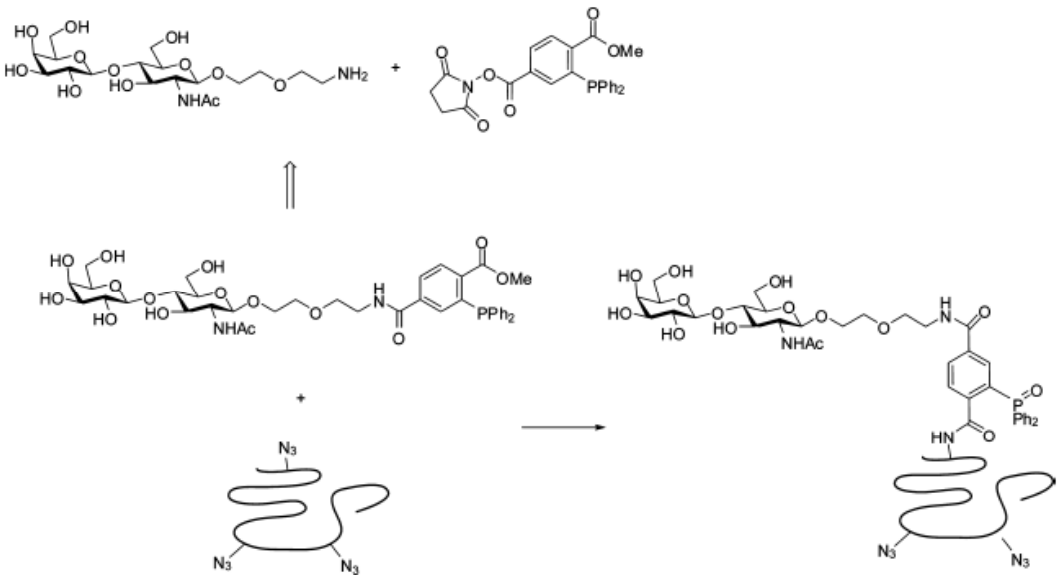
molecular weight protein will have diminished shift due to drag-tags, making resolution more challenging. A second dimension of resolution, either by molecular weight,[12] or isoelectric focusing,[13] could help overcome the resolution-associated challenges of high molecular weight glycoproteins. This approach could also be combined with other methodologies such as pulse-chase metabolic labeling, to learn more about the function of protein glycoforms. Moreover, this strategy is not limited to studies of N-glycans. Rexach et al. have recently reported the use of drag-tags to study O-linked glycosylation.[14]

3.3 Staudinger ligation to introduce synthetic carbohydrate epitopes

One of the major challenges when studying protein glycosylation is the heterogeneity of protein glycoforms. To overcome this challenge, we are interested in developing general strategies to produce synthetic glycoconjugates with uniform glycosylation. The generation of defined glycoconjugates, though challenging, is necessary for studying glycan function, and the development of therapeutics. A variety of strategies have been reported for forming protein glycoconjugates, including amine-cross-linking, native chemical ligation, solid-phase peptide synthesis, unnatural amino acid incorporation, chemoenzymatic synthesis, and chemoselective ligation.[15,16,17] Chemoselective ligation of glycans allows for the combination of a biologically-isolated protein with a synthetic glycan.[16] Chemoselective modifications of glycoproteins have been demonstrated using purified glycoproteins *in vitro*, as well as cell surface glycoproteins in vivo.[3,18,19,20]

The ability to modify glycoproteins in living cells permits identification of specific roles for different glycan epitopes. Chemoselective modification of cell-surface glycans, known as glycoform remodeling, has been used to target specific cells,[21] and to modify the function of glycoproteins.[22] Cell surface glycoform remodeling has been used to alter biological responses, including cellular adhesion.[23,24] These findings suggest that chemoselective ligation strategies can be used to determine the functional basis for specific changes in glycosylation within complex environments.

We chose to employ the Staudinger ligation, discussed in Chapter 2, to introduce galactose (Gal), lactose (Lac, Gal-β(1,4)-Glc), and *N*-acetyl-lactosamine (LacNac, Gal-β(1,4)-GlcNAc) on an azide-bearing model protein, lactoglobulin (Scheme 3.2). We chose to target LacNAc epitopes, which are part of type 2 lactosamine repeats of N- and O-linked glycans.[25]



Scheme 3.2 Bioconjugation of synthetic carbohydrate epitopes. A carbohydrate epitope linked to a phosphine reagent is introduced on azide-labeled proteins by the Staudinger ligation.[1]

The first step was development of phosphine derivatives for introducing the desired carbohydrate epitopes onto azide-containing proteins. Although our primary interest was introduction of LacNAc, related carbohydrate structures (Gal and Lac) were synthesized for use as controls. Each structure consisted of a carbohydrate epitope, a linker, and the phosphine. These reagents can react with azide-contaning biomolecules, such as chemically or metabolically labeled proteins, in aqueous environments. We chose to install the phosphine late in the synthesis to avoid commonly-observed issues with oxidation of phosphorous.[26]

We selected β-lactoglobulin as a model protein due to its small size and lack of glycosylation. The protein has 178 amino acids and a molecular weight of 18 kDa. Azides were synthetically introduced onto the free amines of lysine

residues on lactoglobulin. The carbohydrate-containing phosphine reagents were then reacted with azide-modified lactoglobulin to generate Gal-, Lac-, and LacNAc-conjugated lactoglobulin. These glycoconjugates were then used in a lectin-blotting experiment to test the availability of the installed glycan residues as recognition elements.[27] The proteins were blotted onto nitrocellulose then probed with the *Maackia amurensis* lectin-1 (MAL-1), which binds LacNAc and sialyl-LacNAc glycans. The MAL-I lectin blot (**Figure 3.2**), revealed strong preference for labeling LacNAc-conjugated lactoglobulin. Reduced staining was observed for the Lac conjugate, while neither the Gal conjugate nor the unreacted azido-lactoglobulin conjugates showed any reactivity to the lectin. These results confirm that our chemoselective ligation strategy was successful, and that the carbohydrate residues attached in this method are capable of recognition by lectins. Subsequent SPR studies by Loka et al. support this observation.[1]

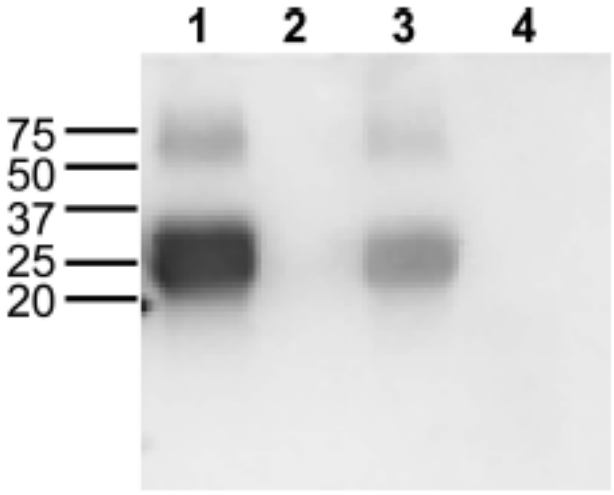


Figure 3.2 Lectin blotting characterization of glyconjugates. Lactoglobulin glycoconjugates were probed with biotinylated MAL-I lectin to confirm the attachment of the desired glycans. Proteins were loaded on an SDS-PAGE gel (5/15%), transferred to nitrocellulose, and probed with MAL-I. The blot was developed with streptavidin-HRP. Lanes shown are as follows: 1, lactoglobulin-LacNAc; 2, lactoglobulin-Gal; 3, lactoglobulin-Lac; 4, azidoacetic acid labeled lactoglobulin. The expected MW for the azidoacetic acid modified lactoglobulin is 18 kDa. MW markers are 75, 50, 37, 25, and 20 kDa.

3.4 Expression of GFP-NEU3 in HeLa cells

A variety of enzymes alter glycoprotein structure. In Chapter 2, we proposed that the sialic acids of a glycoprotein, LFA-1, could modulate its function. Sialic acids may be added or removed from glycoproteins by sialyltransferases, or sialidases, respectively. To date, four mammalian sialidases have been identified, NEU1, NEU2, NEU3, and NEU4.[28,29,30,31] These recently identified, sialic acid-cleaving enzymes are found in various cellular

compartments, including the cytosol (NEU2), lysosome (NEU1), and associated with membranes (NEU3 and NEU4). Among the family, NEU1 and NEU3 have demonstrated activity at the cell surface, and both have been shown to modify glycoproteins.[28,29,30] Our group has been focused on the plasma membrane-associated sialidase, NEU3.[32,33] Due to its localization, NEU3 could play a role in cellular signaling, and in the regulation of sialoforms at the cell surface.

In work from our group, Albohy et al. utilized molecular modeling and site-directed mutagenesis of recombinant NEU3 to gain insight into NEU3 structure.[32] These studies identified the key catalytic residues of the enzyme, including a nucleophilic pair involving Y370 and E225. To study NEU3 in a cellular context, we sought to generate cells stably expressing GFP-fusions of NEU3. To this end, we generated GFP-NEU3 fusion constructs in vector suitable for transfection of mammalian cells (pcDNA 3.1). We also generated a construct for the catalytically inactive mutant, Y370F,[32] as a negative control fusion protein.

We first attempted to express NEU3 in Jurkat cells, using a hemagglutinin-NEU3 (HA-NEU3) fusion construct in a pcDNA 3.1 vector. Using electroporation, we observed expression of HA-NEU3 in Jurkat cells. However, T cells are notoriously difficult to transfect. Although expression of HA-NEU3 was observed (by Western blot detection of HA), the low transfection efficiency of Jurkat cells, as well as the poor viability of the cells after transfection, forced the selection of alternative cell types. We therefore chose to use HeLa, a human endothelial cell model, for studies of NEU3 by fluorescence microscopy. HeLa cells are much easier to transfect, and, unlike Jurkat cells, which are cultured in

suspension, HeLa cells are adhesive. This difference also made HeLa cells more attractive, providing an easier means to isolate individual clones for the establishment of stable cell lines.

We generated three constructs, GFP-NEU3, GFP-NEU3(Y370F) and GFP, each in a pcDNA 3.1 vector. The GFP construct is to control for differences observed due to transfection and selection, as opposed to NEU3 activity. We used lipofectamine as the liposomal transfection reagent. The pcDNA 3.1 vector contains a geneticin-resistance cassette, allowing for selection of transfected cells with this antibiotic. As shown in **Figure 3.3** stable lines expressing GFP, GFP-NEU3, and GFP-NEU3(Y370F) have been established. Cells expressing GFP exhibit fluorescence throughout the cell, while those expressing GFP-NEU3 and GFP-NEU3(Y370F) show localization at the cell surface, consistent with literature reports.[31]Although some intracellular staining is observed in the GFP-NEU3, and GFP-NEU3(Y370F) expressing cells, we observed more staining at the membrane when compared to the GFP-expressing cells. One concern is that the morphology of all transfected cells has changed, from the expected kite-shape, to rounder cells. This change in morphology might be expected for detached cells, but not for attached cells, as we have observed. This change in morphology may be due to NEU3, or due to the selection conditions. Although initial images of GFP-NEU3 showed specific localization in the plasma membrane excluding the cell-cell contact areas (see appendix, Figure A1), we found that the cells were somewhat inconsistent. Nonetheless, these cells could serve as an in vivo model system for studying NEU3 localization and activity. Moreover, if proposed targets

of NEU3 are metabolically labeled and linked to fluorophores, they could be tested as targets for NEU3 in vivo.

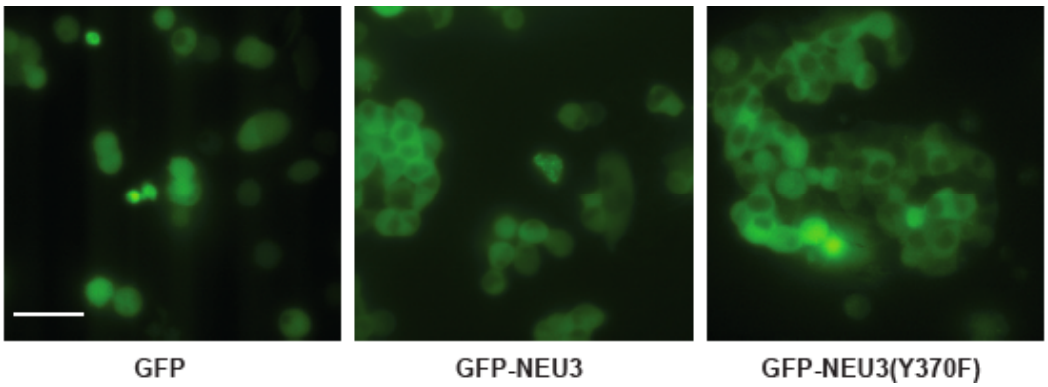


Figure 3.3 Expression of GFP, GFP-NEU3, and GFP-NEU3(Y370F) in HeLa cells. HeLa cells were transfected using liposomes (as described in section 3.6.6). The transfected cells were selected using geneticin, and individual clones were screened for expression of GFP, GFP-NEU3, or GFP-NEU3(Y370F) by fluorescence microscopy. Scale bar, 100 μm.

3.5 Conclusions and future directions

We have successfully demonstrated the use of drag-tags to label sialic acids of glycoprotein sialoforms. These experiments suggest an application for metabolic incorporation of azides into cell surface sialic acids as a viable strategy for studying glycoprotein sialoforms. We have also successfully demonstrated the use of the Staudinger ligation for the generation of glycoconjugates with uniform glycosylation, capable of recognition by lectins.[1,25] Both strategies have been successfully applied to model proteins, and must now be optimized for use in more complex systems. Initial results with the drag-tag bioconjugation strategy to determine protein sialoforms is encouraging (**Figure 3.1**), but the remaining

challenges include resolution at high molecular weight, and sample heterogeneity. This strategy could also be used in conjunction with pulse-chase labeling (**Figure 2.6**) to determine a functional role for sialoforms in glycoprotein function. Finally, we have established a set of stable HeLa cell lines that express a GFP-fused NEU3, and a catalytically inactive mutant, Y30F, as a negative control. These cells will be useful for future *in vivo* studies of NEU3 localization and activity, as well as the identification of new substrates for NEU3.

3.6 Materials and Methods

3.6.1 Metabolic labeling and purification of TS2/4 mAb

HB244 hybridoma cells were obtained from ATCC (Manassas, VA) and cultured in DMEM supplemented with 10% IgG-depleted FBS and 100 μg/mL penicillin and streptomycin, and cultured in at 37 °C in a humidified, 5% CO_2 incubator. For antibody production, the NUNC OptiCell Max 2100 Starter Kit (NUNC, Rochester, NY) was used according to the manufacturer's instructions. OptiCell chambers were seeded with 30 mL of media (supplemented with 30 μM ManNAz, **2.1**) containing 1 x 10^6 HB244 cells in log phase. Cells were cultured at 37 °C in a humidified, 5% CO_2 incubator. 20 mL harvests were performed and replaced at three-day intervals for one month per chamber. Antibodies were precipitated by ammonium sulfate precipitation, and then purified using a Bio-Rad MAPS II Protein A Purification Kit (Bio-Rad, Hercules, CA) according to the supplier's directions.

3.6.2 Conjugation of azide-modified TS2/4 with PEG drag-tag

Solutions of anhydrous copper[20] sulphate (8.08 mM) and bathophenanthroline disulphonic acid (13.0 mM) were prepared using deoxygenated ddH_2O. 30 μL of the copper solution was added to 56 μL of the bathophenanthroline disulphonic acid solution and diluted with 360 μL of deoxygenated milliQ water. Approximately 30 mg of copper powder was added to the solution. The flask was then sealed and flushed with argon for 30 min. The solution was then sonicated for 30 m resulting in a deep forest green solution that

was mixed immediately with a solution of IgG in buffer combined with the deep green prepared Cu (I) solution a solution of mPEGA-2000 containing 0.05% Triton X-100 in deoxygenated ddH_2O (20 μL, 15.7 mM, 20 equiv.). The solutions were mixed under inert atmosphere for 24 h yielding a PEG-labeled IgG, which was immediately analyzed by SDS-PAGE.

3.6.3 SDS-PAGE analysis of PEG-labeled TS2/4

Reaction mixtures of PEG-labeled IgG were combined with Laemmli buffer, then heated at 40 ºC for 15 min. The samples were then subjected to SDS-PAGE using commercial gels (Any kD Mini-PROTEAN TGX Gels, Bio-Rad, Hercules, CA). The gel was stained with fluorescent protein stain (sypro Ruby, Invitrogen, Carlsbad, CA) and visualized with a 300 nm transilluminator. The gel image was analyzed using ImageJ. Speckling was removed by using the outlier removal functions. The image was background corrected by selecting a neutral section of the image and using the subtract background function. Profile plots were generated using the gel analysis and dynamic profiler functions.

3.6.4 Staudinger ligation of azide-labeled proteins

Samples of azide-labeled lactoglobulin (200 μg) were dissolved in 100 μL of buffer (10 mM ammonium acetate, pH 8.9). Gal-, Lac-, and LacNAc-containing phosphines in DMSO/H_2O (1:15, 1 mg/mL; 300 μL) were added to the protein solution and allowed to react for 12 h at room temperature. The mixture

was lyophilized and dissolved in 100 μL of buffer (10 mM, ammonium acetate, pH 8.9) and used to lectin blotting.

3.6.5 Lectin blotting of glycoconjugates

Labeled glycoprotein samples were separated by SDS-PAGE (5%/15%) and transferred to nitrocellulose. The membrane was blocked for 2 h with CarboFree Blocking Solution (Vector Laboratories, Burlingame, CA) and incubated with biotinylated MAL-I lectin (Vector Laboratories) diluted in blocking solution (1 μg/mL) for 1 h. The membrane was washed with TTBS (tris-buffered saline, 0.1% tween 20) and incubated with streptavidin-HRP (Bio-Rad, Hercules, CA) in blocking solution for 1 h. The membrane was washed again with TTBS, and positive signals were then detected by enhanced chemilumiescence (Amersham, Little Chalfont, Bucks). All incubations of protein transfer to nitrocellulose were performed at room temperature.

3.6.6 Establishment of HeLa cell lines expressing GFP, GFP-NEU3, and GFP-NEU3(Y370F)

GFP-NEU3 expression vectors were generated by inserting (GFP-NEU3) between *Bam*HI and *Xho*I sites of pcDNA 3.1. The fusing protein, GFP-NEU3 was modified to have *Bgl*II and *Xho*I restriction sites using a PCR reaction using the *Neu3* template, PfuUltra II Fusion HS DNA Polymerase (Stratagene, Santa Clara, CA), a forward primer for *Bgl*II (5'-GGG ATA T AGA TCT ATC GAA GTG ACA ACA TGC-3') and a reverse primer for *Xho*I (5'-ATT TAC TCG

AGT TAA TTG CTT TTG AAT TGG CTT GGG TT-3'). The reaction was cycled once at 95 °C for 2 min followed by 30 cycles of 95 °C 20 s, 50 °C for 20 s, and 72 °C for 25 s, and 1 cycle of 72 °C for 3 m. The modified *Gfp-Neu3* was then digested with *Bgl*II *Xho*I restriction enzymes and the pcDNA 3.1 vector was digested with *Bam*HI and *Xho*I. The digested *Gfp-Neu3* and pcDNA 3.1 were ligated together with T4 DNA ligase at a 3:1 insert to vector ratio. The resulting expression vector was transformed into electroporation competent XL1-Blue *E. coli* (Stratagene, Santa Clara, CA). Competent cells (50 μL) were mixed with 1 mL LB media, grown with shaking at 37 °C, 240 rpm, for 1 h, and plated on LB agar with kanamycin (26 μg/mL). Clones were selected and grown in 25 μg/mL kanamycin LB medium and the plasmids were isolated using the GeneJET Plasmid Miniprep Kit (Fermantas, Burlington, ONT). Clones were verified by agarose gel electrophoresis, to check plasmid size, and then by gene sequencing with BigDye Terminator Kit V 3.1 (Applied Biosystems, Foster City, CA).

For generating the Y370F mutant, site directed mutagenesis was carried out using the Quikchange II XL Site Directed Mutagenesis Kit (Stratagene, Santa Clara, CA), according to the supplier's protocol, using the pcDNA 3.1-*Gfp-Neu3* template described above.

For transfection, HeLa cells were cultured in DMEM supplemented with 10% FBS, and 100 μg/mL penicillin and streptomycin, and cultured in at 37 °C in a humidified, 5% CO_2 incubator. Prior to transfection, cells were washed, and seeded in media void of antibiotics. Plasmids were diluted in Opti-MEM, and mixed with Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the

supplier's instructions. After 3 h the cells were stimulated with PHA 50 ng/mL and cultured in antibiotic free media for 24 h. The resulting cells were cultured in media containing geneticin (800 μg/mL) as a selection reagent. Selection media was replaced every 48 hours until individual clones could be isolated. After isolation, clones were subcultured in media containing 200 μg/mL geneticin, and subsequently screened by fluorescence microscopy.

3.6.7 Fluorescence microscopy of transfected HeLa cells

Transfected HeLa cells were seeded on glass coverslips and allowed to attach overnight. Cells were analyzed using a Nikon Eclipse Ti inverted fluorescence microscope equipped with DIC optics on a 60X oil objective. Images were captured using a Photometrics QuantEM 512SC camera. Cells were imaged while without removal of culture media, using a 465-495 nm band pass excitation filter.

3.7 Appendix

3.7.1 Microscopy of GFP-NEU3 transfected HeLa cells

HeLa cells stably expressing GFP-NEU3 showed a rounded morphology, and increased membrane localization. The cells shown in **Figure A1** below show membrane localization, but not in the areas of cell-cell contact. This exclusive localization of GFP-NEU3 was not observed in future analyses.

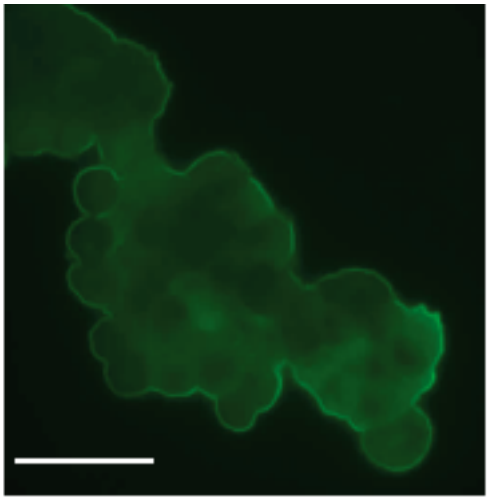


Figure A1. Membrane-localized GFP-NEU3 in HeLa cells. HeLa cells were transfected with GFP-NEU3. The transfected cells were selected using geneticin, and individual clones were screened for expression of GFP-NEU3 by fluorescence microscopy. Scale bar, 100 μm.

Figure A2. GFP, GFP-NEU3, and GFP-NEU3(Y370F) construct sequences

GFP (pcDNA 3.1)

3'atggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacg
gccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgca
ccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgcta
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ctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaac
gtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggca
gcgtgcagctcgccgaccactaccagcagaacaccccatcggcgacggccccgtgctgctgcccgacaaccact
acctgagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgac
cgccgccgggatcactctcggcatggacgagctgtacaag

GFP-NEU3 (pcDNA 3.1)

NEU3

3’atggaagaagtgacaacatgctccttcaacagccctctgttccggcaggaagatgacagagggattacctaccgg
atcccagccctgctctacataccccccacccacaccttcctggcctttgcagagaagcgttccacgaggagagatga
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ccatggtgaaggctttcagagactggccctgagtcgacagctctgtgagcccccacatggttgccaagggagtgtgg

taagtttccggcccctggagatcccacataggtgccaggactctagcagcaaagatgcacccaccattcagcagagc

tctccaggcagttcactgaggctggaggaggaagctggaacaccgtcagaatcatggctcttgtactcacacccaac

cagtaggaaacagagggttgacctaggtatctatctcaaccagacccccttggaggctgcctgctggtcccgcccct

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gtgggaccaagcaagagtgtgagcagattgccttccgcctgtttacacaccgggagatcctgagtcacctgcaggg

ggactgcaccagccctggtaggaacccaagccaattcaaaagcaattaa

GFP-NEU3(Y370F) (pcDNA 3.1)

NEU3(Y370F)

3’atggaagaagtgacaacatgctccttcaacagccctctgttccggcaggaagatgacagagggattacctaccgg

atcccagccctgctctacataccccccacccacaccttcctggcctttgcagagaagcgttccacgaggagagatga

ggatgctctccacctggtgctgaggcgagggttgaggattgggcagttggtacagtgggggcccctgaagccactg

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ggactgcaccagccctggtaggaacccaagccaattcaaaagcaattaa

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