Tn and STn are Members of a Family of Carbohydrate Tumor Antigens that Possess Carbohydrate-Carbohydrate Interactions

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Abstract. The mucin-type O-glycome in cancer aberrantly expresses the truncated glycans Tn (GalNAcα1-Ser/Thr) and STn (Neu5Acα2,6GalNAcα1Ser/Thr). However, the role of Tn and STn in cancer and other diseases is not well understood. Our recent discovery of the self-binding properties (carbohydrate-carbohydrate interactions) of Tn (Tn – Tn) and STn (STn – STn) provides a model for their possible roles in cellular transformation. We also review evidence that Tn and STn are members of a larger family of glycan tumor antigens that possess carbohydrate-carbohydrate interactions, which may participate in oncogenesis.

Key words: carbohydrate tumor antigens, Tn and STn cancer antigens, Neu5Gc-GM3, optical tweezers, carbohydrate-carbohydrate interactions, mechanism of action

Introduction. The human glycome is altered in many diseases including cancers (Ju, T., Wang, Y., et al. 2013). For example, the mucin-type O-glycome expresses the truncated glycans Tn (GalNAcα1-Ser/Thr) and STn (Neu5Acα2,6GalNAcα1-Ser/Thr) in over 80% of carcinomas (Ju, T., Lanneau, G.S., et al. 2008; Springer, G.F. 1984). Indeed, Tn and STn have been tumor markers for colorectal, lung, breast, cervical and gastric carcinomas for over three decades, and the expression level of Tn correlates with the metastatic potential and poor prognosis of patients (cf. (Ju, T., Lanneau, G.S., et al. 2008)). Tn and STn are also expressed in other human diseases including the Tn syndrome and IgA nephropathy (Ju, T., Wang, Y., et al. 2013). However, the role of Tn and STn in cancer and other diseases is not well understood. In this paper, we review the recent discovery of the self-binding properties of Tn (Tn – Tn) and STn (STn – STn) (Haugstad, K.E., Hadjialirezaei, S., et al. 2016), and how the binding and cross-linking activities of these two cancer antigens provide a model for their possible roles in cellular transformation.
and malignancy as well as other diseases. Finally, we discuss a family of carbohydrate tumor antigens that include Tn, STn and Neu5Gc-GM3, which possess carbohydrate-carbohydrate interactions and participate in cancer development.

Carbohydrate-Carbohydrate Interactions (CCIs). CCIs have been observed for over 40 years, and have been largely associated with oligosaccharides and polysaccharides. For example, the oligosaccharide chains of specific glycosphingolipids such as GM3 interact with other glycolipid oligosaccharide chains (Gg3), and with the N-glycans of membrane glycoproteins such as EGFR (heterotypic CCI) (Kojima, N. and Hakomori, S. 1989; Kojima, N. and Hakomori, S. 1989; Hayashi, N., Chiba, H. et al. 2013). This and many other examples led to the definition of the “glycosynapse”, which is a membrane microdomain of glycosphingolipids that was shown to be responsible for carbohydrate-dependent cell adhesion and signaling via transmembrane glycoprotein receptors (Hakomori, S.I. 2002). This concept is analogous to the “immunological synapse”, which interconnects adhesion and signaling. CCIs are often characterized by lower affinities than carbohydrate-protein (lectin) interactions, but are often multivalent resulting in enhanced avidity (Handa, K. and Hakomori, S.-i. 2012). Zhao and co-workers later made important contributions to this field by using fluorescent silica nanoparticles functionalized with carbohydrates in studies of carbohydrate-carbohydrate interactions (Zhao, J., Liu, Y., et al. 2012). By studying the binding of nanoparticles coated with galactosyl (Gal), its 3-sulfo derivative (SGal) or Glc to galactolipids and glycolipids that had been immobilized in a multiwell plate they revealed that the carbohydrate-carbohydrate interactions between the nanoparticles and the glycolipid is extremely specific for Gal - SGal. However, the number of published quantitative studies of carbohydrate self-interactions is limited. Tromas and co-
workers studied the self-interaction (homotypic CCI) of the trisaccharide Lewis\(^x\) determinant (Galβ1,4[Fucα1,3]GlcNAcα) using atomic force microscopy (AFM), (Figure 1) (Tromas, C., Rojo, J., et al. 2001). The AFM force distance curves revealed multiple interactions, characterized by a bond strength between two Lewis\(^x\) molecules equal to 20 ± 4 pN. CCI interactions as observed for the cell adhesion proteoglycans display an average adhesive force of 40 ± 15 pN (Dammer, U., Popescu, O., et al. 1995). Our AFM studies on the porcine submaxillary mucins (PSM) decorated with Tn (Tn-PSM) (i.e. trimmed to the Tn structure) shows self-interactions characterized by unbinding forces in the range of 30-50 pN when probed using loading rates below 10 nN/s (Haugstad, K.E., Gerken, T.A., et al. 2012). Furthermore, the observed bond strength was found to decrease with decreasing force loading rate (Haugstad, K.E., Hadjialirezaei, S., et al. 2016), in accordance with the dynamic force spectroscopy (DFS) theory (Evans, E. 1998). These interaction strengths are lower than those reported for specific protein – carbohydrate (103 – 402 pN for the SBA – mucin interaction (Sletmoen, M., Dam, T.K., et al. 2009) and 73 – 144 pN for the alginate epimerase – alginate interaction (Sletmoen, M., Skjak-Bræk, G., et al. 2004) or protein-protein interactions probed using the same range of loading rates (an overview is provided in (Bizzarri, A.R. and Cannistraro, S. 2010)). In comparison, the strength of single ionic bonds between charged groups in aqueous solution are about 180 pN in physiological ionic strength (Spruijt, E., Van Den Berg, S.A., et al. 2012), hydrogen bonds are reported to be 10 pN (Hoh, J.H., Cleveland, J.P., et al. 1992) while forced dissociation of duplex DNA yields unbinding forces in the range 20-50 pN (Strunz, T., Oroszlan, K., et al. 1999). Using AFM the average lifetime of the Tn-PSM self-interaction was determined to be 0.6 s (Haugstad, K.E., Gerken, T.A., et al. 2012). However, both the strength and the lifetime of these interactions are expected to increase significantly due to bond multiplicity.
CCIs have been demonstrated to be involved in cis and trans interactions on cells and in dynamic processes such as adaptive immune responses, cell adhesion and recognition (Handa, K. and Hakomori, S.-i. 2012). However, the scope and role of CCIs in both normal and disease states is not well understood.

**Mucins with Tn and STn in Cancer.** Mucins represent a class of high molecular weight membrane and secreted glycoproteins that contain long heavily O-glycosylated domains, which are commonly composed of tandem repeats. Both membrane bound and secreted mucins are present on the cell surfaces that line body cavities including the respiratory, digestive and urogenital tracts. The mucosal surfaces have a close relationship with innate and adaptive immunity, and in interactions between internal and external environments including the microbiome. Several types of cancers are accompanied by overexpression of aberrantly glycosylated mucins such as the membrane tethered MUC1 (Corfield, A.P. 2015). Overly expressed MUC1 in colon cancer, for example, is highly substituted with Tn and STn structures, which are believed to be due to changes in glycosyltransferase expression, their relative subcellular localization and even their activity. In addition, the loss of the chaperone COSMC is key to the aberrant over expression of Tn and STn in some cancers (Ju, T., Lanneau, G.S., et al. 2008; Radhakrishnan, P., Dabelsteen, S., et al. 2014). This is because COSMC controls the folding and activity of T-synthase (C1GalT-1 or core1 β1-3galactosyltransferase 1), which is required for the elongation of Tn to longer O-linked oligosaccharides (Aryal, R.P., Ju, T., et al. 2010; Wang, Y., Ju, T., et al. 2010).
Self-binding Studies of Tn and STn. Our laboratory recently examined the effects of the Tn antigen on the self-interactions of mucins using AFM (Figure 1) on a group of differently glycosylated porcine submaxillary mucins (PSM) ranging from only Tn to the elongated core 1 blood group A antigen. Our results showed enhanced self-binding interactions for PSM possessing the Tn antigen (i.e. Tn-PSM) (Haugstad, K.E., Gerken, T.A., et al. 2012; Haugstad, K.E., Stokke, B.T., et al. 2015). Using optical tweezers (OT) experiments (Figure 2), we more recently showed that the enhanced binding of Tn-PSM is solely due to GalNAc – GalNAc interactions (Haugstad, K.E., Hadjialirezaei, S., et al. 2016). Indeed, several mucins including the MUC1 human mucin possessing the Tn structure (Tn-MUC1) all showed similar self-binding interactions. Self-interactions were also present in STn decorated mucins, indicating that the α2,6-sialic acid group was not inhibitory to the GalNAc-GalNAc CCI, or that it indeed may also undergo CCI interactions (see below). However, the addition of β1,3-Gal to the Tn structure to form the T-antigen disaccharide (Galβ1-3GalNAcα1-Ser/Thr) or the ST-antigen trisaccharide (Neu5Accα2-3Galβ1-3GalNAcα1-Ser/Thr) failed to show similar self-interactions (Haugstad, K.E., Hadjialirezaei, S., et al. 2016). Accordingly, self-interactions were observed only after treatment of ST-MUC1 with both neuraminidase and β-galactosidase, which results in a MUC1 possessing only Tn (Haugstad, K.E., Hadjialirezaei, S., et al. 2016). The GalNAc – GalNAc interactions observed for the Tn- and STn-mucins were also independent of the aglycone scaffold since polyethyleneglycol and polyacrylamide conjugates of Tn (in the α-linked form) showed similar binding strength and energy landscape characteristics. Control experiments with mucins or polymers lacking Tn or STn failed to show any such self-interactions. The GalNAc – GalNAc interactions for mucins and polymers possessing Tn also showed density dependent effects, while experiments performed with the addition of non-interacting ST-MUC1 molecules.
to dilute out the self-binding molecules allowed the observation of single binding events (Haugstad, K.E., Hadjialirezaei, S., et al. 2016).

Interestingly, polyacrylamide conjugated with Neu5Gc, the sialic acid present in many mammals in which the N-acetyl methyl group in humans (Neu5Ac) is replaced by the -CH₂OH group, also showed self-binding interactions, and possessed higher rupture forces than the GalNAc self-binding interactions (Haugstad, K.E., Hadjialirezaei, S., et al. 2016). Exogenous Neu5Gc incorporation into the tissues of humans via ingestion of red meat such as beef has been associated with inflammation and cancer (Samraj, A.N., Pearce, O.M.T., et al. 2015).

**A Proposed Mechanism of Action of Tn and STn Involving CCI in Cancer.** The ability of Tn and STn to promote self-interactions may be important for understanding their possible molecular roles for initiating oncogenic pathways leading to many types of cancers. The self-interaction activities of Tn and STn are consistent with the hypothesis that they may be drivers of secondary promoters of carcinogenesis by their aggregation and subsequent activation of heavily O-glycosylated cell surface receptors that may be involved in a range of cellular signaling, including MUC1 and other O-glycosylated receptors. Furthermore, the observed density dependent avidity of Tn and STn on mucins and glycoconjugates (Haugstad, K.E., Hadjialirezaei, S., et al. 2016) is consistent with their elevated expression levels observed in cancer associated MUC1 found in patients with poor prognosis. Indeed, engineered increased expression of STn in cancer cell lines increases their tumorigenicity (Julien, S., Krzewinski-Recchi, M.A., et al. 2001; Ozaki, H., Matsuzaki, H., et al. 2012)

Wandall and coworkers (Radhakrishnan, P., Dabelsteen, S., et al. 2014) investigated the effects of epigenetic silencing of COSMC, the chaperone for T-synthase, and overexpression of
Tn and STn in a pancreatic cell line (T3M4). COSMC knockout cells exhibited enhanced invasive properties in culture, and enhanced growth and invasion as xenografts. These results show that truncation of O-glycans to give Tn and STn enhances malignant and metastatic tumor behavior in pancreatic cell line T3M4. Several signaling pathways regulating cellular homeostasis and oncogenesis were identified, including adhesion and signaling molecules, which indicate that truncation and expression of O-glycans on cancer cells affect multiple pathways simultaneously to enhance tumor growth. However, a system wide mechanism linking the presence of Tn and STn structures to the signaling pathways promoting tumor growth has not been suggested.

We suggest that such a system wide mechanism may involve density dependent glycosylation of specific cell surface glycoproteins and receptors, such as MUC1, by Tn and STn, which via their carbohydrate-carbohydrate interactions induces hetero-/homotypic oligomerization and/or cross-linking. This could lead to the activation of multiple pathways associated with enhanced tumor growth and metastases. Importantly, the over-expression effects of Tn and STn in the pancreatic cancer cell line observed by Wandall and coworkers were fully reversible by re-expression of COSMC (Radhakrishnan, P., Dabelsteen, S., et al. 2014), which suggest a possible therapeutic mechanism for reversing cancers driven by COSMC silencing and Tn and STn overexpression.

Binding of Tn and STn glycosylated proteins to the C-type lectin MGL, a Gal/GalNAc specific lectin, may also play a role in cancer. The role of lectins on immune cells is thought to be in the recognition of pathogens however, it is now clear that these glycan-binding proteins can recognize self-antigens. However, the engagement of MGL in the absence of a danger signal, for
example the triggering of a Toll receptor, can lead to anergy (Beatson, R., Maurstad, G., et al. 2015; van Vliet, S.J., Gringhuis, S.I., et al. 2006).

**Tn and STn Are Members of a Family of Glycan Tumor Antigens Possessing Carbohydrate-Carbohydrate Interactions (CCI).** The observations that Tn and STn show self-binding activities (homotypic CCI) that may be involved in oncogenesis appear to represent two members of a larger family of glycan tumor antigens that possess CCIs. Recently, Gildersleeve and coworkers reported that whole-cell cancer vaccines administered to human patients induce large antibody responses to carbohydrate and glycoprotein antigens (Xia, L., Schrump, D.S., et al. 2016). Using GVAX Pancreas (a granulocyte macrophage colony-stimulating factor-modified whole-cell tumor vaccine), they showed that the pancreatic cancer vaccine induces large immunoglobulin G and M responses in human patients including responses to tumor-associated carbohydrates and blood group antigens, many of which have been seen in other pancreatic cancer screens (Remmers, N., Anderson, J.M., et al. 2013). Table 1 lists the glycan tumor and blood group antigens and their structures found in the Gildersleeve study. The largest and most frequent IgG responses to known tumor-associated antigens were directed toward the blood group determinant sialyl Lewis\(^x\), the Neu5Gc variant of the glycolipid GM3 and STn. The largest and most frequent IgM responses were directed toward the blood group determinant Lewis\(^y\). Large but infrequent IgG and IgM responses were also observed for glycopeptides from the cell surface tethered MUC4 carrying the T antigen, as well as glycopeptides from MUC1 carrying the Tn antigen. In addition, the glycolipid GD3, the Neu5Gc variant of sialyl \(\alpha2,6\)LacNAc and the Neu5Gc variant of sialyl \(\alpha2,3\)LacNAc also induced...
antibody responses. Interestingly, many of these carbohydrate tumor and blood group epitopes have been shown to undergo CCIs (Table 1).

Endogenous Glycan Tumor Antigens in Table 1. The list of endogenous glycan tumor antigens in Table 1 includes Tn and STn, which have been recently demonstrated by us to undergo homotypic CCIs (Haugstad, K.E., Hadjialirezaei, S., et al. 2016). Both Tn and STn are known to be early pan-carcinoma markers including in pancreatic cancer (Remmers, N., Anderson, J.M., et al. 2013). Tn and STn glycosylated proteins have been shown to bind to the C-type lectin MGL, a Gal/GalNAc specific lectin, which also may also play a role in oncogenesis (Beatson, R., Maurstad, G., et al. 2015; van Vliet, S.J., Gringhuis, S.I., et al. 2006).

Lewis\(^x\) in Table 1 has been demonstrated by Hakomori and coworkers (Hakomori, S. 2014; (Handa, K. and Hakomori, S.-i. 2012) to undergo homotypic CCI and has been shown to be involved in embryonic compaction in the mouse, and autoaggregation of mouse embryonal carcinoma F9 cells. It is also an embryonic stage antigen in the mouse (Fenderson, B.A., Eddy, E.M., et al. 1990).

We are unaware of any CCI data for sialyl Lewis\(^x\) in Table 1. On the other hand, sialyl Lewis\(^x\) is a known ligand for E-selectin, and their interactions are associated with promotion of tumor cell invasion and metastasis (Ono, M. and Hakomori, S. 2003). In addition, sialyl Lewis\(^x\) with neighboring sulfated tyrosine residues on the mucin PSGL-1 is a ligand for the P-, L- and E-selectins that are involved in leukocyte, endothelial cells and platelet adhesion interactions (Cummings, R. and McEver, P. 2017).

Lewis\(^y\) in Table 1, which is similar in structure to Lewis\(^x\), has been shown to undergo heterotypic CCI with a blood group H glycosphingolipid (Hakomori, S. 2004). Lewis\(^y\), like
Lewis\(^x\), is also an embryonic stage antigen (Fenderson, B.A., Eddy, E.M., et al. 1990), and is found in a variety of tumors including pancreatic cancer cells (Remmers, N., Anderson, J.M., et al. 2013).

The disialoganglioside GD3 is similar in structure to GM3 in Table 1, but with an extra Neu5Ac moiety attached to the glycan. We are unaware of any CCI data for GD3, but it is a ligand for siglec7 (Nicoll, G., Avril, T., et al. 2003). Hakomori and coworkers have suggested that the interaction of GD3 with siglec7 may play a role in the metastatic potential of renal cell carcinoma, particularly in the lung (Ito, A., Handa, K., et al. 2001).

The T antigen is also found as an endogenous tumor glycan in Table 1. However, we recently showed that the T antigen does not undergo homotypic CCI (Haugstad, K.E., Hadjialirezaei, S., et al. 2016). However, it is a ligand for certain lectins such as galectin-3 (Zhao, Q., Guo, X., et al. 2009). Indeed, the role of some the CCIs above may have in common certain lectin (galectin) lattices, which have been shown to regulate the diffusion and turnover of cell surface receptors, the dynamics and turnover of the formation of the immune synapse and the formation of focal adhesions (Dennis, J.W. 2015). Thus, four of the seven endogenous tumor glycans in Table 1 are known to undergo CCI.

**Exogenous Glycan Tumor Antigens in Table 1.** Neu5Gc, the sialic acid analog found in red meat from a variety of mammals but not humans, is present in several of the exogenous tumor glycans in Table 1. Importantly, Neu5Gc has been recently shown by us to undergo homotypic CCI (Haugstad, K.E., Hadjialirezaei, S., et al. 2016). Interestingly, the Neu5Gc variant of GM3 is present in Table 1. Neu5Ac-GM3, which is present in humans, has been reported to be involved in heterotypic CCIs with other gangliosides such as Gg3, and in...

Importantly, the Neu5Gc variant of GM3 is likely to undergo CCI as reported for Neu5Ac-GM3 (Hakomori, S.-I. and Handa, K. 2015; Kojima, N. and Hakomori, S. 1989). However, the interaction of Neu5Gc-GM3 with EGFR appears different from that of Neu5Ac-GM3 (Casadesus, A.V., Fernandez-Marrero, Y., et al. 2013; Hayashi, N., Chiba, H., et al. 2013), which may contribute to the former's greater oncogenic properties and its presence in other tumors. Thus, the role of the Neu5Gc moiety in Neu5Gc-GM3 appears important in its role in oncogenesis.

Neu5Gc is also incorporated into the structures of sialyl α2,6LacNAc and sialyl α2,3LacNAc shown in Table 1. Due to the presence of Neu5Gc in these glycans, they are also likely to undergo CCIs. This suggests that the CCIs of the three exogenous Neu5Gc modified tumor glycans in Table 1 may contribute to the increased cancer and elevated inflammatory response associated with the Neu5Gc epitope in engineered mice as observed by Varki and coworkers (Samraj, A.N., Pearce, O.M.T., et al. 2015).

Importantly, the list of glycan tumor antigens or markers in Table 1 is not exhaustive of the literature (Stowell, S.R., Ju, T.Z., et al. 2015). For example, sialyl Lewis is another Lewis
blood group determinant, is a well-known gastrointestinal and pancreatic tumor marker (Stowell, S.R., Ju, T.Z., et al. 2015).

**Conclusions.** Our analysis indicates that seven of the ten glycan-tumor antigens in Table 1 either exhibit or are likely to exhibit CCIs. Of the seven endogenous glycan tumor antigens, two are O-linked glycans (Tn and STn), and two are Lewis blood group determinants that may be N- or O-linked or glycolipid (Lewis\(^a\), Lewis\(^\gamma\)). Of the three exogenous glycan tumor antigens, one is a glycosphingolipid (Neu5Gc variant of GM3), and the other two (Neu5Gc variant of sialyl \(\alpha 2,6\)LacNAc and Neu5Gc variant of sialyl \(\alpha 2,3\)LacN\(\alpha\)c) may be associated with N- or O-linked carbohydrate structures. **All three exogenous tumor glyans are likely to undergo CCI.** Thus, it is possible that the variety of N- and O-glycan, glycolipid and blood group epitopes present in Table 1 that undergo CCI and lectin mediated interactions may participate in a variety of cellular signaling pathways leading to oncogenic transformation. Furthermore, the presence of both endogenous and exogenous glycan tumor antigens (Table 1) raises the question of the possible role(s) of these two types of glycans in cellular transformation.

Importantly, IgG responses by patients to the Neu5Gc variant of GM3 and sialyl Lewis\(^a\) in Table 1 showed a trend toward a correlation with survival, and continued evaluation of these responses has been suggested (Xia, L., Schrump, D.S., et al. 2016). Hence, further work is required to investigate the physical and biological roles of these and the other glycan tumor antigens in Table 1.

Lastly, the current model for Tn and STn self-binding of glycoconjugates in cancer may also help to explain the roles of these epitopes in other diseases such as the Tn syndrome and IgA nephropathy (Ju, T., Lanneau, G.S., et al. 2008).
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Conflict of interest statement

None declared.

Abbreviations

AFM, atomic force microscopy; OT, optical tweezers; CCI, carbohydrate-carbohydrate interaction; Tn, GalNAcα1-Ser/Thr; STn, Neu5Acα2,6GalNAcα1-Ser/Thr; PSM, porcine submaxillary mucin.
References


FIGURE LEGENDS

FIGURE 1.
Schematic illustration of quantitative determination of carbohydrate-carbohydrate interactions employing AFM. One of the carbohydrates potentially involved in a carbohydrate-carbohydrate interaction is immobilized onto a small tip that is mounted onto the cantilever used in the AFM microscope. The other carbohydrate is immobilized onto a flat surface. The two molecules are brought into contact by, with the use of a piezo scanner, decreasing the distance between the tip and the surface. If the two carbohydrates have formed an interaction, this will be disrupted upon retraction of the tip from the surface. The degree of deflection of the cantilever holding the tip prior to bond rupture will reflect the strength of the interaction.

FIGURE 2.
Schematic illustration of quantitative determination of carbohydrate-carbohydrate interactions employing dual beam optical tweezer. A: A polystyrene bead functionalized with glycans or glycosylated molecules (the example depicts mucins of different but well-defined glycosylation pattern) is trapped in each of the two optical traps of the dual beam optical tweezers instrument. During the experiment the two beads are brought into contact, allowing the molecules immobilized onto the bead surfaces to interact, before being separated. B and C: Optical micrographs of the two optically trapped beads prior to (B) and in contact (C). The net displacements of the polystyrene beads from the center of the calibrated optical trap are continuously recorded and used for quantifying the force acting on the beads. D: Examples of force distance curves determined when separating two mucin-functionalized polystyrene beads.
The selected force-distance curves reveal force jumps reflecting the rupture of CCIs formed between the immobilized mucin molecules.
Table 1. Endogenous and exogenous glycan tumor and blood group antigens with known or potential carbohydrate-carbohydrate interactions. The list is derived from the large IgM and IgG carbohydrate antibody responses observed upon vaccination of human patients using GVAX pancreas (a granulocyte macrophage colony-stimulating factor-modified whole-cell tumor vaccine) (Xia, L., Schrump, D.S., et al. 2016).

<table>
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<th>Glycan Tumor Antigens</th>
<th>Structure</th>
<th>Graphical structure representation</th>
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