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Thermodynamic Switch in Binding of Adhesion/Growth Regulatory Human Galectin-3 to Tumor-Associated TF Antigen (CD176) and MUC1 Glycopeptides

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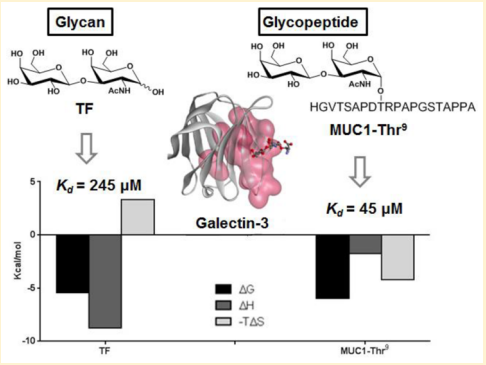
Supporting Information

ABSTRACT: A shift to short-chain glycans is an observed change in mucin-type O-glycosylation in premalignant and malignant epithelia. Given the evidence that human galectin-3 can interact with mucins and also weakly with free tumor-associated Thomsen-Friedenreich (TF) antigen (CD176), the study of its interaction with MUC1 (glyco)peptides is of biomedical relevance. Glycosylated MUC1 fragments that carry the TF antigen attached through either Thr or Ser side chains were synthesized using standard Fmoc-based automated solid-phase peptide chemistry. The dissociation constants (K_d) for interaction of galectin-3 and the glycosylated MUC1 fragments measured by isothermal titration calorimetry decreased up to 10 times in comparison to that of the free TF disaccharide. No binding was observed for the nonglycosylated control version of the MUC1 peptide. The most notable feature of the binding of MUC1 glycopeptides to galectin-3 was a shift from a favorable enthalpy to an entropy-driven binding process. The comparatively diminished enthalpy contribution to the free energy (ΔG) was compensated by a considerable gain in the entropic term. $^{1}H-^{15}N$ heteronuclear single-quantum coherence spectroscopy nuclear magnetic resonance data reveal contact at the canonical site mainly by the glycan moiety of the MUC1 glycopeptide. Ligand-dependent differences in binding affinities were also confirmed by a novel assay for screening of low-affinity glycan−lectin interactions based on AlphaScreen technology. Another key finding is that the glycosylated MUC1 peptides exhibited activity in a concentration-dependent manner in cell-based assays revealing selectivity among human galectins. Thus, the presentation of this tumor-associated carbohydrate ligand by the natural peptide scaffold enhances its affinity, highlighting the significance of model studies of human lectins with synthetic glycopeptides.



Mucins of cancer cells are subject to malignancy-associated alterations in their glycan presentation.[1,2] With similar changes also occurring upon activation of T cells, the respective glycans not only are viewed as markers but also are being delineated to have a functional dimension by serving as docking sites for endogenous receptors.[3,4] In this respect, the Thomsen-Friedenreich (TF) antigen (Galβ1,3GalNAcα Ser/Thr) is a prominent tumor-associated antigen (CD176), found in ∼90% of all human cancers, including precancerous lesions.[5−7] The major protein carrier of this disaccharide in malignancy is the MUC1 glycoprotein, which plays important roles in the adhesion/anti-adhesion processes and immune suppression, a complex series of coordinated events ultimately contributing to tumor progression and metastasis.[8,9] MUC1 is a TF-dependent counterreceptor for the endogenous lectin galectin-3, this association mediating tumor cell interactions.[7,10,11] However, the molecular details of the mechanism, by which recognition of the TF antigen by galectin-3, an inherently weak binding, reaches an affinity level to functionally be involved in metastasis, are poorly understood. One of the important barriers to explaining the functional significance of glycan-based changes in cancer is the natural epitope heterogeneity found on the surface of cancer cells. After all, the extracellular part of MUC1 contains a domain of numerous tandem repeats of 20 amino acids (HGVTSAPD-TRPAPGSTAPPA), each harboring five possible glycosylation

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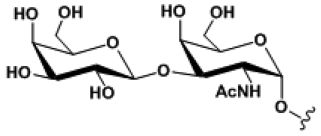
sites (Thr[4], Ser[5], Thr[9], Ser[15], and Thr[16]). In addition to examination of epitope heterogeneity by synthetic permutations, the glycoside cluster and steric hindrance effects of neighboring glycans on binding to their receptor could be thoroughly studied, if appropriate, structurally defined model compounds become available. Considering the emerging complexity of the galectin network with the possibility of functional overlap and/or divergence,[12−14] elucidation of the affinity and specificity of the binding of galectin to the TF antigen, as it exists in its natural cellular context conjugated to MUC1, can thus be accomplished, starting with galectin-3. Of relevance, the interaction of a glycoconjugate with a lectin may not be confined to the glycan but could involve sections of the scaffold.[15−18] With a focus on TF antigen, our initial NMR analysis and molecular dynamics simulations of recognition of the TF-Thr conjugate by avian galectin-3 indicated bioactivity of this compound, making a detailed study possible.[19] On the basis of systematic glycopeptide synthesis, we here report binding analysis of these model compounds, characterizing thermodynamic, structural, and cell biological parameters.

In detail, the synthesis of the glycosylated MUC1 fragments that carry TF antigen attached through either Thr (Thr[4], Thr[9], or Thr[16]) or Ser (Ser[5]) side chains is presented (Scheme 1).

Scheme 1. Glycosylated MUC1 Peptides Used in This Study



T*/S* = Thr/Ser O-linked TF disaccharide

MUC1:	HGVTSAPDTRPAPGSTAPPA
MUC1-Thr[4]:	HGV**T***SAPDTRPAPGSTAPP
MUC1-Thr[9]:	HGVTSAPD**T***RPAPGSTAPP
MUC1-Thr[16]:	HGVTSAPDTRPAPGS**T***APP
MUC1-Ser[5]:	HGVT**S***APDTRPAPGSTAPP
$MUC1_{1-8}$-Thr[4]:	HGV**T***SAPD
$MUC1_{1-15}$-Thr[4]:	HGV**T***SAPDTRPAPGS

The binding affinities of MUC1 glycopeptide fragments for full-length galectin-3 and its carbohydrate recognition domain (CRD) obtained by proteolytic truncation were assessed by an AlphaScreen assay and isothermal titration calorimetry (ITC). Interestingly, the measured dissociation constants (K_d) for galectin-3 and glycosylated MUC1 fragments were consistently lower in comparison to that for the free TF disaccharide. Tested as a control, no binding for the glycan-free MUC1 peptide was seen. NMR HSQC experiments with the ^{15}N-labeled galectin-3 CRD indicate a glycan-dependent response of the lectin structure to ligand accommodation. Of potential biomedical relevance, the glycosylated MUC1 peptides were bioactive and exhibited selectivity among galectins, when their relative capacity as inhibitors of binding to tumor cell surfaces was tested.

EXPERIMENTAL PROCEDURES

Reagents. Asialofetuin (ASF), silver perchlorate ($AgClO_4$), activated powdered molecular sieves, and Celite (Celite 521) were purchased from Sigma-Aldrich. *N*-Acetyllactosamine (LacNAc; Galβ1−4GlcNAc), Fmoc-protected amino acids, and coupling reagents for peptide synthesis 2-(6-chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and 1-hydroxybenzotriazole (HOBt) were obtained from EMD Biosciences (San Diego, CA). *N*,*N*′-Diisopropylcarbodiimide (DIC) and thioacetic acid were purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA). All solvents were purchased from Fisher Scientific (Atlanta, GA) or Sigma-Aldrich and were analytical reagent grade or better. A Spectra/Por Float-A-Lyzer with cellulose ester membrane with a molecular weight cutoff (MWCO) of 0.1−0.5 kDa was purchased from SpectrumLabs. TentaGel S RAM resin was obtained from Advanced ChemTech (Louisville, KY). D-Galactal was purchased from Carbosynth Ltd. (Berkshire, U.K.), 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-galactopyranose (TF; Galβ1−3GalNAc) from Toronto Research Inc., and methyl 2-azido-2-deoxy-β-D-galactopyranoside from Senn Chemicals (San Francisco, CA).

The progress of the reaction was monitored by thin-layer chromatography (TLC) on Merck Silica Gel 60 F_{254} plates and visualized by charring with 10% aqueous sulfuric acid and/or by UV light when applicable. Flash column chromatography was performed on Silica Gel columns (Merck, 230−400 Mesh).

General reagents for microbiology, protein expression, and purification work were purchased from Fisher Scientific (Pittsburgh, PA). The AlphaScreen Histidine (Nickel Chelate) detection kit and solid white polystyrene microplates (Proxiplate-384 PLUS) were purchased from PerkinElmer (Walthan, MA). Biotin-labeled asialofetuin (biotin-ASF) was purchased from Axxora (San Diego, CA).

Synthesis of Glycosylated Thr/Ser Building Blocks. Compounds **1**−**6** were prepared according to the protocol described previously.[20,21] Compounds **9**−**12** were synthesized according to the method described by Shao et al.[22]

*Synthesis of N$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-3-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-di-O-acetyl-2-deoxy-α-D-galactopyranosyl]-L-threonine Pentafluorophenyl Ester (**7**). Based on Scheme 2.* Bromide **6** (500 mg, 0.73 mmol) and Fmoc-Thr-OPfp (0.75 g, 1.46 mmol) were dissolved in a mixture of dry CH_2Cl_2 and toluene [1:1 (v/v), 15 mL]. Activated powdered molecular sieves (4 Å) were added, and the reaction mixture was stirred at room temperature for 1 h under an argon atmosphere. Then the reaction flask was cooled to −40 °C, and $AgClO_4$ (150 mg, 0.73 mmol) was added. The progress of the glycosylation reaction was monitored by TLC [2:1 (v/v) EtOAc/toluene]. Once the complete disappearance of bromide **6** had been observed, usually in 2 h, the reaction mixture was allowed to warm to room temperature, diluted with chloroform (15 mL), and filtered through Celite. The organic filtrate was washed with water, separated, dried over Na_2SO_4, and concentrated. The residue was purified by flash chromatography [2:1 (v/v) EtOAc/toluene] to obtain the activated Fmoc-protected O-glycosylated Thr **7** (500 mg, 62%). MALDI-MS: $[M + Na]^+$ = 1131.66 Da (expected, 1130.92 Da). The HPLC chromatogram and the NMR spectra (^{1}H and ^{13}C) are provided in the Supporting Information (Figures S1, S4, and S5, respectively).

Based on Scheme 3. TF antigen precursor **12** (370 mg, 0.66 mmol) was dissolved in acetonitrile (16 mL), and the reaction mixture was cooled to −40 °C. Ferric chloride hexahydrate (534 mg, 1.98 mmol), sodium azide (86 mg, 1.32 mmol), and hydrogen peroxide (30% aqueous solution, 285 μL) were added, and the reaction mixture was stirred at −40 °C for 8 h. The progress of the glycosylation reaction was monitored by TLC [1:1 (v/v) hexane/EtOAc]. The solution was diluted with diethyl ether and washed with water, a saturated sodium

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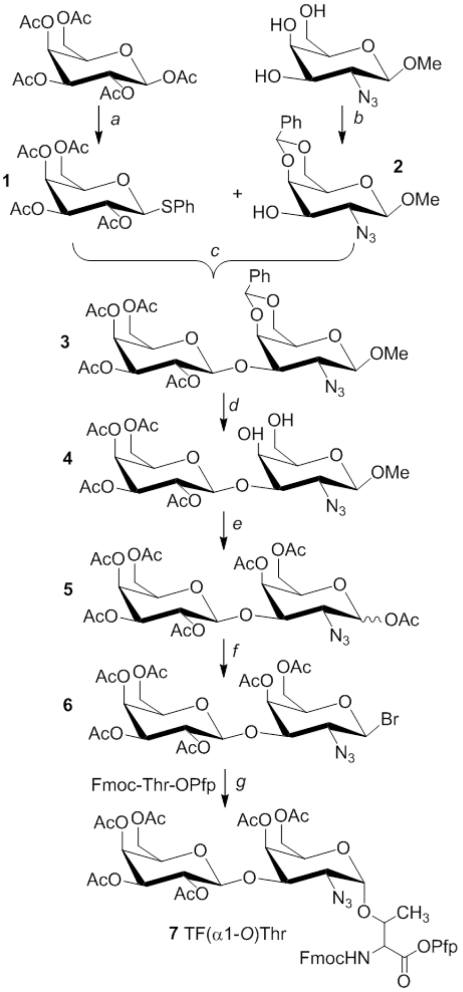
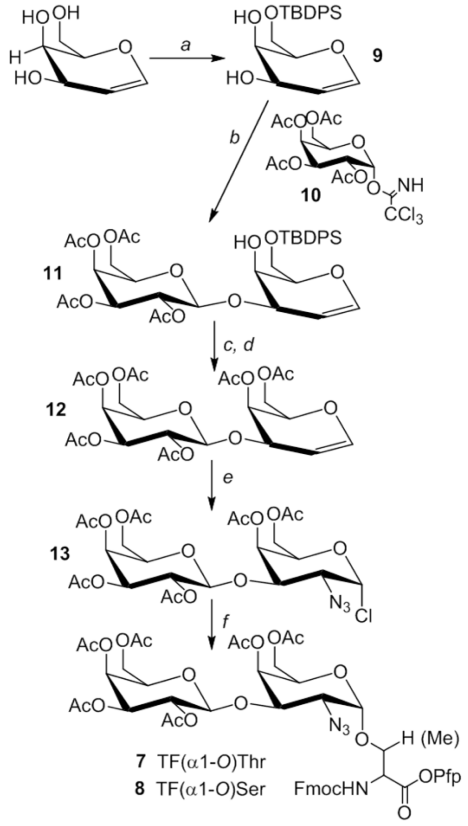
Scheme 2. Synthesis[a] of Fmoc-Protected *O*-Glycosylated Thr Starting from Methyl 2-Azido-2-deoxy-β-D-galactopyranoside[20]



[a]Conditions: (a) PhSh, BF_3-OEt_2; (b) $PhCH(OMe)_2$, TsOH; (c) NIS, TfOH, −45 °C; (d) 80% AcOH, 80 °C; (e) Ac_2O-H_2SO_4, −20 °C; (f) $TiBr_4$; (g) $AgClO_4$, Fmoc-Thr-OPfp, −45 °C.

Scheme 3. Synthesis[a] of Fmoc-Protected *O*-Glycosylated Thr/Ser Based on the One-Pot Azidochlorination Procedure Described by Plattner et al.[35]



[a]Conditions: (a) TBDPS-Cl, Et_3N, DMF, room temperature; (b) **9**, CH_2Cl_2 at −30 °C then TMSOTf at room temperature; (c) TBAF, THF, AcOH, pH 7, room temperature; (d) Ac_2O, pyridine, CH_2Cl_2, room temperature; (e) NaN_3, $FeCl_3$, H_2O_2, CH_3CN, −30 °C; (f) $AgClO_4$, Fmoc-Thr/Ser-OPfp, −45 °C.

bicarbonate solution ($NaHCO_3$), and brine (NaCl) until the organic layer was discolored. The organic filtrate was dried over Na_2SO_4 and concentrated under vacuum, and desired azido chloride **13** (450 mg crude) was used in the next step without further purification.

Compound **13** (450 mg, 0.71 mmol) and Fmoc-Thr-OPfp (0.74 g, 1.46 mmol) were dissolved in a mixture of dry CH_2Cl_2 and toluene [1:1 (v/v), 15 mL]. Activated powdered molecular sieves (4 Å) were added, and the reaction mixture was stirred at room temperature for 1 h under an argon atmosphere. Then the reaction flask was cooled to −40 °C, and $AgClO_4$ (146 mg, 0.73 mmol) was added. The progress of the glycosylation reaction was monitored by TLC [2:1 (v/v) EtOAc/toluene]. Once the complete disappearance of azido chloride **13** had been observed, usually in 2 h, the reaction mixture was allowed to warm to room temperature, diluted with chloroform (15 mL), and filtered through Celite. The organic filtrate was washed with water, separated, dried over Na_2SO_4, and concentrated. The product was purified by flash chromatography [2:1 (v/v) EtOAc/toluene] to obtain the activated Fmoc-protected O-glycosylated Thr **7** (320 mg, 40%). MALDI-MS: $[M + Na]^+$ = 1130.9 Da (expected, 1130.92 Da). The HPLC chromatogram and the NMR spectra (^1H and ^{13}C) are provided in the Supporting Information (Figures S2, S4, and S5, respectively).

*Synthesis of Nα-(Fluoren-9-ylmethoxycarbonyl)-3-O-[2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→3)-2-azido-4,6-di-O-acetyl-2-deoxy-α-D-galactopyranosyl]-L-serine Pentafluorophenyl Ester (**8**). Based on Scheme 3.* Azido chloride **13** (450 mg, 0.71 mmol) and Fmoc-Ser-OPfp (0.72 g, 1.46 mmol) were dissolved in a mixture of dry CH_2Cl_2 and toluene [1:1 (v/v), 15 mL]. Activated powdered molecular sieves (4 Å) were added, and the reaction mixture was stirred at room temperature for 1 h under an argon atmosphere. Then the reaction flask was cooled to −40 °C, and $AgClO_4$ (146 mg, 0.73 mmol) was added. The progress of the glycosylation reaction was monitored by TLC [2:1 (v/v) EtOAc/toluene]. Once the complete disappearance of azido chloride **13** had been observed, usually in 2 h, the reaction mixture was allowed to warm to room temperature, diluted with chloroform (15 mL), and filtered through Celite. The organic filtrate was washed with water, separated, dried over Na_2SO_4, and concentrated. The residue was purified by flash chromatography [2:1 (v/v) EtOAc/toluene] to obtain the activated Fmoc-protected O-glycosylated Ser **8** (295 mg, 38%). MALDI-MS: $[M + Na]^+$ = 1116.89 Da (expected, 1117.26 Da). The HPLC chromatogram and the

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NMR spectra (^{1}H and ^{13}C) are provided in the Supporting Information (Figures S3, S4, and S7, respectively).

Synthesis of Glycosylated MUC1 Fragments. MUC1 glycopeptide analogues were synthesized on a PS3 automated peptide synthesizer (Protein Technologies Inc., Tucson, AZ). Standard Fmoc chemistry was used throughout with a 4-fold molar excess of the acylating amino acids, and HCTU and 1-HOBt as coupling reagents.[23] The pentafluorophenyl ester of the glycoamino acid was coupled manually in a 1.5-fold molar excess to reduce consumption of this amino acid in the presence of *N*,*N*-diisopropylethylamine (DIPEA). Upon completion of the assembly of the peptide chain, the N-terminal Fmoc protection was retained in the case of glycopeptides. The azido group was converted into the desired acetamido group by treatment of the resin with thioacetic acid for 4 days with daily changes of the reagent. The Fmoc protective group was removed with 20% piperidine in dimethylformamide (DMF). The resulting glycopeptides as well as the MUC1 peptide were cleaved from the resin using a thioanisole/water/trifluoroacetic acid mixture (5:5:90) for 2 h. The cleavage solution was extracted with methyl *tert*-butyl ether. Deacetylation of the sugar hydroxyl groups was accomplished by a treatment with 0.01 M NaOH for 15 min.[20] Reversed-phase high-performance liquid chromatography (RP-HPLC) purification was performed on a 1260 Infinity Agilent Technologies liquid chromatography system with a Grace Vydac monomeric C_{18} column (250 mm × 22 mm, 10 μm, 120 Å) at a flow rate of 10.0 mL/min. Eluents were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The elution gradient was 2% B for the first 5 min followed by 0 to 50% B over 80 min. Detection was at λ = 214 nm. Fractions were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and by analytical RP-HPLC. Analytical RP-HPLC was performed on a 1260 Infinity Agilent Technologies liquid chromatograph equipped with a Grace Vydac monomeric C_{18} monomeric column (250 mm × 4.6 mm, 5 μm, 120 Å). Eluents were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The elution gradient was from 2 to 30% B over 20 min with a flow rate of 1.0 mL/min. Detection was at λ = 214 nm. MALDI-TOF MS was performed on a Voyager MALDI-TOF-DE STR mass spectrometer (Applied Biosystems, Foster City, CA) using an α-cyano-4-hydroxycinnamic acid matrix. The HPLC and MALDI-TOF MS analyses of purified glycosylated peptides are provided in the Supporting Information (Figures S8−S14). The peptides were dialyzed against water to remove all salt content by using a Spectra/Por Float-A-Lyzer with a cellulose ester membrane with a MWCO of 0.1−0.5 kDa.

Competition Binding Assay. Mixtures of His-tagged galectin-3 expressed in our laboratory[24] (1.25 μL) and biotin-ASF (1.25 μL) were added to the wells containing varied concentrations (2.5 μL, final concentrations of 0−1 mM) of the MUC1 glycopeptide in assay buffer [25 mM Hepes, 100 mM NaCl, and 0.05% Tween 20 (pH 7.4)]. The final concentration of His-tagged galectin-3 was 200 nM and that of biotin-ASF 5 nM in the assay. The reaction mixture was incubated for 1 h at room temperature; then 5 μL of nickel-chelate-Acceptor and 5 μL of streptavidin-conjugate Donor beads were simultaneously added to a final concentration 25 μg/mL incubated for 1 h in dark at room temperature, and the assay plate was subsequently read at 22 °C in the AlphaScreen mode on the Synergy H4 Hybrid plate reader (BioTek). Data, AlphaScreen signal counts (counts per second) versus log [inhibitor, M], were expressed as means of five replicate measurements. The IC_{50} values were obtained by nonlinear regression analysis using Graph Pad Prism version 5.04.

Isothermal Titration Calorimetry (ITC) Measurements. Calorimetric measurements were recorded using an iTC_{200} calorimeter (MicroCal). In brief, a solution of 40 μL of the ligand, at a concentration 10-fold greater than that of galectin-3, was titrated in aliquots of 1 μL into the calorimetric cell at 1000 rpm, containing 203 μL of galectin-3 (full-length or CRD). Both, ligand and galectin-3, were prepared in exactly the same buffer containing 20 mM phosphate, 0.15 M NaCl, and 10 mM BME (pH 7.2). Injections were performed every 180 or 240 s at 25 °C. A titration of each ligand (glycan or glycopeptide) in the sample cell containing only buffer was subtracted from the actual binding experiment before data analysis. The thermodynamic analysis was performed using the MicroCal analysis software (Origin 7.0).

NMR Experiments. All experiments were conducted either on a Bruker Avance 600 MHz or on a Bruker AVIII 700 MHz spectrometer, each equipped with a cryogenically cooled z-gradient triple resonance probe at 298 K. Samples for $^{1}H-^{15}N$ HSQC experiments contained the ^{15}N-labeled human galectin-3 CRD, obtained by recombinant production using *Escherichia coli* strain BL21 (DE3)-pLysS (Promega, Mannheim, Germany) and the pET12a expression vector (Novagen, Darmstadt, Germany) at a concentration of 100 μM, with or without 10 (or 20) equiv of the tested peptides, in PBS in 90% H_2O and 10% D_2O for field-frequency lock. Chemical shift perturbations were monitored, using the sequence-specific assignments for the ^{1}H and ^{15}N resonances previously reported.[25]

Assessment of Binding Affinities in the Cell-Based Assay. The human galectins (i.e., galectins-1, -3, -4, and -8 as well as the N-terminal domain of galectin-9) were produced using suitable combinations of bacteria and expression vector [i.e., the BL21 (DE3)-pLysS *E. coli* strain with pGEMEX-1 (galectin-1), pET12a (galectin-3, -8, and -9N), and pET24a (galectin-4)], isolated by affinity chromatography on lactose-bearing Sepharose 4B as a crucial step, and controlled for purity by one- and two-dimensional gel electrophoresis/mass spectrometric fingerprinting.[13,26−29] The lectins were either biotinylated or fluoresceinylated under activity-preserving conditions and controlled for maintained activity by solid-phase and cell binding analyses using stringent controls.[30,31] Cell assays were quantitated by cytofluorometry following probe incubation at 4 °C for 30 min with cells using the Chinese hamster ovary parental line (Pro^{-5}) and the human SW480 colon adenocarcinoma and Capan-1 pancreas adenocarcinoma lines, the latter reconstituted for expression of tumor suppressor $p16^{INK4a}$ as described previously.[26,27,31] Controls included processing without the incubation step with lectin (or second-step reagent) and in the presence of cognate sugar, flanked by osmolarity controls with noncognate carbohydrates (mannose). Cell binding data are expressed as a percentage of positive cells and mean fluorescence intensity.

RESULTS

Synthesis of MUC1 Fragments That Carry Thomsen-Friedenreich (TF) Antigen Attached through Thr/Ser Side Chains. The assembly of glycosylated MUC1 peptides (Scheme 1) was performed using standard Fmoc-based automated solid-phase peptide chemistry, and the building block approach was used for the incorporation of the Fmoc-protected *O*-glycosylated Thr/Ser. We have evaluated two synthetic approaches toward the glycosylated amino acid building block that rely on the synthesis of a suitable protected 2-azido disaccharide glycosyl donor. The azide at C-2 was found

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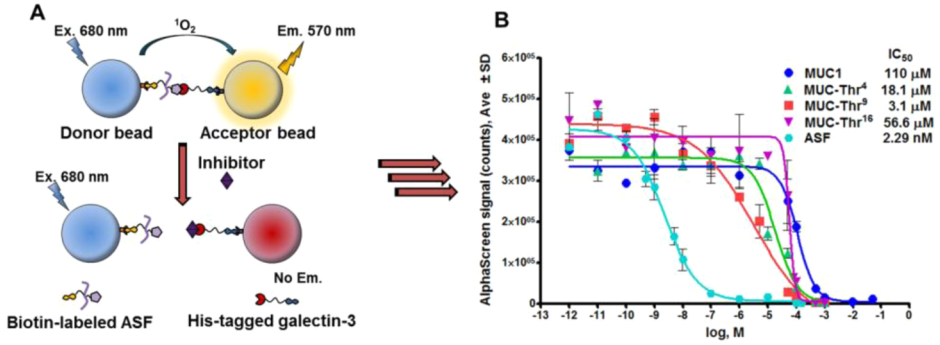

Figure 1. (A) Competitive binding assay. (B) Inhibition of binding of biotinylated ASF (5 nM) to galectin-3 (200 nM) by MUC1 glycopeptides (final concentrations of 0−1 mM). The final concentration of the beads was 25 μg/mL. The assay buffer consisted of 25 mM Hepes (pH 7.4) containing 100 mM NaCl and 0.05% Tween 20. Curves, AlphaScreen signal counts (counts per second) vs log [inhibitor, M], were plotted as means of five replicate measurements. The IC_{50} values were obtained by nonlinear regression analysis using Graph Pad Prism 5.04.

to be a very efficient nonparticipating group, resulting in predominantly α-glycosidic bond formation. It can then be easily converted into the desired acetamido group, reduced, and subsequently acetylated,[32] or reductive acetylation can be performed in a one-step procedure with thioacetic acid, before[33] or after on-resin peptide assembly.[20,34] The first synthetic strategy was based on the protocol previously described for the synthesis of Fmoc-Ser/Thr-OPfp building blocks containing α-linked linear tri- and heptasaccharides (Scheme 2).[20] In this approach, phenyl-β-1-thiogalactoside **1** was coupled to 2-azido-4,6-di-*O*-benzylidene-2-deoxy-β-D-galactopyranoside **2** in the presence of *N*-iodosuccinimide-triflic acid to exclusively afford β-anomer **3** (80% yield). The benzylidine group was removed in aqueous acetic acid at 80 °C, followed by acetylation of the hydroxyl groups and acetolysis of anomeric methyl glycoside **4** using a mixture of acetic acid and sulfuric acid [50:1 (v/v)]. The peracetylated TF disaccharide **5** was converted into α-glycosyl bromide **6** using titanium tetrabromide ($TiBr_4$). Fmoc-protected pentafluorophenyl ester of *O*-glycosylated Thr **7** was prepared by coupling Fmoc-Thr-OPfp with donor **6** in the presence of silver perchlorate ($AgClO_4$) at −40 °C. The stereochemically pure α-anomer was obtained in 62% yield after purification by silica gel chromatography. The purity of the Thr building block **7** was confirmed by RP-HPLC and MALDI-MS (see Figure S1 of the Supporting Information). The NMR spectra are in agreement with the previously published data[21,34] and confirmed the anomeric purity of **7** (see Figures S4 and S5 of the Supporting Information).

The second approach explored the one-pot azidochlorination procedure recently described by Plattner et al.,[35,36] because the synthesis of 2-azido glycosyl donors, in this case 2-azido-2-deoxy-β-D-galactopyranoside, via the azidonitration procedure introduced by Lemieux[37] is rather laborious (Scheme 3). The key starting compound in this approach is D-galactal. The selective protection of C-6 hydroxyl groups of D-galactal by treatment with *tert*-butylchlorodiphenylsilane (TBDPS-Cl) in the presence of triethylamine (Et_3N) afforded the suitably monoprotected derivative of D-galactal **9**.[38] The equatorial 3-OH group is more reactive than the 4-OH group, so it was possible to regioselectively glycosylate **9** by using the proper glycosyl donor. The trichloroacetimidate donor[39] was employed for the formation of the β-1,3-glycosidic bond of TF antigen precursor **11** (53% yield).[22] The silyl ether protective group was removed by treatment with tetrabutylammonium fluoride (TBAF), followed by acetylation of C-4 and C-6 hydroxyl groups to yield the corresponding peracetylated analogue **12**.[22] The azido chloride derivatives **13**, obtained by the one-pot azidochlorination procedure (70% yield, crude),[35] were coupled with the pentafluorophenyl ester of Fmoc-protected Thr/Ser under Koenigs−Knorr activation conditions as described above. The desired *O*-glycosylated Thr/Ser building blocks **7** and **8** were obtained in 40 and 38% yields, respectively, after purification by silica gel chromatography. The purity of these building blocks was confirmed by RP-HPLC and MALDI-MS (see the Supporting Information, Figure S2 for compound **7** and Figure S3 for compound **8**). NMR spectra ascertained the α-linkage (see the Supporting Information, Figures S4 and S5 for compound **7** and Figures S6 and S7 for compound **8**).

Upon completion of glycopeptide chain assembly, the resin was treated with thioacetic acid for direct one-pot reductive acetylation of the azido group on the sugar moiety. Glycopeptide cleavage was performed using TFA in the presence of thioanisole and H_2O as scavengers, followed by deprotection of acetyl groups from the sugar moieties under basic conditions. The synthesized nonglycosylated MUC1 peptide and the corresponding glycopeptides were purified by preparative RP-HPLC, and all were characterized by MALDI-MS and analytical RP-HPLC (see Figures S8−S14 of the Supporting Information).

Assessment of Binding Affinities for Galectin-3 by an AlphaScreen-Based Assay. The IC_{50} values of the glycosylated MUC1 fragments that carry the TF antigen for binding to galectin-3 were evaluated using the AlphaScreen competitive binding assay. We have recently demonstrated that the AlphaScreen assay in a competitive setting is suited to detection of inhibitors of galectin-3 and offers several advantages over existing methods for monitoring low-affinity glycan−lectin interactions.[24] The key components of the assay were His-tagged galectin-3 bound to nickel-chelate Acceptor beads (chemiluminescer) and the biotinylated glycoprotein ASF (biotin-ASF) bound to streptavidin-coated Donor beads (photosensitizer). The three complex-type N-glycans of ASF are ligands for galectins. In the competitive binding mode, increasing the concentration of the inhibitor leads to disruption of the association between the beads and therefore to a decrease in the intensity of the AlphaScreen signal (Figure 1A). The resulting data points can be used to calculate the IC_{50} value for each inhibitor. Unlabeled ASF, titrated in a concentration range from 1 pM to 1 mM, was used as a positive control. An IC_{50} value of 2.3 nM was measured for ASF, as is expected for this receptor−ligand pair.[40] The glycosylated MUC1 fragments MUC1-Thr^4, MUC1-Thr^9, and MUC1-Thr^{16} inhibited His-tagged galectin-3−biotin-ASF interaction with IC_{50} values of 18.1, 3.1, and 56.6 μM,

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Table 1. Thermodynamic Parameters for the Binding of Carbohydrates and (Glyco)peptides to Galectin-3 (full-length and CRD) Determined by ITC[a]

	K_a ($\times 10^{-4}$ M^{-1})	$-\Delta G$ (kcal/mol)	$-\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	n	K_d (μM)
			Galectin-3			
LacNAc	3.06	6.14	12.74	6.60	0.94	33
TF	0.40	5.44	8.74	3.30	0.82	245
Thr-TF	0.35	4.85	8.78	3.93	1.02	283
MUC1	no binding	–	–	–	–	–
MUC1-Thr^4	3.60	6.26	0.56	−5.70	0.76	28
MUC1-Thr^9	2.22	5.95	1.75	−4.20	1.03	45
MUC1-Thr^{16}	2.21	5.93	0.73	−5.20	0.86	45
MUC1-Ser^5	1.60	5.74	1.33	−4.41	0.70	63
MUC1$_{1-15}$-Thr^1	0.76	5.29	1.79	−3.50	0.87	132
MUC1$_{1-8}$-Thr^4	0.29	4.72	1.32	−3.40	0.97	346
			Galectin-3 CRD			
LacNAc	3.31	6.10	13.70	7.60	1.08	30
TF	0.40	4.94	14.05	9.11	0.93	248
Thr-TF	0.37	4.88	12.63	7.75	0.90	272
MUC1-Thr^4	1.06	5.46	0.56	−4.90	0.80	94
MUC1-Thr^9	0.97	5.44	0.94	−4.50	0.98	103
MUC1-Ser^5	1.00	5.44	4.11	−1.34	0.92	103

[a]The binding isotherms are presented in Figures 2 and 3 and Figures S15 and S16 of the Supporting Information.

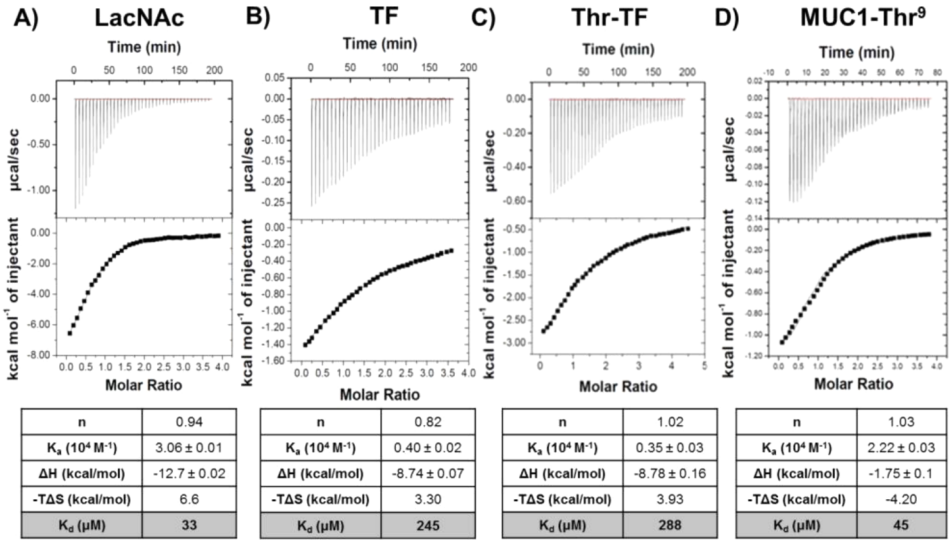

Figure 2. ITC titration profile of (A) galectin-3 (140 μM) with LacNAc (2.6 mM), (B) galectin-3 (280 μM) with TF disaccharide (3.0 mM), (C) galectin-3 (140 μM) with Thr-TF (3.0 mM), and (D) galectin-3 (280 μM) with MUC1-Thr^9 (2.0 mM) in buffer containing 20 mM phosphate, 0.15 M NaCl, and 10 mM BME (pH 7.2). Injections of ligand were performed every 240 s at 298 K. The top panels show the experimental ITC data and bottom panels a fit to a one-site model of the binding data using MicroCal analysis software (Origin 7.0). Resulting values for the stoichiometry (n), binding affinity (K_a), dissociation constant (K_d), enthalpy (ΔH), and change in entropy with respect to temperature ($T\Delta S$) are shown in the tables.

respectively (Figure 1B). Even the nonglycosylated MUC1 peptide interfered with binding, showing the highest IC_{50} value (110 μM) (Figure 1B). In summary, especially the low micromolar value of MUC-1-Thr^9 indicates a lectin association via TF binding.

Assessment of Binding Affinities for Galectin-3 by ITC. The binding affinities of free disaccharides (TF antigen and LacNAc, a positive control) and the glycopeptides were assessed by ITC to infer enthalpic and entropic contributions. The measured K_d and thermodynamic parameters for the binding process are summarized in Table 1. The low micromolar values of K_d determined by the AlphaScreen for MUC1 glycopeptides are in relatively good agreement with the measured ITC data. No binding was observed for the glycan-free control version of the MUC1 peptide under these conditions. The observed discrepancy in reactivity of nonglycosylated MUC1 peptide with galectin-3 in the two assays can be attributed to special conditions of the AlphaScreen assay. This procedure was run in competitive binding mode with ASF as a binding partner of galectin-3. High concentrations of a test compound may establish interactions beyond the lectin's CRD, causing bead dissociation and a decrease in the magnitude of the Alpha signal; for example, on the side of the glycoprotein-carrying beads and also the lectin's, the basic pI value may play a role. On the other hand, in the case of ITC experiments, direct binding of ligand to galectin-3 was measured. Interestingly, the K_d of the MUC1-Thr^9 glycopeptide carrying TF antigen [K_d = 45 μM (Figure 2D)] for galectin-3 was 6 times lower than that of either Thr-TF

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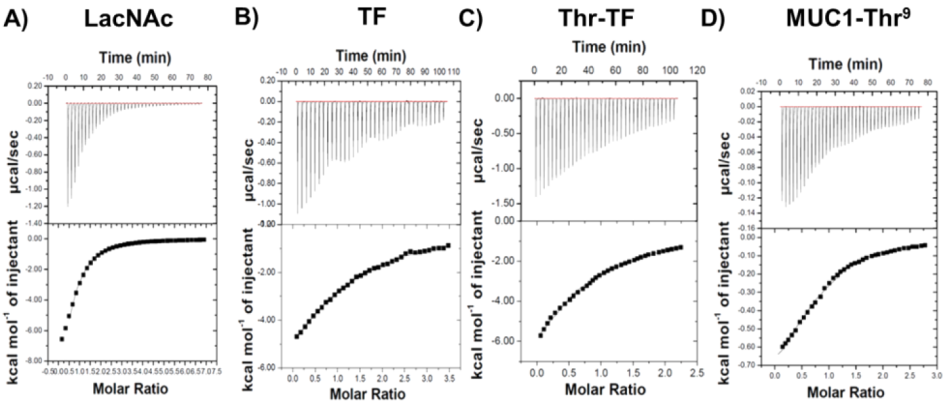

A) LacNAc	
n	1.08
K_a (10^4 M^{-1})	3.31 ± 0.04
ΔH (kcal/mol)	-13.7 ± 0.01
-TΔS (kcal/mol)	7.50
K_d (μM)	30

B) TF	
n	0.93
K_a (10^4 M^{-1})	0.40 ± 0.06
ΔH (kcal/mol)	-14.1 ± 0.07
-TΔS (kcal/mol)	9.11
K_d (μM)	248

C) Thr-TF	
n	0.90
K_a (10^4 M^{-1})	0.37 ± 0.01
ΔH (kcal/mol)	-12.6 ± 0.43
-TΔS (kcal/mol)	2.55
K_d (μM)	272

D) MUC1-Thr9	
n	0.98
K_a (10^4 M^{-1})	0.97 ± 0.09
ΔH (kcal/mol)	-0.94 ± 0.1
-TΔS (kcal/mol)	-4.50
K_d (μM)	103

Figure 3. ITC titration profile of (A) the galectin-3 CRD (140 μM) with LacNAc (2.6 mM), (B) the galectin-3 CRD (140 μM) with TF disaccharide (2.6 mM), (C) the galectin-3 CRD (250 μM) with Thr-TF (3.0 mM), and (D) the galectin-3 CRD (280 μM) with MUC1-Thr^9 (3.1 mM) in buffer containing 20 mM phosphate, 0.15 M NaCl, and 10 mM BME (pH 7.2). Injections of ligand were performed every 240 s at 298 K. The top panels show the experimental ITC data and bottom panels a fit to the one-site model of the binding data using MicroCal analysis software (Origin 7.0). Resulting values for the stoichiometry (n), binding affinity (K_a), dissociation constant (K_d), enthalpy (ΔH), and change in entropy with respect to temperature ($T\Delta S$) are shown in the tables.

[K_d = 288 μM (Figure 2C)] or free TF disaccharide [K_d = 245 μM (Figure 2B)] and comparable to that of LacNAc [K_d = 33 μM (Figure 2A)]. The measured K_d values for MUC1-Thr^4, MUC1-Thr^{16}, and MUC1-Ser^5 were 28, 45, and 63 μM, respectively (Figure S15 of the Supporting Information). Thus, the site of attachment of the TF antigen (Thr^4, Thr^9, Thr^{16}, or Ser^5) did not seem to have a significant effect on the affinities of glycopeptide for galectin-3 (Table 1). These findings indicate that the presentation of the carbohydrate ligand by the natural peptide scaffolds (and not just the Thr residues) contributes to the observed enhanced affinity for galectin-3. To further investigate the possible role of the peptide backbone in the increased affinity for galectin-3, we have synthesized two truncated versions of the Thr^4 TF-bearing peptide, i.e., MUC1$_{1-15}$-Thr^4 and MUC1$_{1-8}$-Thr^4 (Scheme 1). The C-terminal five-amino acid truncation of the MUC1 peptide sequence resulted in an increase in the measured K_d for MUC1$_{1-15}$-Thr^4 [K_d = 132 μM (Table 1 and Figure S15 of the Supporting Information)]. Additional truncation of the peptide sequence caused an even larger decrease in affinity for galectin-3, the measured K_d for MUC1$_{1-8}$-Thr^4 being 346 μM (Table 1 and Figure S15 of the Supporting Information). The integrity of the peptide scaffold is thus a key parameter for high-affinity binding.

Proteolytic truncation of galectin-3, removing its N-terminal tail composed mostly of non-triple-helical collagen-like repeats, is a physiological process, generating the CRD. Thus, we have also performed binding studies with the CRD of galectin-3 (Table 1 and Figure 3A–D) to examine whether the presence of the N-terminal section of galectin-3 has any impact on binding. The K_d values obtained for LacNAc [K_d = 30 μM (Figure 3A), close to 33 μM for the full-length protein, similar slight affinity decreases have previously been reported for ASF and LacNAc binding[40]], TF disaccharide [K_d = 248 μM (Figure 3B)], and the Thr-TF conjugate [K_d = 272 μM (Figure 3C)] were slightly altered or unaffected, and the measured K_d of the glycosylated MUC1-Thr^9 [K_d = 103 μM (Figure 3D)] was increased compared to the K_d measured for full-length galectin-3 [K_d = 45 μM (Figure 2D)]. Overall, a similar trend of an affinity increase was observed for all TF-bearing MUC1 peptides used in this study (Table 1 and Figure S16 of the Supporting Information). The N-terminal section of galectin-3, known to participate in the self-association of the chimera-type lectin,[41] appears to have a modest effect on glycopeptide–galectin-3 binding.

Upon examination of the thermodynamic balance sheet, a conspicuous difference in the thermodynamic profiles of the binding processes for the model carbohydrate, LacNAc, and MUC1 glycopeptides was revealed (Figure 4A). LacNAc binding

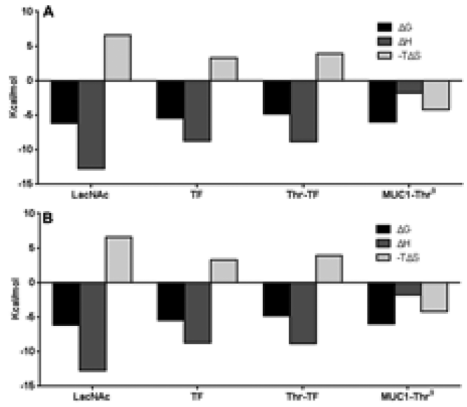

Figure 4. (A) Thermodynamic signature of binding of full-length galectin-3 to LacNAc, TF disaccharide, Thr-TF, and MUC1-Thr^9. (B) Thermodynamic signature of binding of the galectin-3 CRD to LacNAc, TF, Thr-TF, and MUC1-Thr^9.

was enthalpically driven (ΔH = −12.74 kcal mol^{-1}), typical for interactions between lectins and carbohydrate ligands[42−44] and also seen for ASF binding.[40] The thermodynamic profile of the TF-Thr conjugate resembled the LacNAc profile. Of particular

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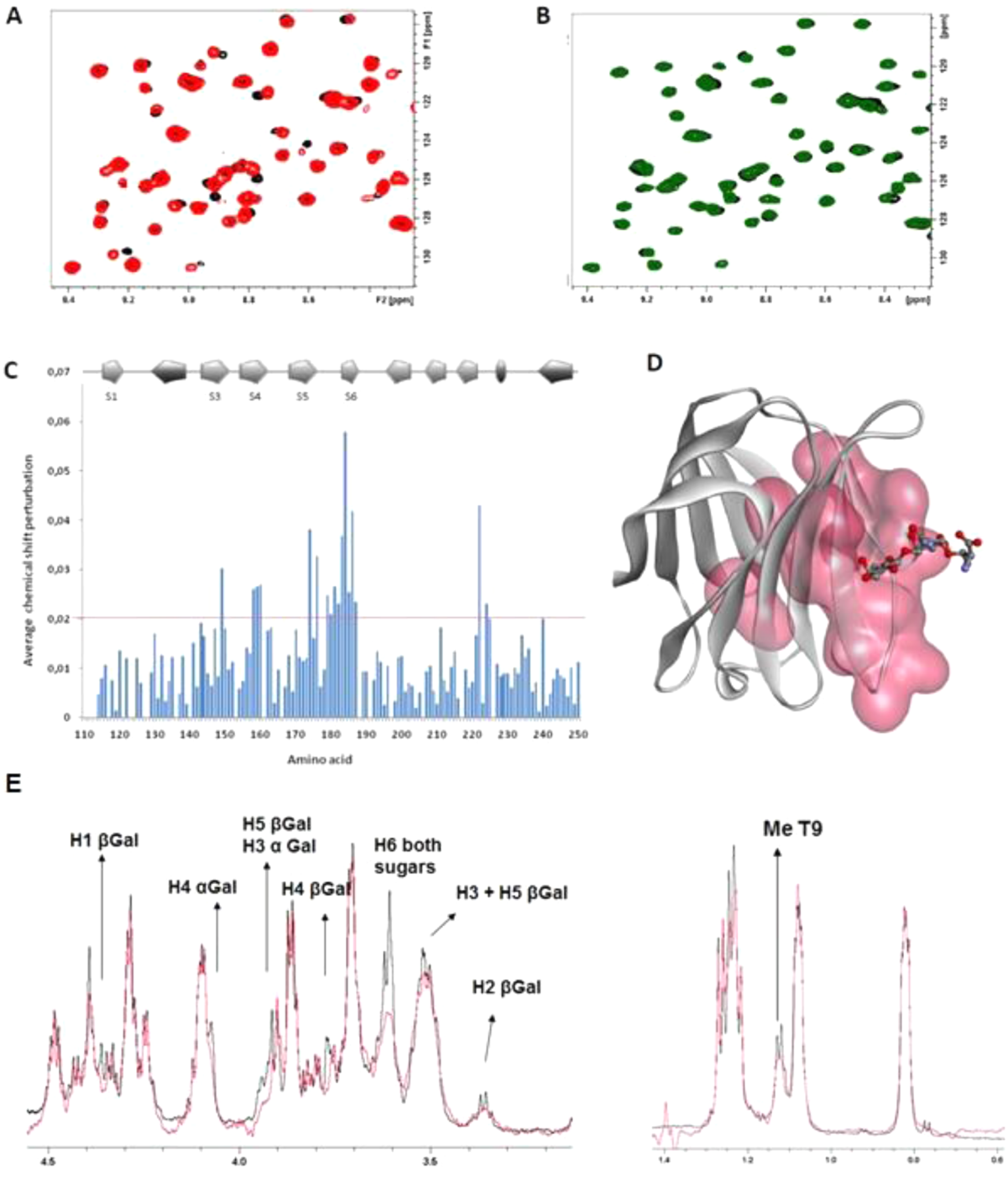

Figure 5. (A) Overlay of the $^{1}H-^{15}N$ HSQC spectra of the galectin-3 CRD: black for the apo form and red upon addition of MUC1-Thr^{9} (10 equiv). (B) Overlay of the $^{1}H-^{15}N$ HSQC spectra of the galectin-3 CRD: black for the apo form and green upon addition of MUC1 (10 equiv). (C) Average ^{1}H and ^{15}N chemical shift perturbation $\{[\Delta H^2 + (\Delta N/5)^2/2]^{1/2}\}$[68,69] plotted for each amino acid of the galectin-3 CRD upon addition of MUC1-Thr^{9} (20 equiv). The horizontal line indicates a significant (>0.02) chemical shift perturbation. Secondary structural elements and corresponding names are shown *at* the top. (D) Ribbon diagram of the galectin-3 CRD complexed with the TF antigen (represented as balls and sticks) as deposited in the PDB (3AYA). The amino acids with a significant (>0.02) chemical shift perturbation for the interaction with MUC1-Thr^{9} are represented with a pink surface. (E) Comparison of the ^{1}H NMR spectra of the free and bound state of MUC1-Thr^{9} showing the protons affected by binding. Overlay of the ^{1}H NMR spectrum of MUC1-Thr^{9} in D_2O (black) and bound MUC1-Thr^{9} (red), obtained by the subtraction of the ^{1}H NMR spectrum of MUC1-Thr^{9}–galectin-3CRD (2:1) and the ^{1}H NMR spectrum of the galectin-3 CRD, acquired under the same conditions. The red spectrum shows how certain proton resonances, corresponding to the Galβ1−3GalNAcα-Thr moiety, broaden significantly while the protons of the peptide part are hardly affected.

note for the binding of the MUC1 glycopeptide to galectin-3 was the shift from a favorable enthalpy toward an entropy-driven binding interaction. In fact, a marked decrease in the favorable enthalpy was observed and the overall free energy (ΔG) maintained by a gain in the entropic contribution (Figure 4A). A similar effect was observed for binding of the galectin-3 CRD to MUC1 glycopeptides (Figure 4B), indicating that the observed entropy-driven recognition process is not related to the presence of the collagenase-sensitive N-terminal section in the lectin. To gain a view of structural aspects, we proceeded to NMR

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experiments, facilitated by full-scale assignments of ^{13}C and ^{15}N resonances.[25]

Interaction between the Galectin-3 CRD and MUC1 Glycopeptides Analyzed by NMR. $^{1}H-^{15}N$ HSQC spectra of ^{15}N-labeled galectin-3 CRD were recorded in the presence of (glyco)peptides. The interaction between MUC1-Thr^{9} and galectin-3 CRD was evident (Figure 5A), while no interaction was observed with the peptide lacking the *O*-glycan, MUC1 (Figure 5B). Only a few $^{1}H-^{15}N$ cross-peaks of the ^{15}N-labeled human galectin-3 shift significantly upon ligand addition (Figure 5C), indicating a localized binding site. In fact, most perturbations take place at the canonical site, involving residues in β-strands S4−S6 (Figure 5C,D), as defined by NMR spectroscopy and crystallography.[45−47] In detail, amino acids R186, R162, R184, N160, and H158 through hydrogen bonding, and W181 through CH-π stacking, as is also the case for the TF antigen according to X-ray crystallography[46] [Protein Data Bank (PDB) entry 3AYA (Figure 5D)], serve as contact sites. Notable additions to this panel based on the chemical shift perturbation data are residues N222 and R224, far from the carbohydrate-binding site, an observation previously reported when using synthetic derivatives.[47] On the level of the glycopeptide, only localized effects at the TF epitope were observed for the MUC1 glycopeptide upon binding to galectin-3. The comparison of the proton NMR spectra of the free and bound state indicates that protons of the carbohydrate moiety and Thr^{9} methyl protons were the only ones affected (extensive line broadening) by the binding process (Figure 5E). Therefore, it is mostly the disaccharide that directly interacts with human galectin-3, whose $^{1}H-^{15}N$ HSQC spectrum presents changes similar to those seen upon lactose binding, an enthalpically driven process. In principle, protein conformational entropy can significantly contribute to the free energy of ligand binding.[48−51] In this case, the observed thermodynamic changes are probably caused by the chemical nature of the relatively large peptide portion of the ligand, the special context-dependent presentation of the glycan, the flexibility of the peptide chain, and interactions with the surrounding water molecules.

Assessment of the Reactivity of MUC1 Glycopeptides for Galectins in the Cell-Based Assay. Several members of the galectin family have been detected immunohistochemically, e.g., in colon cancer,[52,53] and very often in the form of a network.[54] To give an example, expression in colon cancer has been delineated for galectin-1, -3, -4, -7, -8, and -9.[26,52] Evidence of an overlap of staining by galectin-4 and plant lectins due to core 1 *O*-glycans points to potential *in situ* interaction of TF-bearing MUC1 and galectins beyond galectin-3 in colon cancer.[55] In addition to the *N*-glycan of the MUC1 glycoprotein at Asn-36, a ligand for galectin-3,[56] the mucin-type O-glycosylation with TF glycosylation can thus be likely reactive with several galectins, a question addressed by a cell assay using synthetic glycopeptides as inhibitors.

Routinely, these assays were performed in parallel, with aliquots of the same cell suspension to ensure comparability. Each galectin had first been tested in titration series to ascertain concentration dependence and determine the optimal lectin concentration for the inhibition assays. To ensure respective sensitivity, this concentration was selected to be in the range of the linear increase of the two staining parameters. In this setting, haptenic saccharides lactose (Lac) and *N*-acetylgalactosamine (GalNAc) were tested along with osmolarity controls to determine the dependence of binding on protein−carbohydrate interaction.

The concentration dependence for the lectin and Lac as an inhibitor for galectin-3 is exemplarily shown in panels A and B of Figure 6. GalNAc (Figure 6C) is apparently less active than Lac

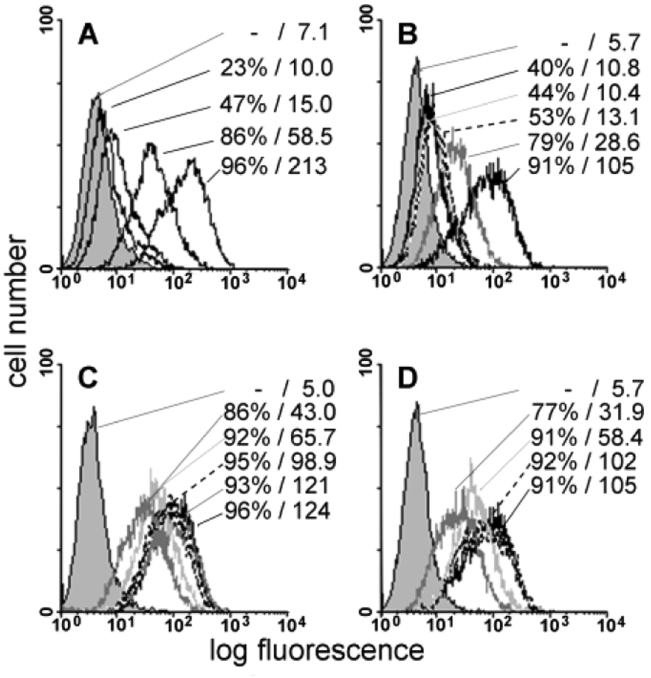

Figure 6. Semilogarithmic representation of fluorescent surface staining by galectin-3 of human SW480 colon adenocarcinoma cells. The control value of cell positivity by the second-step reagent in the absence of lectin is given as a gray-shaded area and the 100% value (lectin staining in the absence of inhibitor) as a thick black line. Quantitative characteristics of binding (percentage of positive cells/mean fluorescence intensity) in each panel are given in the order of listing (from bottom to top, in coding of gray scaling or using dashed lines): (A) dependence of binding on lectin concentration (10, 4, 2, and 1 μg/mL), (B and C) inhibition of the extent of binding at 5 μg/mL galectin-3 by increasing the concentration of lactose (B, 0.2, 0.5, 1, and 2 mM) or *N*-acetylgalactosamine (C, 0.5, 1, 2, and 5 mM), and (D) inhibition of galectin-3 binding (5 μg/mL) by the nonglycosylated peptide (MUC1) at 1 mM and glycopeptide MUC1-Thr^{4} at 0.5 and 1 mM.

as an inhibitor (Figure 6B), as known from solid-phase assays.[57] This result documents the inherent validity of comparison between different assays under the given conditions. Whereas the glycan-free peptide does not affect binding at 1 mM, the glycopeptide MUC1-Thr^{4} was clearly inhibitory, much more than free sugar (Figure 6D). In direct comparison, no major difference was found in the inhibitory capacity for the three glycopeptides (not shown). Lac and GalNAc are also both inhibitory for galectin-9 (Figure 7A). Again, the MUC1 peptide did not affect the level of binding at the test concentrations, but the three glycopeptides, i.e., MUC1-Thr^{4}, MUC1-Thr^{9}, and MUC1-Thr^{16}, exhibited rather similar levels of inhibitory capacity (Figure 7B). Tested at 1 mM, galectin-4 binding was only weakly responsive to the presence of sugars, while the glycopeptides tested reached the extent of activity of Lac (Figure 7C,D). A similar situation was encountered for galectin-1, whereas galectin-8 binding was not affected by the presence of any glycopeptide (not shown). The position of the monosubstitution on the peptide scaffold appears to be of minor significance in this respect, and the data clearly reveal the sensitivity of galectin binding to the presence of glycopeptides beyond galectin-3.

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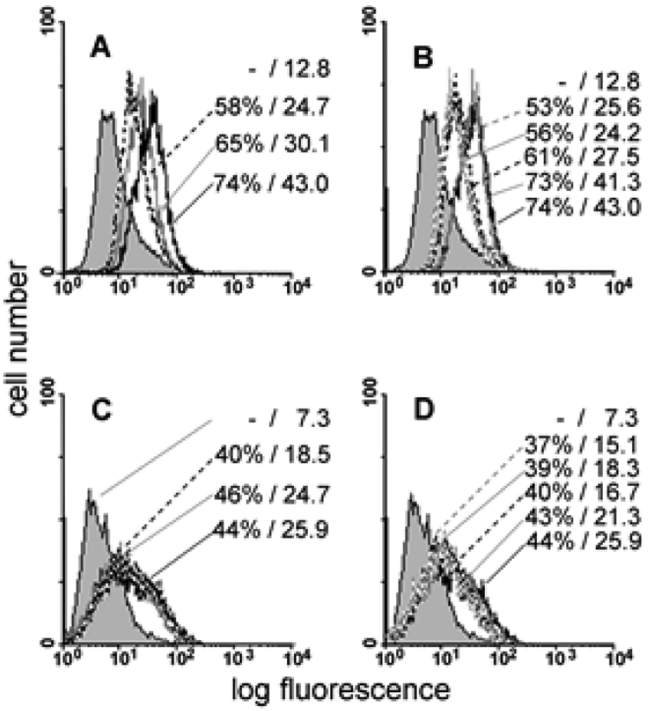

Figure 7. Semilogarithmic representation of fluorescent surface staining by the N-terminal domain of galectin-9 (A and B) or galectin-4 (C and D) using CHO wild-type cells (A and B) or human Capan-1 pancreatic adenocarcinoma cells expressing tumor suppressor $p16^{INK4a}$ (C and D). (A and B) Inhibition of binding of the N-terminal domain of galectin-9 (2 μg/mL) by test compounds used at a constant concentration of 1 mM (A, GalNAc, Lac; B, MUC1, MUC1-Thr^9, MUC1-Thr^4, MUC1-Thr^{16}). (C and D) Inhibition of binding of galectin-4 (5 μg/mL) by test compounds at a constant concentration of 1 mM (C, GalNAc, Lac; D, MUC1, MUC1-Thr^9, MUC1-Thr^4, MUC1-Thr^{16}).

DISCUSSION

Because of the complexity of a cell's glycosylation machinery, the structural and functional analysis of surface-displayed glycans remains challenging (for further information, please see refs 58 and 59). In this study, we have examined the interaction of galectin-3 with TF antigen (CD176), as it exists in its natural cellular context, presented by MUC1, as a step to characterize the molecular origin of the high selectivity of endogenous lectins for their natural counter-receptors.[60] On the basis of the ITC data, the position of the attachment, i.e., Thr (Thr^4, Thr^9, or Thr^{16}) or Ser (Ser^5) side chain, did not play a major role. However, the presence of the peptide backbone and its length significantly influenced the thermodynamic binding profile. The measured K_d values of the MUC1 glycopeptides carrying TF antigen for galectin-3 were 5−10 times lower in comparison to those of the TF disaccharide alone. This affinity increase by length extension of the peptide portion was based on a shift from a favorable enthalpy toward an entropy-driven binding interaction. HSQC titrations of the ^{15}N-labeled galectin-3 with MUC1 glycopeptide ligands revealed minor structural changes beyond the immediate contact site. Of note, on the glycopeptide side, exclusively protons of the carbohydrate moiety and Thr^9 methyl protons were affected by binding. Thus, galectin-3 binds TF presented in a MUC1 context in an entropically driven process, without involving additional peptide−galectin interactions.

The obtained data highlight that the driving force for the recognition of mucin-type *O*-glycan glycopeptides by this human lectin remains largely unknown, contrary to the molecular processes for oligosaccharides. In this particular case, the "structural" NMR data indicate that major interactions (in principle, related to enthalpy) arise from the recognition of the TF epitope by the lectin. No other major interactions are observed at either entity. However, the enthalpy gain is significantly smaller than that observed for the free TF antigen (or for LacNAc). Although at this moment speculative, this fact may suggest that the sugar moiety maintains high-level flexibility (unlikely according to the NMR data), that intramolecular stabilizing contacts between the saccharide portion and the peptide within MUC1 are lost upon galectin-3 binding, and/or that the enthalpy gain provided by water molecules to the binding process is more favorable for the small glycan than for the large glycopeptide, with compensating effects on entropy. Binding of the small ligands is penalized by entropy, usually explained by the formation of a molecular complex from two individual molecules. The rigidity of the interacting moieties, as well as some of their torsional degrees of freedom, has the same effect. For the TF antigen, this entropic penalty is not compensated by the release of water molecules to the bulk. Therefore, the process is enthalpy-driven, as it is typically for sugar−lectin interactions. In contrast, for the large glycosylated MUC1, the process, as shown by ITC here, is driven by entropy. When the complex has formed through the typical CH/π and hydrogen bond interactions, no major contacts between lectin and peptide are observed, as deduced from the NMR measurements. Despite the absence of major and long-lasting contacts between the glycopeptide and the lectin, we can assume that the two entities are relatively close in space with transient contacts and that water molecules may thus be released to the bulk, with a concomitant entropy gain. This event could probably be at the heart of this recognition process. This reasoning makes it tempting to introduce sequence derivations into the peptide part and analyze the resulting glycopeptide variants accordingly to answer the question of so far not realized special properties of the MUC1 repeat scaffold.

Other factors should also be considered. The special context of presentation of the carbohydrate ligand by the natural peptide scaffolds appears to be relevant, as is also underscored by a report that galectin-1 and -3 did not bind significantly to the TF disaccharide when presented on a glycan array.[12] The authors have suggested that the natural presentation of this glycan may be required for proper galectin recognition. In addition to glycan presentation at single sites, the density patterns of O-linked glycans, too, can have a major impact on recognition, a feature that was not tested by our panel, giving some direction for further work. Of note, the arrangement but not the quantity of GalNAc residues had been found to determine the affinity of *Vicia villosa* B4 agglutinin for undecapeptides with up to six sugar units.[61] On the basis of elegant calorimetric work with porcine submaxillary mucin and processed forms thereof, using two plant lectins as model receptors, a "jump-and-bind" mechanism with negative cooperativity has been developed.[62−64] Because galectin-3 has an inherent tendency for ligand-induced aggregation when binding to multivalent cell surfaces,[41] extending such measurements to this TF receptor is relevant. If we turn further to the galectin network, the question of whether the glycopeptide may also interact with other galectins arises.

The presented data document that TF-bearing glycopeptides can interfere with the binding of human galectins to cell surfaces, with particular activity on galectin-3 and -9. Interestingly, analysis of the interaction of GST-based fusion proteins of canine

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galectin-1, -3, -4, -7, -8, -9N, and -9C with immunopurified human MUC1 expressed in Madin Darby canine kidney cells has also revealed a preference for binding by GST-galectin-3 and -9.[65] Thus, certain members of the galectin family are capable of being receptors for this type of epitope, in a physiologically relevant setting, on the cell surface. If one focuses on the differential binding of galectins to MUC1 glycopeptides, cell association of galectin-3 and -4, which can recognize Tn clusters,[57,66,67] and the N-domain of galectin-9, reacting with the Forssmann epitope,[26] is susceptible to the presence of a glycopeptide, albeit to variable degrees. Galectin-1, as galectin-4, shows sensitivity at a low level, while the extent of galectin-8 binding was not reduced. Thus, the observed selectivity among galectins calls for a detailed elucidation of the affinity and specificity among human lectins for glycans as they exist in their natural cellular context, conjugated to a protein.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as mentioned in the text. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00555.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ASF, asialofetuin; BME, β-mercaptoethanol; CRD, carbohydrate recognition domain; EtOAc, ethyl acetate; Fmoc, fluorenylmethoxycarbonyl; Gal, galactose; GalNAc, *N*-acetylgalactosamine; HSQC, heteronuclear single-quantum coherence spectroscopy; ITC, isothermal titration calorimetry; Lac, lactose; LacNAc, *N*-acetyllactosamine; Pfp, pentafluorophenyl; TF, Thomsen-Friedenreich.

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