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Further characterization of the combining sites of *Bandeiraea (Griffonia) simplicifolia* lectin-I, isolectin A_4

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***Bandeiraea (Griffonia) simplicifolia* lectin-I, isolectin A_4 (GS I-A_4), which is cytotoxic to the human colon cancer cell lines, is one of two lectin families derived from its seed extract. It contains only a homo-oligomer of subunit A, and is most specific for GalNAcα1→. In order to elucidate the GS I-A_4-glycoconjugate interactions in greater detail, the combining site of this lectin was further characterized by enzyme linked lectino-sorbent assay (ELLSA) and by inhibition of lectin-glycoprotein interactions. This study has demonstrated that the Tn-containing glycoproteins tested, consisting of mammalian salivary glycoproteins (armadillo, asialo-hamster sublingual, asialo-ovine, -bovine, and -porcine submandibular), are bound strongly by GS I-A_4. Among monovalent inhibitors so far tested, p-NO2-phenylα GalNAc is the most potent, suggesting that hydrophobic forces are important in the interaction of this lectin. GS I-A_4 is able to accommodate the monosaccharide GalNAc at the nonreducing end of oligosaccharides. This suggests that the combining site of the lectin is a shallow cavity. Among oligosaccharides and monosaccharides tested as inhibitors of the binding of GS I-A_4, the hierarchy of potencies are: GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glc (Forssman pentasaccharide) > GalNAcα1→3(LFucα1→2)Gal (blood group A) > GalNAc > Galα1→4Gal > Galα1→3Gal (blood group B-like) > Gal.**

Key words: carbohydrate specificities/*Bandeiraea (Griffonia) simplicifolia* lectin-I isolectin A_4, (GS I-A_4)/glycoprotein binding/lectins

Introduction

Bandeiraea (Griffonia) simplicifolia lectin-I (GS I) is one of two lectin families that is extracted from the seeds of *Bandeiraea (Griffonia) simplicifolia* (GS) (Mäkelä, 1957). It agglutinates human blood group A and B cells and is specific for GalNAcα1→ and Galα1→ at different strengths (Hayes and Goldstein, 1974). The GS I family is a glycoprotein of MW 114,000 consisting of four subunits (Hayes and Goldstein, 1974). This family is composed of five tetrameric isolectins with different binding specificities, which result from the combination of two different glycoprotein (A and B) subunits (A_4, A_3B, A_2B_2, AB_3, and B_4) (Murphy and Goldstein, 1977). GS I-A_4 contains only A subunits, and is specific for GalNAc, but also reacts with Galα1→ containing glycoproteins (Wood *et al.*, 1979). This lectin is cytotoxic to the human colon cancer cell lines LS174t and SW1116 (Chen *et al.*, 1994) and has affinity for almost all human ovarian cyst blood group A active glycoproteins (Wood, 1979; Wu *et al.*, 1996). However, our knowledge related to the binding profiles of GS I-A_4 to mammalian structural units (Wu and Sugii, 1988, 1991; Wu *et al.*, 1992a, 1996; Chen *et al.*, 1998) is very limited, especially the relative reactivities of I-A_4 to Forssman and Tn related ligands. In order to understand better the biological roles of GS I-A_4 and for it to be useful as an investigative tool for biochemical and immunochemical studies, it is important to establish its fine binding properties. In the present communication, we characterized the combining sites of GS I-A_4 by enzyme linked lectino-sorbent assay (ELLSA) and by inhibition of lectin binding by ELLSA, using glycoconjugates that have been used in our laboratory to group lectins for over a decade (Wu and Sugii, 1988; Wu *et al.*, 1992a, 1997a,b; Chen *et al.*, 1998). The results showed that GS I-A_4 has strong affinity for GalNAc located at both the **Tn** determinant and at nonreducing ends (as Forssman pentasaccharide, GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glc and human blood group A active trisaccharide, GalNAcα1→3[LFucα1→ 2]Gal).

Results

GS I-A_4-glycoform interaction

The interaction patterns of GS I-A_4 with glycoproteins, as studied by a microtiter plate lectin-enzyme binding assay (ELLSA), are shown in Figure 1 and its binding profile for glycoproteins is illustrated in Table I. The binding data are expressed as ng glycoprotein required for binding at 1.5 A_{405} and as maximum A_{405} absorbance after 2 h incubation. Among 18 glycoproteins tested, GS I-A_4 reacted best with the mammalian salivary **Tn** (GalNAcα1→Ser/Thr) containing glycoproteins. These include armadillo submandibular **Tn** glycoprotein (native ASG-**Tn**, Figure 1b), asialo hamster sublingual mucin

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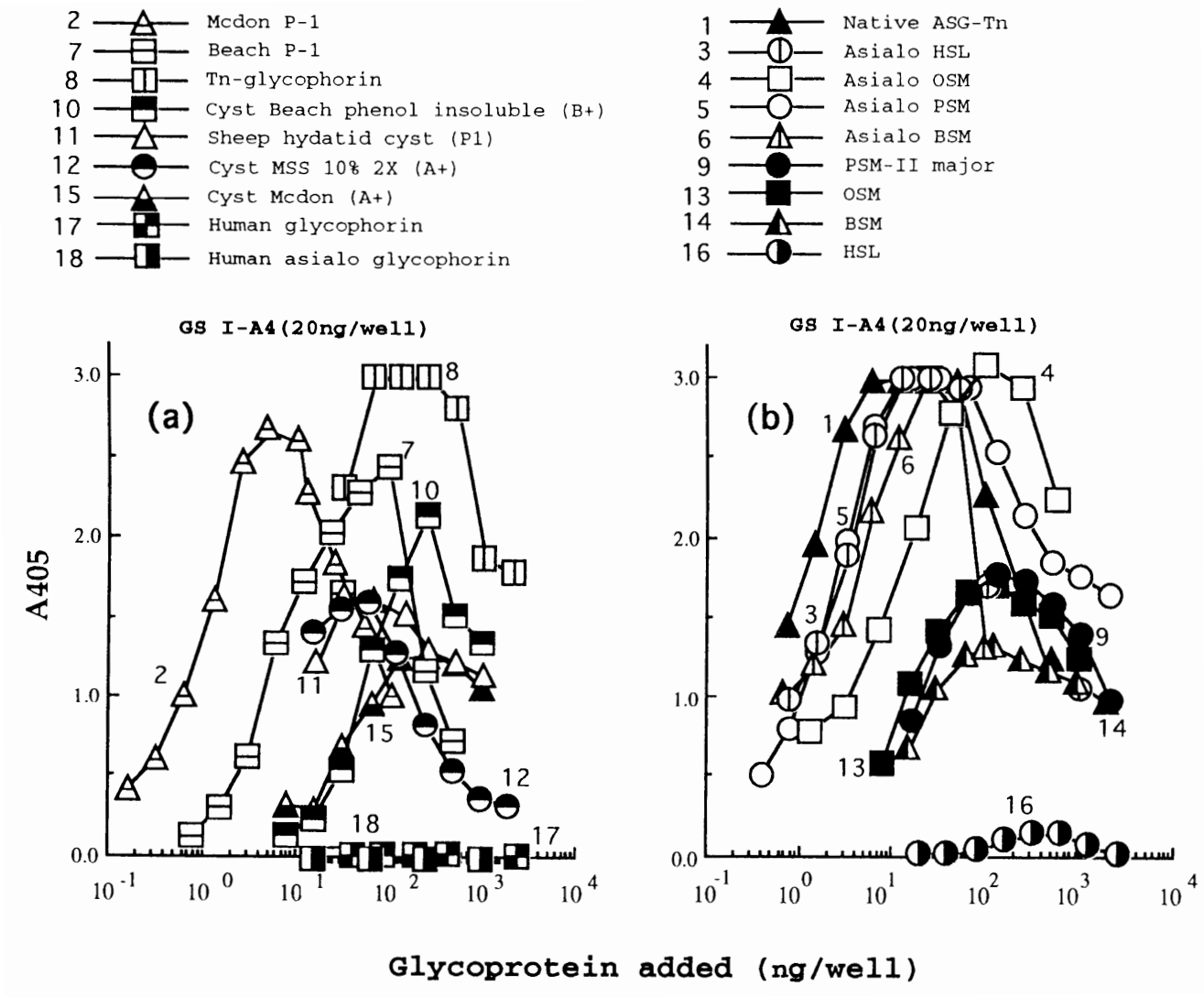

Fig. 1. Binding of GS I-A_4 to microtiter plates coated with serially diluted glycoproteins. The lectin (GS I-A_4) was used at a constant amount of 20 ng/well. Total volume 50 µl. A_{405} was read at 2 h.

(asialo HSL, Figure 1b), asialo ovine (asialo OSM, Figure 1b), bovine (asialo BSM, Figure 1b), porcine (asialo PSM, Figure 1b) submandibular glycoproteins, and a human blood group ABO precursor equivalent glycoprotein from human ovarian cyst fluid (Mcdon P-1, Figure 1a), in which less than 3.1 ng of the glycoproteins coated was required to interact with 20 ng of lectin to yield a A_{405} of 1.5 within 2 h. Although the percentage of absorbance of the glycoproteins in the microtiter plate assay has not been established, the amount of glycoprotein required to give 1.5 (A_{405}) units with this lectin has to be equal to or less than 3.1 ng. This lectin also bound to a human blood group P_1 active glycoprotein purified from sheep hydatid cyst fluid (Figure 1a), human blood group A or B active glycoproteins (cyst MSS, cyst Mcdon, and cyst Beach phenol insoluble in Figure 1a and Table I), human **Tn** glycophorin (Figure 1a), sialylated **Tn** containing gps (PSM-major and OSM-major; Figure 1b) and other blood group precursor equivalent gp (Beach P-1, Figure 1a), but was inactive with both native and asialo human glycophorin and hamster sublingual glycoprotein.

Inhibition of GS I-A_4–glycoform interaction by various glycoproteins

The ability of various glycoproteins to inhibit the binding of GS I-A_4 with asialo ovine salivary glycoprotein by ELLSA was also analyzed. The amounts of glycoprotein (nanogram) required for 50% inhibition are shown in Figure 2 and Table II.

Among the glycoproteins tested for inhibition of interaction, Mcdon P-1, a mild acid hydrolyzed glycoprotein (cyst Mcdon) prepared from human ovarian cyst fluid (curve 1 in Figure 2a) (Wu and Sugii, 1988), asialo-OSM, -BSM, and -PSM (curves 2, 3 and 3 in Figure 2b), ASG-Tn (curve 4 in Figure 2a) and asialo HSL (curve 5 in Figure 2b) were the best which required less than 3.1 ng to inhibit 50% of the interaction. All of these **Tn** glycoproteins were more than ten times more active than their native or sialylated compounds. The decreasing order of the reactivities of these glycoforms is Mcdon P-1 (precursor equivalent glycoprotein, curve 1), salivary **Tn**-glycoproteins (curves 2–5) ≥ human blood group P_1 active gp (sheep hydatid cyst gp, curve 6) > cyst MSS 10% 2× (a human blood group A active gp,

Table I. Binding of GS I-A_4 lectin to human blood group A, B, H, and P_1 active glycoproteins(gps), sialo- and asialo glycoproteins analyzed by ELLSA[a]

Curve no.	a,b in Fig. 1	Glycoprotein[b]	1.5 (A_{405}) unit (ng)	Max. A_{405} absorbance	
				Reading	Intensity[c]
1	b	Native ASG-Tn (**Tn**)	0.8	3.0	+++++
2	a	Mcdon P-1 (**Tn,T,I,II**)	1.1	2.6	+++++
3	b	Asialo HSL (**Tn**)	2.0	3.0	+++++
4	b	Asialo OSM (**Tn**)	2.0	3.0	+++++
5	b	Asialo PSM (**Tn,A,Ah,T**)	2.0	3.0	+++++
6	b	Asialo BSM (**Tn**)	3.1	3.0	+++++
7	a	Beach P-1 (**Tn,T,I,II**)	9.5	3.0	+++++
8	a	Tn-glycophorin (**Tn**)	18.0	3.0	+++++
9	b	PSM (sialyl **Tn,A,Ah,T**)	19.5	1.76	+++
10	a	Cyst beach phenol insoluble (**B**)	20.0	2.1	++++
11	a	Sheep hydatid cyst gp (**E**[P1])	26.0	1.70	+++
12	a	Cyst MSS 10% 2× (**A_h**[A1])	28.0	1.57	+++
13	b	OSM (sialyl **Tn**)	40.0	1.71	+++
14	b	BSM (sialyl **Tn**)	—	1.31	++
15	a	Cyst Mcdon (**A_h**)	—	1.24	++
16	b	HSL (Sialyl **Tn**)	—	0.15	—
17	a	Human glycophorin (Sialyl **T**)	—	0	—
18	a	Human asialo glycophorin (**T**)	—	0	—

[a]20 ng of biotinylated GS I-A_4 was added to various concentrations of glycoprotein ranging from 0.031 ng to 10 μg..

[b]Lectin determinants in parentheses; blood group specificity in brackets.The symbols in parentheses indicate the human blood group activity and/or lectin determinants (Wu and Sugii, 1988, 1991; Wu *et al*., 1997a) are expressed in bold: **F**, GalNAcα1→3GalNAc; **A**, GalNAcα1→3Gal; **A_h**, GalNAcα1→3[LFucα1→2]Gal; **B**, Galα1→3Gal; **E**, Galα1→4Gal; **T**, Galβ1→3GalNAc; **Tn**, GalNAcα1→Ser/Thr; **I/II**, Galβ1→3/4GlcNAc.

[c]The results were recorded according to the spectrophotometric absorbance value at 405 nm (i.e., A_{405}) after two hrs incubation and expressed by both ng glycoproteins required at 1.5 A_{405} and maximum A_{405} absorbance as follows: +++++ (O.D. ≥ 2.5), ++++ (O.D.: 2.5–2.0), +++ (O.D.: 2.0–1.5), ++ (O.D.: 1.5–0.75), + (O.D.: 0.75–0.2), and - (O.D.: < =0.2).

curve 7) > sialylated **Tn** glycoproteins (BSM and PSM, curve 8); precursor equivalent gp (cyst Beach P-1, curve 9) > human **Tn** glycophorin and OSM (curves 10 and 11) >> cyst Mcdon and HSL (blood group $\mathbf{A}^+$ and sialylated **Tn** gp from sublingual gland, (curves 12 and 13) >> cyst Beach phenol insoluble ($\mathbf{B}^+$) and **Tn** glycopeptide mixture (curves 14 and 15).

Inhibition of GS I-A_4–glycoform interaction by mono- and oligosaccharides

The ability of various sugar inhibitors to inhibit the binding of GS I-A_4 with asialo ovine salivary glycoprotein by ELLSA is shown in Figure 3 and the nanomoles of ligands required for 50% inhibition of the lectin–glycan interaction are shown in Table III.

Among the monosaccharides and oligosaccharides studied, p-NO_2-phenyl αGalNAc (curve 1 in Figure 3) was the best inhibitor, and 7.7 times more active than the Forssman pentasaccharide (**F_p**, GalNAcα1 →3GalNAcβ1→3Galα1→4Galβ1→4Glc; curve 2 in Figure 3b). It was 15.3 and 115.4 times more active than its β anomer (curve 6) and Gal (curve 14 in Figure 3), respectively, while p-NO_2-phenyl-β-GalNAc and GalNAc were equally active. p-NO_2-phenyl derivatives of Gal were about four times more active than the corresponding methyl derivatives (curve 3, 8, 13, and 16 in Figure 3a). Melibiose (Galα1→6Glc, curve 7) was slightly less active than GalNAc and about 4.2 times more active than Gal (curve 14), and two times more active than raffinose (Galα1→6Glcβ1→2DFru, curve 11) and stachyose (Galα1→6Galα1→6Glcβ1→2DFru, curve 12).

Among the mammalian oligosaccharides tested for inhibition of interaction, the Forssman pentasaccharide (**F_p**, GalNAcα1→3GalNAcβ1 →3Galα1→4Galβ1→4Glc, curve 2 in Figure 3b) was the best—it was 1.7 and 2.0 times more active than the human blood group A trisaccharide (GalNAcα1→3[LFucα1→2]Gal, **A_h**, curve 4 in Figure 3b) and GalNAc (curve 5), respectively, while GalNAc (curve 5) was 2.0 and 3.8 times more active than Galα1→4Gal (**E**, curve 9) and Galα1→3Gal (**B**, curve 10), respectively. Gal (curve 14), which was 7.5 times less active than GalNAc, was 2 and 3 times more active than Galβ1→3GlcNAc (**I**, curve 15) and Galβ1→3GalNAc (**T**, curve 17), respectively, while Galβ1→4Glc (**L**, curve 18), Galβ1→4GlcNAc (**II**) and Glc were poor inhibitors or inactive.

Discussion

Previous studies on the combining sites of *Bandeiraea (Griffonia) simplicifolia* lectin-I, isolectin A_4, (GS I-A_4) indicated that

Table II. Amount of various glycoproteins and Tn containing glycopeptide giving 50% inhibition of binding of GSI-A_4 (20 ng/50µl) by asialo OSM (10 ng/50µl)[a]

Curve	a,b in Fig. 3	Inhibitor	Lectin determinants[c]	Quant. giving 50% inhib.(ng)	Rel. potency
1	a	Mcdon P-1	**Tn**,**T**,**I**,**II**	1.5	1.00
2	b	Asialo OSM	**Tn**	1.6	1.07
3	b	Asialo BSM	**Tn**	1.7	1.13
3	b	Asialo PSM	**Tn**,**A**,$\mathbf{A_h}$,**T**	1.7	1.13
4	a	Native ASG-Tn	**Tn**	2.0	1.33
5	b	Asialo HSL	**Tn**	3.1	2.07
6	a	Sheep hydatid cyst gp	**E**[P_1]	5.5	3.67
7	a	Cyst MSS 10% 2×	$\mathbf{A_h}$[A_1]	10.0	6.67
8	b	BSM	Sialyl **Tn**	20.0	13.33
8	b	PSM	Sialyl **Tn**,**A**,$\mathbf{A_h}$,**T**	20.0	13.33
9	a	Beach P-1	**Tn**,**T**,**I**,**II**	28.0	18.67
10	a	Tn-glycophorin	**Tn**	38.0	25.33
11	b	OSM	Sialyl **Tn**	40.0	26.67
12	a	Cyst Mcdon	$\mathbf{A_h}$	130.0	86.67
13	b	HSL	Sialyl **Tn**	150.0	100.00
14	b	Cyst Beach phenol insoluble	$\mathbf{B}^+$	690.0	460.00
15	b	Tn glycopeptides (Tn mixture)	**Tn**	690.0	460.00
16	a	Human glycophorin	Sialyl **T**	277.8 (6% inhibition)	
17	a	Human asialo glycophorin	**T**	277.8 (5% inhibition)	

[a]The inhibitory activity was estimated from the inhibition curve in **Figure 2** and is expressed as the amount (ng) of inhibitor giving 50% inhibition of the control lectin binding. Total volume 50µl.
[b]Relative potency of glycoproteins when Mcdon P-1 was taken as 1.0.
[c]Blood group specificity.

GS I-A_4 has affinity for **A**(GalNAcα1→3Gal), **Tn**(GalNAcα1→Ser/Thr) > **B**(Galα1→3Gal), and **E**(Galα1→4Gal) (Wood, 1979; Wu *et al.*, 1996). In this report, we further studied the binding reactivity of GS I-A_4 using our recently established ELLSA method, in which the lectin was biotinylated and binding was detected with alkaline phosphatase–conjugated avidin (Duk *et al.*, 1994; Lisowska *et al.*, 1996). Since it is difficult to quantitate the amounts of the glycoproteins absorbed onto the microwells, the interactions of GS I-A_4 with various glycoproteins were examined by three parameters: (1) Amounts of the glycoproteins added to wells that gave 1.5 (A_{405}) (Table I) units; (2) The maximum O.D. for each glycoprotein after 2 h of incubation (Table I); (3) The amount of the glycoproteins required to give 50 % inhibition (Table II). The structures of the sugar chains of the mucins tested have not all been established. Certain conclusions can nevertheless be made.

When the interactions of three parameters of GS I-A4 were compared (Figure 4), it is clearly shown that these salivary **Tn** containing glycoproteins containing clusters of **Tn** residues are most strongly bound by GS I-A4. Mcdon P-1, which is the nondialyzable fraction of a human blood group A active glycoprotein prepared from ovarian cyst fluid (cyst Mcdon, Figure 1a and curve 1 in Figure 2) after mild acid hydrolysis, is also among the those most strongly bound ones. Thus, it can be assumed that the high reactivity of this glycoprotein is due to the exposure of a large number of **Tn** structures following mild acid hydrolysis.

Based on the data of the three parameters, variation of different reactivities were found in some glycoproteins. Human blood group **A** (cyst MSS and cyst Mcdon), **B** (cyst Beach) and $\mathbf{P_1}$ (sheep hydatid gp) active glycoproteins, and sialylated gps (BSM, PSM, OSM, and HSL) showed relatively poor binding (Figure 4a,b), but were active in the inhibition assay (Figure 4a). This can be explained by different absorption (onto the microwell) properties among glycoproteins, especially the sialylated gps.

From the results of both binding and inhibition assays, it is shown that all of the human blood group A or B active glycoproteins (curves 10, 12, and 15 in Figure 1, and curve 7, 12, and 14 in Figure 2) were less active than the mammalian **Tn** containing gps indicating that this lectin favors **Tn** clusters (curves 1–6 in Figure 1 and curves 1–5 in Figure 2).

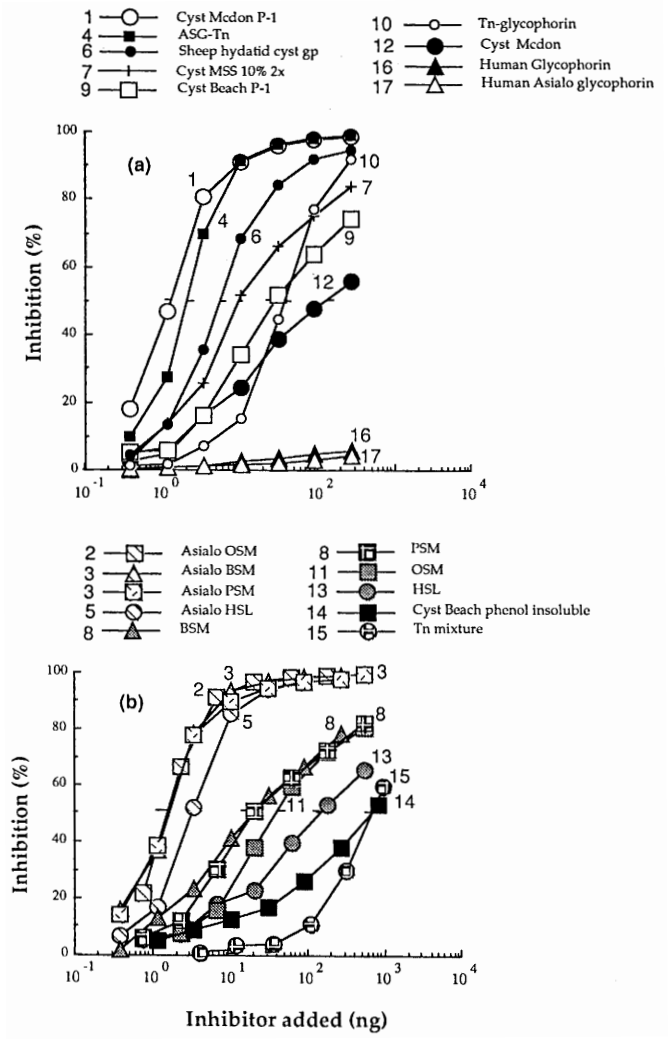

Fig. 2. Inhibition of GS I-A_4 binding to asialo OSM-coated ELLSA plates with various glycoproteins and a mixture Tn containing glycopeptides. The quantity of asialo OSM in the coating solution was 10 ng per well. The amount of lectin used for inhibition assay was 20 ng per well. Total volume: 50 µl. A_{405} was read at 2 h.

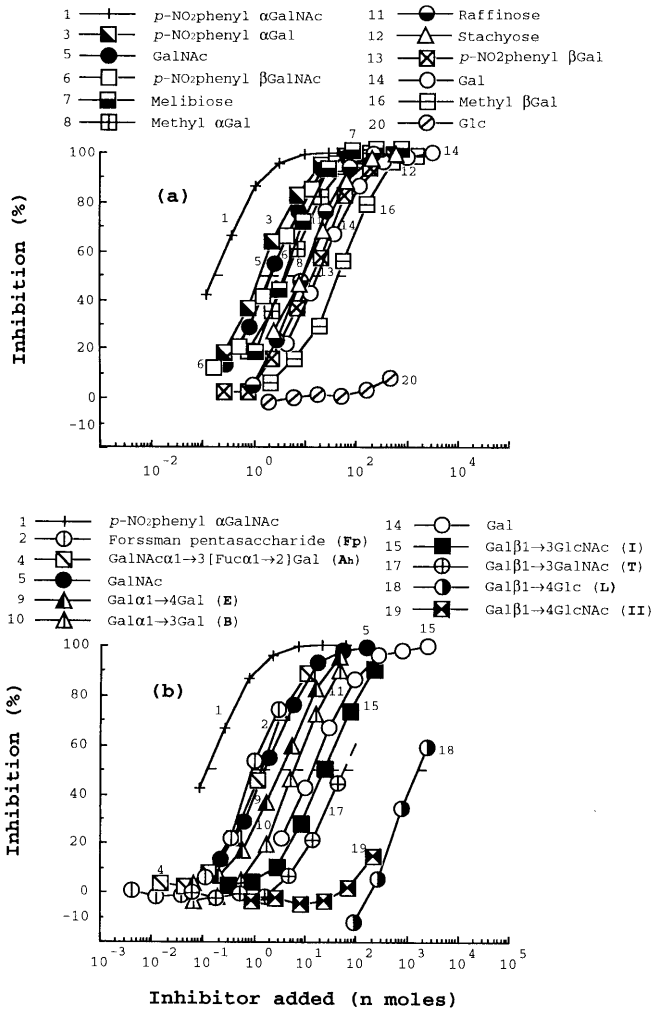

Fig. 3. Inhibition of GS I-A_4 binding to asialo OSM-coated ELLSA plates with monosaccharides, and oligosaccharides. The quantity of asialo OSM in the coating solution was 0.6 ng per well. The quantity of lectin used was 20 ng per well. Total volume: 50 µl. A_{405} was read at 2 h.

Although human **Tn**-glycophorin also reacted well with GS I-A4, its reactivity was about 12–25 times weaker than that of the other **Tn**-containing glycoproteins (curves 1–6 in Figure 2). This suggests that **Tn**-glycophorin contains fewer **Tn** residues (about nine residues) than those of secretory type mucins (Lisowska, 1988; Wu *et al.*, 1994a–c).

Based on the inhibition profile, **Tn**-glycophorin was at least seven times more active than native glycophorin and asialoglycophorin (cure 10, 15, and 16 in Figure 2) implying that the binding of **Tn**-glycophorin can be abolished by substitution of Gal at carbon 3 and of sialic acid at carbon 6 (Table II).

Tn glycopeptides (curve 2 and 14 in Figure 2) from asialo OSM were 430 times less active than the original molecule, showing that the size of **Tn** cluster is an important factor affecting binding.

From the inhibition experiments with defined oligosaccharides, the following conclusion were reached: Forssman pentasaccharide (GalNAcα1→ 3GalNAcβ1→3Galα1→4Galβ1→4Glc, **Fp**, curve 2 in Figure 3b), and GalNAcα1→3[LFucα1→2]Gal (**A_h**, curve 4 in Figure 3b), are the most potent ligands so far identified for GS I-A_4. When the inhibitory reactivities of GS I-A_4-**Tn** glycoprotein binding by mono- and oligo-saccharide were compared, it was shown that p-NO_2-phenyl αGalNAc is the most potent inhibitor. Furthermore, the p-NO_2-phenyl derivatives of GalNAc and Gal were more active than the corresponding methyl derivatives (curves 3, 8, 13, and 16 in Figure 3a; Wood *et al.*, 1979). Thus, it can be deduced that hydrophobic forces are important for binding (Table III). As GS I-A_4 is able to accommodate the monosaccharide at the nonreducing end of an oligosaccharide, the combining site of this lectin is likely to be a shallow cavity (Table III). From these and previous data (Wood *et al.*, 1979; Wu *et al.*, 1996), the binding activities of this lectin toward mammalian structural features can be ranked in decreasing order as follows: **F**, **Fp**, **A** > **Ah** > GalNAc > **E** > **B** > Gal.

The **Tn** determinant is the simplest carbohydrate side chain, in which GalNAcα1→ is linked to the Ser/Thr of the protein core. It has been proposed as a marker for cancerous tissues

Table III. Amount of various saccharides giving 50% inhibition of binding of GSI-A_4 (20 ng/50 μl) by asialo OSM (0.6 ng/50μl)[a]

Curve	a,b in Fig. 3	Inhibitor	Quant. giving 50% inhib. (nmol)	Reciprocal of rel.potency[b]
1	a,b	*p*-NO_2phenyl αGalNAc	0.13	115.4
2	b	GalNAcα1→3GalNAcβ1→3		
		Galα1→4Galβ1→4Glc (**Fp**)	1.0	15.0
3	a	*p*-NO_2phenyl αGal	1.0	15.0
4	b	GalNAcα1→3[Fucα1→2]	1.7	8.8
		Gal (**A_h**)		
5	a,b	GalNAc	2.0	7.5
6	a	*p*-NO_2phenyl βGalNAc	2.0	7.5
7	a	Melibiose	3.6	4.2
8	a	Methyl αGal	4.0	3.8
9	b	Galα1→4Gal (**E**)	4.0	3.8
10	b	Galα1→3Gal (**B**)	7.0	2.1
11	a	Raffinose	7.1	2.1
12	a	Stachyose	7.1	2.1
13	a	*p*-NO_2phenyl βGal	10.0	1.5
14	a,b	Gal	15.0	1.0
15	b	Galβ1→3GlcNAc (**I**)	30.0	0.5
16	a	Methyl βGal	39.0	0.4
17	b	Galβ1→3GalNAc (**T**)	60.0	0.25
18	b	Galβ1→4Glc (**L**)	1900.0	7.9×10^{-3}
19	b	Galβ1→4GlcNAc (**II**)	>217.3	
			(15.0% inhibition)	
20	a	Glc	>376.7	
			(9.0% inhibition)	

[a]The inhibitory activity was estimated from the inhibition curve in **Figure 3** and is expressed as the amount of inhibitor giving 50% inhibition of the control lectin binding. Total volume 50 μl.
[b]Reciprocal of relative potency of sugars when Gal was taken as 1.0 (Wu *et al.*, 1992a).

(Springer, 1984; Hirohashi *et al.*, 1985; Kjeldsen *et al.*, 1988; Itzkowitz *et al.*, 1989, 1990). At the surface of the red cell membrane, the **Tn** transformation indicates an acquired disorder characterized by the exposure of normally cryptic GalNAc residues linked α→ to the hydroxyl group of Ser or Thr of membrane sialoglycoproteins (Dahr *et al.*, 1974; Cartron *et al.*, 1978). The **Tn** antigen can also be detected at the cell surface of erythrocytes, granulocytes, platelets, and B and T lymphocytes of patients presenting the **Tn** syndrome.

The Forssman antigen (GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→ 1′ceramide) is a commonly occurring heterophile antigen and it is thought that it is not present in most humans. It has been demonstrated that the Forssman antigen is found significantly in several forms of human cancer, including gastric, colon, and lung cancer (Hakomori *et al.*, 1977; Yoda *et al.*, 1980; Taniguchi *et al.*, 1981; Hakomori, 1984, 1989). This antigen is one of the tumor-associated glycolipid antigens with blood group A-like epitopes. Since the end terminal of the antigen shares a sugar residue, GalNAcα1→, with the blood group A terminal saccharide as well as Tn antigen, the unusual enhancement of activity of the blood group A-like antigen has been strongly associated with carcinogenesis. It also indicates that an assay for this antigen in tissue sections and in circulating plasma would be of value to detect colon cancer (Ono *et al.*, 1994).

The Galα1→4Gal sequence (**E**, galabiose), which is the isomer of the blood group B active disaccharide (Galα1→3Gal), is frequently found in the carbohydrate chains of many glycosphingolipids located at the surface of mammalian cell membranes, such as intestinal and red blood cells; it is a receptor for the uropathogenic E.coli ligand and for toxin attachment (Bock *et al.*, 1988; Karlsson, 1989; Wu *et al.*, 1992b, 1995a). As shown in Figure 3, these two isomers were about one-fourth as active as **Fp**. However, the reactivity of the cluster form of Galα1→4Gal, as in sheep hydatid cyst gp (curve 6 in Figure 2), was more active than the blood group A active gp (curve 7 and 12). Therefore, the cluster effect of Galα1→4Gal sequence in glycoproteins may also be an important factor influencing binding. To understand the role of attachment of GS I-A_4 onto cancer cells, the binding of Galα1→4Gal containing glycosphingolipids has to be investigated.

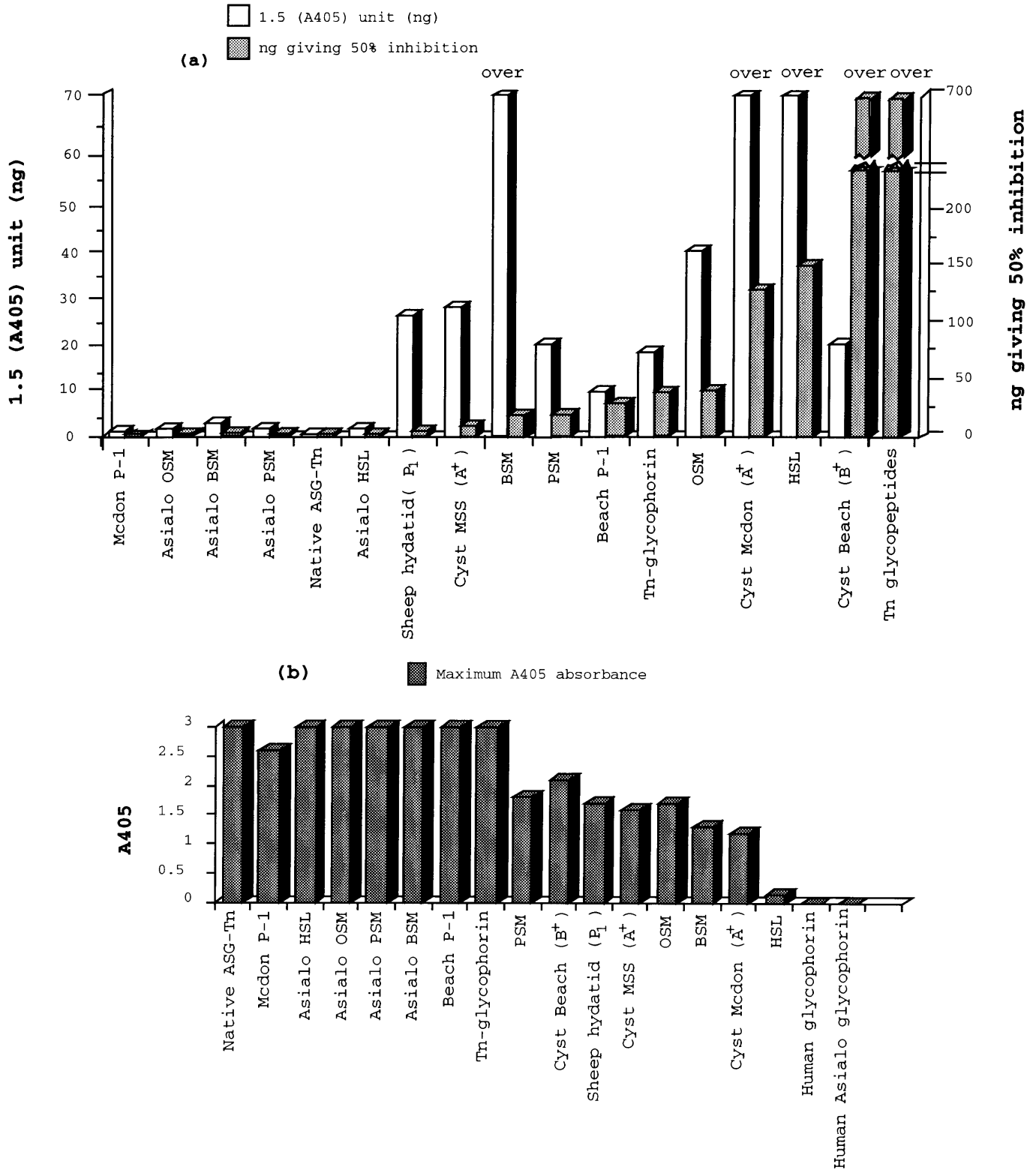

Fig. 4. Comparison of the binding of GS I-A4 with glycoproteins. Two parameters of ng required at 1.5 unit of A_{405} (Figure 1 and Table I) and ng required for 50% inhibition of glycoproteins (Figure 2 and Table II) are illustrated in Figure 4a, and their maximum A_{405} in Figure 4b (Figure 1, Table I).

Most of the GalNAcα1→ specific lectins bind strongly either the nonreducing end (**Fp**, **A**, and $\mathbf{A_h}$) (such as *Dolichos biflorus*, *Helix pomatia*, and *Wistaria floribunda* agglutinins) or the **Tn** residue (such as *Vicia villosa*-B_4 agglutinin) of glycoconjugates (Wu and Sugii, 1988, 1991), while *Codium fragile* subspecies *tomentosoides* (CFT) and GS I-A_4 are lectins that

react well with both, but CFT also reacts with the Galβ1→3GalNAcα1→ (**T**α) determinant and GS I-A_4, with the cluster forms of Galα1→4Gal (**E**) and Galα1→3Gal (**B**) determinants. The binding property of GS I-A_4 has potential to serve as a probe not only to detect **Tn**, **F** and **A** glycotopes but also **B** and **E** clusters. However, it is a poor reagent for detecting Galβ1→3GalNAcα1→Ser/Thr (**T**α), and GalNAcβ1→3/4Gal (**P**/**S**) residues. Summarizing previous data (Wood *et al.*, 1979; Wu *et al.*, 1996; 1997a) and the present results, it is concluded that each of the GalNAc specific lectins has its own binding characteristic. GS I-A_4 has been shown to be toxic to the human colon cancer cell lines (Chen *et al.*, 1994). In this case, **Tn** clusters and/or Forssman glycotopes may be involved.

Materials and methods

The lectin

Bandeiraea (Griffonia) simplicifolia lectin-I isolectin A_4 (GS I-A_4) (L-1509) was purchased from Sigma Chemical Co., St Louis, MO.

Glycoproteins

Ovine and porcine salivary glycoproteins (OSM-major and PSM), and bovine submandibular glycoprotein (BSM-major) were prepared by the method of Tettamanti and Pigman (1968). Hamster submaxillary glycoprotein was prepared by the methods of Downs and Herp (1977). For desialylation, a sample of glycoprotein in 0.01 N HCl was hydrolyzed at 80°C for 90 min and dialyzed against distilled H_2O (Wu *et al.*, 1994c).

Native ASG-**Tn**, an armadillo salivary glycoprotein containing only **Tn** (GalNAcα1→Ser/Thr) as carbohydrate side chains, was isolated from the 0.01 M PBS pH 6.8 gland extract after removal of ASG-A, which is one of the sialic acid containing glycoproteins in armadillo glands (Wu and Pigman, 1977; Wu *et al.*, 1994a, 1995b).

The blood group active glycoproteins from human ovarian cyst fluids were purified by digestion with pepsin and precipitation with ethanol; the dried ethanol precipitates were extracted with 90% phenol, the insoluble fraction being named according to its blood group glycoprotein (e.g., Cyst Beach phenol insoluble). The supernatant was fractionally precipitated by addition of 50% ethanol in 90% phenol to the indicated concentrations. The designation 10 or 20% (ppt.) denotes a fraction precipitated from phenol at an ethanol concentration of 10 or 20%; 2× signifies that a second phenol extraction and ethanol precipitation were carried out (e.g., Cyst MSS 10% 2×). The P-1 fraction represents the nondialyzable portion of the blood group substances after mild hydrolysis at pH 1.5–2.0 and 100°C for 2 h which removed most of the L-fucopyranosyl end groups, as well as some blood group A and B active oligosaccharide side-chains (Lloyd and Kabat, 1968; Wu *et al.*, 1992a, 1995b)

Human blood group P_1-active glycoprotein, isolated from sheep hydatid cyst (Morgan and Watkins, 1964; Cory *et al.*, 1974), was kindly provided by Dr. W.M.Watkins, University of London, Royal Postgraduate Medical School Hammersmith Hospital, London.

Glycophorin A was prepared from the membranes of outdated human blood group O erythrocytes by phenol/saline extraction and was purified by gel filtration in the presence of SDS (Lisowska *et al.*, 1987). Asialo-glycoprotein was prepared by mild acid hydrolysis (Wu and Pigman, 1977; Wu el al., 1994b,c). The Tn-type glycophorin (Tn-glycophorin) was obtained by removing galactose residues from asialo-glycophorin by periodate oxidation and mild acid hydrolysis (Smith degradation) (Duk *et al.*, 1994).

Sugar inhibitors

Monosaccharides, their derivatives and oligosaccharides were purchased from Sigma Chemical Company (St. Louis, MO). GalNAcα1→3 [LFucα1→2]Gal(**Ah**), and GalNAcα1→3GalNAcβ1→3Galα1→ 4Galβ1→4Glc(**Fp**) were from Accurate Chemical & Scientific Corp., Westburg, NY.

Tn glycopeptide preparation: The OSM **Tn** glycopeptides were prepared as described by the method of Wu *et al.* (1997b).

The microtiter plate lectin-enzyme binding assay

Biotinylation of lectin was prepared according to the method described by Duk (1994) and Lisowska (1996). The assay was performed according to the procedures previously described (Duk *et al.*, 1994; Lisowska *et al.*, 1996; Chen *et al.*, 1998). The volume of each reagent applied to the plate was 50 μl/well, and all incubations, except for coating, were performed at 20°C. 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.

All experiments were done in duplicate or triplicate and results are mean values. The standard deviation did not exceed 10% and in most experiments was less than 5% of the mean value. The control wells, where coating or addition of biotinylated lectin was omitted, gave low absorbance values (below 0.1, read against the well filled with buffer) and were used as blank. It showed that blocking the wells before lectin additon was not necessary, when Tween 20 was used in TBS.

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Abbreviations

Lectins: *Bandeiraea (Griffonia) simplicifolia* lectin-I isolectin A_4(GS I-A_4); CFT, Codium fragile subspecies tomentosoides. Monosaccharides: Gal, D-galactopyranose; Glc, D-glucopyranose;

LFuc, L-fucopyranose; DFuc, D-fucopyranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; NeuNAc/Neu5Ac, *N*-acetylneuraminic acid; NeuNGc, *N*-glycolylneuraminic acid; Fru, D-fructofuranose. Glycoprotein (GP): OSM-major, major fraction of ovine submandibular glycoprotein; BSM-major, major fraction of bovine submandibular glycoprotein; HSL, hamster sublingual glycoprotein; PSM, porcine submandibular gp-major; ASG, armadillo submandibular gp.

The carbohydrate structural units (lectin determinants) used to characterize binding properties of applied lectins (Wu and Sugii, 1988, 1991; Wu *et al*., 1997a) are: **F**, Forssman specific disaccharide (GalNAcα1→3GalNAc); **Fp** (Forssman pentasaccharide, GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glc); **A**, human blood group A specific disaccharide (GalNAcα1→3Gal); $\mathbf{A_h}$, GalNAcα1→3 [LFucα1→2]Gal, blood group A specific disaccharide containing crypto H determinant; **Tn**, GalNAcα1→Ser/Thr; **I**, human blood type I precursor sequence (Galβ1→3GlcNAc); **II**, human blood type II precursor sequence (Galβ1→4GlcNAc); **L**, Galβ1→4Glc; **T**, Galβ1→3GalNAc, **T**α, Galβ1→3GalNAcα1→ Ser/Thr, the mucin type sugar sequence on the human erythrocyte membrane; **B**, blood group B specific disaccharide (Galα1→3Gal); **E**, galabiose (Galα1→4Gal) sequence, a receptor of the uropathogenic E.coli ligand; ELLSA, enzyme linked lectinosorbent assay.

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