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Review

Variant glycosylation: an underappreciated regulatory mechanism for β1 integrins

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Abstract

Although it has been known for many years that β1 integrins undergo differential glycosylation in accordance with changes in cell phenotype, the potential role of *N*-glycosylation as a modulator of integrin function has received little attention. One reason for the relatively limited interest in this topic likely relates to the fact that much of the prior research was correlative in nature. However, new results now bolster the hypothesis that there is a causal relationship between variant glycosylation and altered integrin activity. In this review, the evidence for variant glycosylation as a regulatory mechanism for β1 integrins are summarized, with particular emphasis on: (1) outlining the instances in which cell phenotypic variation is associated with differential β1 glycosylation, (2) describing the specific alterations in glycan structure that accompany phenotypic changes and (3) presenting potential mechanisms by which variant glycosylation might regulate integrin function.

Keywords: Integrin; Sialic acid; Glycosylation; Adhesion; Fibronectin

1. Introduction

The activity of integrin adhesion receptors is essential for normal cellular function and survival. The binding of integrins to protein ligands present within the extracellular matrix, or expressed on the surface of other cells, leads to the activation of intracellular signaling cascades that ultimately regulate diverse processes such as embryogenesis, wound healing, and tumorigenesis [1–4]. Because of the importance of integrins in human biology and disease, substantial effort has been directed at understanding the regulation of these receptors.

Integrins are heterodimeric transmembrane glycoproteins composed of α and β subunits. There are multiple α and β species, and the pairing of these species determines the specificity of the integrin for ligand. The β1 integrin subunit is ubiquitously expressed, and is known to pair with at least 12 different α subunits [5]. Some of the more widely expressed β1-containing heterodimers include α5β1 and α6β1, which are selective for fibronectin and laminin, respectively, as well as α2β1 and α3β1, both of which can bind multiple ligands including collagen and laminin.

Much of the work aimed at understanding integrin regulation has focused on functional changes that are induced by the activation of intracellular signaling cascades (“inside-out signaling”) [5,6]. In this model, signaling events trigger some alteration in integrin cytoplasmic tails. These alterations, in turn, either modulate integrin avidity through clustering, or induce altered conformation in the extracellular domain, leading to changes in integrin affinity for ligand. Clearly, inside-out signaling represents an important mechanism for integrin regulation. However, in contrast to the considerable attention currently focused on this model, the potential role of altered glycosylation in modulating integrin function has been largely ignored, despite the widespread observation that cells express variant β1 glycoforms when undergoing significant phenotypic changes. The principal goal of this review is to summarize the evidence supporting differential *N*-glycosylation as a regulatory mechanism, particularly for integrins of the β1 subfamily.

2. Variant β1 integrin glycoforms have been observed in multiple cell types

Numerous studies spanning the last 15 years have reported that cells express a β1 integrin isoform with

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altered electrophoretic mobility during events that dictate a marked change in cell phenotype [7–19]. Treatment of integrins with *N*-glycan-cleaving enzymes prior to electrophoresis eliminated this mobility difference, suggesting that mobility shifts were due to variability in the composition of integrin *N*-linked carbohydrates. Differential β1 integrin glycosylation occurs in conjunction with multiple processes including keratinocyte activation [14], thymocyte maturation [17], Sezary syndrome [20], myeloid differentiation [7,10], cytotrophoblast invasiveness [16] and oncogenic transformation or metastasis [8,11,13,15,21–23]. The finding that variant β1 integrin glycoforms are expressed by diverse cell types, under conditions that promote long-term changes in cell adhesiveness and motility, lends strong support for the supposition that variant glycoforms are functionally important.

Intriguingly, the β1 integrin may be well positioned for regulation by glycosylation. Unlike other integrin subunits, partially glycosylated precursor β1 integrins form a stable pool within the endoplasmic reticulum [24–27]. The cell, therefore, may be able to direct the expression of a variantly glycosylated species by recruiting precursors from the ER, rather than requiring de novo synthesis of the polypeptide backbone. Very little research has been directed at understanding the trafficking of β1 integrins from ER to Golgi, however, this transition obviously represents a potential target for regulation of β1 integrin cell surface expression. Such a transition does appear to have physiologic relevance in at least one instance; one of the early events in keratinocyte terminal differentiation involves a blockade in Golgi-mediated β1 glycosylation, contributing ultimately to a loss in cell surface β1-containing heterodimers [28].

3. An altered β1 integrin carbohydrate profile most commonly results from a change in β1-6 branching of oligosaccharides, or in the degree of sialylation

Although future research will likely underscore the importance of ER–Golgi trafficking in the regulation of β1, most of the prior reports of variable β1 posttranslational processing describe changes that occur within later subcompartments of the Golgi. For example, differential electrophoretic mobility is typically observed in the "mature" (Golgi-modified) integrin species, but not in the ER-resident precursor, suggesting that core glycosylation does not vary significantly in response to changes in cell phenotype. Attempts to define the specific Golgi-mediated modifications that contribute to differential mobility have been limited, most probably due to the technical challenges associated with the analysis of carbohydrate structures. A few recent studies have employed either mass spectrometric methods [29,30] or an HPLC mapping technique [31] to determine that β1 integrins are elaborated with a heterogeneous mixture of *N*-acetyllactosamine type multiantennary structures that are sometimes capped with the negatively charged sugar, sialic acid (see Refs. [32–35] for reviews of *N*-glycan biosynthesis). These data are in good agreement with prior lectin analyses of integrin glycans [9,21,23,31,36,37]. In general, the differential β1 glycosylation observed during cell phenotypic variation seems to result primarily from an altered abundance of sialic acids or from increased β1-6 branching of *N*-linked oligosaccharides [7,8,12,13,17,21,23,30,36,38].

The formation of a β1-6 branched structure is initiated by GnT-V (β1-6 *N*-acetylglucosaminyltransferase V), a trans-Golgi enzyme encoded by the Mgat 5 gene. GnT-V adds *N*-acetylglucosamine to mannose in a β1-6 linkage, and the resulting structure can subsequently be acted on by other glycosyltransferases to form a poly-*N*-acetyllactosamine chain (see Fig. 1 for clarification of oligosaccharide structures). The terminal galactose on the chain can either be left unmodified, or capped with several sugars including sialic acid. It follows that an increase in the total number of poly-*N*-acetyllactosamine chains (due to the addition of the β1-6 branch) is often accompanied by a corresponding increase in sialylation. Sialic acids are most commonly added to *N*-linked glycans in an α2-3 linkage, but can also be added in α2-6 or α2-8 linkages. Multiple sialyltransferases direct the α2-3 linkage, whereas the α2-6 linkage is primarily mediated by ST6Gal I (β-galactoside α2,6-sialyltransferase). Another α2-6 sialyltransferase, ST6Gal II, has been identified; however, this enzyme acts preferentially on oligosaccharides, as compared with *N*-linked glycoprotein glycans [39,40].

While changes in β1-6 branching and/or sialic acid abundance have been noted in several different cell types, the most compelling data suggesting a functional role for these alterations come from studies of tumorigenesis/metastasis and immune cell behavior.

4. Altered glycosylation of β1 integrins is prevalent in tumor cells, and is associated with cell invasiveness and metastasis

An altered profile of cell surface carbohydrates is a well-accepted hallmark of malignant transformation (reviewed in Refs. [41–47]). Aberrant *N*-glycosylation has long been associated with tumor cell behaviors including altered adhesiveness to extracellular matrix ligands, increased cell motility/invasiveness, and anchorage-independent growth. While a number of glycosyltransferases may participate in these processes, evidence supporting the involvement of GnT-V and ST6Gal I in tumorigenesis/metastasis are especially persuasive. Elevated expression and activity of these enzymes have consistently been observed in oncogene-expressing cell lines as well as in human neoplastic tissue. Cell culture studies suggest that GnT-V is up-regulated by src [48], her-2/neu [49], ras [50–54], Ets-1 [55,56], v-sis [54] and polyoma middle T antigen [57,58], whereas ST6Gal I is up-regulated by ras [8,50,59,60]. Animal studies

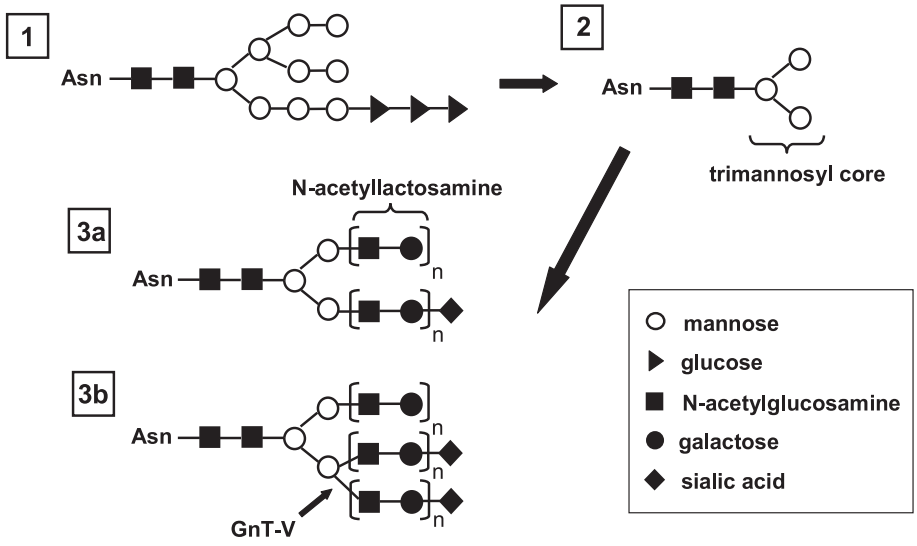

Fig. 1. Synthesis of representative complex *N*-linked oligosaccharides. [1] Within the ER, a pre-formed oligosaccharide is transferred from a lipid carrier to an asparagine residue. [2] Glycosidases within the ER and Golgi remove glucoses and selected mannoses from the oligosaccharide precursor. [3a] Glycosyltransferases within the medial/trans Golgi act sequentially to modify the trimannosyl core. The diagram depicts the addition of an *N*-acetyllactosamine unit, which can be repeated ("poly-*N*-acetyllactosamine"). Following addition of these units, the terminal galactose on the chain can either be left unmodified, or capped with sugars such as sialic acid. (Other types of modifications not shown here include fucosylation.) [3b] In some instances, additional poly-*N*-acetyllactosamine chains are added. This addition is initiated by GnT-V, an enzyme that adds *N*-acetylglucosamine to mannose in a β1-6 linkage (forming a "β1-6 branch"). (Note that a fourth chain can be added to the other mannose, generating a tetraantennary structure).

support a role for ST6Gal I and GnT-V in tumorigenesis/metastasis [58,61–68]. For example, GnT-V-deficient transgenic mice experience attenuated tumor growth and metastasis when challenged with a polyoma virus middle T oncogene [58]. In humans, the activity and/or expression of GnT-V and ST6Gal I are elevated in multiple types of tumors [69–80], and high levels of these enzymes or their cognate sugars are correlated with metastasis and a poor patient prognosis [69,71,74,81–88]. Despite this wealth of data implicating GnT-V and ST6Gal I in cancer progression, our understanding of the molecular mechanisms linking altered glycosylation to tumorigenesis/metastasis has been limited by the lack of knowledge regarding the specific substrates for these enzymes.

Accumulating evidence points to β1 integrins as an important target for GnT-V and ST6Gal I. Increased levels of β1-6 branching and sialylation are present on β1 integrins expressed by several transformed cell types [8,21–23]. Our group has shown that stable transfection of oncogenic ras in colon epithelial cells induces increased ST6Gal I expression, and correspondingly, elevated α2-6 sialylation of β1, but not β3 or β5, integrins [8]. Accordingly, oncogenic ras-expressing cells demonstrate altered adhesiveness to β1 ligands such as collagen, but not to the β3/β5 ligand, vitronectin. This differential preference of ras-transformed cells for selected integrin ligands could influence many aspects of tumor cell behavior including cell recruitment to, or survival within, novel matrix milieus.

Elevated α2-6 sialylation is particularly correlated with changes in cell motility and invasiveness [89–93]. The overexpression of ST6Gal I in mammary carcinoma cells stimulates cell invasion through a fibronectin meshwork [92], whereas invasion through Matrigel is markedly reduced in HT-29 colonocytes transfected with antisense ST6Gal I [90]. Similar to the effects of ST6Gal I, forced expression of GnT-V causes altered cell adhesion to the substratum [65,94], increased haptotactic migration toward fibronectin [94], and enhanced cell invasion through Matrigel [56,94]. In the latter investigations, it was not determined whether the overexpression of GnT-V was associated with any changes in sialylation, an important consideration given that poly-*N*-acetyllactosamine chains are acceptor sites for sialic acid. The possibility that GnT-V mediates at least some of its effects on cell behavior via increased sialylation is a topic that merits further exploration.

5. Altered sialylation of β1 integrins regulates immune cell behavior

Another major area of investigation suggesting that altered integrin glycosylation has functional relevance centers on studies of immune cell behavior. It is well known that glycoconjugates regulate many aspects of leukocyte function (reviewed in Refs. [95–97]), and sialyl Lewis structures (which contain α2-3 linked sialic acids) are important mediators of leukocyte/endothelial cell interactions [98]. However, evidence implicating α2-6 linked sialic acids in immune cell function is also mounting. Data from our laboratory [7] suggest that variant α2-6 sialylation of β1 integrins regulates some aspects of myeloid differentiation along the monocyte/macrophage lineage. Phorbol ester

(PMA)-stimulated monocytic differentiation of U937 and THP-1 myeloid cells induces down-regulation of ST6Gal I, and correspondingly leads to the expression of hyposialylated β1 integrins. The expression of hyposialylated β1 integrins is temporally correlated with elevated cell binding to β1 integrin ligands such as fibronctin, and a U937 cell variant that does not down-regulate ST6Gal I in response to PMA does not exhibit PMA-dependent cell adhesiveness. Further implicating sialylation as a direct modulator of integrin function, we reported that the enzymatic de-sialylation of purified α5β1 integrins significantly enhances fibronectin-binding in a cell-free assay system. Thus, the behavior of purified α5β1 integrins recapitulates that of α5β1 integrins expressed on the myeloid cell surface; integrins lacking sialic acids bind better to fibronectin. Supporting our results, the enzymatic removal of sialic acids from the surface of HL60 myeloid cells [99], MDCK cells [100] and MDAY-D2 murine metastatic cells [101] stimulates fibronectin binding to cell surface integrins, although in one instance de-sialylation was reported to inhibit fibronectin binding [12], a discrepancy not currently understood. Final evidence suggesting that sialylation plays a causal role in regulating integrin function has been provided by Villavicencio-Lorini et al. [102]. In this study, HL60 myeloid cells were engineered to express an unnatural variant of sialic acid; the α5β1 and α4β1 integrins of these cells displayed elevated binding activity.

Studies using genetically engineered mice unequivocally show that immune cell responses depend upon the activity of ST6Gal I. ST6Gal I knock-out mice are immunocompromised, primarily due to a defect in B cell development [103]. However, an ST6Gal I-deficient (but not null) mouse, generated by mutation of one of the several ST6Gal I promoter sequences, demonstrates an enhanced inflammatory response when challenged with a bacterial pathogen [104]. These latter results fit our model quite well; hyposialylated β1 integrins expressed by leukocytes have better ligand binding activity, leading to increased leukocyte adhesiveness (and therefore recruitment to sites of inflammation).

6. Potential mechanisms linking variant integrin glycosylation to alterations in integrin function

Some of the earliest studies of β1 glycosylation focused on the relatively straightforward question of whether *N*-glycosylation was required for integrin activity. In these studies, adhesion was examined in cells treated with either tunicamycin, a reagent that prevents the addition of glycans to asparagine residues by blocking the formation of the lipid-linked oligosaccharide precursor, or with compounds that inhibit mannosidase activity (e.g. swainsonine or deoxymannojirimycin). Results generated from this type of approach strongly suggested that integrin glycosylation was necessary for normal cell adhesion and spreading on integrin ligands [36,105–107], although this has been disputed [108]. Further examination revealed that at least some of the inhibitory effects of these compounds (particularly tunicamycin) on integrin function were due to impaired trafficking of integrins through the Golgi [106], leading to a loss in cell surface receptors. *N*-glycosylation was also reported to be essential for αβ subunit pairing [106]. As noted above, however, under physiologic conditions, changes in integrin glycosylation typically involve the more terminal carbohydrate structures added within the trans-Golgi (e.g. sialic acid or β1-6 branched chains). The presence or absence of these glycans does not significantly affect integrin maturation or cell surface expression; rather, these structures seem to directly modulate integrin function.

The mechanisms that link altered glycosylation to altered function have not been defined, although many mechanisms can be speculated. One of the obvious possibilities is that *N*-glycosylation influences protein conformation. Spatial conformation studies indicate that β1-6-linked poly-*N*-acetyllactosamine chains bend back toward the protein rather than extending into the extracellular milieu, implying that this structure could interact with the polypeptide backbone [109,110]. Other studies, based on NMR analyses, suggest that glycosylation restricts dynamic fluctuations in protein conformation [111–113]. This is due, at least in some cases, to stabilizing interactions between the glycan and selected amino acid residues [111,112]. Finally, exciting new work from Luo et al. [114] provides very convincing evidence for conformational regulation by *N*-glycans. In this study, an asparagine residue was substituted for a proline in the β1 integrin subunit at amino acid #333. Upon expression in CHO-K1 cells, this asparagine was *N*-glycosylated, and it was elegantly shown that α5β1 integrins with these aberrantly glycosylated β1 subunits assumed an active ligand-binding conformation. Although the introduction of an asparagine residue at site 333 does not mimic any known naturally occurring mutation, clearly these studies provide proof of principal that altered glycosylation can affect integrin conformation.

Another possibility is that the large size (or charge) of *N*-glycans masks critical functional domains within the integrin heterodimer. In fact, masking of peptide, or particularly carbohydrate (e.g., galactose), epitopes is thought to be one of the important functions of sialic acid [96,115]. However, our research suggests that any relationship between sialic acid masking and integrin function may be complex. We find that the enzymatic de-sialylation of purified α1β1 integrins inhibits α1β1 binding to collagen (the preferred ligand for this receptor), whereas de-sialylation of α5β1 stimulates fibronectin binding [7,8]. Like α5β1, de-sialylated α3β1 integrins exhibit enhanced ligand binding [30]. It is tempting to speculate that the fundamental difference in the effects of sialylation on the function of α1β1, versus α5β1 or α3β1, has something to do with the fact that α1, but not α5 or α3, subunits have

an "I" domain, a domain thought to bind directly to integrin ligands [116,117]. The β1 subunit has an "I-like" domain, and it is hypothesized that, in heterodimers lacking I domain-containing α subunits, ligand binds directly to the β1 I-like domain [116]. Conversely, in heterodimers that have an α I domain, the conformation of the β1 subunit is thought to allosterically regulate α I domain/ligand interactions. Hence, the mechanisms regulating ligand binding appear to be fundamentally different for integrin heterodimers with and without I-domain-containing α subunits. Surprisingly, the β1 integrin asparagine residues that carry *N*-linked carbohydrates have not yet been identified, so it is currently difficult to speculate how altered sialylation (with the corresponding change in negative charge) could affect integrin conformation and/or ligand recognition. However, two of the 12 asparagine residues that have consensus for *N*-glycosylation [118] lie within the β1 integrin "I-like" domain, and therefore, carbohydrate modifications at these sites would likely affect ligand binding.

While results from purified integrin/ligand binding assays suggest that carbohydrate moieties directly modulate ligand/receptor interactions, altered glycosylation could affect integrin function via less direct mechanisms as well. Variant glycosylation may alter the lateral association of integrins with other membrane-associated proteins that coordinately regulate downstream integrin-dependent processes (for review of integrin-associated proteins, see Ref. [119]). Some examples of membrane-associated proteins that interact with the extracellular domain of integrins include the urokinase-type plasminogen activation receptor, uPAR, [120], and certain members of the tetraspanin family [121,122]. Notably, the interaction between tetraspanin CD82 and the integrin heterodimers α3β1 or α5β1 appears to depend upon the glycosylation state of both CD82 and the integrin species [123]. Similarly, glycosylation of α3β1 influences the formation of a complex comprised of α3β1, the CD9 tetraspanin, and the GM3 glycosphingolipid [124].

N-glycosylation could also potentially regulate the association between integrins and membrane glycolipids, and thereby direct localization of integrins to plasma membrane microdomains. It has been known for many years that glycosphingolipids play some role in modulating cell adhesiveness (reviewed in Refs. [125–127]), although the molecular mechanisms underlying this phenomenon are not well understood. Recently, Wang et al. [128] reported that the glycosphingolipid, GT1b, binds directly to α5β1, and subsequently alters the binding of this integrin species to fibronectin. *N*-glycosylation of α5β1 is necessary for this interaction, as the enzymatic removal of *N*-glycans from α5β1 blocked GT1b association. Complementary studies suggested that the glycosylation of α3 and α5 subunits may regulate the translocation of these integrins to glycosphingolipid-enriched plasma membrane microdomains [129].

Localization of integrins to specific subdomains may depend upon the degree of β1-6 branching. Yamamoto et al. [56] reported that α3β1 integrins with an elevated level of β1-6 linked poly-*N*-acetyllactosamine chains (due to forced expression of GnT-V) were targeted to the leading lamellipodia of migratory glioma cells. It was not determined in this study whether elevated β1-6 branching was on the α3 or β1 subunit. β1-6 linked poly-*N*-acetyllactosamine structures may also regulate integrin clustering. In work from Pierce's group, transfection of GnT-V into fibrosarcoma cells caused increased β1-6 branching on β1 integrins, and the acquisition of these structures was, in turn, associated with reduced α5β1 integrin clustering [94]. The α5 subunit of GnT-V-transfected cells did not carry β1-6 branched chains, suggesting that α5 is not a substrate for this enzyme. Likewise, elevated β1-6 branching was observed on β1, but not α5, integrins expressed by GnT-V-transfected hepatocarcinoma cells [130]. These results, combined with our results indicating that ST6Gal I modifies β1, but not β3 or β5 integrins, highlight a concept that tends to be underappreciated by those outside of the glycobiology community, namely, that glycosyltransferases, particularly those acting late in *N*-glycan synthesis, have a selected substrate specificity, and may therefore regulate specific cellular responses.

7. Summary

The hypothesis that variant glycosylation represents an important regulatory mechanism for β1 integrins is founded on two general observations. First, the direct manipulation of β1 glycosylation (via enzymatic methods or introducing glycosylation sites through mutagenesis) clearly alters integrin conformation and ligand-binding activity. These data provide proof of principal that *N*-linked glycans modulate integrin function. However, perhaps more importantly, there is an impressive body of literature indicating that β1 integrins undergo altered glycosylation in response to physiologic cues. In light of these findings, it is reasonable to assume that the altered cell adhesiveness and motility induced by events such as cell differentiation or transformation are regulated, at least in part, by the variant glycosylation of β1-containing integrin heterodimers. In sum, the evidence supporting variant glycosylation as a regulatory mechanism for β1 integrins are compelling, however, much work remains to be done with regard to understanding the specific mechanisms by which variant glycosylation regulates integrin function. For example, identifying specific glycosylation sites, as well as defining carbohydrate structures, would allow a better understanding of how altered glycosylation might affect integrin conformation or ligand recognition. In addition to direct effects on integrin structure, there is a need to evaluate the role of variant glycosylation in regulating processes such as integrin subcellular localization or interaction with known integrin-associating proteins.

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