BINDING STUDIES OF α-GALNAC SPECIFIC LECTINS TO THE α-GALNAC (TN-ANTIGEN) FORM OF PORCINE SUBMAXILLARY MUCIN AND ITS SMALLER FRAGMENTS*
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Isothermal titration microcalorimetry (ITC) and hemagglutination inhibition measurements demonstrate that a chemically and enzymatically prepared form of porcine submaxillary mucin that possesses a molecular mass of ~10⁶ daltons and ~2300 α-GalNAc residues (Tn-PSM) binds to the soybean agglutinin (SBA) with a Kd of 0.2 nM which is ~10³-fold enhanced affinity relative to GalNAcα1-0-Ser (Tn), the pancarcinoma carbohydrate antigen. The enzymatically derived 81 amino acid tandem repeat domain of Tn-PSM containing ~23 α-GalNAc residues binds with ~10³-fold enhanced affinity, while the enzymatically derived 38/40 amino acid cleavage product(s) of Tn-PSM containing ~11-12 α-GalNAc residues shows ~10²-fold enhanced affinity. A natural carbohydrate decorated form of PSM (Fd-PSM) containing 40% of the core 1 blood group type A tetrasaccharide, and 58% peptide linked GalNAcα1-0-Ser/Thr residues, with 45% of the peptide linked α-GalNAc residues linked α(2,6) to N-glycolylineuraminic acid, shows ~10¹ enhanced affinity for SBA. Vatairea macrocarpa lectin (VML), which is also a GalNAc binding lectin, displays a similar pattern of binding to the four forms of PSM, although there are quantitative differences in its affinities as compared to SBA. The higher affinities of SBA and VML for Tn-PSM relative to Fd-PSM indicates the importance of carbohydrate composition and epitope density of mucins on their affinities for lectins. The higher affinities of SBA and VML for Tn-PSM relative to its two shorter chain analogs demonstrate that the length of a mucin polypeptide and hence total carbohydrate valence determines the affinities of the three Tn-PSM analogs. The results suggest a binding model in which lectin molecules “bind and jump” from α-GalNAc residue to α-GalNAc residue along the polypeptide chain of Tn-PSM before dissociating. The complete thermodynamic binding parameters for these mucins including their binding stoichiometries are presented. The results have important implications for the biological activities of mucins including those expressing the Tn cancer antigen.

The interactions of lectins which are carbohydrate binding proteins with the glycan chains of cell surface glycoprotein receptors often leads to binding and cross-linking of the receptors and subsequent signal transduction effects including cell growth and cell death (cf. 1,2). For example, binding of galectin-1, a dimeric β-Gal¹ specific lectin produced by thymic epithelia cells, leads to selective cross-linking of multiple glycoprotein receptors and induction of cell death in thymocytes, activated T cells and T-lymphoblastoid cell lines (3). The galectin-1 counter receptors in these cells include several different glycoproteins including globular and linear mucin-type glycoproteins. For example, CD45 RO, a counter receptor for galectin-1 on T cells (3), is expressed as a globular transmembrane glycoprotein that possesses multiple N-linked glycosylation chains with LacNAc epitopes. CD43, another galectin-1 counter receptor on T cells (3), is a mucin-type transmembrane glycoprotein that possesses approximately 80 O-
linked chains with terminal LacNAc epitopes (4). The presence of multiple carbohydrate epitopes is known to facilitate cross-linking of these glycoproteins with lectins including galectin-1 (3). However, the presence of multiple carbohydrate epitopes in CD43 and CD45 on their mechanisms of binding to galectin-1 and other lectins is not well understood. Details of the mechanisms of binding of multivalent globular and linear glycoproteins to lectins in general have been lacking until recently.

Recently, isothermal titration microcalorimetry (ITC) has been used to investigate the binding of asialofetuin (ASF), a globular glycoprotein that possesses nine LacNAc epitopes on three N-linked triantennary chains, to a number of galectins including galectin-1 (5). The observed Ka values for ASF binding to the galectins are 50- to 80-fold greater than that of monovalent LacNAc. These results are similar to the increases in observed Ka values obtained by ITC for binding of a synthetic tetravalent carbohydrate to the lectins concanavalin A (ConA) and *Dioclea grandiflora* (DGL) relative to monovalent carbohydrate binding (6). Furthermore, Hill plots of the ITC data for the binding of synthetic di-, tri- and tetravalent carbohydrates to ConA and DGL showed evidence for large gradients of microscopic binding constants of the different epitopes of the carbohydrates to the two lectins (6). Similar observations were observed in the Hill plots of ASF binding to the galectins (5). The latter study indicates that galectins bind to the nine LacNAc epitopes of ASF with a gradient of microscopic affinity constants ranging from 3000- to 6000-fold for the first to the ninth galectin molecule. Thus, the observed macroscopic Ka values for the galectins are derived from the average free energy of binding of the microscopic binding constants of the nine LacNAc epitopes of ASF (5). These observations serve as models for the mechanisms of binding and affinity enhancements of multivalent synthetic carbohydrates and a nonavalent globular glycoprotein to lectins. However, little information is known about the mechanisms of binding of mucins and mucin-type glycoproteins to lectins.

The glycoprotein receptors for a number of lectins including selectins (7), siglecs (8) and galectins (3) possess mucin domains with large number of glycan epitopes. Mucins are heavily O-glycosylated glycoproteins that are secreted by higher organisms to protect and lubricate epithelia cell surfaces from biological, chemical and mechanical insults. Mucin and mucin-type molecules exist as soluble and membrane attached molecules that are involved in modulating immune response, inflammation, adhesion and tumorigensis (cf. 9-11). The O-glycosylated domains of mucins and mucin-type glycoproteins contain 50 – 80% carbohydrate and possess expanded conformations. Polypeptide tandem repeats are found in mucins that contain clusters of Ser and Thr residues in high content. The O-linked carbohydrate chains in these domains are attached via α-GalNAc residues to Ser and Thr residues which have been shown to induce a 3-fold expansion of the polypeptide chain of these molecules (12).

Studies of porcine submaxillary gland mucins demonstrate that their O-glycans make up ~60 – 70% of their masses. The cDNA sequence of porcine submaxillary mucin (PSM) has been determined (13), along with the composition of carbohydrate chains of PSM as determined by chemical (14) and NMR techniques (15). Furthermore, Gerken and colleagues (15) have isolated the O-glycosylated domain of PSM that possesses a molecular mass of ~10^6 daltons and is fully decorated with naturally occurring carbohydrates (Fd-PSM). The O-glycosylated domain of PSM possessing only α-GalNAc residues (Tn-PSM) has also been obtained using chemical and enzymatic treatments (16). The GalNAcα1-O-Ser/Thr residue(s) in Tn-PSM is the pancerinoma carbohydrate antigen Tn that is aberrantly expressed in mucins such as MUC1 in adenocarcinomas (17). Binding of galectin-3, a member of the galectin family of β-Gal binding animal lectins, to cancer associated MUC1 causes increased endothelial cancer cell adhesion (18). Furthermore, immunization of mice with desialylated ovine submaxillary mucin, which expresses large amounts of Tn, protected mice against TA3-Ha tumor challenge and produced a high-antibody titer (19). Thus, it is important to compare the binding properties of Tn-PSM with the naturally occurring fully decorated PSM (Fd-PSM) containing core 1 blood group type A oligosaccharides. The 81-mer tandem repeat
domain of Tn-PSM (81-mer TnPSM) and the 38/40-mer digest of this domain (38/40-mer TnPSM) also have been obtained using enzymatic digests (16). These shorter fragments of Tn-PSM allow investigation of altering the length of Tn-PSM on its binding interactions with lectins.

The present study reports ITC and hemagglutination inhibition studies of the binding of soybean agglutinin (SBA) and Vatairea macrocarpa lectin (VML), which are both tetrameric GalNAc-specific lectins (20,21), to Fd-PSM, Tn-PSM, 81-mer Tn-PSM, and 38/40-mer Tn-PSM. The results are discussed in terms of the mechanisms of binding of α-GalNAc specific lectins to PSM, and the effects of the Tn antigen and the length of the Tn-PSM polypeptide chain on these interactions.

**Experimental Procedures**

*Materials*- SBA was obtained from EY Biochemicals, Inc. The concentration of SBA was determined spectrophotometrically using A1%1cm = 12.8 (20) and expressed in terms of monomer. VML was purified by affinity chromatography on a guar gum column (22). The concentration of VML was determined spectrophotometrically using A1%1cm = 13.0 and expressed in terms of monomer. The preparations of Fd-PSM, Tn-PSM, 81-mer Tn-PSM and 38/40-mer Tn-PSM have been previously described (16). The 81-mer Tn-PSM was obtained by trypsin digestion of Tn-PSM, and 38/40-mer Tn-PSM obtained by GluC digestion of 81-mer PSM. GalNAcc1-O-Ser was obtained from Sigma Chemical Company. All other reagents were of analytical grade.

*Hemagglutination Inhibition Assay*- The assay was performed at 22 °C using a 2-fold serial dilution technique (23) and 3% (v/v) rabbit erythrocytes in Hepes buffer (0.1 M Hepes, 0.15 M NaCl, 1mM CaCl2 and 1mM MnCl2, pH 7.2). The minimum concentration of saccharide required for complete inhibition of four hemagglutination doses was determined.

*Isothermal Titration Microcalorimetry*- ITC experiments were performed using a VP-ITC instrument from Microcal, Inc. (Northampton, MA). Injections of 4 µl of carbohydrate solution were added from a computer-controlled micro syringe at an interval of 4 min into the sample solution of lectin (cell volume = 1.43 ml) with stirring at 350 rpm. An example of an ITC experiment is shown in Fig. 1 for Tn-PSM and SBA at 27 °C. Control experiments were performed by injections of the mucin into a cell containing only buffer. The concentration range of the lectins was 5 µM - 100 µM and the mucins 0.6 µM – 0.50 mM based on the dry weight of the latter molecules. Concentration of the mucins was based on their molecular weights. Titrations were done at pH 7.2 using 20 mM PBS buffer. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (binding enthalpy in kcal mol⁻¹) (lectin monomer units), Ka (association constant) and n (number of binding sites per monomer), as adjustable parameters. Kd (dissociation constant) was calculated from 1/Ka. The quantity c = KaMt(0), where Mt(0) is the initial macromolecule concentration, is of importance in titration microcalorimetry (24). All experiments were performed with c values 1 < c < 200. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2’-monophosphate (2’-CMP) supplied by the manufacturer. Thermodynamic parameters were calculated from the Gibbs Free Energy equation, \( \Delta G = \Delta H - \Delta S = -RT \ln K_a \), where \( \Delta G \), \( \Delta H \) and \( \Delta S \) are the changes in free energy, enthalpy, and entropy of binding, respectively. T is the absolute temperature and R = 1.98 cal mol⁻¹ K⁻¹.

**RESULTS**

*Structures of Fd-PSM, Tn-PSM, 81-mer Tn-PSM and 38/40-mer Tn-PSM*. The structure of PSM is composed of a very large central O-glycosylated domain flanked by small Cys-rich globular domains at both the N and C termini (13). The O-glycosylated domain consists of approximately 100 81-residue tandem repeats with the sequence shown in Fig. 2A (25). Each tandem repeat contains 31 Ser/Thr O-glycosylation sites, and about 75% or more of the Ser and Thr residues are glycosylated to varying extents (15). Hence, there are ~2300 carbohydrate chains in the O-glycosylated domain of PSM. The carbohydrate side chains described for PSM range from the monosaccharide GalNAcc1-O-Ser/Thr, the pancarcinoma carbohydrate antigen Tn, to the core 1 blood group A tetrasaccharide α-GalNAc(1-
3)[α-Fuc(1-2)]-β-Gal(1-3)-α-GalNAc-O-Ser/Thr (14). Each of the mono- through tetrasaccharides is potentially glycosylated by an NeuNGl residue attached α(2-6) to the peptide-linked GalNAc and therefore up to eight possible oligosaccharides are found in PSM. Carbon-13 NMR (15) indicates that the oligosaccharide side chain composition of the present sample of Fd-PSM consist of 58% mono α-GalNAc residues, 2% of the trisaccharide and 40% of the tetrasaccharide. The percentage of α-NeuNGl was determined to be 45% in Fd-PSM, and thus ~45% of the peptide-linked α-GalNAc residues in Fd-PSM have NeuNGl attached to them (illustrated in Fig. 2B).

The structures of Tn-PSM possessing ~2300 α-GalNAc residues (Fig. 2C), 81-mer Tn-PSM possessing ~23 α-GalNAc residues (Fig. 2D), and 38/40-mer Tn-PSM possessing ~11-12 α-GalNAc residues (Fig. 2E) that were obtained by chemical and/or enzymatic digests were confirmed by NMR and column chromatography (16).

Hemagglutination Inhibition Data. Hemagglutination inhibition data in Table 1 shows that SBA possesses different affinities for Fd-PSM, Tn-PSM, 81-mer Tn-PSM, and 38/40-mer Tn-PSM. For comparison, the concentration for 50% inhibition by α-GalNAcc1-O-Ser is given a relative inhibition value of 1.0. SBA is inhibited by Tn-PSM at 0.3 nM which is 3 x 10^6 lower concentration than GalNAcc1-O-Ser. 81-mer Tn-PSM inhibits SBA at 0.15 μM which is 7 x 10^3 lower concentration than GalNAcc1-O-Ser, and 38/40-mer Tn-PSM inhibits at 5.6 μM which is ~600 times lower concentration than GalNAcc1-O-Ser. Thus, Tn-PSM is a more effective inhibitor than 81-mer Tn-PSM, which, in turn, is more effective than 38/40-mer Tn-PSM. SBA is inhibited by Fd-PSM at 0.08 μM that is a 10^6 lower concentration than GalNAcc1-O-Ser.

Hemagglutination inhibition data for VLM are also shown in Table 1. VLM is inhibited by Tn-PSM at 0.2 nM which is a 3 x 10^6 lower concentration than GalNAcc1-O-Ser. 81-mer Tn-PSM inhibits VLM at 0.02 μM which is a 3 x 10^4 lower concentration than GalNAcc1-O-Ser, and 38/40-mer Tn-PSM inhibits at 1.2 μM which is a 500 times lower concentration than GalNAcc1-O-serine. VLM is inhibited by Fd-PSM at 2 nM, which is a 3 x 10^4 lower concentration than GalNAcc1-O-Ser.

ITC Data. ITC data for the binding of SBA to Tn-PSM, 81-mer Tn-PSM, 38/40-mer Tn-PSM, and Fd-PSM are shown in Table 2. Kd values for Tn-PSM, Fd-PSM, 81-mer Tn-PSM and 38/40-mer Tn-PSM binding to SBA are 0.2 x 10^-9 M, 2.4 x 10^-8 M, 6.0 x 10^-8 M and 1.4 x 10^-6 M, respectively. The results show that SBA possesses the highest affinities for the Fd-PSM and Tn-PSM, and decreasing affinities for 81-mer Tn-PSM and 38/40-mer Tn-PSM. The free energy of binding (ΔG), enthalpy of binding (ΔH) and entropy of binding (ΔS) for the four forms of PSM to SBA are also shown in Table 2 as well as the respective value of n, the number of binding sites per subunit of SBA. ITC data for SBA binding to GalNAcc1-O-Ser is also shown in Table 2.

ITC data for the binding of VLM to Tn-PSM, 81-mer Tn-PSM, 38/40-mer Tn-PSM, and Fd-PSM are also shown in Table 2. The observed Kd values for Tn-PSM, Fd-PSM, 81-mer Tn-PSM, and 38/40-mer Tn-PSM are 0.1 x 10^-9 M, 1.4 x 10^-9 M, 1.2 x 10^-8 M and 0.2 x 10^-6 M, respectively. These values are similar to the respective inhibition constants found by hemagglutination inhibition in Table 1. The results show that VLM possesses the highest affinities for Fd-PSM and Tn-PSM, and decreasing affinities for 81-mer Tn-PSM and 38/40-mer Tn-PSM. The ΔG, ΔH and ΔS values for the four forms of PSM are also shown in Table 2, as well as the respective values of n. ITC data for GalNAcc1-O-Ser is also shown in Table 2.

**DISCUSSION**

Hemagglutination Inhibition Data for SBA and VML Binding to Tn-PSM, 81-mer PSM, 38/40-mer PSM and Fd-PSM. The hemagglutination inhibition data in Table 1 indicate that SBA and VML bind to Tn-PSM with affinities greater than the other three forms of PSM. The difference in affinities between Tn-PSM and Fd-PSM may be due to the masking of GalNAc residues in the latter by the presence of NeuNGl residues that are linked α(2-6) to 45% of the total peptide linked GalNAc residues. As a consequence, the structural valence of GalNAc residues in Fd-PSM may be ~1300 as compared to
~2300 in Tn-PSM. The lower affinities of SBA and VML for Fd-PSM may also reflect differences in the presentation of the nonreducing terminal α-GalNAc residue in the blood group type A tetrasaccharide as compared to the peptide linked α-GalNAc residue.

Both SBA and VML bind with greater affinities to Tn-PSM and Fd-PSM as compared to 81-mer Tn-PSM. In turn, both lectins bind to 81-mer Tn-PSM with greater affinities than to 38/40-mer Tn-PSM. This indicates that the longer polypeptide chain forms of PSM bind with higher affinities to the two lectins, and that the total number of free GalNAc residues in Tn-PSM and Fd-PSM are important for binding to the lectins. The hemagglutination inhibition data also suggest differences in the specificities of SBA and VML for the four forms of PSM. These differences are examined in detail by ITC measurements described below.

**ITC Data for SBA Binding to Tn-PSM.** ITC data for SBA binding to Tn-PSM (Table 2) shows a Kd value of 0.2 nM that agrees well with the hemagglutination inhibition concentration of 0.3 nM in Table 1. The ITC derived K(rel) for Tn-PSM is 850,00-fold greater than that of GalNAccα1-O-Ser, which can be compared with the K(rel) of 3 × 10⁸ obtained by hemagglutination inhibition.

The n value for SBA binding to Tn-PSM, which is the binding stoichiometry expressed as number of binding sites per subunit of lectin, is 0.0018 (Table 2). We previously demonstrated that 1/n provides the functional valence of multivalent carbohydrates and glycoproteins binding to lectins (5,26). The 1/n value for Tn-PSM binding of SBA is 540, which indicates that 540 α-GalNAc residues of the ~2300 α-GalNAc residues of Tn-PSM bind to 540 monomers of SBA since each monomer of SBA binds one GalNAccα1-O-Ser molecule (Table 2). The 1/n value indicates that the functional valence of Tn-PSM for SBA is less than the structural valence of Tn-PSM. The reason for the fractional binding of SBA to the carbohydrate epitopes in Tn-PSM appears to be the size of the SBA tetramer, which possesses a molecular mass of 120 kDa and one N-linked Man-9 oligomannose chain per monomer (27,28). Kiessling and coworkers have reported similar fractional occupancies for the binding of ConA, a tetrameric Man-specific lectin similar in size to SBA, to synthetic polymers containing α-mannose residues (29). Fractional occupancy has also been reported for SBA binding to the nine LacNAc epitopes of ASF (30).

SBA binding to Tn-PSM yields a ΔH of -4310 kcal/mol as compared to -7.9 kcal/mol for GalNAccα1-O-Ser (Table 2). If the ΔH for SBA binding to Tn-PSM is divided by the 1/n value of 540 α-GalNAc residues on Tn-PSM that bind to the lectin, the resulting ΔH per α-GalNAc residue of Tn-PSM is -7.98 kcal/mol, which is very similar to the ΔH of -7.9 kcal/mol for GalNAccα1-O-Ser. This indicates that each α-GalNAc residue of Tn-PSM that binds to SBA possesses the same enthalpy of binding as that of GalNAccα1-O-Ser. The observed ΔH for SBA binding to Tn-PSM is thus the sum of the individual ΔH values of the α-GalNAc binding residues of the mucin. Similar observations have been made for the binding of ConA and DGL to synthetic bi-, tri- and tetraantennary glycosides (6).

The calculated TΔS for SBA binding to Tn-PSM is -4297 kcal/mol (Table 2). If TΔS is divided by the 1/n value of 540, the resulting TΔS value per α-GalNAc residue of Tn-PSM is -7.96 kcal/mol, which is more unfavorable than the -2.7 kcal/mol for GalNAccα1-O-Ser (Table 2). If TΔS were proportional to the number of binding epitopes in Tn-PSM, the observed TΔS value would be -1458 kcal/mol, and ΔG would therefore be equal to ΔH – TΔS or -2852 kcal/mol, an impossibly large value. The observation that, unlike ΔH, TΔS does not scale in proportion to the number of binding epitopes in multivalent carbohydrates binding to lectins but instead is much more negative has been previously observed in the binding of ConA and DGL to bi-, tri- and tetraantennary carbohydrates (6). The nonlinearity of TΔS is characteristic of separate lectin molecules binding to different epitopes of a multivalent carbohydrate instead of a single lectin binding to multiple epitopes of a multivalent carbohydrate (31). In the latter case, both ΔH and TΔS increase in proportion to the number of binding epitopes in the multivalent ligand, with concomitantly larger increases in affinity (31). Nevertheless, the observed TΔS value of -4297 kcal/mol when subtracted from the observed ΔH value of -4310 kcal/mol for SBA binding to Tn-
PSM gives a ΔG value of -13.1 kcal/mol, which is -7.9 kcal/mol greater than the ΔG of -5.2 kcal/mol for GalNAcα1-O-Ser (Table 2).

**ITC Data for SBA Binding to 81-mer Tn-PSM.** The ITC derived Kd value of 0.06 μM for SBA binding to 81-mer Tn-PSM can be compared with the value of 0.15 μM obtained by hemagglutination inhibition (Table 1). K(α) for 81-mer Tn-PSM is 2800 which can be compared to 7000 obtained by hemagglutination inhibition (Table 1). The affinity of SBA for 81-mer Tn-PSM is nearly 300-fold weaker than that of Tn-PSM. Thus, the shorter length of 81-mer Tn-PSM results in weaker binding of SBA as compared to Tn-PSM. The n value for SBA binding to 81-mer Tn-PSM is 0.12, and 1/n = 8, which suggests that ~8 α-GalNAc residues of 81-mer Tn-PSM bind to ~8 monomers of SBA. Hence, the functional valence of 81-mer PSM is less than its structural valence for SBA binding, as observed for Tn-PSM.

The ΔH for SBA binding to 81-mer Tn-PSM is -56.1 kcal/mol. If the observed ΔH is divided by the 1/n value of 8, the number of bound SBA monomers per ~23 α-GalNAc residues, the resulting ΔH is -7.0 kcal/mol per α-GalNAc binding residue which is close to the value of -7.9 kcal/mol for GalNAcα1-O-Ser. This indicates that each α-GalNAc binding residue of 81-mer Tn-PSM binds with nearly the same ΔH as that of GalNAcα1-O-Ser. This finding is similar to that observed for SBA binding to Tn-PSM.

SBA binding to 81-mer Tn-PSM gives a TΔS of -46.3 kcal/mol which is greater than the calculated value of -21.6 kcal/mol if TΔS were proportional to the number α-GalNAc residues involved in binding to SBA. Thus, TΔS for SBA binding to 81-mer Tn-PSM does not increase in proportion to the number of α-GalNAc residues that bind, but rather has a larger negative value. Similar results are observed for SBA binding to Tn-PSM.

**ITC Data for SBA Binding to 38/40-mer Tn-PSM.** ITC data for the binding of SBA to 38/40-mer Tn-PSM shows a Kd of 1.4 μM (Table 2) which can be compared with ~6 μM obtained by hemagglutination inhibition (Table 1). K(α) from ITC measurements is 120 which can be compared to a K(α) of 600 from hemagglutination inhibition (Table 1). The Kd for 38/40-mer Tn-PSM is reduced nearly 20-fold relative to that for 81-mer Tn-PSM. Thus, SBA shows lowest affinity for the shortest fragment of Tn-PSM. The n value for SBA binding to 38/40-mer Tn-PSM is 0.2, and 1/n = 5. This indicates that ~5 α-GalNAc residues of 38/40-mer Tn-PSM binds to five SBA monomers.

The ΔH and TΔS values of -32.2 kcal/mol and -24.2 kcal/mol, respectively, are consistent with the lower affinity of SBA for 38/40-mer Tn-PSM as compared to the longer polypeptide chain analogs. If ΔH is divided by the 1/n value of 5, then ΔH per α-GalNAc residue of 38/40-mer is -6.44 kcal/mol, which is somewhat lower than the -7.9 kcal/mol for GalNAcα1-O-Ser. The lower ΔH per α-GalNAc residue may be due to the lower affinity of SBA for 38/40-mer Tn-PSM as compared to Tn-PSM and 81-mer Tn-PSM (discussed further below). If the observed TΔS is divided by 1/n value of 5, the TΔS per α-GalNAc residue of 38/40-mer is -4.84 kcal/mol, which is larger than -2.7 kcal/mol for GalNAcα1-O-Ser. Similar results are observed for SBA binding to Tn-PSM and 81-mer Tn-PSM, suggesting similar binding mechanisms of SBA with all three PSM analogs.

**ITC Data for SBA binding to Fd-PSM.** The ITC derived Kd for SBA binding to Fd-PSM is 0.024 μM (Table 2), which can be compared to the inhibition constant of 0.08 μM obtained by hemagglutination inhibition (Table 1). The ITC K(α) for Fd-PSM is 7,100 which is ~100-fold lower than that of Tn-PSM. The loss in affinity of SBA for Fd-PSM relative to Tn-PSM may be due to the lower density of free α-GalNAc residues in Fd-PSM, due to NeuGCl present on ~45% of the total α-GalNAc residues.

The n value for SBA binding to Fd-PSM is 0.008 (Table 2), and 1/n is 125, which is the number of α-GalNAc residues of Fd-PSM bound to SBA monomers. This number of α-GalNAc residues is consistent with a reduced number of binding sites for SBA on Fd-PSM relative to the 540 α-GalNAc residues on Tn-PSM. The factor of ~four reduction in the number of binding sites on Fd-PSM suggest that in addition to ~50& of the total α-GalNAc residues capped with NeuGCl, binding of SBA to the non-reducing α-GalNAc residue of the tetrasaccharide chains of PSM may alter the accessibility of SBA binding to the single
peptide linked α-GalNAc residues by a further factor of two. Similar effects are observed for VML binding to Tn-PSM and Fd-PSM (below).

The ΔH and TΔS values for Fd-PSM binding to SBA are -703 kcal/mol and -693 kcal/mol, respectively, and are lower than the corresponding values for Tn-PSM (Table 2). If ΔH is divided by 1/n = 125, the resulting ΔH per binding α-GalNAc residue is -5.6 kcal/mol which is somewhat lower than that for GalNAcα1-O-Ser (Table 2). If TΔS is divided by 1/n = 125, the resulting TΔS per binding α-GalNAc residue is -5.54 kcal/mol which is greater than that for GalNAcα1-O-Ser. Similar results are observed for SBA binding to Tn-PSM, 81-mer Tn-PSM and 38/40-mer PSM.

ITC Data for VML Binding to Tn-PSM. The ITC data for VML binding to Tn-PSM is similar to that for SBA. The main difference is the stoichiometry of binding of VML which shows that 1/n = 833 α-GalNAc residues bind to 833 monomers of VML. This can be compared with 540 α-GalNAc residues of Tn-PSM binding to 540 monomers of SBA. Importantly, the size of the VML tetramer is similar to that of SBA (32), and VML also possesses covalently bound carbohydrate like SBA (32).

ITC Data for VML Binding to 81-mer Tn-PSM and 38/40-mer Tn-PSM. The ITC data for VML binding to 81-mer Tn-PSM and 38/40-mer Tn-PSM in Table 2 are similar to that observed for SBA. Differences between VML and SBA binding to these two fragments of Tn-PSM are principally in their K(rel) values which are somewhat greater for VML. The binding stoichiometries of VML to the two fragments are also similar to those observed for SBA. The results support the conclusion that the affinity of VML, like SBA, decreases with shorter fragments of Tn-PSM.

ITC Data for VML Binding to Fd-PSM. The ITC data for VML binding to Fd-PSM is also similar to that for SBA. Differences between the two lectins are in their K(rel) values which are greater for VML. Like SBA, VML binds with greater affinity to Fd-PSM than to 81-mer Tn-PSM and 38/40-mer PSM, but with less affinity to Fd-PSM than to Tn-PSM. The binding stoichiometry of VML to Fd-PSM indicates that there are 200 α-GalNAc residues of Fd-PSM that bind to VML monomers. This can be compared to the 125 α-GalNAc residues of Fd-PSM that bind to SBA.

Mechanisms of Binding of SBA and VML to Fd-PSM, Tn-PSM and Shorter Chain Analogs of Tn-PSM. The increasing affinities of SBA and VML for 38/40-mer Tn-PSM, 81-mer Tn-PSM and Tn-PSM, respectively, indicate that the lengths of the polypeptide chains and hence total carbohydrate valences of these mucin analogs regulate their affinities for the lectins. The higher affinities of SBA and VML for Tn-PSM relative to Fd-PSM also indicates the importance of carbohydrate composition and epitope density of the mucins on their affinities for lectins. The similarities in the ΔH per α-GalNAc binding residue of Tn-PSM and Fd-PSM with that of GalNAcα1-O-Ser for the two lectins, respectively, and similar correlations with 81-mer Tn-PSM and 38/40-mer TnPSM, suggest that the binding mechanisms of these PSM analogs are common.

A model to explain these results is similar to that proposed for the binding of lectins to multivalent carbohydrates (6) and globular glycoproteins (5). Namely, binding of a lectin to a multivalent carbohydrate or glycoprotein involves internal diffusion “jumps” of the lectin from carbohydrate epitope to epitope before complete dissociation. Kinetically, this has the effect of reducing the macroscopic off-rate of the lectin and hence increasing its affinity, since the affinity constant is the ratio of the forward and reverse rate constants. The forward rate constant for lectin binding may also be enhanced as a result of the larger number of binding epitopes on the glycoprotein.

The diffusion jump model for SBA and VML binding to Tn-PSM and the other PSM analogs can be envisioned as occurring with one subunit of SBA or VML bound to one α-GalNAc residue of Tn-PSM at a time (Fig. 3A). (Two subunits of individual SBA or VML molecules simultaneously binding to a single Tn-PSM chain is not supported by the enhanced affinities of both lectins to 38/mer Tn-PSM relative to GalNAcα1-O-Ser (Tables 1 and 2). If two subunits of an SBA tetramer bound to 38-mer Tn-PSM, the affinity enhancement would be approximately the product of the individual GalNAcα1-O-Ser dissociation constant which would be ~10^-8 M instead of the observed 10^-6 M.) This model provides a molecular
mechanism to account for the dependence of the affinities of the two lectins on the total carbohydrate valences of Tn-PSM, 81-mer Tn-PSM and 38/40-mer Tn-PSM. This model further suggests that as more lectin molecules bind to a mucin chain, the affinity of the lectin will decrease because of steric crowding and shorter diffusion distances on the polypeptide chain of the mucin (Fig. 3B, 3C). Indeed, Hill plots for SBA binding to Tn-PSM show evidence for negative binding cooperativity (data to be shown in a later paper). Similar Hill plots have been interpreted as evidence for increasing negative cooperativity in the binding of galectins to ASF (5) and ConA and DGL to synthetic di-, tri- and tetravalent carbohydrates (6). In these cases, binding of the lectins to the multivalent carbohydrates and glycoprotein were associated with gradients of decreasing microaffinity constants of the lectins for the multiple epitopes of the ligands. This suggest that the observed dissociation constants for SBA and VML binding to the four PSM analogs in Table 2 represent a composite of the gradient of binding constants present in each case. Such gradients have been estimated to be as large as 3000- to 6000-fold for galectins binding to ASF (5). These gradient effects may explain the lower average ∆H values per α-GalNAc residue for 81-mer Tn-PSM and 38/40-mer PSM binding to SBA and VML.

The model for SBA and VML binding must also agree with the final saturation density of SBA and VML molecules bound to single PSM analogs as reflected in ITC n values. For example, the 1/n value for SBA binding to Tn-PSM indicates 540 α-GalNAc residues bound to 540 SBA monomers. The 1/n value for VML binding to Tn-PSM indicates 833 α-GalNAc residues bound to 833 VML monomers. Another requirement of the binding model is the observation that at the end of ITC experiments, solutions of SBA and VML with all four PSM analogs begin to precipitate out of solution (data not shown). This indicates that lectin mediated cross-linking of the mucins occurs following saturation binding.

Both of these observations are accounted for in the schematic representation in Fig. 3D of SBA cross-linked with Tn-PSM under saturation conditions. The schematic shows individual SBA molecules cross-linked to four different Tn-PSM molecules. Importantly, in order to form the cross-linked complex shown in Fig. 3D, lectin molecules bind to α-GalNAc residues on all four sides of a Tn-PSM polypeptide chain. This allows staggering of individual SBA molecules along the Tn-PSM polypeptide chain, with concomitant reduction in steric interactions between lectin molecules. This is important since calculations of the density of SBA molecules bound to Tn-PSM (knowing the diameter of SBA from x-ray crystal studies (28) and the length of the Tn-PSM polypeptide chain) suggests that only ~300 SBA tetramers can bind to the same side of a Tn-PSM polypeptide chain, which is less than the 540 bound monomers of SBA and 833 bound monomers of VML derived from ITC n values. The apparent steric crowding is overcome by having lectin molecules bound to all four sides of Tn-PSM.

In summary, the binding models first show a fraction of SBA or VML molecules that bind to Tn-PSM and “jump” between different α-GalNAc residues of the mucin (Fig. 3A). As the number of bound lectin molecules increase, the affinity of the lectin decreases because of shorter diffusion distances on the mucin chain due to steric crowding and cross-linking by multiple bound lectin molecules (Fig. 3B, C). Finally, upon saturation binding, full lectin mediated cross-linking and precipitation of the complexes takes place (Fig. 3D).

Implications and Summary. The model for lectin binding to mucins suggests that the length of mucin chains and mucin-type domains in glycoproteins modulates the affinities of lectins and related ligands for such glycoprotein receptors. Indeed, the length of mucins and mucin-type domains in glycoproteins is regulated in biological systems (cf. 9-11). Furthermore, mucins and mucin-type domains are also expressed with different amino acid sequences, different degrees of clustered carbohydrates, and different decorations of carbohydrates (cf. 9). All of these structural variations are expected to affect the affinities of lectins and other ligands including glycosyl transferases for mucins and mucin-type domains.

The Kd values of 0.2 nM and 0.1 nM for SBA and VML binding to Tn-PSM, respectively, are among the lowest reported Kd values for glycoprotein-lectin interactions. For comparison, the observed Kd values for galectins-1, -2, -3, -4, -
5 and -7 binding to the multivalent globular glycoprotein ASF which has nine LacNAc epitopes are in the range of ~1 \( \mu M \) (5). SBA and VML bind to 38/40-mer Tn-PSM, which has a structural valence of ~11 \( \alpha\)-GalNAc residues, in the Kd range of the galectins binding to ASF. Thus, the large difference in Kd values for the binding of SBA and VML to Tn-PSM and the galectins binding to ASF appear to reflect the much greater carbohydrate valence of Tn-PSM (~2300 \( \alpha\)-GalNAc residues) as compared to ASF (nine LacNAc residues). Indeed, differences in the affinities of the N-terminal domain of galectin-4 for globular glycoproteins and mucins have been reported (33). The differences in geometry between globular versus linear glycoproteins permits higher carbohydrate valence in mucins that, in turn, is associated with higher affinities toward ligands such as lectins.

Importantly, the internal diffusion model for lectins binding to mucins is distinct from the classical “lock and key” model of ligand binding to a receptor. The internal diffusion model is dynamic and allows a small fraction of lectin molecules to bind to the entire length of the receptor (mucin) and facilitate cross-linking interactions with other mucin molecules. For transmembrane bound mucins, such as CD-43, cross-linking by lectins (galectin-1) can lead to signal transduction effects including apoptosis (3). The polyvalency of long, linear chain molecules such as mucins also facilitates the uptake of low concentration gradients of lectin molecules that are released from the surface of cells.

The observation that SBA and VML bind with higher affinities to Tn-PSM relative to Fd-PSM has implications for the aberrant expression of the Tn epitope in molecules such as MUC1 that is found with overexpression of the Tn epitope in adenocarcinomas (34). The present data indicate that clustering of the Tn epitope on a mucin such as PSM can result in high affinity binding of lectins with \( \alpha\)-GalNAc specificities. Interestingly, a recent study of the binding of galectin-3 to MUC1 from epithelial cancer cells which expresses the TF antigen (Gal\( \beta\)1,3GalNAc-\( \alpha\)) as well as the Tn antigen causes increased cancer cell endothelial adhesion (18). The mechanism of binding of galectin-3 to MUC1 may be similar to that observed for SBA and VML to the PSM analogs in the present study.

Lastly, the internal diffusion model of a lectin jumping from carbohydrate epitope to epitope in a mucin chain is similar to that for a variety of ligands binding to DNA in which binding and sliding occur along the DNA backbone (cf. 35). Indeed, the present results suggest a possible fusion between the mechanisms of binding of lectins to mucins and, in general, ligands binding to linear biopolymers including glycosaminoglycans, polysaccharides, polypeptides and nucleic acid polymers.

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**FOOTNOTES**

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1Abbreviations: SBA, soybean agglutinin; VML, *Vatairea macrocarpa* lectin; ConA, concanavalin A; DGL, *Dioclea grandiflora* lectin; PSM, porcine submaxillary mucin; Fd-PSM, fully carbohydrate decorated porcine submaxillary mucin; Tn-PSM, porcine submaxillary mucin containing GalNAcα1-O-Ser/Thr residues; 81-mer Tn-PSM; 81-residue amino acid repeat of domain of porcine submaxillary mucin containing GalNAcα1-O-Ser/Thr residues; 81-mer Tn-PSM containing only GalNAcα1-O-Ser/Thr residues; Tn-antigen, GalNAcα1-O-
Ser/Thr; ASF, asialofetuin; GalNAc, N-acetyl-D-galactosamine; Fuc, L-fucose; Gal, D-galactose; NeuNGl, N-glycoloylneuraminic acid or sialic acid; LacNAc; N-acetyl-D-lactosamine; ITC, isothermal titration microcalorimetry; NMR, nuclear magnetic resonance.

**FIGURE LEGENDS**

Fig. 1. ITC titration profile (top panel) of SBA (10μM monomer concentration) titrated with Tn-PSM (0.7μM) in pH 7.2 buffer at 27 °C. The lower panel shows a fit of the binding data using Origin software.

Fig. 2. Structural representations of A) the amino acid sequence of the 100-repeat 81-residue polypeptide O-glycosylation domain of intact PSM; B) the fully carbohydrate decorated form (described in the text) of the 100-repeat 81-residue polypeptide O-glycosylation domain of PSM (Fd-PSM); C) the 100-repeat 81-residue polypeptide O-glycosylation domain of PSM containing only peptide linked α-GalNAc residues (Tn-PSM); D) the single 81-residue polypeptide O-glycosylation domain of PSM containing peptide linked α-GalNAc residues (81-mer Tn-PSM); E) the 38/40-residue polypeptide(s) derived from the 81-residue polypeptide O-glycosylation domain of PSM containing peptide linked α-GalNAc residues (38/40-mer Tn-PSM). The number of glycan chains in Fd-PSM and Tn-PSM is ~2,300. The number of α-GalNAc residues in 81-mer Tn-PSM is ~23, while the number of α-GalNAc residues in 38/40-mer Tn-PSM is ~11-12.

Fig. 3. Schematic representations of A) SBA or VML binding at low density to Tn-PSM; B) SBA or VML binding at higher density to Tn-PSM; C) SBA and VML binding at higher density to Tn-PSM and initiating cross-linking of the complexes; D) SBA cross-linked complexes with Tn-PSM under saturation binding conditions. The view is end on of the polypeptide chains of Tn-PSM in Fig. 3C. α-GalNAc residues extend out from the polypeptide chains of Tn-PSM in 3-dimensions. Lectin tetramers are bound to four separate Tn-PSM chains, with staggered binding down the length of the mucin chains.
Table 1. Hemagglutination inhibition data for SBA and VML at pH 7.2 27 °C

| Ligand                | SBA (µM) | K(rel)<sup>b</sup> | VML (µM) | K(rel)<sup>b</sup> |
|-----------------------|----------|--------------------|----------|--------------------|
| GalNAcα1-O-Ser        | 1000     | 1                  | 600      | 1                  |
| 38/40-mer Tn-PSM      | 5.6      | 180                | 1.2      | 500                |
| 81-mer Tn-PSM         | 0.15     | 7000               | 0.02     | 30,000             |
| Tn-PSM                | 0.0003   | 3,000,000          | 0.0002   | 3,000,000          |
| Fd-PSM                | 0.08     | 10,000             | 0.002    | 300,000            |

<sup>a</sup>Errors are ±50%.  <sup>b</sup>Inhibition concentration relative to GalNAcα1-O-Ser.
Table 2. Thermodynamic binding parameters for SBA and VML at pH 7.2, 27 °C

| Ligand                  | Kd (µM) | K(rel) | -ΔG (kcal/mol) | -ΔH (kcal/mol) | -TΔS (kcal/mol) | n |
|-------------------------|---------|--------|----------------|----------------|-----------------|---|
|                         |         |        |                |                |                 |   |
| **SBA**                 |         |        |                |                |                 |   |
| GalNAcα1-O-Ser          | 170     | 1      | 5.2            | 7.9            | 2.7             | 1.0 |
| 38/40-mer Tn-PSM        | 1.4     | 120    | 8.0            | 32.2           | 24.2            | 0.2 |
| 81-mer Tn-PSM           | 0.06    | 2,800  | 9.8            | 56.1           | 46.3            | 0.12 |
| Tn-PSM                  | 0.0002  | 850,000| 13.1           | 4310           | 4297            | 0.0018 |
| Fd-PSM                  | 0.024   | 7,100  | 10.4           | 703            | 693             | 0.008 |
| **VML**                 |         |        |                |                |                 |   |
| GalNAcα1-O-Ser          | 130     | 1      | 5.3            | 6.4            | 1.1             | 1.0 |
| 38/40-mer Tn-PSM        | 0.20    | 650    | 9.1            | 36.8           | 27.7            | 0.2 |
| 81-mer Tn-PSM           | 0.012   | 11,000 | 10.8           | 52.7           | 41.9            | 0.1 |
| Tn-PSM                  | 0.0001  | 1,300,000| 13.6          | 5274           | 5260            | 0.0012 |
| Fd-PSM                  | 0.0014  | 93,000 | 12.1           | 1251           | 1240            | 0.005 |

*Errors in Kd range from 1-7%; bRelative to GalNAcα1-O-Ser; cerrors in ΔG are less than 2%; derrors in ΔH are 1% to 4%; eerrors in TΔS are 1% to 7%; ferrors in n are less than 4%.
Figure 1
Figure 2
Figure 3
Binding studies of alpha-GalNAc specific lectins to the alpha-GalNAc (Tn-antigen) form of porcine submaxillary mucin and its smaller fragments
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