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Polyvalent GalNAcα1→Ser/Thr (Tn) and Galβ1→3GalNAcα1→Ser/Thr (T_α) as the most potent recognition factors involved in *Maclura pomifera* agglutinin–glycan interactions

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Summary

The agglutinin isolated from the seeds of *Maclura pomifera* (MPA) recognizes a mucin-type disaccharide sequence, Galβ1→3GalNAc (T) on a human erythrocyte membrane. We have utilized the enzyme-linked lectinosorbent assay (ELLSA) and inhibition assay to more systematically analyze the carbohydrate specificity of MPA with glyco-recognition factors and mammalian Gal/GalNAc structural units in lectin–glycoform interactions. From the results, it is concluded that the high densities of polyvalent GalNAcα1→Ser/Thr (Tn) and Galβ1→3GalNAcα1→Ser/Thr (T_α) glycotopes in macromolecules are the most critical factors for MPA binding, being on a nanogram basis 2.0×10^5, 4.6×10^4 and 3.9×10^4 more active than monovalent Gal, monomeric T and Tn glycotope, respectively. Other carbohydrate structural units in mammalian glycoconjugates, such as human blood group Sd (a^+) related disaccharide (GalNAcβ1→4Gal) and P^k/P_1 active disaccharide (Galα1→4Gal) were inactive. These results demonstrate that the configurations of carbon-4 and carbon-2 are essential for MPA binding and establish the importance of affinity enhancement by high-density polyvalencies of Tn/T glycotopes in MPA–glycan interactions. The overall binding profile of MPA can be defined in decreasing order as: high density of polyvalent Tn/T_α (M.W. $> 4.0 \times 10^4$) >> Tn-containing glycopeptides (M.W. $< 3.0 \times 10^3$) > monomeric T/Tn and P (GalNAcβ1→3Gal) > GalNAc > Gal >> Man, L-Ara, D-Fuc and Glc (inactive). Our findings should aid in the selection of this lectin for elucidating functions of carbohydrate chains in life processes and for applications in the biomedical sciences.

Abbreviations: ASG-Tn – native Tn glycoprotein from armadillo salivary gland; BSM – bovine submandibular glycoprotein-major; ELLSA – enzyme-linked lectinosorbent assay; D-Fuc – D-fucopyranose; Gal – D-galactopyranose; GalNAc – 2-acetamido-2-deoxy-D-galactopyranose; Glc – D-glucopyranose; GlcNAc – 2-acetamido-2-deoxy-D-glucopyranose; gp – glycoprotein; HSM – hamster submaxillary sialyl Tn gp; L-Fuc – L-fucopyranose; Man – D-mannopyranose; MPA – *Maclura pomifera* agglutinin; NeuAc – *N*-acetylneuraminic acid; OSM – ovine submandibular glycoprotein-major; PSM – porcine salivary glycoprotein; TBS-T – Tris–HCl buffered saline containing 0.05% Tween 20; THGP – Tamm–Horsfall glycoprotein.
The mammalian carbohydrate structural units in glycans used to define the binding properties of MPA are: T, Galβ1 → 3GalNAc, Thomsen–Friedenreich disaccharide; T_α, Galβ1 → 3GalNAcα1 → Ser/Thr; Tn,

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GalNAcα1 → Ser/Thr; A, GalNAcα1 → 3Gal, human blood group A-specific disaccharide; A_h, GalNAcα1 → 3(L-Fucα1 → 2)Gal, human blood group A-specific trisaccharide containing crypto H determinant; H, L-Fucα1 → 2Gal, human blood group H-specific disaccharide; L, Galβ1 → 4Glc; F, GalNAcα1 → 3GalNAc; P, GalNAcβ1 → 3Gal; S, GalNAcβ1 → 4Gal; B, Galα1 → 3Gal; E, Galα1 → 4Gal; I, Galβ1 → 3GlcNAc, human blood group type I precursor sequence; and II, Galβ1 → 4GlcNAc, human blood group type II precursor sequence.

Introduction

The seeds of *Maclura pomifera* contain a lectin (MPA) with a molecular weight of $4.0–4.3 \times 10^4$ that can recognize Thomsen–Friedenreich (T_α, Galβ1→3GalNAcα1→Ser/Thr) and Tn (GalNAcα1→Ser/Thr) antigens [1–5]. MPA, like Jacalin, has a $\alpha_4\beta_4$ structure that shows far greater microheterogeneity than plant lectins from other families, mainly due to its multiple genetic isoforms and posttranslational processing [2, 6, 7]. The subunits have been sequenced [7, 8] and are highly homologous to that of Jacalin. It was reported that this lectin reacts more strongly with polyagglutinable red cells from patients with hereditary erythroblastic multinuclearity than with En (a^-) erythrocytes which lack glycophorin [9]. MPA can interact with certain bacterial polysaccharides [10] and can also be used as a specific marker for a glycoprotein on type 2 aveolar cells [11]. It is known to have an affinity for the membranocystic lesions of membranous lipodystrophy (Nasu–Hakola disease) [12].

Although the structural nature of MPA required for binding has been documented previously [3, 4], this information was limited to monosaccharides, Galβ1→3GalNAc (T) and Galβ1→ related oligosaccharides [3, 5]. The effects of polyvalencies of natural glycotopes on the lectin binding and other mammalian carbohydrate structural units present in glycoconjugates have not been extensively elucidated. Therefore, in this study, we characterized the glyco-recognition factors involved in MPA–glycan interactions using our collection of ligands and glycans by enzyme-linked lectinosorbent assay (ELLSA) and lectin–glycan inhibition assay [13, 14]. Based on the results of interaction and inhibition data, it is concluded that the high densities of polyvalent Tn and T_α glycotopes in macromolecules are the most critical factors for MPA binding and that overall binding profile of MPA can be expressed in decreasing order as: high density of polyvalent Tn/T_α (M.W. $> 4.0 \times 10^4$) >> Tn-containing glycopeptides (M.W. $< 3.0 \times 10^3$) > monomeric T/Tn and P (GalNAcβ1→3Gal) > GalNAc > Gal >> Man, L-Ara, D-Fuc and Glc (inactive). The binding properties of six Galβ1→3GalNAc (T) and/or GalNAcα1→Ser/Thr (Tn)-specific lectins toward glycoproteins were also compared. These distinct binding features of MPA establish a valuable concept of affinity enhancement by high-density polyvalencies of glycotopes relevant to ligand–lectin recognition and will undoubtedly facilitate selection of this lectin for biological applications.

Materials and methods

Lectin preparation and biotinylation

Maclura pomifera agglutinin (MPA) was prepared as previously described [4]. For biotinylation by biotinamidocaproate-*N*-hydroxy-succinimide ester (biotin ester; Sigma Chemical, St. Louis, MO, USA), the lectin (200 μg/250 μl phosphate-buffered saline, PBS; 0.14 M NaCl, 0.027 M KCl, 0.081 M Na_2HPO_4, 0.0014 M KH_2PO_4, pH 7.3) was mixed with 400 μl of the biotin ester solution (100 μg biotin ester/200 μg lectin) for 30 min at room temperature. The biotinylated lectin was dialyzed for 2–3 h against ddH_2O and overnight against Tris-buffered saline (TBS; 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.35). After dialysis, the sample volume was adjusted to 1 ml with TBS, and 20 μl of 5% sodium azide was added (yielding a 200 μg/ml solution of MPA in 0.1% NaN_3) [13, 14].

Glycoproteins and polysaccharides

Tn-containing glycoproteins (gps) and their related glycans used for this study are listed in Table 1. Native ASG-Tn [15], a salivary glycoprotein of the nine-banded armadillo (*Dasypus novemcinctus mexicanus*) containing only Tn (GalNAcα1→Ser/Thr) as carbohydrate side chains, was isolated from

Table 1. Carbohydrate structures in MPA reactive glycoproteins [47].

1. Native armadillo salivary Tn gp (Native ASG-Tn)
   GalNAcα1→Ser/Thr (Tn)

2. Armadillo salivary gp-A (ASG-A)
   GalNAcα1→Ser/Thr (Tn ≈ 65%)
   NeuAcα2→6GalNAcα1→Ser/Thr (Tn ≈ 35%)

3. Asialo hamster submandibular gp (Asialo HSM)
   GalNAcα1→Ser/Thr (Tn)

4. Asialo ovine submandibular gp (Asialo OSM)
   Major: GalNAcα1→Ser/Thr (Tn > 75%)
   Others: Galβ1→3GalNAcα1→Ser/Thr (T_α)
   Fucα1→2Galβ1→3GalNAcα1→Ser/Thr
   Galβ1→3[GlcNAcβ1→6]GalNAcα1→Ser/Thr (GlcNAcβ1→6T_α)
   Galβ1→3[Galβ1→4GlcNAcβ1→6]GalNAcα1→Ser/Thr (II_β1→T_α)

5. Asialo bovine submandibular gp (Asialo BSM)
   (i) GalNAcα1→Ser/Thr (Tn > 53%)
   (ii) GlcNAcβ1→3GalNAcα1→Ser/Thr (GlcNAcβ1→3Tn > 22%)
   (iii) Galβ1→3GalNAcα1→Ser/Thr (T_α)
   (iv) Galβ1→3[GlcNAβ1→6]GalNAcα1→Ser/Thr (GlcNAβ1→6T_α)

   Longer chains containing GalNAc, GlcNAc, Fuc, and Gal

6. Asialo porcine submandibular gp (Asialo PSM)
   (i) GalNAcα1→Ser/Thr (Tn)
   (ii) Galβ1→3GalNAcα1→Ser/Thr (T_α)
   (iii) GalNAcα1→3Galβ1→3GalNAcα1→Ser/Thr (A)
   (iv) LFucα1,2Galβ1→3GalNAcα1→Ser/Thr (H)
   (v) GalNAcα1→3Galβ1→3GalNAcα1→Ser/Thr (A_h)

   ↑ α1,2
   LFuc

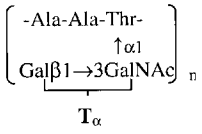
7. Human glycophorin
   (i) Native glycophorin

   Galβ1 → 3GalNAcα1 → Ser/Thr (sialyl **T**$_\alpha$, crypto **T**)

   ↑α2,3 NeuAc ↑α2,6 NeuAc

   (ii) Asialo glycophorin
   Galβ1→3GalNAcα1→Ser/Thr (T_α)
   (iii) Tn-glycophorin
   Gal NAcα1→Ser/Thr (Tn)

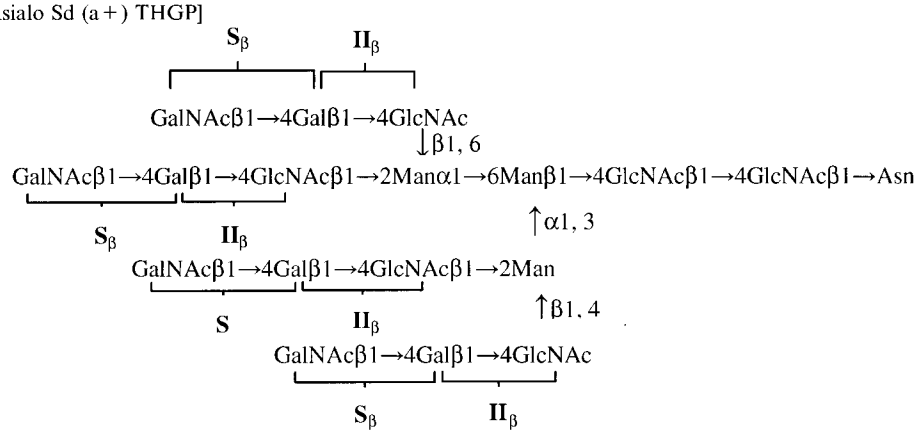
8. Repeating units of active and inactive antifreeze glycoproteins



Structure of the active antifreeze glycoproteins. Glycoproteins from the Antarctic fish *Trematomus borchgrevinki* are composed of repeating units of a diglycosyltripeptide with Tα as carbohydrate side chains [21, 49, 50]. The molecular weights of these glycoproteins vary from 10,500 to 21,000. The inactive antifreeze glycoproteins have a molecular weight ranging from 2600 to 3800. Their structures are similar to the active preparations except that proline is present in the peptide chain. The inactive glycoproteins were initially thought not to lower the freezing point, but in later work were reported to have some antifreeze activity.

Table 1. Continued

9. Mammalian Carbohydrate Structural units present in Asialo Sd (a+) Tamm-Horsfall urinary glycoprotein [Asialo Sd (a+) THGP]



the 0.01 M PBS pH 6.8 gland extract after removal of ASG-A, which is one of the sialoglycoproteins in armadillo glands [15]. About two-thirds of the carbohydrate side chains of armadillo submandibular gland mucin (ASG-A) is GalNAcα1→Ser/Thr (Tn) and one third is Neu5Acα2→6GalNAcα1→ *O*→Ser/Thr (Sialyl Tn) [16–18].

Hamster submaxillary glycoprotein, one of the simplest glycoproteins among mammalian salivary mucins containing sialyl-Tn (NeuAc→GalNAc) and Tn (GalNAcα1→Ser/Thr) residues as carbohydrate side chains, was obtained by a modification of the methods previously described [16]. The sialyl-Tn and Tn-containing gp isolated from hamster submaxillary gland contained 28% GalNAc, 35% NeuAc and 33% total amino acids, in which six amino acids (Thr + Ser, 54%; Pro, 16.2%; Glu, 8.7%; Lys, 8.3% and Ala, 6.7%) constituted 94 mole% of the protein core.

Ovine, bovine, and porcine submandibular/salivary glycoproteins were purified according to the method of Tettamanti and Pigman [19] with modifications [16]. About 75% of the carbohydrate side chains of asialo OSM were GalNAc α 1→ Ser/Thr (Tn). Asialo PSM contains Galβ1→3GalNAcα1 (T_α) together with Tn and GalNAc α 1→ 3Gal (A) sequences, as most of the outer fucosyl residues and sialic acids are cleaved by mild acid hydrolysis.

Glycophorin A, a mucin-type *O*-glycan containing sialylated T_α (Table 1), 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 [14]. Asialo-glycophorin (T_α) was prepared by mild acid hydrolysis [17, 19]. The Tn-type glycophorin (Tn-glycophorin) was obtained by removing galactose residues from asialo-glycophorin by periodate oxidation and mild acid hydrolysis (Smith degradation) [13, 20].

The active (M.W. 1.0–2.1 × 10^4) and inactive (M.W. 2.0–3.8 × 10^3) antifreeze glycoproteins from an Antarctic fish (*Trematomus borchgrevinki*) which contain only Galβ1→3GalNAcα1 (T_α) as carbohydrate chains [21] were provided by Dr. R.E. Freeney, Dept. of Food Science and Technology, University of California, Davis, CA, USA through the late Dr. E.A. Kabat (Columbia Medical Center, New York, NY, USA). Their structural differences are described in the legend of Structure 8, Table 1. Desialylation of sialo-glycoproteins was performed by mild acid hydrolysis in 0.01 N HCl at 80 °C for 90 min, and dialysis against distilled water for 2 d to remove small fragments [17, 19].

Blood group A, B, H, Le^a, Le^b and Ii active substances were purified from human ovarian cyst fluid (HOC) by digestion with pepsin and precipitation with increasing concentrations of ethanol [22–25]; the dried ethanol precipitates were extracted with 90% phenol, the insoluble fraction being named after the blood group substance (e.g., cyst Beach phenol insoluble). Codes such as Mcdon, MSS, MSM, etc. represent different patients from which the samples were taken.

Supernatants were fractionally precipitated by addition of 50% ethanol in 90% phenol to the indicated concentrations [22]. The designation '10 (or 20)% (ppt)' denotes a fraction precipitated from phenol at an ethanol concentration of 10 or 20%. The carbohydrate chains of HOC consist of multiple saccharide branches attached by *O*-glycosidic linkages at their internal reducing ends to serine or threonine residues of the polypeptide backbone [23–25]. In general, the P-1 fractions (e.g. Cyst Mcdon P-1, Beach P-1 or Tighe P-1) represent the nondialyzable portion of the blood group substances after mild hydrolysis at pH 1.5–2.0 for 2 h, which removes most of the L-fucopyranosyl end groups, as well as some blood group A and B active oligosaccharide side-chains [26–28]. P-1 fractions from HOC gps, which expose the internal structures equivalent to those on the blood group precursors, are defined as 'precursor equivalent gps'.

Fetuin (Gibco, Grand Island, NY, USA), which is the major glycoprotein in fetal calf serum, has six oligosaccharide side chains per molecule – three of them (of two types) are *O*-glycosyl-linked to Ser or Thr residues of the protein core, and the others are tri-antennary II *N*-glycosyl-linked to Asn and a small amount of Gal β 1,3-linked isomer at the nonreducing terminal ends [29].

Tamm-Horsfall glycoprotein (THGP) with Sd (a^+) blood group (Structure 9 in Table 1) [30] was kindly provided by the late Dr. W.M. Watkins, University of London, Royal Postgraduate Medical School, Hammersmith Hospital, London, UK.

The *pneumococcus* type 14 polysaccharide, isolated from *Streptococcus pneumoniae* capsule [31], was a generous gift from the late Dr. E.A. Kabat.

Yeast high-mannose type glycan (mannan), polygalacturonic acid-containing pectin from the cell wall matrix of the apple (pectin-A) and poly-2, 8-*N*-acetylneuraminic acid capsular polysaccharide from *E. coli* (colominic acid) were purchased from Sigma.

Sugars used for the inhibition studies

Mono, di- and oligosaccharides used were purchased from Sigma and Dextra (Berkshire, UK). Tri-antennary Galβ1→4GlcNAc (II) glycopeptides were prepared from asialo fetuin by pronase digestion and repeatedly fractionated by BioGel P-4 400 mesh, column chromatography [32]. The Tn clusters used for this study were mixtures of Tn-containing glycopeptides from OSM in the filterable fraction (molecular mass cut-off < 3000) [33].

The microtiter plate lectin-enzyme binding assay

The assay was performed according to the procedures described by Duk et al. [13,14]. 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 (pH 7.4) or 0.15 M NaCl containing 0.05% Tween 20 was used for washing the plate between incubations.

The 96-well microtiter plates (Nunc, MaxiSorp, Vienna, Austria) were coated with gps ranging from 10 ng to less than 0.08 ng (Figure 1a), and 500 ng to less than 50 ng per well (Figure 1f) in 0.05 M carbonate buffer, pH 9.6, and incubated overnight at 4 °C. After washing the plate, biotinylated lectins (5 ng) were added to each well and incubated for 30 min. The plates were washed to remove unabsorbed lectin and the ExtrAvidin/alkaline phosphatase solution (Sigma, diluted 1:10,000) was added. After 1-h, the plates were washed at least four times and incubated with *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate 5 mg tablets) in 0.05 M carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$ (1 tablet/5 ml). The absorbance was read at 405 nm in a microtiter plate reader, after a 2-h incubation with the substrate.

For inhibition studies, serially diluted inhibitors were mixed with an equal volume of lectin solution containing a fixed amount of MPA (10.0 ng/well). The control lectin sample was diluted 2-fold with TBS-T. After 30 min at room temperature, samples were tested by the binding assay [13, 14]. The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor (ng or nmol/well) giving 50% inhibition of the control lectin binding.

All experiments were done in duplicates or triplicates, and data are presented as the mean values of the results. The standard deviation did not exceed 12% and in most experiments was less than 5% of the mean value. For the binding experiment, the control wells, where coating or addition of biotinylated lectin was omitted, gave low absorbance values (below 0.1). This showed that blocking the

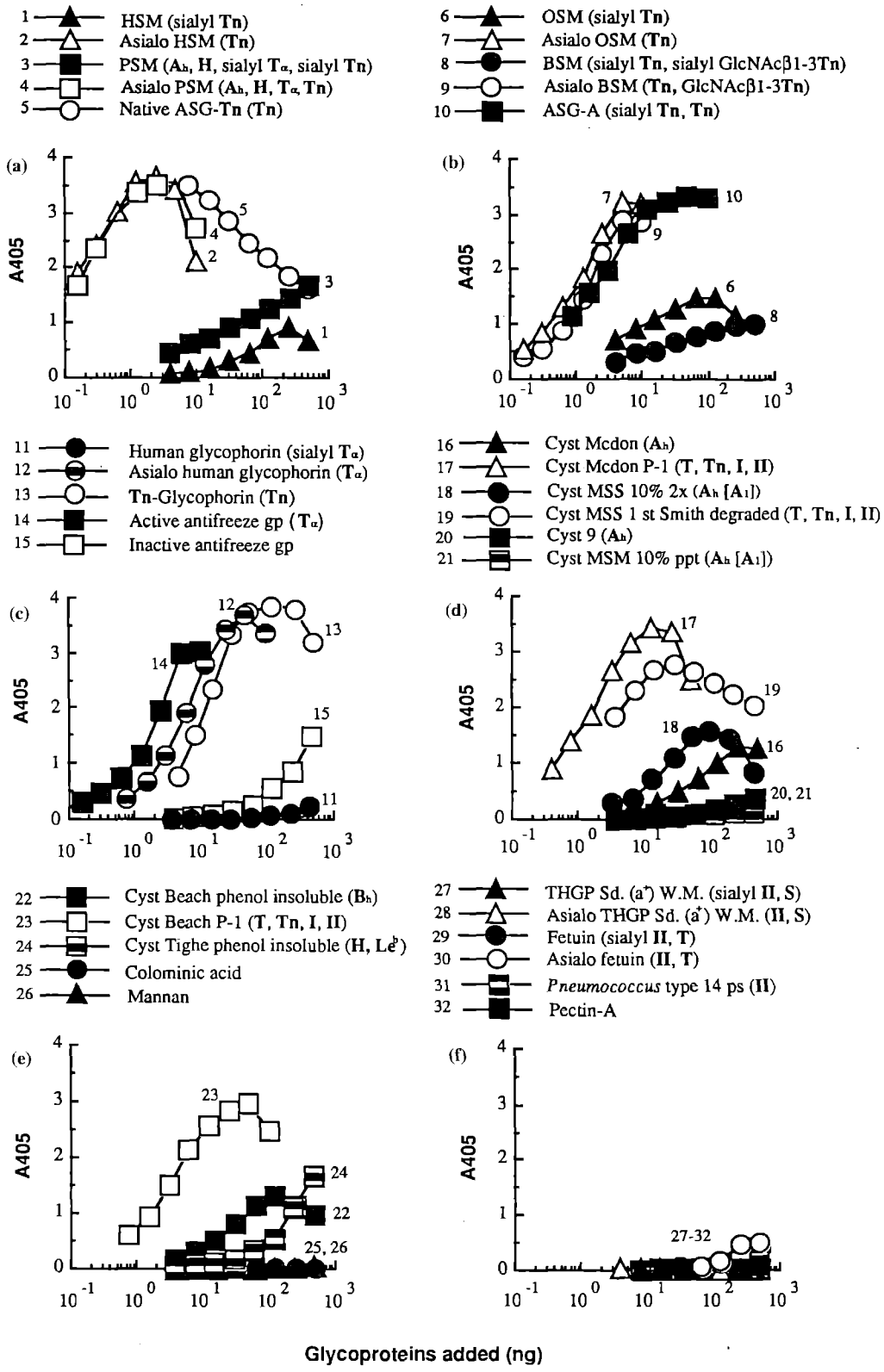

1 HSM (sialyl Tn)
2 Asialo HSM (Tn)
3 PSM (Ah, H, sialyl Tα, sialyl Tn)
4 Asialo PSM (Ah, H, Tα, Tn)
5 Native ASG-Tn (Tn)
6 OSM (sialyl Tn)
7 Asialo OSM (Tn)
8 BSM (sialyl Tn, sialyl GlcNAcβ1-3Tn)
9 Asialo BSM (Tn, GlcNAcβ1-3Tn)
10 ASG-A (sialyl Tn, Tn)
(a)
(b)
A405
11 Human glycophorin (sialyl Tα)
12 Asialo human glycophorin (Tα)
13 Tn-Glycophorin (Tn)
14 Active antifreeze gp (Tα)
15 Inactive antifreeze gp
16 Cyst Mcdon (Ah)
17 Cyst Mcdon P-1 (T, Tn, I, II)
18 Cyst MSS 10% 2x (Ah [A1])
19 Cyst MSS 1 st Smith degraded (T, Tn, I, II)
20 Cyst 9 (Ah)
21 Cyst MSM 10% ppt (Ah [A1])
(c)
(d)
22 Cyst Beach phenol insoluble (Bh)
23 Cyst Beach P-1 (T, Tn, I, II)
24 Cyst Tighe phenol insoluble (H, Le^b)
25 Colominic acid
26 Mannan
27 THGP Sd. (a+) W.M. (sialyl II, S)
28 Asialo THGP Sd. (a+) W.M. (II, S)
29 Fetuin (sialyl II, T)
30 Asialo fetuin (II, T)
31 Pneumococcus type 14 ps (II)
32 Pectin-A
(e)
(f)
27-32
Glycoproteins added (ng)

Figure 1. Binding of MPA to microtiter plates coated with serially diluted human blood group A, B, H, Le^a and Le^b active glycoproteins, sialo- and asialoglycoproteins. The amount of lectin used was 5 ng/well. Total volume of the assay was 50 µl. A_{405} was recorded after a 2-h incubation. MPA reacted best with high-density polyvalent GalNAcα1→Ser/Thr (Tn)-containing gps [asialo HSM, asialo PSM and native ASG-Tn] in Figure 1a. MPA bound strongly with Tn and T_α [Galβ1→3GalNAcα1-]-containing gps [asialo OSM, asialo BSM and ASG-A] in Figure 1b; Tn-glycophorin in Figure 1c etc; T_α-containing asialo human glycophorin and active antifreeze glycoprotein (M.W. 1.0–2.1 × 10^4) in Figure 1c. MPA reacted weakly or was inactive with sialylated T/Tn-containing gps [HSM and human glycophorin in Figure 1a and c, respectively]; human blood group Sd (a^+) THGP from urine in Figure 1f; *pneumococcus* type 14 polysaccharide in Figure 1f; *N*-linked multiantennary II-containing plasma gp [fetuin and its asialo product in Figure 1f]; human blood group A active gps [cyst 9 and cyst MSM 10% ppt in Figure 1d]; and natural polysaccharides.

wells before lectin addition was not necessary when Tween 20 was present in the TBS. On the other hand, for the inhibition experiment, two controls were set up. The first control was treated the same as the experimental group except that the inhibitor was left out; the absorbance value recorded at 2-h was between 3.0 and 3.3 (A405). For the second control (coating only or negative control), both lectin and inhibitors were left out while other conditions were kept the same as in the experimental group. The absorbance value of this control was below 0.1, which was used as the subtracted background value.

Results

Lectin–glycoform interaction

The avidity of MPA for various glycoproteins (gps) studied by ELLSA is summarized in Table 2 according to the interaction profiles shown in Figure 1. It is clear that MPA reacted best with high density polyvalent GalNAcα1→Ser/Thr (Tn)-containing gps (asialo hamster [asialo HSM], porcine [asialo PSM] and armadillo salivary mucin [native ASG-Tn] in Tables 1 and 2, Figure 1a), where less than 0.35 ng of glycoprotein coating was required to interact with 5.0 ng of lectin to yield a A_{405} value of 1.5 within 2 h. MPA bound strongly with other Tn and T_α (Galβ1→3GalNAcα1)-containing gps. These include polyvalent Tn-containing gps (Table 2; asialo ovine [asialo OSM] , bovine [asialo bovine] and armadillo submandibular Tn glycoproteins [ASG-A] in Figure 1b); Tn-glycophorin prepared from human erythrocytes (Figure 1c); human blood group precursor equivalent Tn-containing gps (Mcdon P-1 in Figure 1d; Beach P-1 and MSS 1st Smith degraded gp in Figure 1d and e); T_α-containing asialo human glycophorin and active antifreeze glycoprotein (M.W. 1.0–2.1 × 10^4 in Figure 1c). The absorbance values at 405 nm of all of these T/Tn-containing glycoforms reached over 2.5 within 2-h. Nevertheless, MPA also reacted modestly with crypto-Tn-containing gps (OSM, PSM and BSM, Figure 1a and b), inactive antifreeze gp (M.W. 2.0–3.8 × 10^3, Figure 1c), and many glycosylated human blood group ABH active gps (cyst Mcdon and cyst MSS 10% 2× in Figure 1d, cyst Beach and Tighe phenol insoluble in Figure 1e). But it reacted weakly or was inactive with few highly sialylated T/Tn-containing gps (HSM and human glycophorin in Figure 1a and c), Sd (a^+) gp from urine (Figure 1f), *pneumococcus* type 14 polysaccharide (Figure 1f), *N*-linked multiantennary II-containing plasma gp (fetuin and its asialo product in Figure 1f), human blood group A active gps (cyst 9 and cyst MSM 10% ppt in Figure 1d) and natural polysaccharides – mannan, pectin-A and colominic acid (Figure 1e and f).

Inhibition of MPA–glycoform interaction by various glycans

To exclude the possibility that the affinity differences of these gps were due to the plate adsorption discrepancies, the binding affinity was further confirmed by the inhibition assay described in Section 'Materials and methods'. The ability of various glycans to inhibit the binding of MPA to a Tn-containing glycoprotein (asialo OSM) was analyzed by ELLSA and is shown in Table 3, and Figure 2. Among the glycans tested for inhibition of this interaction, three mammalian salivary-containing gps (asialo HSM, native ASG-Tn and asialo BSM) were the most potent inhibitors, requiring less than 1.0 ng to inhibit 50% of the lectin–glycan interaction. They were up to 2.0 × 10^5 and 8.0 × 10^4 times more active than Gal and GalNAc, respectively (curves 1–3 vs. curves 20 and 19, Table 3, Figure 2a). The MPA–glycan interaction was also inhibited strongly by many other high-density polyvalent T/Tn-containing

Table 2. Binding of MPA to human blood group A, B, H, P_1, Le^a and Le^b active glycoproteins, sialo- and asialo glycoproteins by ELLSA[a].

Graph in Figure 1	Glycoprotein (Lectin determinants[b]; blood group specificity)	1.5 (A405) unit (ng)	Maximum A_{405}	
			Absorbance reading[c]	Binding intensity[c]
Exposed polyvalent Tn/Tα glycoproteins				
a	Asialo HSM (**Tn**)	0.08	3.6	+ + + + +
a	Asialo PSM (**Tn**, **Tα** > **A_h**, **H**)	0.15	3.6	+ + + + +
a	Native ASG-**Tn** (**Tn**)	0.35	3.6	+ + + + +
b	Asialo OSM (**Tn**)	0.8	3.2	+ + + + +
d	Cyst Mcdon P-1 (**T**, **Tn**, **I/II**)	0.9	3.4	+ + + + +
b	Asialo BSM (**Tn**, GlcNAcβ1-3**Tn**)	1.1	2.9	+ + + + +
b	ASG-A (sialyl **Tn**)	1.5	3.3	+ + + + +
c	Active antifreeze gp (**Tα** M.W. 1.0–2.1 × 10^4)	1.9	3.0	+ + + + +
d	Cyst MSS 1st Smith degraded (**T**, **Tn**, **I**, **II**)	2.8	2.8	+ + + + +
e	Cyst Beach P-1 (**T**, **Tn**, **I/II**)	3.1	3.0	+ + + + +
c	Asialo human glycophorin (**Tα**; M.W. 1.0–2.1 × 10^4)	4.3	3.7	+ + + + +
c	**Tn**-glycophorin (**Tn**)	8.0	3.9	+ + + + +
c	Inactive antifreeze gp (**Tα**; M.W. 2.6–3.8 × 10^3)	500.0	1.5	+ + +
Cryptic Tn / Tα glycoproteins				
b	OSM (sialyl **Tn**)	70.0	1.5	+ + +
a	PSM (sialyl **Tn**, **Tα** > **A_h**, **H**)	280.0	1.7	+ + +
b	BSM (sialyl **Tn**, sialyl GlcNAcβ1-3**Tn**)	–	1.0	+ +
a	HSM (sialyl **Tn**)	–	0.9	+
c	Human glycophorin (sialyl **Tα**)	–	0.2	±
Other glycoproteins and polysaccharides				
e	Cyst Tighe phenol insoluble (**H**, **Le^b**)	450.0	1.7	+ + +
d	Cyst MSS 10% 2× (**A_h** [**A_1**])	70.0	1.5	+ + +
d	Cyst Mcdon (**A_h**)	–	1.3	+ +
e	Cyst Beach phenol insoluble (**B_h**)	–	1.3	+ +
f	Asialo fetuin (**II**, **T**)	–	0.5	+
f	*Pneumococcus* type 14 ps (**II**)	–	0.3	±
d	Cyst 9 (**A_h**)	–	0.4	±
d	Cyst MSM 10% ppt (**A_h** [**A_1**])	–	0.1	–
f	Fetuin (sialyl **II**, **T**)	–	0.1	–
f	Asialo THGP Sd. (a^+) W.M. (**II**, **S**)	–	0.0	–
f	THGP Sd. (a^+) W.M. (sialyl **II**, **S**)	–	0.0	–
e	Mannan	–	0.0	–
e	Colominic acid (Poly α2→8 *N*-acetylneuraminic acid)	–	0.0	–
f	Pectin-A (Poly GalUAc)	–	0.0	–

[a]5 ng of biotinylated lectin was added to various glycoprotein concentrations ranging from 0.008 ng to 1 μg. [b]The symbol in parentheses indicates the human blood group activity and/or lectin determinants [47]. Expressed in bold are: T (Galβ1-3GalNAc); Tn (Gal NAcα1-Ser/Thr); I/II (Galβ1-3/4GlcNAc); A (GalNAcα1-3Gal); A_h (GalNAcα1-3[LFucα1-2]Gal); B (Galα1-3Gal); B_h (Galα1-3[LFucα1-2]Gal); H (LFucα1-2Gal). [c]The results were interpreted according to the measured A_{405} after 2 h incubation as follows: + + + + + (O.D. ≥ 2.5), + + + + (2.5 > O.D. ≥ 2.0), + + + (2.0 > O.D. ≥ 1.5), + + (1.5 > O.D. ≥ 1.0), + (1.0 > O.D. ≥ 0.5), ± (0.5 > O.D. ≥ 0.2), and – (O.D. < 0.2).

gps. Their activities were up to 6.2 × 10^4, 2.5 × 10^4 and 1.5 × 10^4 times higher than monomeric Gal, GalNAc and T disaccharide, respectively (curves 5–15 vs. curves 20, 19 and 18). The Tn glycopeptide (M.W. < 3.0 × 10^3) from ovine salivary gp was 76 and 1.6 × 10^2 times more active than

Table 3. Amounts of various glycoproteins giving 50% inhibition of binding of MPA (5 ng/50 μl) to a Tn-containing gp (asialo OSM 2 ng/50 μl)[a].

Curves or identification no.	Curves in Figure 2	Inhibitor (lectin determinants or blood group specificity)[c]	Quantity giving 50% inhibition (ng)	Relative potency[b]
1	a	Asialo HSM (Tn)	0.8	2.0×10^5
2	a	Native ASG-Tn (Tn)	0.9	1.8×10^5
3	a	Asialo BSM (Tn , GlcNAcβ1→3Tn)	1.0	1.6×10^5
4	b	Cyst Mcdon P-1 (T, Tn, I/II)	2.0	8.0×10^4
5	a	Asialo PSM (Tn, Tα > A_h, H)	2.6	6.2×10^4
6	b	Cyst Beach P-1 (T, Tn, I/II)	3.0	5.3×10^4
7	a	ASG-A (sialyl Tn)	7.0	2.3×10^4
8	a	Asialo OSM (Tn)	9.0	1.8×10^4
9	b	Cyst MSS 1st Smith degraded (I, II, T, Tn)	9.0	1.8×10^4
10	a	Asialo human glycophorin (Tα)	9.5	1.7×10^4
11	a	Tn Glycophorin (Tn)	30.0	5.3×10^3
12	a	HSM (sialyl Tn, Tn)	65.0	2.5×10^3
13	b	Cyst Beach phenol insoluble (B_h)	98.0	1.6×10^3
14	b	Cyst Mcdon (A_h)	190.0	8.4×10^2
15	a	Active antifreeze gp (Tα; M.W. $1.0–2.1 \times 10^4$)	300.0	5.3×10^2
16	a	Tn containing glycopeptides (M.W. $< 3.0 \times 10^3$)	406.0	3.9×10^2
17	a, b	GalNAcα1→Ser/Thr (Tn)	3.1×10^4	5.1
18	a, b	Galβ1→3GalNAc (T)	3.6×10^4	4.4
19	a, b	GalNAc	6.4×10^4	2.5
20	a, b	Gal	1.6×10^5	1.0
21	a	OSM (sialyl Tn)	$>2.8 \times 10^2$ (32%) [d]	–
22	a	PSM (sialyl Tn, Tα > A_h, H)	$>2.8 \times 10^2$ (29%)	–
23	b	Cyst MSM 10% ppt (A_h [A_1])	$>2.8 \times 10^2$ (18%)	–
24	a	BSM (sialyl Tn, sialyl GlcNAcβ1-3Tn)	$>2.8 \times 10^2$ (16%)	–
25	b	*Pneumococcus* type 14 ps (II)	$>2.8 \times 10^2$ (13%)	–
26	b	Fetuin (sialyl II, T)	$>2.8 \times 10^2$ (10%)	–
27	b	Asialo fetuin (II, T)	$>2.8 \times 10^2$ (10%)	–
28	a	Inactive antifreeze gp (Tα; M.W. $2.6–3.8 \times 10^3$)	$>2.8 \times 10^2$ (9%)	–
29	a	Human glycophorin (sialyl Tα)	$>2.8 \times 10^2$ (6%)	–
30	b	Asialo THGP Sd. (a^+) W.M. (II, S)	$>2.8 \times 10^2$ (7%)	–
31	b	THGP Sd. (a^+) W.M. (sialyl II, S)	$>2.8 \times 10^2$ (15%)	–
32	b	Mannan	$>2.8 \times 10^2$ (14%)	–
33	b	Colominic acid (Poly α2→8 *N*-acetylneuraminic acid)	$>2.8 \times 10^2$ (13%)	–
34	b	Pectin-A (Poly GalUAc)	$>2.8 \times 10^2$ (0.3%)	–

[a]The inhibitory activity was estimated from the inhibition curve in Figure 2 and is expressed as the amount of inhibitor giving 50% inhibition of the control lectin binding. Total volume is 50 μl.
[b]Relative potency (RP) = quantity of Gal (curve 20) required for 50% inhibition (taken as 1.0)/quantity of sample required for 50% inhibition.
[c]Listed in footnote of Table 2.
[d]The inhibitory potency of inactive glycoproteins is expressed as the maximum amount of glycans tested that yield inhibition (in parentheses) below 50%.

monomeric Tn and GalNAc (curve 16 vs. curves 17 and 19), respectively, and was 5.0×10^2 times less active than the corresponding high-density polyvalent Tn glycans (curve 16 vs. 1, Figure 2a). These results imply that a high density of polyvalent T/Tn glycotopes is required for strong MPA-carbohydrate binding. The sialylated T/Tn glycotopes of *O*-glycans (curves 21, 22, 24 and 29, Figure 2a), such as salivary glycoproteins, multiantennary II-containing *N*-glycans (25–27, 30–31,

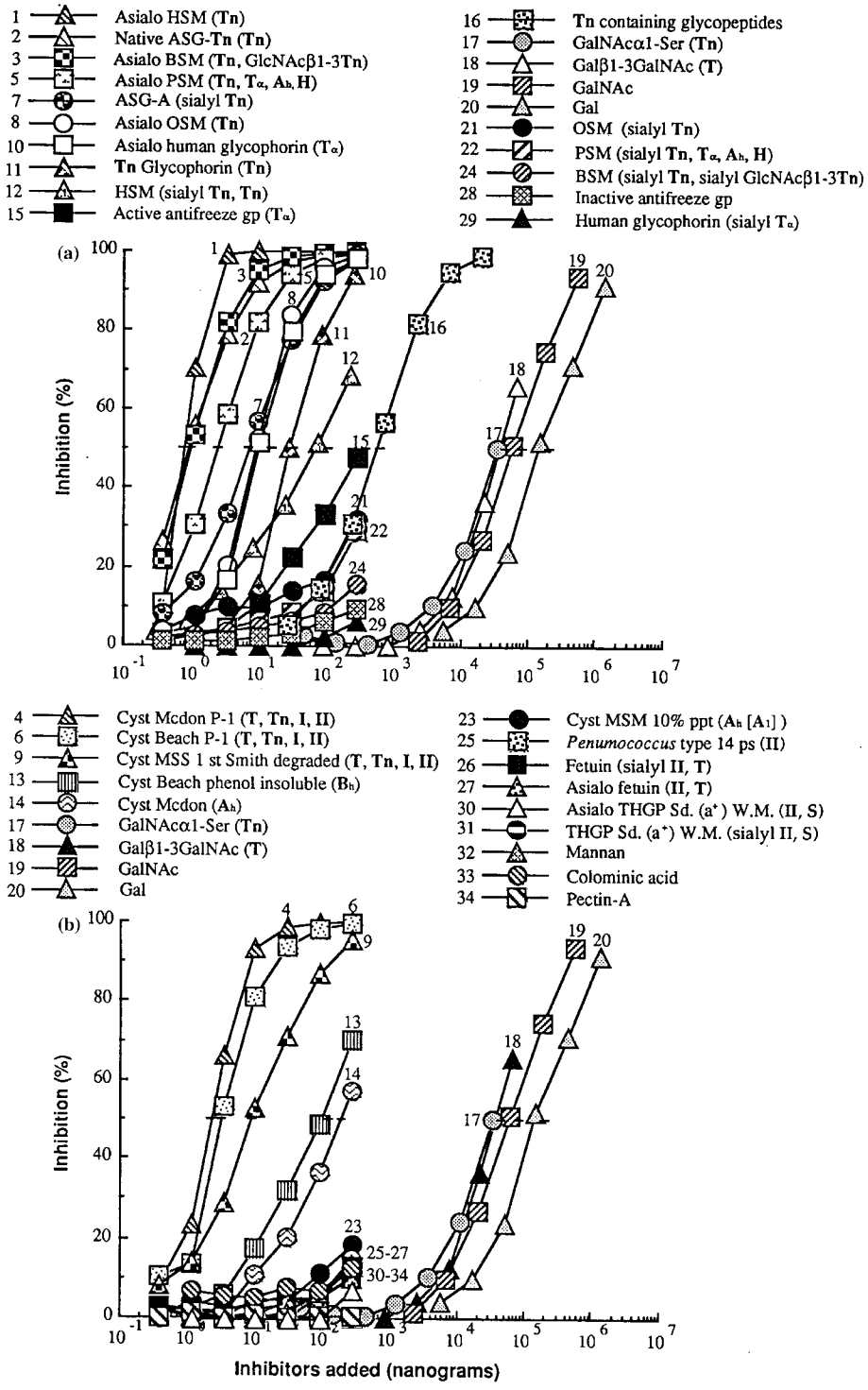


Figure 2. Inhibition of MPA binding to a Tn-containing glycoprotein (asialo OSM) coated on ELLSA plates by various glycoproteins. The quantity of glycoprotein in the coating solution was 2.0 ng/well. The lectin (10.0 ng/well) was pre-incubated with an equal volume of serially diluted glycoproteins. The final MPA content was 5.0 ng/well. Total volume was 50 µl. A_{405} was recorded after a 2-h incubation. The amount (ng) of glycoprotein required to induce 50% inhibition was determined.

Figure 2b) and polysaccharides (curves 32–34, Figure 2b) were tested up to 278 ng, but did not yield 50% inhibition (Table 3).

Inhibition of MPA-glycoform interaction by mono- and oligosaccharides

The ability of various sugar ligands to inhibit the binding of MPA to a Tn-containing glycoprotein (asialo OSM) was determined by ELLSA. The amounts in nanomoles required for 50% inhibition of the binding of MPA to asialo OSM are listed in Table 4. Details of the inhibition by individual mono- and oligosaccharides are presented in Figure 3. Among the oligosaccharides and mammalian glycoconjugates tested, the Tn-containing glycopeptide prepared from OSM was

Table 4. Amounts of various saccharides giving 50% inhibition of binding of MPA (5 ng/50 µl) to a Tn-containing gp (asialo OSM 2 ng/50 µl)[a].

Curves no.	Curves in Figure 3	Saccharide	Quantity giving 50% inhibition (nmol)	Relative potency[b]
Monosaccharides and their derivatives				
1	b	*p*-NO_2-phenyl-α-Gal	5.0	180.0
2	b	*p*-NO_2-phenyl-β-Gal	85.0	11.0
3	b	*p*-NO_2-phenyl-α-GalNAc	6.0	150.0
4	b	*p*-NO_2-phenyl-β-GalNAc	100.0	9.0
5	b	Methyl-α-GalNAc	13.0	69.2
6	b	Methyl-β-GalNAc	500.0[c]	1.8
7	b	Methyl-α-Gal	30.0	30.0
8	b	Methyl-β-Gal	3.1×10^3	0.3
9	a, b	GalNAc	290.0	3.1
10	a, b	Gal	900.0	1.0
11	b	Man	$> 2.7 \times 10^4$ (21%)[d]	–
12	b	L-Ara	$> 1.6 \times 10^4$ (19%)	–
13	b	D-Fuc	$> 2.3 \times 10^4$ (25%)	–
14	b	Glc	$> 2.1 \times 10^4$ (15%)	–
Tn/T, P glycotopes and simple Tn clusters				
15	a	Tn-containing glycopeptides (M.W. $< 3.0 \times 10^3$)	1.3	692.3
16	a	GalNAcα1→Ser/Thr (Tn)	100.0	9.0
17	a	Galβ1→3GalNAc (T)	95.0	9.5
18	a	Galβ1→3Gal NAcα1→Ser (Tα)	250.0[c]	3.6[c]
19	a	GalNAcβ1→3Gal (P)	110.0[c]	8.1[c]
Other mammalian carbohydrate structural units				
20	a	Galα1→3Gal (B)	$> 1.5 \times 10^2$ (21.0%)[d]	–
21	a	GalNAcα1→3Gal (A)	$> 1.3 \times 10^2$ (16.4%)	–
22	a	Galα1→4Gal (E)	$> 2.4 \times 10^2$ (12.0%)	–
23	a	Galβ1→4Glc (L)	$> 7.1 \times 10^3$ (9.0%)	–
24	a	Galβ1→3GlcNAc (I)	$> 3.6 \times 10^2$ (0.0%)	–
25	a	Galβ1→4GlcNAc (II)	$> 3.6 \times 10^2$ (−8.0%)	–
26	a	GalNAcβ1→4Gal (S)	$> 1.3 \times 10^2$ (−7.0%)	–
27	a	GalNAcα1→3GalNAc (F)	$> 1.2 \times 10^2$ (−3.1%)	–

[a]The inhibitory activity was estimated from the inhibition curve in Figure 3 and is expressed as the amount of inhibitor giving 50% inhibition. Total volume was 50 µl.

[b]Relative potency = quantity of Gal (curve 10) required for 50% inhibition is taken as 1.0/quantity of sample required for 50% inhibition.

[c]Extrapolation.

[d]The inhibitory potency of inactive saccharides is expressed as the maximum amount of sugars tested that yield inhibition (in parentheses) below 50%.

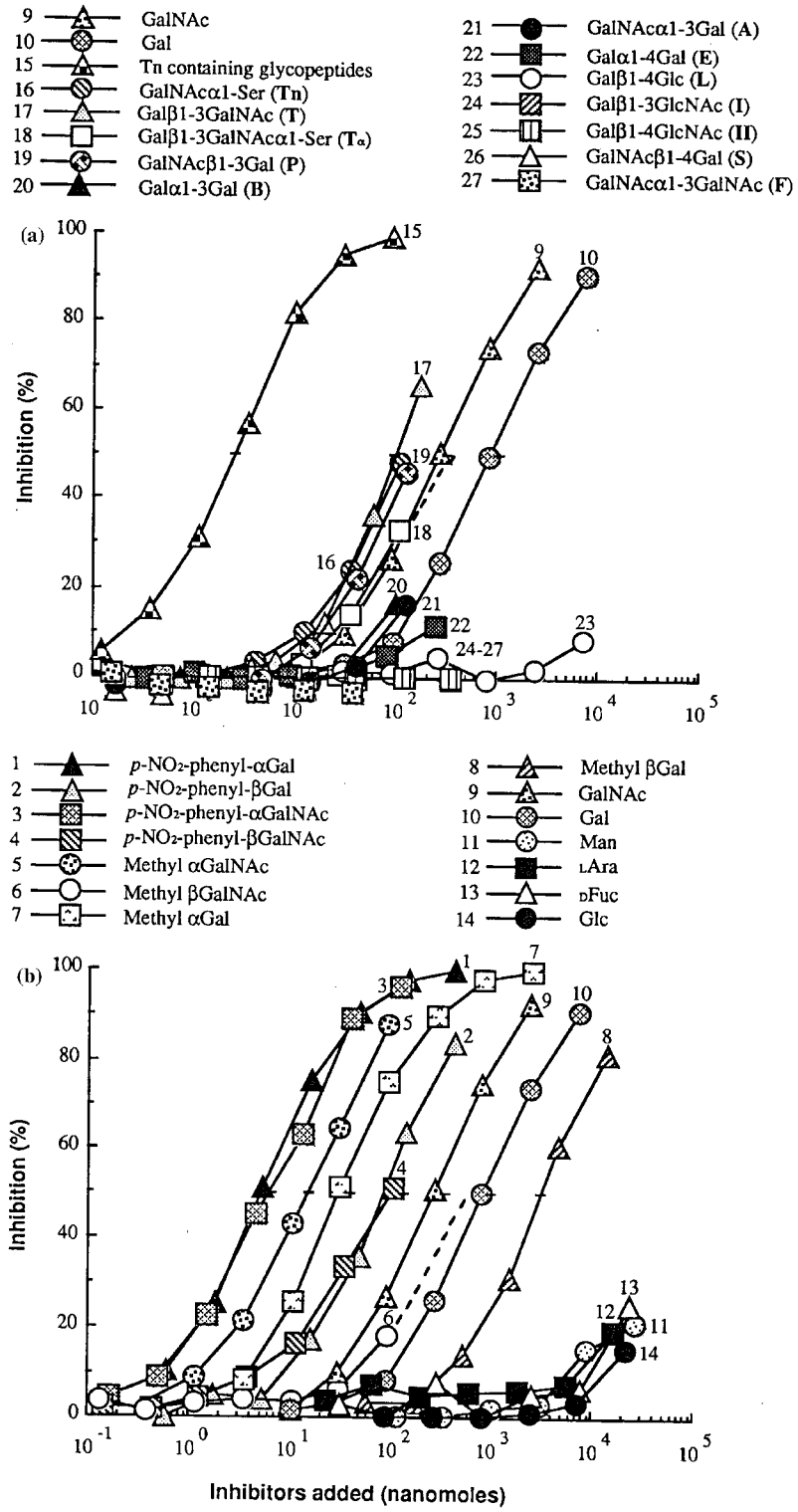


Figure 3. Inhibition of MPA binding to a Tn-containing glycoprotein (asialo OSM) coated on ELLSA plates by various saccharides. The amount of glycoprotein in the coating solution was 2.0 ng/well. The lectin (10.0 ng/well) was pre-incubated with an equal volume of serially diluted inhibitor. The final MPA content was 5.0 ng/well. Total volume was 50 µl. A_{405} was recorded after a 2-h incubation.

the best inhibitor being 6.9×10^2, 2.2×10^2 and 77 times more active than monovalent Gal, GalNAc and Tn (curve 15 vs. curves 10, 9 and 16), respectively, suggesting that cluster forms of Tn contributed significantly to binding.

T-disaccharide (Gal β1 → 3GalNAc, curve 17) was approximately 3 times more active than T_α (Gal β1 → 3GalNAc α1 → Ser, curve 18), suggesting that α1 → serine at the reducing end blocks the binding. Moreover, GalNAcβ1→3Gal (P) was as active as T-disaccharide (curve 19 vs. 17, Figure 3a). The other mammalian disaccharide structural units such as Galβ1→3GlcNAc (I), Galβ1→4GlcNAc (II), Galα1→3Gal (B), Galα1→4Gal (E), Galβ1→4Glc (L), GalNAcα1→3GalNAc (F), GalNAcβ1→4Gal (S) and GalNAcα1→3Gal (A) (curves 20–27, Table 4) failed to reach 50% inhibition, demonstrating that the α-anomer of Gal with the *N*-acetamido group at carbon-2 in Tn-epitope and the conformation of the hydroxyl group at carbon-4 and the carbon-2 acetamido group associated with the T-disaccharide are important for binding with MPA.

Of the monosaccharides studied, *p*-NO_2-phenyl α-Gal and GalNAc (Figure 3b, curves 1 and 3, Table 4) were the best inhibitors, being 180 and 48 times more active than Gal and GalNAc (curves 10 and 9), respectively. When the value of methyl β-GalNAc was extrapolated to 50% inhibition, its inhibitory power was less then that of *p*-NO_2-phenyl β-GalNAc (curve 4 vs. curve 6). Both, *p*-NO_2-phenyl α- and β-Gal and GalNAc were more active than their counterparts' methyl α- and β-Gal and GalNAc derivatives, suggesting that the hydrophobicity surrounding Gal and GalNAc is important for binding. The α-anomer of Gal and GalNAc is preferred over the β-anomers for the glycosides methyl and *p*-NO_2-phenyl (curves 1 vs. 2, 3 vs. 4, 5 vs. 6 and 7 vs. 8 in Table 4, Figure 3b), indicating that α-anomer is essential for binding. All Man and Man-related oligosaccharides were inactive (Table 5).

Discussion

The remarkably strong affinity of MPA for T/Tn gps observed in this study, with only minor cross reaction with other glycoproteins, suggests that MPA can be a useful tool; (i) as a structural probe

Table 5. Maximal quantities of various saccharides giving negligible or weak inhibition of MPA binding (5 ng/50 µl) to a Tn-containing gp (asialo OSM 2 ng/50 µl)[a,b].

Curves or identification no.	Saccharide	Maximum amount of inhibitor (nmol)	Inhibition (%)
28	Neu5Acα2→6GalNAcα1→3Ser (sialyl Tn)	18.3	6.1
29	Galβ1 → 4GlcNAcβ1 →2Man ↓ α1 − 6 Galβ1 → 4GlcNAcβ1 → 2Manα1 →3Manβ1 → 4GlcNAcβ1 → 4GlcNAcβ1Asn ↑ β1 − 4 Galβ1 → 4GlcNAc Tri-antennary Galβ 1-4GlcNAc (Tri-**II**)	4.4	6.0
30		99.0	4.8
31		60.3	4.8
32	Manα1→6Man (α1-6 Mannobiose)	146.0	1.1
33	*p*-NO_2-phenyl αMan	147.5	37.7
34	*p*-NO_2-phenyl βMan	192.5	24.2
35	Methyl αMan	1.6×10^3	0.6
36	Methyl βMan	3.2×10^3	5.7

[a]Consisting of 5 ng MPA + 2 ng asialo OSM. Total volume was 50 µl.

[b]The inhibitory activity was estimated from the inhibition curve, which was not shown in Figure 3. Curve no. used here is only for identification.

to detect the aberrant expression of T/Tn epitopes in tissues and cells; (ii) in the analysis of *O*-linked glycoproteins; and (iii) for the identification and fractionation of glycopeptides and oligosaccharides.

Dozens of assays [13, 32–37] have demonstrated that enzyme-linked lectinosorbent assay (ELLSA) is a rapid and reagent-saving method in which the binding profiles can be easily elucidated. To avoid potential problems caused by differences in adsorption of the glycoproteins to the microplate wells, the reactivities of the glycoproteins used in Figure 1 (Table 2) were tested for their ability to inhibit lectin–glycoprotein binding (Figure 2 and Table 3). The results were expressed as the amount of glycopeptides or glycans (in nanograms) required to inhibit 50% of the MPA–glycoprotein interaction (Table 3), as it is very difficult to obtain accurate numbers and structures of carbohydrate chains present in many complex-*O*-linked glycoproteins [25, 40]. Furthermore, the data expressed on a nanogram basis should be more conservative than nanomoles as amino acid contents are also included in the gram weight.

Numerous studies have demonstrated that polyvalent display of carbohydrates can lead to remarkably increased binding avidities [35, 38–41]. Therefore, it becomes important to estimate the polyvalent effects on binding. From the results in Table 3, it is demonstrated that high density Tn-containing glycoproteins, such as asialo HSM and native ASG-Tn, are up to 2.0×10^5, 8.0×10^4 and 4.0×10^4 times more active than monomeric Gal, GalNAc and Tn, respectively (curves 1 and 2 vs. 20, 19 and 17, Figure 2). Although a mixture of Tn-containing glycopeptides (Tn cluster; M.W. $< 3.0 \times 10^3$) was the most active ligand among various saccharides (curve 15, Table 4), it was, on a nanogram basis, about 76 times more active than monomeric Tn (curve 16 vs. curve 17), and about 460 times less active than native ASG-Tn or asialo HSM (curve 16 vs. high-density polyvalent Tn, curve 1 or curve 2). These results suggest that when Tn is in cluster form it is a potent ligand and when Tn is in its high-density polyvalent form (curves 1 and 2, Table 3), it plays the major role in binding. Indeed, these types of *O*-glycans intrinsically have an exceedingly high density of glycotopes, which are different from other types of glycoconjugates due to their tandem-repeat peptide sequences, and it is this that contributes to their high affinity for binding. Therefore, it is suggested that the concept of glycoside cluster effect be classified into two groups: (a) the '*Multi-antennary* or simple glycoside cluster effect', as in galactosides with hepatic lectin [42, 43] and triantennary II sequences as in a galectin from chicken liver (CG-16) [44] or Tn glycopeptides (curve 15 in Table 4); and (b) the '*High-density polyvalent* effect' as in macromolecular interaction of polyvalent Tn glycotope [the α-anomer of Gal with $NHCH_3CO$ at carbon-2] in asialo HSM which generates an enhancement in affinity with MPA by about 2.0×10^5 times over Gal (curve 1 vs. 20 in Table 3). This increase in inhibitory potency from free monovalent sugars to glycoproteins for MPA suggests the importance of polyvalency. However, the effect of polyvalencies of glycotopes on the carbohydrate–protein binding is not always so important. For example, the potency in the interaction of *Pseudomonas aeruginosa*-II lectin (PA-IIL) with L-Fuc α1-polyvalent glycans is about as strong as or weaker than the incremental increase by carbohydrate specificity of monomers [45] (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) affinity for monosaccharide specificity (Gal, GalNAc, GlcNAc, and/or Man) and its derivatives, (ii) expression of reactivities toward mammalian disaccharides and Tn structural units (in decreasing order) [46, 47], (iii) the most active ligand, (iv) simple multivalent or cluster effects of carbohydrate structured units such as Tn glycopeptides and multi-antennary glycotopes to inhibit binding, and (v) complex multivalent or cluster effects present in macromolecules with known glycotopes. In previous studies, the specificity of MPA was mainly limited to scales of (i)-(iii) and within only the T-related spectrum. From previous studies and our current results, it can be concluded that: (a) the α-anomer of Gal with $NHCH_3CO$ at carbon-2 enhances the reactivity by 5.1 (curve 17 vs. curve 20 in Table 3), while high-density polyvalent Tn increases the reactivity up to 2.0×10^5 (curve 1 vs. curve 20), thus glycotope polyvalency (high density polyvalent glycoside effect) is the most important factor for MPA binding. A similar observation was also seen in other agglutinins from *Sclerotium rolfsii* (SRL) [41], *Agaricus*

bisporus (ABA) [35] and *Vicia villosa* B_4 (VVL-B_4) [40]; (b) for MPA (Table 4), Galβ1→3GalNAc (T), GalNAcα1→Ser/Thr (Tn) and GalNAcβ1→3Gal (P) were the most potent mammalian carbohydrate structural units, whereas other disaccharide ligands, such as GalNAcα1→3Gal (A), GalNAcα1→3GalNAc (F), GalNAcβ1→4Gal (S), Galβ1→ 3/4GlcNAc (I/II), Galβ1→4Glc (L) and Galα1→3/4 (B/E) were poor inhibitors; (c) hydrophobicity enhances the reactivity significantly (curve 3 vs. curve 5 in Table 4); (d) the configurations of carbon-4 and carbon-2 are required for MPA binding; (e) MPA recognized mainly exposed Tn and Tn glycosylated by Galβ1→3 residues (T), but reacts weakly or poorly with other glycosylated Tn; (f) the α-anomers of Gal and GalNAc were more active than their β-anomers (curve 1 vs. curve 2; curve 3 vs. curve 4 in Table 4); (g) on a nanogram basis, the overall binding profile of MPA can be expressed in

Table 6. Comparison of binding activities of MPA with T/Tn, and II specific lectins with glycoproteins[a] [35–37, 40, 46, 47, 50].

Glycoprotein	MPA (T/Tn)	Jacalin (T,Tn)	ABA (T,Tn/ II)	BPA (T, Tn/II)	VVL-B_4 (Tn)	APA (T, II)
Polyvalent T/Tn-containing gps						
Native ASG-Tn (Tn only)	5+[b]	5+[b]	5+[b]	3+[b]	5+[b]	–
Asialo OSM (Tn > > Tα)	5+[b]	5+[b]	5+[b]	5+[b]	5+[b]	2+
Polyvalent Tα-containing gp						
Active antifreeze gp (Tα)	5+[b]	4+[b, c]	5+[b]	5+[b]	–[b, c]	4+
Inactive antifreeze gp (Tα)	3+[b]	±[b, c]	5+[b, c]	+[b]	–[b, c]	ND
Polyvalent I/II or multi-antennary II-containing gps						
Polyvalent II (O-linked)						
Cyst Mcdon P-1 (I, II, T, Tn)	5+[b]	5+[b, c]	5+[b, c]	5+[b]	4+[b]	4+
Cyst Beach P-1 (I, II, T, Tn)	5+[b]	4+[b]	5+[b]	5+[b]	+[b]	4+
Multi-antennary II (N-linked)						
Asialo human α_1-acid gp (II)	4+[b]	5+[b]	5+[b]	5+[b]	–[b]	5+
Asialo fetuin (II)	+[b]	5+[b]	5+[b]	5+[b]	–[b]	5+
Pneumococcus type 14 ps (II)	±[b]	2+[b]	4+[b]	+[b]	–[b]	4+
Masking effect of advanced glycosylation						
Blood group ABH active gps						
Cyst MSS 10% 2× (A_h [A_1])	3+[b]	2+[b]	3+[b]	±[b]	–[b]	4+
Cyst Mcdon (A_h)	2+[b]	5+[b, c]	5+[b, c]	+[b]	–[b]	4+
Cyst 9 (A_h)	±[b]	+[b]	5+[b, c]	+[b, c]	–[b, c]	4+
Cyst MSM 10% ppt (A_h [A_1])	–[b]	–[b, c]	5+[b, c]	±[b, c]	±[b]	5+
Cyst Beach Phenol-insoluble (B_h)	2+[b]	2+[b]	3+[b]	+[b]	–[b]	4+
Cyst Tighe phenol-insoluble (H, Le^b)	3+[b]	+[c]	5+[b]	2+[b]	–[b]	5+
Cyst N-1 Le^a 20% 2× (Le^a)	4+[b, c]	3+[b, c]	5+[b]	4+[b, c]	±[b, c]	ND
Sialylated gps						
OSM (sialyl Tn)	3+[b]	4+[b]	3+[b]	±[b]	2+[b]	–
Human α_1-acid gp (sialyl II)	2+[b]	5+[b]	4+[b]	–[b]	–[b]	2+
Fetuin (sialyl II)	–[b]	5+[b]	5+[b]	–[b]	–[b]	+

Lectin abbreviations: MPA – *Maclura pomifera* agglutinin; BPA – *Bauhinia purpurea agglutinin*; Jacalin (from *Artocarpus integrifolia*); ABA (*Agaricus bisporus agglutinin*); VVL-B4 – *Vicia villosa* isolectin B4; APA – *Abrus precatorius agglutinin*.

[a]Lectins were used for the quantitative precipitin assays. –, less than 3% of lectin precipitated; ±, less than 15% of lectin precipitated; +, between 16 and 25% of lectin precipitated; + +, between 26 and 50% of lectin precipitated; + + +, between 51 and 75% of lectin precipitated; + + + +, between 76 and 95% of lectin precipitated; + + + + +, completely precipitated.

[b]The results were interpreted according to the spectrophotometric absorbance value at 405 nm (i.e. O.D.405) after 2 h incubation as follows: + + + + +, (O.D. ≥ 2.5); + + + +, (2.5 > O.D. ≥ 2.0); + + +, (2.0 > O.D. ≥ 1.5); + +, (1.5 > O.D. ≥ 1.0); +, (1.0 > O.D. ≥ 0.5); ±, (0.5 > O.D. ≥ 0.2); –, (O.D. < 0.2).

[c]From our unpublished data.

ND: not determined.

decreasing order as: high-density polyvalent Tn/T clusters >> Tn-glycopeptides > monovalent Tn/T and P > GalNAc > Gal, while Man, L-Ara, D-Fuc and Glc were inactive.

The GalNAcβ1→3Galα1→ (P_α) disaccharide structural unit is present at the nonreducing end of the human blood group P (GalNAcβ1→3Galα1→4Galβ1→ 4Glcβ1→1Cer) sequence in human erythrocytes, kidney and meconium and also exists as an internal structural unit of many glycosphingolipids [47, 48]. As it is only present in glycosphingolipids, the interaction of MPA with this ligand is not included in this study. However, it will be further characterized when a suitable analytical method is established and reagents become available.

It is of interest to compare the binding of MPA–glycotopes with the other Tn, T or II-reactive lectin families [46, 47, 49, 50]: (a) for proportional contributions of individual residues of T disaccharides (Gal and GalNAc of Galβ1→3GalNAc), it was found that GalNAc > Gal in *Maclura pomifera* (MPA), Jacalin (from *Artocarpus integrifolia* lectin), *Bauhinia purpurea* agglutinin (BPA) and *Agaricus bisporus* agglutinin (ABA), but Gal >> GalNAc in *Abrus precatorius* and *Arachis hypogea* agglutinins (APA and PNA). When Galβ1→3 was added to Tn (T glycotope; curve 16 vs. curve 18 in Table 3), the reactivity of MPA toward Tn was decreased about 60% (curve 16 vs. curve 18 in Table 4) and that of *Vicia villosa* isolectin B_4 (VVL-B_4) was totally lost, indicating that glycosylation of Galβ1→3 functioned as a blocking factor [40]. However, the reactivity of MPA can be recovered and enhanced by the polyvalent effect of T (curves 5, 10 and 15 in Table 3); (b) as shown in Table 6, the reactivities of binding toward poly Tn can be ranked as VVL-B_4 ≥ MPL, Jacalin, ABA > BPA >> APA and PNA; (c) the affinities for antifreeze gps (poly T) are MPA, ABA, BPA and PNA ≥ Jacalin, APA >> VVL-B_4; (d) the effect of ABH key sugars and sialic acids on binding can be summarized as VVL-B_4 ≥ BPA > MPA > Jacalin > APA and ABA; (e) MPA, ABA and Jacalin can be used as probes to detect both exposed and crypto T/Tn, while VVL-B_4 and BPA should be used as ideal reagents to identify the exposed Tn and/or T glycotopes. When two or three of the above lectins are combined to examine T/Tn glycotopes in glycoproteins, a satisfactory depiction of the glycan profile can be reached.

From the results of the present study, it is illustrated that each lectin with a unique amino acid sequence has its own binding character (Table 6) [46, 49–51]. Furthermore, this study also defines essential knowledge for the future selection of these structural probes in medical and biotechnological applications.

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