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Analysis of the fine specificity of Tn-binding proteins using synthetic glycopeptide epitopes and a biosensor based on surface plasmon resonance spectroscopy

Eduardo Osinaga[a,*], Sylvie Bay[b], Diana Tello[c], Alvaro Babino[a], Otto Pritsch[a], Karine Assemat[c], Daniele Cantacuzene[b], Hiroshi Nakada[d], Pedro Alzari[c]

[a] *Departamento de Bioquímica, Facultad de Medicina, Av. Gral. Flores 2125, CP 11800 Montevideo, Uruguay*
[b] *Unité de Chimie Organique, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, France*
[c] *Unité de Biochimie Structurale, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris, France*
[d] *Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, Kyoto 603, Japan*

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Abstract Using synthetic Tn (GalNAc-*O*-Ser/Thr) glycopeptide models and a biosensor based on surface plasmon resonance spectroscopy we have determined that isolectin B4 from *Vicia villosa* (VVLB4) binds to one Tn determinant whereas the anti-Tn monoclonal antibodies 83D4 and MLS128 require at least two Tn residues for recognition. When an unglycosylated amino acid is introduced between the Tn residues, both antibodies do not bind. MLS128 affinity was higher on a glycopeptide with three consecutive Tn residues. These results indicate that Tn residues organized in clusters are essential for the binding of these antibodies and indicate a different Tn recognition pattern for VVLB4.

Key words: Tn antigen; *O*-Glycosylation; Lectin; Monoclonal antibody; Surface plasmon resonance spectroscopy; Cancer

1. Introduction

Aberrant glycosylation of tumor mucins results in expression of unusual glycosidic structures and exposure of the peptide backbone [1]. The Tn determinant (GalNAcα-*O*-Ser/Thr), normally cryptic in mucin-type *O*-glycans, is a tumor-associated marker which has attracted particular interest in cancer biology: (a) it may be a useful diagnostic marker since it is poorly expressed in normal tissues but is widely expressed in a variety of adenocarcinomas [2]; (b) a direct correlation has been shown between carcinoma aggressiveness and the density of this antigen, e.g. extent of tissue spread and vessel invasion [3]; (c) Tn antigen can be recognized by the immune system [4] and thus provides a potentially important therapeutic target [5]; (d) Tn has been implicated in the metastasis of tumor cells [6]. Furthermore, Tn is found in the envelope glycoprotein gp120 of different HIV isolates where it constitutes an immunoneutralization epitope [7].

The Tn antigen was characterized using both monoclonal antibodies (mAb) [8–11] and lectins [12,13], but the specific reactivities displayed by these proteins are not identical. This raises questions about the exact molecular structure of the epitope. For example, it would be of interest to determine if these Tn-binding proteins require more complex epitopes than the GalNAc-Ser/Thr structure such as the involvement of other amino acids in the antigenic determinant, conformational elements or high density of Tn residues. It has been reported that clusters of Tn are an essential part of the epitope recognized by the mAb MLS128 (an IgG), which is poorly reactive or unreactive with glycopeptides bearing unclustered Tn structures [14]. Under different conditions of deglycosylation of Tn-rich mucins we have previously shown [11] that binding of mAb 83D4 (an IgM) to the mucin was much more affected by mild alkaline treatment than was the isolectin B4 from *Vicia villosa* (a lectin with specificity for Tn antigen).

Because of the potential involvement of Tn antigen in the metastatic process and its use as target for immunotherapy, a definition of the exact nature of the epitope is important. The notion of epitope is a functional concept and binding measurements are essential for the definition of the Tn epitope. In this paper we describe and measure the affinity constants of Tn clusters with two monoclonal antibodies, MLS128 and 83D4, as well as with the isolectin B4 from *Vicia villosa* (VVLB4) using well-defined glycopeptide models and a biosensor based on surface plasmon resonance spectroscopy (BIAcore™).

2. Materials and methods

2.1. Monoclonal antibodies and isolectin B4 from V. villosa

The mAb 83D4 (IgM) was produced from a mouse immunized with cell suspensions obtained from formalin-fixed paraffin-embedded sections of an invasive human breast carcinoma [15]. mAb was precipitated from ascitic fluids by dialysis against demineralized water at 4°C, dissolved in a small volume of 0.5 M NaCl in phosphate buffer saline (PBS), applied to a Sephacryl S-200 gel column (2.5×85 cm), and eluted with PBS. IgM was excluded from the gel and recovered in the first elution peak. The mAb MLS128 (IgG_1), established by immunizing mice with human colonic cancer cells (LS180) [16], was purified by affinity chromatography on protein A-Sepharose. The Tn-binding isolectin B4 from *V. villosa* seeds was purchased from Sigma Chemical Co., St Louis, MO, USA. The purity of the three anti-Tn proteins was demonstrated by SDS–PAGE analysis.

2.2. Synthesis of Tn glycopeptides

2.2.1. General methods. Reagents were purchased from Aldrich or Sigma. All the solvents were high grade and dry. CH_2Cl_2 was distilled over P_2O_5 before use. Glycosylated serine and threonine derivatives

*Corresponding author. Fax: (598)-2-924 95 63.
E-mail: eosinaga@lobbm.fmed.edu.uy

Abbreviations: mAb, monoclonal antibody; VVLB4, isolectin B4 from *Vicia villosa*

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[Fmoc-Ser(α-GalNAc(OAc)$_3$) and Fmoc-Thr(α-GalNAc(OAc)$_3$)] were prepared by classical methods [17,18] involving glycosylation of N^{α}-(fluoren-9-yl-methoxycarbonyl)-L-serine/threonine *tert*-butyl esters [19,20] with 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl chloride (obtained from tri-*O*-acetyl-D-galactal [21]) using AgOTf as catalyst [22]; this condensation was followed by the reduction/acetylation of the azido group in the 2-position [23]. At the end, the *t*-butyl esters were deprotected in formic acid [24]. For the peptide synthesis, Fmoc-protected amino acid derivatives and Wang resin were obtained from Bachem or Novabiochem. When unglycosylated, the side chains of the amino acids serine and threonine were protected by a *t*-butyl group and the lysine residues by a *t*-butyloxycarbonyl group. DMF and acetonitrile for HPLC were purchased from Merck. The final compounds were purified by reverse phase high performance liquid chromatography (HPLC) using a Perkin-Elmer pump system with a UV detector (230 nm). A column (250×10 mm) of Nucleosil C_{18} (5 mm, 300 Å) was used and the products were eluted with a gradient of MeCN/0.1% trifluoroacetic acid buffer during 20 min (flow rate 6 ml/min). Mass spectra were measured by electrospray on a Platform spectrometer (VG-Biotech-Micromass, Manchester, UK). Amino acid analyses were obtained using a Beckman 6300 analyzer, after hydrolysis of the peptides with 6 N HCl at 110°C in sealed glass tubes for 20 h.

2.2.2. Solid phase syntheses. The solid phase peptide and glycopeptide syntheses were performed manually using the standard Fmoc chemistry protocol on a polystyrene resin functionalized with *p*-benzyloxybenzyl alcohol (Wang resin) esterified with a glycine residue. The N^{α}-Fmoc amino acids (carrying standard side chain protective groups) and the glycosylated building blocks (3 equivalents) were incorporated to the peptide chain using TBTU/HOBT/DIEA 1/1/1.7 as an activating agent and DMF as solvent [25]. The couplings were monitored by the Kaiser test [26] and usually completed within 20 min. All Fmoc cleavages were carried out by treatment of the resin with 20% piperidine in DMF (2 min then 8 min). Following each deprotection, the resin was successively rinsed with DMF, CH_2Cl_2, DMF. At the end of the synthesis, the resin was extensively washed with DMF and CH_2Cl_2, dried, and treated with an aqueous TFA solution (95%) for 2 h. After filtration of the resin, the solution was concentrated and the crude product precipitated with diethyl ether. The precipitate was filtered, dissolved in water and lyophilized. The peptides were purified by HPLC (gradient from 10% to 40% in 20 min) and obtained with an overall yield of 25%. Finally the acetyl groups of the sugar moiety were removed by adding, dropwise, a solution of 1% MeONa (pH 10.8). After 8 h, methanol was evaporated and the medium neutralized to neutral pH. The peptides were purified by HPLC on a reverse phase column (C_{18}) using a gradient of 0.1% TFA in water (solution A) and acetonitrile (solution B), and characterized by mass spectrometry and amino acid analysis.

2.2.3. Characteristics of peptides. Tn0: Lys-(Gly)$_4$-Ser-Thr-Thr-(Gly)$_3$, elution with a gradient from 0% to 17% (retention time 8.4 min); ESMS: 834.47 (calc 834.38). Amino acid analysis: Lys 1.06 (1), Gly 6.66 (7), Ser 0.85 (1), Thr 2.

Tn1: Lys-(Gly)$_4$-Ser(α-GalNAc)-Thr-Thr-(Gly)$_3$, elution with a gradient from 0% to 17% (retention time 8.6 min); ESMS 1037.64 (calc 1037.46). Amino acid analysis: Lys 1, Gly 6.97 (7), Ser 0.87 (1), Thr 1.85 (2).

Tn2: Lys-(Gly)$_4$-Ser(α-GalNAc)-Thr(α-GalNAc)-Thr-(Gly)$_3$, elution with a gradient from 0% to 15% (retention time 8.8 min); ESMS 1240.41 (calc 1240.54). Amino acid analysis: Lys 1.07 (1), Gly 7, Ser 0.93 (1), Thr 1.90 (2).

Tn3: Lys-(Gly)$_4$-Ser(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-(Gly)$_3$, elution with a gradient from 0% to 10% (retention time 11.6 min); ESMS 1443.86 (calc 1443.62). Amino acid analysis: Lys 1, Gly 6.48 (7), Ser 0.93 (1), Thr 1.74 (2).

Tn2t: Lys-(Gly)$_4$-Ser(α-GalNAc)-Thr-Thr(α-GalNAc)-(Gly)$_3$, elution with a gradient from 0% to 8% in 10 min (retention time 9.6 min); ESMS 1240.49 (calc 1240.54). Amino acid analysis: Lys 1, Gly 6.68 (7), Ser 0.89 (1), Thr 1.85 (2).

Tn2p: Lys-(Gly)$_4$-Ser(α-GalNAc)-Pro-Thr(α-GalNAc)-(Gly)$_3$, elution with a gradient from 0% to 8% (retention time 15.2 min); ESMS 1236.80 (calc 1236.55). Amino acid analysis: Lys 1, Gly 6.88 (7), Ser 0.90 (1), Thr 0.92 (1), Pro 1.03 (1).

2.3. Surface plasmon resonance studies

The BIAcore™ biosensor (Pharmacia, Uppsala, Sweden), which uses surface plasmon resonance detection and permits real-time kinetic analysis of two interacting species [27], was used to measure the binding kinetics of Tn to the proteins. Immobilization of peptides on the sensor surface (a CM5 dextran sensor chip) was performed through lysine residue according to standard procedures described previously [28]. The dextran layer of the sensor chip was first activated by injecting 35 μl of 0.2 M *N*-ethyl-*N*-(3-diethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccinimide. Next, each peptide (30 μl) at a concentration of 2.5 mg/ml in 10 mM sodium acetate, pH 4.5, was injected. Excess reactive groups were blocked by injection of 30 μl of 1 M ethanolamine, pH 8.5. All binding analyses were conducted in HBS buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3.4 μM EDTA, 0.005% surfactant P20) at a flow rate of 5 μl/min at 25°C. The surface was regenerated with 1 M NaCl with no loss of activity as reflected by the identical binding evidenced when an equivalent sample was reintroduced in the next cycle (data not shown). Samples for binding analysis were diluted into the binding buffer (HBS). Kinetic studies were performed by injecting 20 μl of each protein, at a flow of 5 μl/min. Each anti-Tn protein was analyzed at five different concentrations (0.062, 0.125, 0.250, 0.5, and 1.0 μM). The kinetic constants for association (K_A) and dissociation (K_D) were evaluated using BIAevaluation 3.0 software supplied by the manufacturer (Pharmacia).

3. Results and discussion

Trying to better understand the molecular structure of what is called the Tn epitope, we have performed kinetic studies by surface plasmon resonance spectroscopy to evaluate the affinities between Tn and different Tn-binding proteins. In order to analyze the clustering effect of GalNAc residues *O*-linked to serine or threonine, we have synthesized three Tn glycopeptides (Tn1, Tn2 and Tn3) as well as the Tn-free peptide (Tn0) as a control. The peptide backbone sequence is made of glycine residues to minimize steric hindrance and side chain interactions. An additional N-terminal lysine residue was introduced for binding to the sensor surface. For the kinetic analysis, each Tn-binding protein (mAbs MLS128, 83D4 and lectin VVLB4) was added at five concentrations (0.062–1.0 μM) to each flow cell; association and dissociation were monitored in real time. As can be seen in the representative sensorgrams, VVLB4 bound Tn1, Tn2 and Tn3 glycopeptides (Fig. 1A), while no binding to the GalNAc-free peptide (Tn0) was observed. Using the same flow cells we found that anti-Tn mAbs MLS128 and 83D4 did not bind to the GalNAc-free

Table 1
Kinetic parameters obtained from the interaction analysis of Tn1, Tn2 and Tn3 glycopeptides and Tn-binding proteins by a biosensor

	Tn1			Tn2			Tn3		
	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_{off}/K_{on} (M)	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_{off}/K_{on} (M)	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_{off}/K_{on} (M)
VVLB4	2.5×10^{4}	5.4×10^{-3}	2.2×10^{-7}	1.4×10^{4}	4.5×10^{-3}	3.2×10^{-7}	2.7×10^{4}	3×10^{-3}	1.1×10^{-7}
83D4	–	–	–	7.6×10^{5}	7.6×10^{-3}	1×10^{-8}	2.1×10^{5}	2.5×10^{-3}	1.2×10^{-8}
MLS128	–	–	–	2.7×10^{4}	3.6×10^{-3}	1.3×10^{-7}	4.5×10^{4}	1.1×10^{-3}	2.4×10^{-8}

Each parameter was determined as described in Section 2, evaluating each protein (mAbs MLS128, 83D4 and lectin VVLB4) at five concentrations (0.062, 0.125, 0.250, 0.5, and 1.0 μM).

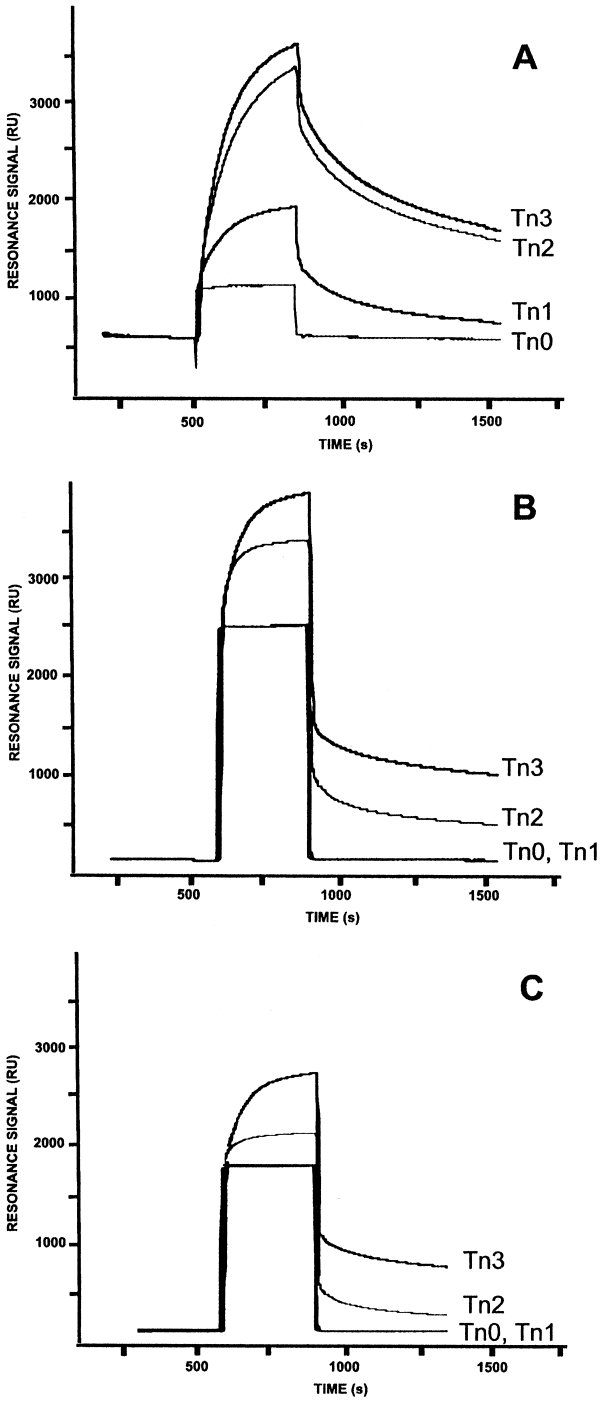

Fig. 1. Sensorgrams showing the interactions between the three Tn-binding proteins and the synthetic Tn glycopeptides. In these representative sensorgrams each protein was injected onto the surface-bound glycopeptide at 0.5 μM. A: Isolectin B4 from *V. villosa* seeds. B: mAb 83D4. C: mAb MLS128.

and the Tn1 peptides, but both did interact with the Tn2 and Tn3 peptides (Fig. 1B,C). These results indicate one Tn residue is enough for VVLB4 binding whereas the anti-Tn antibodies require at least two consecutive Tn residues for recognition.

The association, dissociation and affinity constants for the interactions between Tn glycopeptides and proteins are shown in Table 1. It appears that the VVLB4 binding does not depend on the density of the Tn determinant since the three glycopeptides Tn1, Tn2 and Tn3 show similar affinities. The mAb 83D4 recognizes both the di-Tn and the tri-Tn glycopeptides, while the mAb MLS128, although reactive with the di-Tn glycopeptide, has a higher affinity with the tri-Tn glycopeptide Tn3.

These observations suggest that a dimeric Tn cluster appears to be the 'minimum recognized structure' for MLS128 and 83D4 antibodies, and raise the question whether these monoclonal antibodies could bind to peptides where the Tn residues are not consecutive in the peptide sequence. To investigate this aspect, we synthesized glycopeptides Tn2t and Tn2p where the two Tn residues are separated by one unglycosylated amino acid. In order to evaluate the influence of the unglycosylated threonine in peptide Tn2t, proline was introduced between the two Tn units (peptide Tn2p). The results of the kinetics studies using the three Tn binding proteins are shown in the Table 2. It is observed that when an unglycosylated amino acid is introduced between the Tn residues, both MLS128 and 83D4 do not bind. This result clearly shows that two consecutive Tn structures are essential for the binding of these antibodies. By contrast, VVLB4 bound to the glycopeptides Tn2t and Tn2p with affinities similar to those observed with the glycopeptides Tn1, Tn2 and Tn3. Considering that in Tn1 glycopeptide GalNAc is *O*-linked to a serine residue, and that in both Tn2t and Tn2p glycopeptides one GalNAc is *O*-linked to serine and the other to threonine, these results indicate that the unreactivity of anti-Tn monoclonal antibodies on mono-Tn glycopeptide motifs is independent of whether GalNAc is *O*-linked to serine or threonine residues.

Our results using mAb MLS128 agree with the previous observation that this antibody preferentially binds with a cluster of three adjacent serine or threonine residues, each linked to GalNAc by a α linkage, but does not recognize mono-Tn glycopeptides [14]. Here, using synthetic model Tn glycopeptides and a biosensor based on surface plasmon resonance spectroscopy, we show that mAb MLS128 also binds glycopeptides bearing two adjacent Tn residues, although with less affinity. Furthermore, mAb 83D4 binds with similar affinity to dimeric as well as to trimeric clusters of Tn, suggesting that the fine specificity of both anti-Tn monoclonal antibodies is not the same. The clustering effect appears to be not restricted to the epitope identified by antibodies. Recently it has been reported that Tn clusters are essential for the recognition by the Tn-specific human macrophage C-type lectin, which reacts poorly with glycopeptides bearing mono-Tn motifs [29].

The clustering effect has also been reported on anti-sialyl-Tn monoclonal antibodies. Reddish et al. [30] have shown that some antibodies recognize the sialyl-Tn as an isolated structure (mAb 195.3) whereas some others need a cluster for binding (mAbs B72.3 and CC49). Recently, Tanaka et al. [31] found that a cluster composed of four sialyl-Tn antigens is the essential epitopic structure for mAb MLS132. The biological role of clustered versus single Tn or sialyl-Tn epitopes on cancer cells is still not understood. Ogata et al. [32] reported that sialyl-Tn clusters appear during malignant transformation of human colonic mucosa. The ability to synthesize monomer or cluster of Tn/sialyl-Tn structures on mucin polypeptide backbones could depend in part on the sequence of *O*-glycosylation sites of the apomucin, as well as on the repertoire and specificity of glycosyltransferases required for Tn/sialyl-Tn synthesis that exist in normal versus

Table 2
Kinetic parameters obtained from the interaction analysis of Tn2p and Tn2t glycopeptides and Tn-binding proteins by a biosensor

	Tn2p			Tn2t		
	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_{off}/K_{on} (M)	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})	K_{off}/K_{on} (M)
VVLB4	2.2×10^{4}	3×10^{-3}	2.0×10^{-7}	1.9×10^{4}	4.3×10^{-3}	2.3×10^{-7}
83D4	–	–	–	–	–	–
MLS128	–	–	–	–	–	–

Each parameter was determined as described in Section 2, evaluating each protein (mAbs MLS128, 83D4 and lectin VVLB4) at five concentrations (0.062, 0.125, 0.250, 0.5, and 1.0 μM,)

cancerous cells. The initial transfer of GalNAc to the apomucin peptide can be performed by several UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl transferases (EC 2.4.1.41) which control the *O*-linked glycosylation [33]. Studies using the MUC1 tandem repeat peptide as acceptor showed that site-specific glycosylation could regulate the addition of GalNAc to Ser/Thr at adjacent or distant positions [34], indicating that the addition of GalNAc can be affected by previous glycosylation of other sites of the peptide substrate.

The fact that Tn binding exhibited by VVLB4 is not dependent on the density of Tn determinants is in favor of different Tn recognition patterns for this lectin and the two antibodies studied, MLS128 and 83D4. These results, using model glycopeptides immobilized on a solid phase, are consistent with the reactivity observed for two plant lectins (*Salvia sclarea* and VVLB4) and mAb 83D4 using the same glycopeptides but in solution [35]. In that case also, both lectins bind similarly to Tn1, Tn2, Tn3 whereas mAb 83D4 only recognized the di- and tri-Tn glycopeptides. As part of our structural studies of Tn-binding proteins, we have constructed a single-chain Fv fragment of the anti-Tn mAb 83D4, and have demonstrated that this recombinant polypeptide retains Tn-binding properties [36]. The monoclonal antibodies directed against four sialyl-Tn and mAb 83D4 use the same V_H germline gene segment, V_HαTAG-1 [36], indicating that the heavy chain plays a predominant role in determining the antigen binding affinity. Molecular modeling of the 83D4 molecule indicated that the antibody-combining site might be primarily defined by the CDR H1 and H2 loops. The differences in specificity between anti-Tn and anti-sialyl-Tn antibodies could be explained by significant sequence variations which occur in both the light chain and the CDR H3 loop. On the other hand, we have reported the amino acid sequence and the crystal structure of the isolectin B4 from *V. villosa* seeds [37]. The amino acid residues defining the metal- and sugar-binding sites of GalNAc-specific lectins are highly conserved in the VVLB4 structure, indicating that residues outside the carbohydrate-binding pocket and/or quaternary association determinants may modulate the affinity for the Tn glycopeptide. Crystallographic studies of these Tn-binding proteins in complex with various Tn-containing molecules are in progress, and should provide invaluable information about the specific recognition of Tn determinant. Furthermore, considering that the amino acid sequence near the *O*-linked GalNAc could participate in the determinant identified by monoclonal antibodies, we are evaluating several Tn glycopeptides in order to determine the role of the peptide moiety on their Tn-binding reactivity.

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