Polyvalency of **Tn** (GalNAcα1→Ser/Thr) glycotope as a critical factor for *Vicia villosa* B_4 and glycoprotein interactions

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Abstract ***Vicia villosa*** **B_4 (VVL-B_4) is an important lectin for detecting exposed Tn (GalNAcα1-Ser/Thr) determinants on cancer cells. In order to elucidate the binding factors involved in VVL-B_4 and glycotope interaction, the binding properties of this lectin were analyzed by enzyme-linked lectinosorbent and inhibition assays. From the results, it is concluded that the most critical factor affecting VVL-B_4 binding is polyvalency at the α anomer of Gal with –NH CH_3CO at carbon-2 (Tn epitope), which enhances the reactivity by 3.3×10^5 times over monovalent Gal. The reactivities of glycotopes can be ranked as follows: high density Tn cluster ≫ Tn glycopeptides (MW < 3.0×10^3) ≫ monomeric Tn to tri- Tn glycopeptides ⋙ other GalNAcα/β-related structural units > Gal and Galα- or β-linked ligands, demonstrating the essential role of the polyvalency of Tn glycotopes in the enhancement of the binding.**

Key words: Polyvalency; Multivalent effect; Carbohydrate specificity; Glycoprotein binding; Lectin; *Vicia villosa* B_4

1. Introduction

The **Tn** determinant (GalNAcα-*O*-Ser/Thr), normally a cryptic structure in the peptide core of *O*-glycoproteins (gps), is expressed in an unmasked form in about 90% of human carcinomas [1–3]. A direct link has been shown to exist between carcinoma aggressiveness and the density of this antigen, including extent of tissue spread and vessel invasion [4]. Therefore, the **Tn** determinant (GalNAcα-*O*-Ser/Thr) has been considered to be the most specific human tumor-associated structure [4]. The **Tn** antigen has been characterized using both monoclonal antibodies and plant lectins [5–7]. Among these, a plant lectin from the seeds of *Vicia villosa* (VVL-B_4) has been recognized as one of the most useful probes. This lectin is a tetramer composed of two different subunits. Three related isolectins, composed of different amounts of the two subunits, have been purified [8]. The A_4 isolectin (composed of four A subunits) is responsible for the anti-A_1 blood group activity. The B_4 isolectin, which is composed of four B subunits, does not agglutinate normal human erythrocytes, but does agglutinate **Tn** erythrocytes. VVL-B_4 also agglutinates erythrocytes expressing the Cad determinant [9,10]. The **Tn** antigen is a terminal *O*-linked α-GalNAc residue, while the Cad determinant is an *O*-linked chain with a sialylated β-GalNAc. Many papers have described the binding properties of VVL-B_4 [11–16]. However, these studies were limited to the interaction/inhibition of GalNAc-related saccharides [11,12], **Tn** monomer to tri-**Tn** synthetic glycopeptides [13], simple sugar crystal lectin binding [16], and histochemical detection [17]. The recognition profiles with natural glycotope clusters in macromolecules and binding factors [18,19] have not been extensively examined. Therefore, we characterized the binding properties of this lectin by enzyme-linked lectinosorbent (ELLSA) and inhibition assays using our library of ligands [20]. From the results, it is concluded that five important factors are involved in VVL-B_4 binding, with the configuration of Gal being the most essential; the cluster form of Gal with –NH CH_3CO at carbon-2 (high density **Tn** cluster) enhanced the reactivity by more than 3.3×10^5 times over monovalent Gal. Apart from the **Tn** glycotope, all other mammalian glycotopes reacted weakly with VVL-B_4, and the reactivities of glycotope clusters and monomeric glycotopes can be ranked in decreasing order as: polyvalent **Tn** ≫ **Tn** glycopeptides ≫ monomeric **Tn** to tri-**Tn** glycopeptides > GalNAc ≫ Gal, thus giving an excellent example of affinity enhancement by glycotope polyvalencies relevant to carbohydrate–protein recognition processes.

2. Materials and methods

2.1. Glycoproteins

Tn and sialyl-**Tn** containing ovine, bovine, armadillo, and porcine submandibular gps were purified according to the procedure of Tettamanti and Pigman [21] and its modifications [22]. Native ASG-**Tn**, a salivary gp of the nine-banded armadillo containing only **Tn** as carbohydrate side chains, was isolated as described by the method of Wu et al. [23].

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Abbreviations: Sugars: Gal, D-galactopyranose; Glc, D-glucopyranose; D-Fuc, D-fucopyranose; L-Ara, L-arabinose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; Man, D-mannopyranose; NeuNAc/Sialic acid, *N*-acetylneuraminic acid; Glycoproteins (gp): HSM, hamster submaxillary sialyl **Tn** gp; ASG-Tn, native **Tn** glycoprotein from armadillo salivary gland; OSM, ovine submandibular glycoprotein-major; BSM, bovine submandibular glycoprotein-major; HOC, human ovarian cyst fluid; PSM, porcine salivary glycoprotein; THGP, Tamm–Horsfall glycoprotein; Lectin: VVL-B_4, **Tn**-specific lectin from seeds of *Vicia villosa*; The mammalian carbohydrate structural units in glycans used to define binding properties of VVL-B_4 and expressed in parentheses after glycoproteins are listed in Table 2; **II**, Galβ1→4GlcNAc; m**II**, poly (multi) valent **II**; ELLSA, enzyme-linked lectinosorbent assay; Reagent: TBS-T, Tris–HCl-buffered saline containing 0.05% Tween 20

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Glycophorin A, a sialyl T_α-containing gp, was prepared from the membranes of human blood group O erythrocytes [24,25]. Asialoglycophorin (T_α glycophorin) was prepared by mild acid hydrolysis [21,22]. **Tn**-glycophorin was prepared from asialo-glycophorin by Smith degradation [20,24].

ABH blood group active gps (e.g. Cyst MSS, Beach and Tighe) were prepared from human ovarian cyst fluid [26–29]. Human α_1-acid gp and fetuin, which contain multi-antennary **II** containing *N*-glycans, were purchased from Sigma Chemical (St. Louis, MO, USA).

Desialylation of sialo gps was performed by mild acid hydrolysis in 0.01 N HCl at 80°C for 90 min and dialyzed against distilled H_2O for 2 days to remove small fragments [21,22].

2.2. Sugars used for inhibition studies

Monosaccharides and oligosaccharides were purchased from Sigma Chemical and Dextra (Berkshire, UK). **Tn** glycopeptides (MW $< 3.0 \times 10^3$) and tri-antennary Galβ1→4GlcNAc glycopeptides were prepared from asialo fetuin and asialo OSM, respectively [30,31].

2.3. Lectin

VVL-B_4 was purchased from Sigma Chemical. Biotinylation of lectin was performed as described by Duk et al. [20].

2.4. Lectinochemical assays

The assay was performed according to the procedures described by

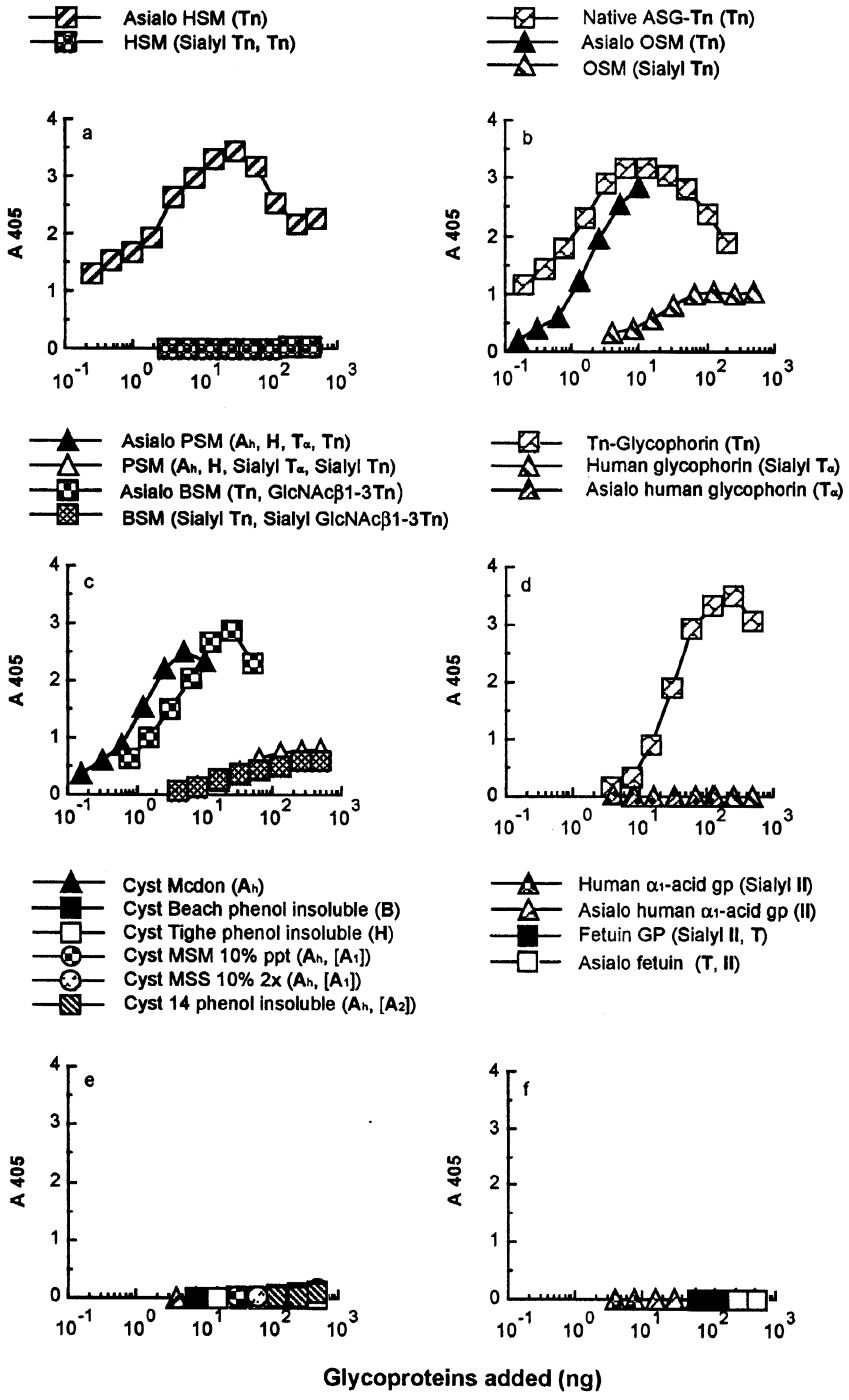

Fig. 1. Binding of VVL-B_4 to microtiter plates coated with serially diluted human blood group ABH and **Tn** active gps, sialo and asialo gps. The amount of lectin used was 5 ng/well. Total volume of the assay was 50 μl. A_{405} was recorded after 2 h incubation.

Duk et al. [20]. The volume of each reagent applied to the plate was 50 μl/well, and all incubations, except for coating, were performed at room temperature. The reagents, if not indicated otherwise, were diluted with TBS containing 0.05% Tween 20 (TBS-T). The TBS buffer or 0.15 M NaCl containing 0.05% Tween 20 was used for washing the plate between incubations. For inhibition studies, the serially diluted inhibitor samples were mixed with an equal volume of lectin solution containing a fixed amount of VVL-B_4. The control lectin sample was diluted twofold with TBS-T. After 30 min at room temperature, the samples were tested by the binding assay, as described by Duk et al. [20]. The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor (nmol/well in Table 2 and ng in Table 3) giving 50% inhibition of the control lectin binding.

3. Results

3.1. VVL-B_4–glycoform interaction

The interaction profiles of VVL-B_4 with various gps, as studied by a microtiter plate ELLSA, are illustrated in Fig. 1 and the maximum A_{405} value and the amount required for 1.5 (A_{405}) units are shown in Table 1. Among the ligands tested, VVL-B_4 bound most strongly to polyvalent **Tn** containing gps (asialo hamster, porcine, ovine, and bovine submaxillary mucin in Fig. 1a–c and native armadillo submandibular **Tn** gps in Fig. 1b) [18,19]. These reached absorbance values of over 2.5 (A_{405}) within 2 h and required less than 3.1 ng gp for 1.5 units of absorbance. VVL-B_4 also reacted well with the human **Tn**-glycophorin (Fig. 1d) but required 20.0 ng of gp to reach 1.5 units of absorbance. VVL-B_4 reacted poorly with sialylated **Tn** containing gps (OSM, PSM, BSM, HSM in Fig. 1a–c), $\mathbf{T}_\alpha$ gp (asialo human glycophorin, Fig. 1d), ABH blood group gps (Cyst Mcdon ($\mathbf{A}_h$), Cyst Beach phenol-insoluble ($\mathbf{B}_h$), Cyst Tighe phenol-insoluble (**H**); Fig. 1e) as well as native and asialo fetuin/human α_1-acid gp (m**II**). These results indicate that the unmasked polyvalent **Tn** glycotopes, but not the other mammalian structures (**I/II**, **ABH** and **T**) are the essential factors for VVL-B_4 binding.

3.2. Inhibition of VVL-B_4–glycoform interaction by mono- and oligosaccharides

The ability of various sugar ligands to inhibit the binding of VVL-B_4 with a **Tn**-containing gp (asialo OSM) as measured by ELLSA is shown in Fig. 2 and the nanomoles of ligands required for 50% inhibition of the lectin–glycan interaction are listed in Table 2. Among the monosaccharides and oligosaccharides studied, the **Tn** glycopeptide fraction ($MW < 3.0 \times 10^3$) from ovine submandibular gp was the best inhibitor. It was about 1.2×10^3, 13.3 and 3.3 times more active than Gal, GalNAc and GalNAcα1→Ser/Thr (**Tn**), respectively. This fraction was about 5–28 times more active than other GalNAc-related ligands – $\mathbf{P}_\alpha$ (GalNAcβ1-3Galα1-*O*-Me), **S** (GalNAcβ1-4Gal), **A** (GalNAcα1-3Gal), **P** (GalNAcβ1-3Gal) and **F** (GalNAcα1-3GalNAc) – while **L** (Galβ1-4Glc), **I** (Galβ1-3GlcNAc), **II** (Galβ1-4GlcNAc), **E** (Galα1-4Gal), $\mathbf{T}_\alpha$ (Galβ1-3GalNAcα1-benzyl), **T** (Galβ1-3GalNAc), **B** (Galα1-3Gal) and $\mathbf{T}_\beta$ (Galβ1-3GalNAcβ1-*O*-Me) (curves 24–31) were poor inhibitors.

3.3. Inhibition of VVL-B_4–glycoform interaction by various glycoproteins

The abilities of various gps to inhibit the binding of VVL-B_4 with asialo OSM were analyzed by ELLSA and are shown

Table 1
Binding of VVL-B_4 (5 ng/well) to human blood group A, B, H, P_1 Le^a and Le^b active gps, sialo and asialo gps by ELLSA[a]

Graph in Fig. 1	Glycoprotein (lectin determinants[b]; blood group specificity)	1.5 (A_{405}) unit (ng)	Maximum A_{405}	
			Absorbance reading[c]	Binding intensity[c]
Exposed polyvalent **Tn** gps				
a	Asialo HSM (**Tn**)	0.4	3.4	+++++
b	Native ASG-**Tn** (**Tn**)	0.5	3.2	+++++
c	Asialo PSM (**Tn**, $\mathbf{T}_\alpha$, $\mathbf{A}_h$, **H**)	1.1	2.5	+++++
b	Asialo OSM (**Tn**)	1.7	2.9	+++++
c	Asialo BSM (**Tn**, GlcNAcβ1-3 **Tn**)	3.1	2.9	+++++
d	**Tn**-glycophorin (**Tn**)	20.0	3.5	+++++
Cryptic **Tn** and other gps				
b	OSM (sialyl **Tn**, **Tn**)	–	1.0	++
c	PSM ($\mathbf{A}_h$, **H**, sialyl $\mathbf{T}_\alpha$, sialyl **Tn**)	–	0.8	+
c	BSM (sialyl **Tn**, sialyl GlcNAcβ1-3 **Tn**)	–	0.6	+
e	Cyst MSM 10% ppt ($\mathbf{A}_h$, [$\mathbf{A}_1$])	–	0.2	±
e	Cyst MSS 10% 2× ($\mathbf{A}_h$, [$\mathbf{A}_1$])	–	0.1	−
e	Cyst 14 phenol-insoluble ($\mathbf{A}_h$, [$\mathbf{A}_2$])	–	0.1	−
e	Cyst Mcdon ($\mathbf{A}_h$)	–	0.1	−
e	Cyst Beach phenol-insoluble (**B**)	–	0.1	−
a	HSM (Sialyl **Tn**, **Tn**)	–	0	−
d	Human glycophorin (sialyl $\mathbf{T}_\alpha$)	–	0	−
d	Asialo human glycophorin ($\mathbf{T}_\alpha$)	–	0	−
f	Human α_1-acid gp (sialyl **II**)	–	0	−
f	Asialo human α_1-acid gp (**II**)	–	0	−
e	Cyst Tighe phenol-insoluble (**H**)	–	0	−
f	Fetuin gp (sialyl **II**, **T**)	–	0	−
f	Asialo fetuin (**T**, **II**)	–	0	−

[a]5 ng of biotinylated lectin was added to various gp concentrations ranging from 0.001 ng to 5 μg.
[b]The symbol in parentheses indicates the human blood group activity and/or lectin determinants [19]. Expressed in bold are: **A** (GalNAcα1-3Gal); $\mathbf{A}_h$ (GalNAcα1-3[Fucα1-2]Gal); **B** (Galα1-3Gal); **H** (Fucα1-2Gal); **T** (Galβ1-3GalNAc); **Tn** (GalNAcα1-Ser/Thr); **I/II** (Galβ1-3/4GlcNAc).
[c]The results were interpreted according to the measured A_{405} after 2 h incubation as follows: +++++ ($OD \geq 2.5$), ++++ ($2.5 > OD \geq 2.0$), +++ ($2.0 > OD \geq 1.5$), ++ ($1.5 > OD \geq 1.0$), + ($1.0 > OD \geq 0.5$), ± ($0.5 > OD \geq 0.2$), and − ($OD < 0.2$).

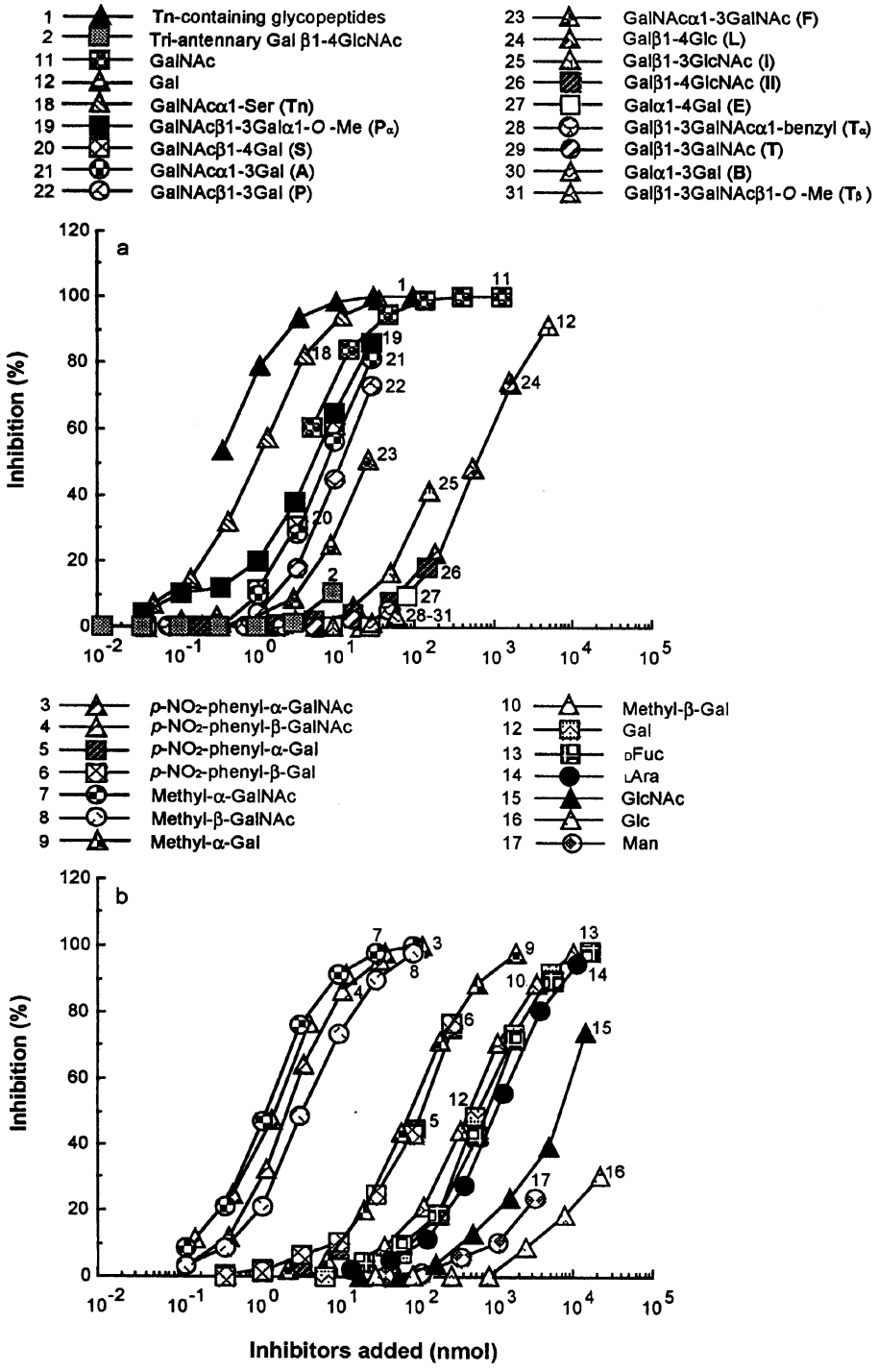

Fig. 2. Inhibition of VVL-B_4 binding to a **Tn**-containing gp (asialo OSM)-coated ELLSA plate by various saccharides. The amount of gp in the coating solution was 10.0 ng/well. The lectin (10.0 ng/well) was pre-incubated with an equal volume of serially diluted inhibitor. The final VVL-B_4 content was 5.0 ng/well. The total volume was 50 μl. A_{405} was recorded after 2 h incubation. a: Inhibition profile of mammalian structural units. b: Inhibition profile of monosaccharides and their derivatives.

in Fig. 3. The amounts of gp required for 50% inhibition are listed in Table 3. Among the gps tested for inhibition of interaction, three **Tn**-containing gps (asialo OSM, native ASG-**Tn** and asialo BSM) were the best (Fig. 3), requiring less than 0.6 ng to inhibit 50% of the interaction. They were up to 3.3×10^5, 4.4×10^3 and 1.5×10^3 times more active than Gal, GalNAc, and **Tn** monomer, respectively, while the **Tn** glycopeptide fraction ($MW < 3.0\times10^3$) was only 5.2 times more active than **Tn** monomer (curves 1–4 vs. 10 and 11). These results establish that polyvalency of **Tn** plays an important role in VVL-B_4 and gp binding. Proline-rich **Tn** gp (asialo HSM, curve 4), and a low density of **Tn** gp (curve 5, **Tn**-glycophorin, containing less than 15 **Tn** residues/mol) reduced the reactivity significantly. Apart from asialo OSM and asialo BSM, most crypto-**Tn** glycans and **I/II**-containing gps were inactive.

Table 2
Amount of various saccharides giving 50% inhibition of binding of VVL-B_4 (5 ng/50 μl) by asialo OSM (2 ng/50 μl)[a]

Curve No.	Curve in Fig. 2	Saccharide	Quantity giving 50% inhibition (nmol)	Relative potency[b]
Simple **Tn** cluster				
1	a	**Tn**-containing glycopeptides ($MW < 3.0\times10^3$)	0.3	1200.0
2	a	Tri-antennary Galβ1→4GlcNAc	>8.9 (10.0%)	–
Monosaccharides and their derivatives				
3	b	*p*-NO_2-phenyl-α-GalNAc	1.9	189.5
4	b	*p*-NO_2-phenyl-β-GalNAc	2.3	156.5
5	b	*p*-NO_2-phenyl-α-Gal	102.0	3.5
6	b	*p*-NO_2-phenyl-β-Gal	102.0	3.5
7	b	Methyl-α-GalNAc	1.2	300.0
8	b	Methyl-β-GalNAc	3.5	102.9
9	b	Methyl-α-Gal	80.0	4.5
10	b	Methyl-β-Gal	410.0	0.9
11	a	GalNAc	4.0	90.0
12	a, b	Gal	360.0	1.0
13	b	D-Fuc	700.0	0.5
14	b	L-Ara	910.0	0.4
15	b	GlcNAc	7.0×10^3	0.051
16	b	Glc	$>2.0\times10^4$ (30.0%)[c]	–
17	b	Man	$>3.0\times10^3$ (23.7%)	–
Mammalian carbohydrate structural units				
18	a	GalNAcα1-Ser (**Tn**)	1.0	360.0
19	a	GalNAcβ1-3Galα1-*O*-Me ($\mathbf{P}_\alpha$)	5.0	72.0
20	a	GalNAcβ1-4Gal (**S**)	6.0	60.0
21	a	GalNAcα1-3Gal (**A**)	8.0	45.0
22	a	GalNAcβ1-3Gal (**P**)	11.0	32.7
23	a	GalNAcα1-3GalNAc (**F**)	28.0	12.9
24	a	Galβ1-4Glc (**L**)	540.0	0.7
25	a	Galβ1-3GlcNAc (**I**)	>148.3 (41.1%)	–
26	a	Galβ1-4GlcNAc (**II**)	>145 (17.9%)	–
27	a	Galα1-4Gal (**E**)	>31.1 (9.3%)	–
28	a	Galβ1-3GalNAcα1-benzyl ($\mathbf{T}_\alpha$)	>46.9 (4.6%)	–
29	a	Galβ1-3GalNAc (**T**)	>57.8 (3.4%)	–
30	a	Galα1-3Gal (**B**)	>31.1 (1.4%)	–
31	a	Galβ1-3GalNAcβ1-*O*-Me ($\mathbf{T}_\beta$)	>27.8 (0.8%)	–

[a]The inhibitory activity was estimated from the inhibition curve in Fig. 2 and is expressed as the amount of inhibitor giving 50% inhibition. Total volume 50 μl.
[b]Relative potency = quantity of Gal (curve 12) required for 50% inhibition taken as 1.0/quantity of sample required for 50% inhibition.
[c]The inhibitory potency of inactive saccharides is expressed as the maximum amount of sugar tested that yielded inhibition (in parentheses) below 50%.

4. Discussion

During the past two decades, it has been established that many multi-branched oligosaccharides exhibit a significant increment in lectin binding reactivity as compared to their linear counterparts [32], especially 'the glycoside cluster effect' in mammalian hepatic asialo gp receptors [33,34]. Based on the results of this study, the concept of glycoside cluster effect can be classified into two groups: (a) the '*multi-antennary* or simple glycoside cluster effect' as in galactosides with hepatic lectin [33,34] and tri-antennary **II** sequences as in a galectin from chicken liver (CG-16) [35] or **Tn** glycopeptides (curve 7 in Table 3). The molecular sizes of these ligands are usually less than 1.0×10^4; and (b) the '*high-density polyvalent* or complex glycoside cluster effect', such as polyvalent **Tn** in asialo OSM which generates an enhancement in affinity with VVL-B_4 by about 3.3×10^5 times over Gal (curves 1 vs. 11 in Table 3) and is about 1000 times more active than monomeric **Tn**. A similar phenomenon was also observed with some animal lectins [36]. This peculiar effect could be explained by a remarkably extended sugar binding surface area formed by the planar array of four sugar binding sites of VVL-B_4 tetramers, based on the model of another structurally well-studied polyvalent ligand binding protein, the mammalian mannose binding protein A [37]. However, the effect of polyvalencies of glycotopes on the carbohydrate–protein binding does not always make such an important contribution. For example, the potency in the interaction of *Pseudomonas aeruginosa* II lectin with L-Fucα1 polyvalent glycans is about as strong as or weaker than the incremental increase by carbohydrate specificity of monomers [38] (unpublished data). Therefore, to obtain a solid description of the carbohydrate specificities of a lectin in order to elucidate its functional roles and biomedical applications, the following information should be given: (i) monosaccharide specificity (Gal, GalNAc, GlcNAc, and/or Man), (ii) expression of reactivities toward mammalian disaccharides and **Tn** structural units (in decreasing order) [32], (iii) the most active ligand, (iv) simple multivalent or cluster effect of carbohydrate structured unit such as **Tn** glycopeptides and multi-antennary glycotopes to inhibit binding, and (v) complex multivalent or cluster effects present in macromolecules with known glycotopes.

Based on these guidelines, we demonstrated the effects of various factors on VVL-B_4 binding as follows: (a) the configuration of Gal is essential (Table 3), (b) –NH CH_3CO at carbon-2 of GalNAc enhances the reactivity by more than 73 times (Table 3), (c) the α-anomer of GalNAc (**Tn**) is more potent than the β-anomer (GalNAcβ1→) and is the most active ligand, (d) a simple **Tn** cluster (**Tn** glycopeptide; $MW < 3.0\times10^3$; Table 3) can increase the reactivity up to

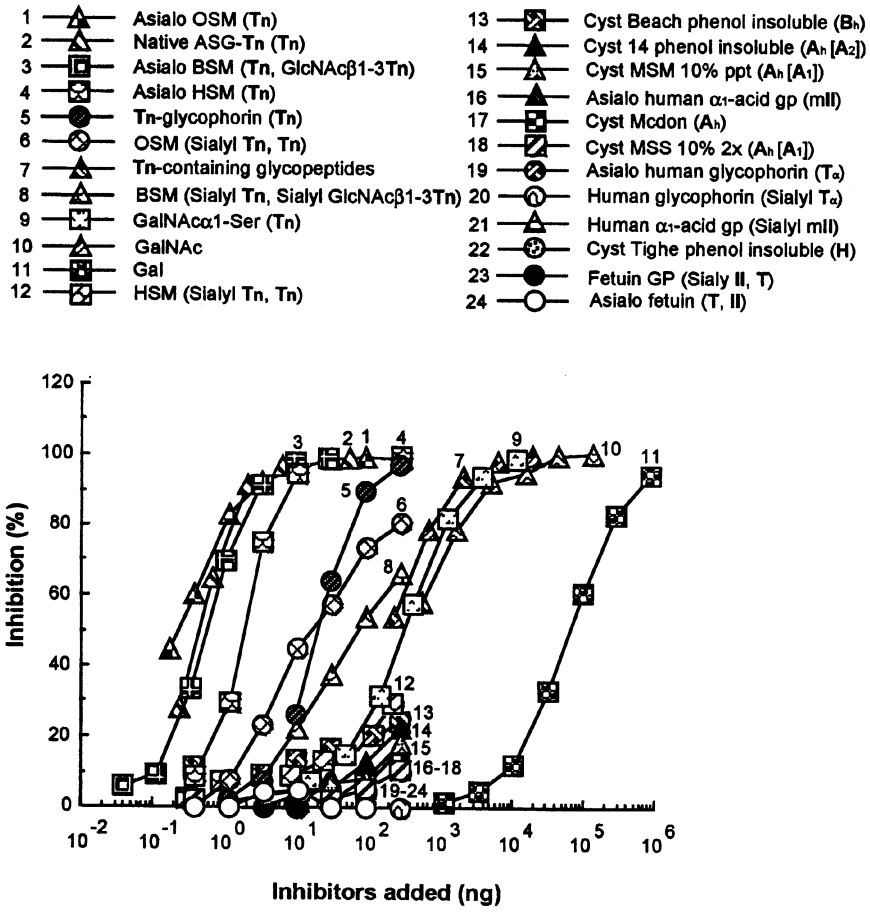

Fig. 3. Inhibition of VVL-B_4 binding to a **Tn**-containing gp (asialo OSM)-coated ELLSA plate with various gps. The quantity of gp in the coating solution was 10.0 ng/well. The quantity of lectin used for the inhibition assay was 5 ng/well. The total volume was 50 μl. A_{405} was recorded after 2 h incubation. The amount (ng) of gp required to induce 50% inhibition was determined.

1.1×10^3 times (curve 7 in Table 3), and (e) high density of **Tn** is $>5.0\times10^3$ times more active than **Tn** monomer.

In previous studies [13,39], the binding of VVL-B_4 was shown to be independent of the density of **Tn** structures. Their conclusions were based on the results of three small synthetic **Tn**s (one to three **Tn** residues) containing the glycopeptides used as inhibitors. Their results, thus, were fitting within an independent zone of the **Tn** density as compared with the known profile. In this study, the unrevealed part of VVL-B_4–gp binding was explored by using our developed method and a wider range (four to 100 residues) of **Tn** polyvalent-containing glycopeptides and gps from natural sources. As a result, the effect of the polyvalency on binding was detected. Combining the results of previous reports and this study, it is concluded that the concept of 'polyvalency of **Tn** (GalNacα1→Ser/Thr) glycotope as a critical factor for *V. villosa* B_4 and gp interactions' becomes more understandable and valuable.

It was reported that VVL-B_4 has been used to differentiate between Sd.(a^+) and Sd.(a^-) Tamm–Horsfall gps (THGP) [40]. The disaccharide structural unit (GalNAcβ1→4Gal residue) of Sd.(a^+) THGP was a poor inhibitor for VVL-B_4 binding. However, the binding forces of THGP Sd.(a^+) and VVL-B_4 interaction can be derived from the following two factors: (a) the amount of lectin added for the interaction was increased 20 times and (b) the polyvalency effect of GalNAcβ1→4Gal enhanced the reactivity. These two factors may also explain the interaction of VVL-B_4 with Cad-carrying erythrocytes [9,10]. In order to establish a reliable differential binding system, it is suggested to quantify the amount of lectin required for detecting **Tn**, Sd.(a^+), and Cad epitopes. However, this is hampered by the lack of availability of related glycans and ligands.

In summary, the binding profile of VVL-B_4 can be expressed in decreasing order as follows: high density of polyvalent **Tn** macromolecules ($MW>1.0\times10^5$) ≫ **Tn** glycopeptides ($MW<3.0\times10^3$) ≫ monomeric **Tn** to tri-**Tn** glycopeptides > GalNAc > GalNAcβ1-3Galα1-*O*-Me ($\mathbf{P}_\alpha$), GalNAcβ1-4Gal (**S**), GalNAcα1-3Gal (**A**), GalNAcβ1-3Gal (**P**), GalNAcα1-3GalNAc (**F**) > Gal ≫ Galβ1-4Glc (**L**), Galβ1-3GlcNAc (**I**), Galβ1-4GlcNAc (**II**), Galα1-4Gal (**E**), Galβ1-3GalNAcα1-benzyl ($\mathbf{T}_\alpha$), Galβ1-3GalNAc (**T**), Galα1-3Gal (**B**), Galβ1-3GalNAcβ1-*O*-Me ($\mathbf{T}_\beta$) (weak or inactive), where polyvalency of **Tn** clusters plays a critical role in binding. Glycoconjugates on the cell surface occur frequently in clustered and polyvalent forms. Among them, many weak ligands, such as the binding ability of GalNAcβ1→4Gal, can be enhanced to reach a biologically significant level by polyvalency effects [40]. Therefore, this binding profile has to be updated whenever new cluster effects on lectin binding are identified. The information obtained from this study not only facilitates understanding of VVL-B_4 interaction with nat-

Table 3
Amounts of different gps giving 50% inhibition of binding of VVL-B_4 (5 ng/50 μl) by asialo OSM (2 ng/50 μl)[a]

Curve No.	Glycoprotein/glycan	Quantity giving 50% inhibition (ng)	Relative potency[b]
1	Asialo OSM (**Tn**)	0.2	3.3×10^5
2	Native ASG-**Tn** (**Tn**)	0.4	1.6×10^5
3	Asialo BSM (**Tn**, GlcNAcβ1-3**Tn**)	0.6	1.1×10^5
4	Asialo HSM (**Tn**)	2.0	3.2×10^4
5	**Tn**-glycophorin (**Tn**)	20.0	3.2×10^3
6	OSM (sialyl **Tn**, **Tn**)	30.0	2.2×10^3
7	**Tn**-containing glycopeptides ($MW < 3.0\times10^3$)	60.9	1.1×10^3
8	BSM (sialyl **Tn**, sialyl GlcNAcβ1-3**Tn**)	100.0	648.0
9	GalNAcα1-Ser (**Tn**)	308.0	210.4
10	GalNAc	884.8	73.2
11	Gal	6.5×10^4	1.0
12	HSM (sialyl **Tn**, **Tn**)	>277.8 (29.5%)[c]	–
13	Cyst Beach phenol-insoluble (**B**)	>277.8 (24.3%)	–
14	Cyst 14 phenol-insoluble ($\mathbf{A_h}$ [$\mathbf{A_2}$])	>277.8 (22.7%)	–
15	Cyst MSM 10% ppt ($\mathbf{A_h}$ [$\mathbf{A_2}$])	>277.8 (18.2%)	–
16	Asialo human α_1-acid gp (m**II**)	>277.8 (16.3%)	–
17	Cyst Mcdon ($\mathbf{A_h}$)	>277.8 (12.3%)	–
18	Cyst MSS 10% 2× ($\mathbf{A_h}$ [$\mathbf{A_1}$])	>277.8 (10.5%)	–
19	Asialo human glycophorin ($\mathbf{T_\alpha}$)	>277.8 (4.1%)	–
20	Human glycophorin (sialyl $\mathbf{T_\alpha}$)	>277.8 (3.8%)	–
21	Human α_1-acid gp (sialyl m**II**)	>277.8 (3.1%)	–
22	Cyst Tighe phenol-insoluble (**H**)	>277.8 (0%)	–
23	Fetuin gp (sialyl **II**, **T**)	>277.8 (0%)	–
24	Asialo fetuin (**T**, **II**)	>277.8 (0%)	–

[a]The inhibitory activity was estimated from the inhibition curve in Fig. 3 and is expressed as the amount of inhibitor giving 50% inhibition. Total volume 50 μl.
[b]Relative potency = quantity of Gal (curve 11) required for 50% inhibition taken as 1.0/quantity of sample required for 50% inhibition.
[c]The inhibitory potency of inactive saccharides is expressed as the maximum amount of sugar tested that yielded inhibition (in parentheses) below 50%.

ural cellular (polyvalent) ligands, but also explains the various biological activities of VVL-B_4 with respect to lectin–receptor recognition processes. These special VVL-B_4 binding features also support the concept that every lectin with a unique amino acid sequence has its own binding characteristics [19].

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