Effect of polyvalencies of glycotopes on the binding of a lectin from the edible mushroom, *Agaricus bisporus*

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Agaricus bisporus agglutinin (ABA) isolated from edible mushroom has a potent anti-proliferative effect on malignant colon cells with considerable therapeutic potential as an anti-neoplastic agent. Since previous studies on the structural requirement for binding were limited to molecular or submolecular levels of Gal β 1-3GalNAc (**T**; Thomsen–Friedenreich disaccharide glycotope; where Gal represents D-galactopyranose and GalNAc represents 2-acetamido-2-deoxy-D-galactopyranose) and its derivatives, the binding properties of ABA were further investigated using our collection of glycans by enzyme-linked lectinosorbent assay and lectin–glycan inhibition assay. The results indicate that polyvalent Gal β 1-related glycotopes, GalNAc α 1-Ser/Thr (**Tn**), and their cryptoforms, are the most potent factor for ABA binding. They were up to 5.5×10^5 and 4.7×10^6 times more active than monomeric **T** and GalNAc respectively. The

affinity of ABA for ligands can be ranked as: multivalent **T_n** (Gal β 1-3GalNAc α 1-), **Tn** and **I/II** (Gal β 1-3GlcNAc/Gal β 1-4GlcNAc, where GlcNAc represents 2-acetamido-2-deoxy-D-glucopyranose) > > > monomeric **T_n** and **Tn** > **I** > > GalNAc > > **II**, **L** (Gal β 1-4Glc, where Glc represents D-glucopyranose) and Gal (inactive). These specific binding features of ABA establish the importance of affinity enhancement by high-density polyvalent (versus multiantennary **I/II**) glycotopes and facilitate our understanding of the lectin receptor recognition events relevant to its biological activities.

Key words: *Agaricus bisporus* agglutinin, carbohydrate specificity, glycoprotein binding, lectin, multivalent effect, polyvalency.

INTRODUCTION

Many lectins abundant in the diet resist heat and digestion [1], remain in active form in colon and faeces [1,2] and have marked effects on the function of gastrointestinal cells [1–4]. *Agaricus bisporus* agglutinin (ABA) isolated from edible mushroom is one of the Gal β 1-3GalNAc (**T**, Thomsen–Friedenreich disaccharide glycotope; where Gal represents D-galactopyranose and GalNAc represents 2-acetamido-2-deoxy-D-galactopyranose)-specific lectins that reacts weakly with monosaccharides and possesses a potent anti-proliferative effect on malignant colonic epithelial cells without any apparent cytotoxicity [1,3,4]. This lectin, with a molecular mass of 6.4×10^4 Da, is a tetramer consisting of four apparently identical subunits (M_r 16 000) [5]. Moreover, four different isoelectric forms with similar carbohydrate-binding specificities were reported [5]. Although its special structural nature required for binding, which is different from that of other **T**-specific lectins, had been documented previously [6], this information was limited to molecular or submolecular **T** and **T**-related oligosaccharides. Its binding relationship towards other mammalian structural units has not been determined. The unusual combination of biological properties described

previously [3–6] prompted us to investigate its binding characteristics. To understand the roles of ABA, we further characterized its binding properties using our collection of glycans by enzyme-linked lectinosorbent assay (ELLSA) and lectin–glycan inhibition assay. The results indicate that GalNAc α 1-Ser/Thr (**Tn**) glycotopes and Gal β 1-3GlcNAc (**I**, human blood group type I precursor sequence), in addition to the **T** sequence, are also active ligands. In most cases, ABA recognizes not only glycans exposing Gal β 1 and **Tn**-glycotopes, but also their cryptoforms masked by blood group determinants or sialic acids. Based on the interaction profiles of the present study and inhibition of the ABA–glycoprotein binding by glycans, polyvalencies of glycotopes should be the most important factor involved in ABA binding; the affinity of ABA for mammalian carbohydrate structural units was examined and the overall binding was defined.

EXPERIMENTAL

Lectin and biotinylation of lectin

ABA was purchased from Sigma (St. Louis, MO, U.S.A.). The biotinylation of lectin was prepared as described previously [7,8].

Abbreviations used: ABA, *Agaricus bisporus* agglutinin; ELLSA, enzyme-linked lectinosorbent assay; Gal, D-galactopyranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; Glc, D-glucopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; HOC, human ovarian cyst fluid; OSM, ovine salivary glycoprotein; PNA, peanut (*Arachis hypogaea*) agglutinin; PSM, porcine salivary mucin; TBS, Tris/HCl-buffered saline; THGP, Tamm–Horsfall glycoprotein. The mammalian carbohydrate structural units in glycans used to define binding properties of ABA are: **A**, GalNAc α 1-3Gal, human blood group A-specific disaccharide; **A_n**, GalNAc α 1-3Gal(L-Fuc α 1-2), human blood group A-specific trisaccharide containing crypto H determinant; **B**, Gal α 1-3Gal, human blood group B-specific disaccharide; **E**, Gal α 1-4Gal, galabiose, the uropathogenic *Escherichia coli* receptor; **F**, GalNAc α 1-3GalNAc; **H**, L-Fuc α 1-2Gal, human blood group H-specific disaccharide; **I**, Gal β 1-3GlcNAc, human blood group type I precursor sequence; **II**, Gal β 1-4GlcNAc, human blood group type II precursor sequence; **L**, Gal β 1-4Glc; **P**, GalNAc β 1-3Gal; **S**, GalNAc β 1-4Gal; **T**, Gal β 1-3GalNAc, Thomsen–Friedenreich disaccharide; **T_n**, Gal β 1-3GalNAc α 1-; **Tn**, GalNAc α 1-Ser/Thr.

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Figure 1 Proposed representative carbohydrate side chains of blood-group-active glycoproteins, prepared from HOC

This structure represents the internal portion of carbohydrate chains to which various human blood group determinants are attached. The four-branched structure (1–4) shown is the representative internal portion of the carbohydrate moiety of blood group substances to which the residues responsible for A, B, H, Le^a and Le^b activities are attached. This structure also represents precursor blood-group-active glycoproteins [12,16] and can be prepared by Smith degradation of A, B, H active glycoproteins, purified from HOC [10–13]. Numbers in parentheses indicate the site of attachment for the human blood group A (1–3 and/or 4–10, 12), B (1–3 and/or 4–10, 12), H (5–12), Le^a (6 and 10) and Le^b (5–12) determinants. These determinants as well as the structural units at the non-reducing end are the sources of lectin-reactive A/A_n [where A_n represents GalNAcα1-3(L-Fucα1-2)Gal, human blood group A-specific trisaccharide containing crypto H determinant; L-Fuc represents L-fucopyranose], B, I/II, T and Tn determinants [35,36]. This megalosaccharide of 24 sugars has not been isolated. However, most of the carbohydrate chains isolated are parts of this structure.

Glycoproteins and polysaccharides

The 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 [9–11]; the dried ethanol precipitates were extracted with 90% phenol, the insoluble fraction being named after the blood group substance (e.g. Cyst Tighe phenol insoluble). The 'Tighe' denotes the code of the HOC sample. A similar principle could be applied to the other HOC collections such as Beach, MSS, JS, N-1, etc. The supernatant was fractionally precipitated by addition of 50% ethanol in 90% phenol at the indicated concentrations [9]. 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 round of phenol extraction and ethanol precipitation was performed (Cyst MSS 10% 2 × and Cyst N-1 Le^a 20% 2 ×). 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 (Figure 1) [10,12].

In general, the 'P-1' fractions (Cyst Beach P-1 and Cyst Tighe phenol P-1) represent the non-dialysable portion of the blood group substances after mild hydrolysis at pH 1.5–2.0 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 [13–15]. The 'Smith-degraded' products of blood-group-active substances (Cyst MSS 1st Smith degraded, Cyst JS 1st Smith degraded), in which almost all of the sugar groups at the non-reducing ends were removed, were prepared as described previously [16,17]. Both P-1 fractions and Smith-degraded products from HOC glycoproteins exposing the internal structures equivalent to those on the blood group precursors are defined as 'precursor equivalent glycoproteins'.

The anti-freeze glycoprotein from the Antarctic fish (*Trematomus borchgrevinkii*) which contains only Galβ1-3GalNAcα1- (T_α) as carbohydrate chains (Figure 2) [18] was provided by Dr R. E. Feeney (Department of Food Science and Technology, University of California, Davis, CA, U.S.A.) through the late Dr E. A. Kabat (Columbia Medical Center, New York, NY, U.S.A.).

Glycophorin A, a mucin type O-glycan containing sialylated T_α, 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 [19]. Asialoglycophorin (T_α) was prepared by mild acid hydrolysis [20,21].

- (i) GalNAcα1-Ser/Thr (Tn)
- (ii) Galβ1-3GalNAcα1-Ser/Thr (T)
- (iii) GalNAcα1-3Galβ1-3GalNAcα1-Ser/Thr (A)

Figure 3 Structure of PSM glycoprotein

The carbohydrate side chains of this glycoprotein are O-glycosidically linked by GalNAc at the reducing end of the carbohydrate side chain to a Ser or Thr residue of the protein core [22,23]. Twelve kinds of carbohydrate side chains have been isolated. They are composed of 1–5 sugar residues with Galβ1-3GalNAcα1-1-O-Thr or -Ser as the carbohydrate core region. They can be masked by N-acetylneuraminic acid (NeuNAc) and NeuNGc. Substitution by GalNAc or L-Fuc exhibits blood group A and H activities respectively. The mild acid-treated product of PSM [20,21] contains mainly mixtures of the above three structures.

Figure 2 Structure of active anti-freeze glycoproteins

The active anti-freeze glycoproteins from the Antarctic fish *Trematomus borchgrevinkii* is composed of repeating units of a diglycosyltripeptide as shown in the above structure [19]. The shaded area (T_α) is proposed to be the active site for ABA attachment. Binding enhancement can be reached by its polyvalency.

Figure 4 Repeating unit of the *Pneumococcus* type 14 capsular polysaccharide [28]

Shaded area (II) is proposed to be the reactive glycotope for ABA. Binding intensity can be increased greatly by its polyvalency.

Figure 5 Structure of fetuin

The glycoprotein has three oligosaccharide side chains with three different structures O-glycosidically linked to Ser or Thr of the protein core as well as three carbohydrate side chains per molecule N-glycosidically linked to asparagine [25,29]. Carbohydrate side chains of mild acid-treated (desialized) fetuin [20,21] contain no NeuNAc.

The **Tn**-type glycoprophorin (**Tn**-glycophorin) was obtained by removing galactose residues from asialo-glycophorin by periodate oxidation and mild acid hydrolysis (Smith degradation) [7,16].

Ovine salivary glycoprotein major (OSM) and porcine salivary mucin (PSM; Figure 3) were purified by the method of Tettamanti and Pigman [20] with some modifications [22,23]. Asialo PSM contains **T_x** [22] together with **Tn** and **A** (GalNAc α 1-3Gal, human blood group A-specific disaccharide) sequences, as most of the outer fucosyl residues and sialic acids are cleaved by mild acid hydrolysis.

The *Pneumococcus* type 14 polysaccharide, isolated from *Streptococcus pneumoniae* capsule (Figure 4) [24], was a gift from the late Dr E. A. Kabat.

Fetuin (Gibco Laboratories, Grand Island, NY, U.S.A.) is the major glycoprotein in foetal calf serum and 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 triantennary **II** (Gal β 1-4GlcNAc, human blood group type II precursor sequence) N-glycans and a small amount of the Gal β 1,3-linked isomer at the non-reducing terminal ends (Figure 5) [25].

Human α_1 -acid glycoprotein was purchased from Sigma. It is a complex-type N-glycan and the carbohydrate units of this asialoglycoprotein can be grouped into compounds with biantennary and triantennary **II** structure with and without a fucose residue (Figure 6) [26].

Desialylation of sialoglycoproteins was performed by mild acid hydrolysis using 0.01 M HCl at 80 °C for 90 min, and

Table 1 Binding of ABA to human blood group A, B, H, P₁, Le^a and Le^b active glycoproteins, sialo- and asialoglycoproteins and polysaccharides by ELLSA

Biotinylated lectin (10 ng) was added to glycoprotein concentrations ranging from 0.001 ng to 5 μ g.

Curve in Figure 7	Glycoprotein lectin determinants* (blood group specificity)	Glycoform (ng) required to reach an A_{405} of 1.5	Maximum A_{405} reading†	Binding intensity†
b	Active anti-freeze glycoprotein (T_x)	0.3	3.0	+++++
f	Cyst Tighe P-1 (I, II, T, Tn)	0.4	3.0	+++++
f	Cyst Beach P-1 (I, II, T, Tn)	0.7	3.0	+++++
a	Cyst JS 1st Smith degraded (I, II, T, Tn)	1.0	3.0	+++++
e	Cyst MSS 1st Smith degraded (I, II, T, Tn)	1.0	3.0	+++++
d	Asialo fetuin (II, T)	3.0	2.7	+++++
b	Tn-Glycophorin (Tn)	4.1	3.0	+++++
d	Fetuin (sialyl II, T)	4.5	3.0	+++++
e	Cyst N-1 Le ^a 20% 2 $\times$ (Le^a)	5.0	2.5	+++++
a	Asialo human α_1 -acid glycoprotein (II)	7.0	3.0	+++++
c	Asialo PSM (A, A₁, T_x, Tn)	11.0	3.0	+++++
b	Asialo human glycophorin (T_x)	14.0	3.0	+++++
e	Cyst MSS 10% $\times$ 2 (A₁, [A₁])	15.0	1.7	+++
c	Asialo ovine salivary glycoprotein major (Tn)	17.0	3.0	+++++
c	PSM (sialyl A, A₁, T_x, Tn)	20.0	1.7	+++
f	Cyst Tighe phenol insoluble (H, Le^b)	25.0	3.0	+++++
c	Ovine salivary glycoprotein major (sialyl Tn)	50.0	2.0	+++
f	Cyst Beach phenol insoluble (B)	51.0	1.6	+++
a	<i>Pneumococcus</i> type 14 polysaccharide (II)	110.0	2.2	++++
a	Human α_1 -acid glycoprotein (sialyl II)	200.0	2.3	++++
e	Arabinogalactan	—	0.2	$\pm$
d	Native and asialo THGP Sd. (a +) W.M. (II, S)	—	< 0.2	$\pm$
a	Mannan	—	0.1	—
b	Human glycophorin (sialyl T_x)	—	0.05	—
b	Pectin A and pectin C	—	—	—
d	Colominic acid	—	—	—

* The symbol in parentheses indicates the human blood group activity (Figure 1) and/or lectin determinants [35,36].

† The results were interpreted according to the spectrophotometric A_{405} value after 2 h incubation as follows: + + + + +, $A \geq 2.5$; + + + +, $2.5 > A \geq 2.0$; + + +, $2.0 > A \geq 1.5$; + +, $1.5 > A \geq 1.0$; +, $1.0 > A \geq 0.5$; $\pm$, $0.5 > A \geq 0.2$; and —, $A < 0.2$.

Figure 6 Structure of human plasma α_1 -acid glycoprotein

The primary structure of classes A, B, BF, C and CF carbohydrate units of the glycosylation site in human plasma α_1 -acid glycoprotein [26] is indicated in the above structure for asialo-orosomucoid. The carbohydrate units of this asialoglycoprotein can be grouped into compounds with biantennary (class A), triantennary (class B) and the triantennary II structure with a fucose residue (class BF). Shaded areas (II) are proposed to be the reactive glycotopes for ABA.

dialysed against distilled water for 2 days to remove small fragments [20,21].

Tamm–Horsfall glycoprotein (THGP) with Sd(a+) blood group [27,28] was kindly provided by Dr W. M. Watkins (Royal Postgraduate Medical School, Hammersmith Hospital, University of London, London, U.K.).

Yeast high-mannose type glycan (mannan), polygalacturonic acid-containing pectins from the cell wall matrix of apple (pectin A) and citrus fruits (pectin C), poly-2,8-*N*-acetylneuraminic acid capsular polysaccharide from *Escherichia coli* (colominic acid) and arabinogalactan (an L-arabino-D-galactan mixture of two monosaccharides in the ratio of L-Araf/D-Galp residues varying from 1:4, with 1:6 being the most prevalent) were purchased from Sigma.

Sugars used for inhibition studies

Mono-, di- and oligosaccharides used were purchased or prepared by Dextra (Reading, Berkshire, U.K.) and Sigma. Triantennary II glycopeptides were prepared from asialofetuin by pronase digestion and repeatedly fractionated by BioGel P-4 400 mesh column chromatography [30]. The Tn clusters used for this study were mixtures of Tn-containing glycopeptides in the filterable fraction (molecular mass cut-off < 3000 Da).

Microtitre plate lectin–enzyme-binding assay

The assay was performed according to the procedures described by Duk et al. [7]. The volume of each reagent applied to the plate was 50 μ l/well, and all incubations, except for coating, were performed at room temperature (20–25 °C). The reagents, if not indicated otherwise, were diluted with Tris/HCl-buffered saline (TBS) containing 0.05 % Tween 20. The TBS (pH 7.4) or 0.15 M NaCl containing 0.05 % Tween 20 was used for washing the plate between incubations.

For inhibition studies, the serially diluted inhibitors were mixed with an equal volume of lectin solution containing a fixed amount of ABA. The control lectin sample was diluted 2-fold with TBS containing 0.05 % Tween 20. After 30 min at room temperature, the samples were tested using the binding assay [7,8]. The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor (nmol/well) giving 50 % inhibition of the control lectin binding.

RESULTS

Lectin–glycan interactions

The avidity of ABA for various glycans studied by ELLSA is summarized in Table 1 according to the interaction profiles shown in Figure 7. It is clear that ABA reacted strongly with high-density polyvalent glycotopes of glycoproteins with Tn, T_z (active anti-freeze glycoprotein in Figures 2 and 7b), I/II (precursor equivalent glycoproteins in Figure 1; Cyst 1st Smith-degraded glycoproteins in Figures 7a and 7e, and Cyst P-1 glycoproteins in Figure 7f) and, where less than 1.0 ng of coated glycoprotein was required to interact with 10.0 ng of lectin to yield an absorbance (A_{405}) value of 1.5 within 2 h.

This lectin also bound well to other exposed and cryptomultivalent II-containing glycoproteins or polysaccharide [human α_1 -acid glycoprotein, fetuin and their asialo products in Figures 5, 6, 7a and 7d; *Pneumococcus* type 14 polysaccharide in Figures 4 and 7a], Tn-containing glycoproteins and blood-group-active cyst glycoproteins (Tn-glycophorin in Figure 7b, asialo PSM in Figures 3 and 7c, Cyst glycoproteins in Figure 7e, etc.). However, it reacted weakly or not at all with GalNAc β 1-containing glycoprotein [Tamm–Horsfall Sd(a+) urinary glycoprotein in Figure 7d], high-mannose type yeast N-glycan (Figure 7a), colominic acid (Figure 7d), etc. Except for human glycophorin, sialylation only slightly interfered with the binding.

Inhibition of ABA–glycoform interactions by mono- and oligo-saccharides

The ability of various sugars to inhibit the binding of ABA with a T/Tn-containing glycoprotein (asialo PSM) is shown in Figure 8 and the amounts of ligands required for 50 % inhibition of the lectin–glycan interaction are listed in Table 2. Among 23 ligands tested, only four reached 50 % inhibition (curves 1–4). T_z (curve 1), which was the best inhibitor, was approx. 3 times more active than T (curve 2), suggesting that α 1-benzyl at the reducing end enhances binding. T (curve 2) was approx. 15 times more active than GalNAc (curve 4), indicating a strong contribution from the Gal β 1-3 residue. I (curve 3) was approx. 5 times more active than GalNAc (curve 4), but approx. 3 times less active than T (curve 2), showing the importance of Gal β 1-3 and *N*-acetyl amino groups at C-2 of GlcNAc in binding, as individually GlcNAc and are inactive (curves 11 and 18). II (curve 7) and Gal β 1-4Glc (L, curve 8) were tested at concentrations up to 2.3 and 26 times respectively more than I (curve 3) required for 50 % inhibition, but were still inactive, implying that the β 1-3 linkage is more efficient than the β 1-4 linkage. The *N*-acetyl amino group of the GalNAc residue (curve 4) should be an essential factor for the binding since Gal (curve 10) was tested at concentrations up to 14.8 times greater amounts than that of GalNAc (curve 4) required for 50 % inhibition, but still showed no reactivity.

Although mammalian carbohydrate structural units [A, F (GalNAc α 1-3GalNAc), P (GalNAc β 1-3Gal), S (GalNAc β 1-4Gal), B (Gal α 1-3Gal, human blood group B-specific disaccharide), L and E (Gal α 1-4Gal, galabiose, the uropathogenic *E. coli* receptor)] were tested at concentrations from 2 to 228 times greater than that required for T_z to achieve 50 % inhibition, they were still inactive (Table 2). Moreover, neither triantennary II glycopeptides nor Tn-containing glycopeptides (Figure 8; curves 6 and 5), which were tested up to 13 and 267 nmol respectively, showed inhibitory activity.

Inhibition of ABA–glycoform interactions by various glycans

The ability of glycans shown in Figure 9 to inhibit the binding of ABA to a T/Tn-containing glycoprotein (asialo PSM) was

Figure 7 Binding of ABA to microtitre plates coated with serially diluted human blood groups A, B, H, Le^b and Ii active glycoproteins (gp), sialo and asialoglycoproteins and polysaccharides (ps)

The amount of lectin used was 10 ng/well. Total volume of the assay was 50 μ l. A_{405} was recorded after 2 h incubation. The following glycans did not bind to ABA and $A < 0.2$: Mannan, THGP Sd. (a+) W.M. (sialyl II, S), human glycophorin (sialyl T_α), pectin A, pectin C and colominic acid.

Figure 8 Inhibition of ABA binding to Tn-containing glycoprotein (asialo PSM)-coated ELLSA plates by various saccharides

The amount of glycoprotein in the coating solution was 5.0 ng/well. The lectin (5.0 ng/well) was preincubated with an equal volume of serially diluted inhibitor. The final ABA content was 2.5 ng/well. The total volume was 50 μ l. A_{405} was recorded after 2 h incubation. Maximal quantities of other sugar inhibitors giving negligible or weak inhibition of ABA are listed in Table 2.

analysed by ELLSA with the representative panel of glycans listed in Table 3. The ABA–glycan interaction was inhibited by most high-density polyvalent T/Tn- and I/II-containing glycoproteins and their cryptofoms masked by blood group determinants or sialic acids. These include Tn (curves 1–4, 8, 9, 12, 13 and 15) and T (curves 5 and 6)-containing glycoproteins, I/II cluster-containing glycoproteins, especially human blood-group-active cyst glycoproteins (curves 7, 10 and 11) and their precursors (curves 2–4 and 8), N-linked multiantennary II-containing glycoproteins (fetuin, human α_1 -acid glycoprotein and their asialo products, in curves 14, 16, 18 and 19) and *Pneumococcus* type 14 polysaccharide (curve 17). Their reactivities were 1.2×10^3 to 4.7×10^6 times higher than that of monomeric GalNAc (Figure 9 and Table 3; curves 1 and 19 versus curve 22) and up to 5.5×10^3 times higher than that of T disaccharide (Table 3). The inhibitory reactivities of glycoforms towards ABA, in general, agree with the maximum absorbances recorded in the binding assay (Figure 7 and Table 1).

DISCUSSION

During the past two decades, it has been established that many multi-branched oligosaccharides exhibit a significant increment in lectin-binding reactivities as compared with their linear counterparts [31,32], especially ‘the glycoside cluster effect’ in mammalian hepatic asialoglycoprotein receptors [31]. Our results indicate that disaccharide II monomer and triantennary II glycopeptides (curves 7 and 6 in Figure 8 and Table 2), which are the major carbohydrate side chains of *Pneumococcus* type 14

polysaccharide, asialo fetuin and asialo human α_1 -acid glycoprotein, were poor inhibitors, whereas their polyvalent carriers were very active (Tables 1 and 3; Figures 4–6; curves 14, 16 and 17 in Figure 9), implying there must be other more complicated structural factors involved in binding besides the multiantennary II sequences. To explain this phenomenon, the present concept of the glycoside cluster effect has to be further defined and classified into two groups: (a) the ‘multiantennary or simple glycoside cluster effect’ as in galactosides with hepatic lectin [31,32] and triantennary II with a galectin from chicken liver (CG-16) [33], as well as (b) the ‘high-density polyvalent or complex glycoside cluster effect’ as in macromolecule interaction of high-density II cluster-containing glycoproteins (*Pneumococcus* type 14 polysaccharide and asialo human α_1 -acid glycoproteins) with ABA. The clustered glycotopes present on macromolecules generate a great enhancement in affinity with ABA of up to 4.7×10^6 times as compared with their monomeric counterparts (Table 3), and provide a good demonstration of the structural importance of complex carbohydrates. Possibly, 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 ABA tetramers, based on the model of another structurally well-studied polyvalent ligand-binding protein, the mammalian mannose-binding protein A (MBP-A) [34]. Such an extended sugar-binding shallow surface allows complex glycoprotein ligands to contact via clusters of glycotopes arranged in the correct geometry, which are absent in mono- and disaccharides and, thus, displays a lower affinity for their corresponding sugar monomers [34].

1 —■— Asialo PSM (A, A _b , T _α , T _n)	14 —□— Asialo fetuin (II, T)
2 —□— Cyst Tighe P-1 (I, II, T, T _n)	19 —●— Fetuin gp (Sialyl II, T)
4 —△— Cyst Beach P-1 (I, II, T, T _n)	20 —△— Galβ1-3GalNAcα- (T _α)
8 —△— Cyst MSS 1st Smith degraded (I, II, T, T _n)	21 —□— Galβ1-3GalNAc (T)
10 —○— Cyst Tighe phenol insoluble (H, Le ^b)	22 —●— GalNAc
11 —□— Cyst Beach phenol insoluble (B)	24 —□— Arabinogalactan
13 —△— PSM (Sialyl A, A _b , T _α , T _n)	25 —○— Pectin-A

3 —△— Cyst JS 1st Smith degraded (I, II, T, T _n)	16 —□— Asialo human α ₁ -acid gp (II)
5 —□— Asialo human glycoporphin (T _α)	17 —□— <i>Pneumococcus</i> type 14 ps (II)
6 —●— Active antifreeze gp (T _α)	18 —△— Human α ₁ -acid gp (Sialyl II)
7 —○— Cyst JS phenol insoluble (H)	20 —△— Galβ1-3GalNAcα- (T _α)
9 —△— Asialo OSM (T _n)	21 —□— Galβ1-3GalNAc (T)
12 —△— Tn-glycoporphin (T _n)	22 —●— GalNAc
15 —△— OSM (Sialyl T _n)	23 —□— Mannan

Figure 9 Inhibition of ABA binding to GalNAc α 1-Ser/Thr-containing glycoprotein (asialo PSM)-coated ELLSA plates with various glycoproteins (gp) and polysaccharides (ps)

The quantity of glycoprotein in the coating solution was 5.0 ng/well. The quantity of lectin used for inhibition assay was 2.5 ng/well. The total volume was 50 μ l. A_{405} was recorded after 2 h incubation. The amount (ng) of glycoprotein required to induce 50% inhibition was determined. In this assay, the following glycoproteins and polysaccharides did not reach 50% inhibition with 277.8 ng glycoprotein as inhibitors: THGP Sd. (a +) W.M. (sialyl II, S), asialo THGP Sd. (a +) W.M. (II, S), human glycoporphin (sialyl T_α), colominic acid, arabinogalactan, pectin A and pectin C.

Table 2 Amounts of various saccharides giving 50% inhibition of ABA (2.5 ng/50 μ l) binding to asialo PSM (5 ng/50 μ l)

The inhibitory activity was estimated from the inhibition curve in Figure 8 and is expressed as the amount of inhibitor giving 50% inhibition of the control lectin binding. Total volume, 50 μ l.

Sample no.	Inhibitor	Quantity giving 50% inhibition (nmol)	Relative potency*
1	Gal β 1-3GalNAc α 1-benzyl (T_α)	32.0	46.9
2	Gal β 1-3GalNAc (T)	100.0	15.0
3	Gal β 1-3GlcNAc (I)	280.0	5.4
4	GalNAc	1500.0	1.0
5	Tn -containing glycopeptides ($M_w < 3000$ Da)	> 266.7 (1.8% inhibition)	
6	Triantennary II glycopeptides	> 13.3 (0% inhibition)	
7	Gal β 1-4GlcNAc (II)	> 652.5 (5.1% inhibition)	
8	Gal β 1-4Glc (L)	> 7300 (28.8% inhibition)	
9	Gal β 1-6GlcNAc	> 160 (12.6% inhibition)	
10	Gal	> 22200 (5.7% inhibition)	
11	GlcNAc	> 14350 (9.1% inhibition)	
12	GalNAc α 1-3Gal (A)	> 86.7 (—0.3% inhibition)	
13	GalNAc α 1-3GalNAc (F)	> 78.3 (—1.1% inhibition)	
14	GalNAc β 1-3Gal (P)	> 86.7 (0.3% inhibition)	
15	GalNAc β 1-4Gal (S)	> 86.7 (0.4% inhibition)	
16	Gal α 1-3Gal (B)	> 97.5 (1.8% inhibition)	
17	Gal α 1-4Gal (E)	> 243.3 (—5.5% inhibition)	
18	Glc	> 21000 (3.9% inhibition)	
19	Man	> 12222.2 (7.3% inhibition)	
20	<i>p</i> -NO ₂ -phenyl α GalNAc	> 122.5 (2.9% inhibition)	
21	<i>p</i> -NO ₂ -phenyl β GalNAc	> 105.0 (8.8% inhibition)	
22	<i>p</i> -NO ₂ -phenyl β Gal	> 645.1 (14.6% inhibition)	
23	Methyl β Gal	> 9445.2 (9.1% inhibition)	

* Relative potency (RP) = quantity of GalNAc (curve 4) required for 50% inhibition is taken as 1.0/quantity of sample required for 50% inhibition.

Most glycoprotein ligands strongly inhibited ABA–glycoprotein binding regardless of the masking blood group determinants or sialic acids on their **T**/**Tn** or **I**/**II** glycotopes (Figure 9 and Table 3), which resembles the binding properties of Jacalin [35,36], but is not seen in other **T**-specific lectins like peanut (*Arachis hypogaea*) agglutinin (PNA) [37] and *Abrus precatorius* agglutinin [38]. This is in agreement with the previous reports that ABA could recognize sialyl **T**-antigen, a characteristic distinct from the behaviour of PNA [37]. However, the present study demonstrates that ABA has a more comprehensive specificity towards ABH blood group glycoproteins and their precursors containing high densities of **I**/**II** and **T**/**Tn** glycotopes. These unusually broad specificities may be ascribed to the water-mediated interactions as in the model proposed by Weis and Drickamer [34]. On the other hand, the poor reactivities of some glycoproteins, like mannan and THGP Sd(a+) that lack the reactive Gal β 1 and **Tn** glycotopes, can rule out the possibility that the broad inhibitions resulted from non-specific protein–protein interactions between glycoproteins and ABA. Moreover, this multispecificity may explain some additionally inexplicable activities of ABA, including vascular smooth-muscle cell and endothelial cell mitogenesis that are absent in the similar **T**-specific PNA [37,39].

In the reaction with **II**, ABA displays a higher affinity for high-density **II** O-glycans [e.g. blood group precursor (equivalent) glycoproteins; curves 2–4 and 8 in Table 3], than for those present with a lesser density in N-glycans (e.g. fetuin, human α_1 -acid glycoprotein and their asialo products; curves 14, 16, 18 and 19 in Table 3). Furthermore, these multi-**II** N-glycans are relatively weaker ligands than most of the **T**/**Tn**-containing O-glycans (curves 14 and 16 versus curves 1, 6 and 9 in Figure 9).

Table 3 Amounts of glycoproteins or polysaccharides giving 50% inhibition of ABA (2.5 ng/50 μ l) binding to asialo PSM (5 ng/50 μ l)

The inhibitory activity was estimated from the inhibition curves in Figure 9 and is expressed as the amount of inhibitor giving 50% inhibition of the control lectin binding. Total volume, 50 μ l.

Curve no.	Curve in Figure 9	Inhibitor (multivalent structural units)	Quantity giving 50% inhibition (ng)	Relative potency*
1	a	Asialo PSM (A , A_n , T_α , Tn)	0.07	4.7×10^6
2	a	Cyst Tighe P-1 (I , II , T , Tn)	0.4	8.3×10^5
3	b	Cyst JS 1st Smith degraded (I , II , T , Tn)	0.5	6.6×10^5
4	a	Cyst Beach P-1 (I , II , T , Tn)	0.6	5.5×10^5
5	b	Asialo human glycophorin (T_α)	0.6	5.5×10^5
6	b	Active anti-freeze glycoprotein (T_α)	1.9	1.7×10^5
7	b	Cyst JS phenol insoluble (H)	2.0	1.7×10^5
8	a	Cyst MSS 1st Smith degraded (I , II , T , Tn)	3.0	1.1×10^5
9	b	Asialo ovine salivary glycoprotein major (Tn)	3.0	1.1×10^5
10	a	Cyst Tighe phenol insoluble (H , Le^a)	3.0	1.1×10^5
11	a	Cyst Beach phenol insoluble (B)	6.0	5.5×10^4
12	b	Tn -Glycophorin (Tn)	7.0	4.7×10^4
13	a	PSM (sialyl A , A_n , T_α , Tn)	10.0	3.3×10^4
14	a	Asialo fetuin (II , T)	20.0	1.7×10^4
15	b	Ovine salivary glycoprotein major (sialyl Tn)	20.0	1.7×10^4
16	b	Asialo human α_1 -acid glycoprotein (II)	22.0	1.5×10^4
17	b	<i>Pneumococcus</i> type 14 polysaccharide (II)	110.0	3.0×10^3
18	b	Human α_1 -acid glycoprotein (sialyl II)	200.0	1.7×10^3
19	a	Fetuin (sialyl II , T)	280.0	1.2×10^3
20	a, b	Monomeric Gal β 1-3GalNAc α 1-benzyl (T_α)	1.5×10^4	22.0
21	a, b	Monomeric Gal β 1-3GalNAc (T)	3.8×10^4	8.6
22	a, b	GalNAc	3.3×10^5	1.0
23	b	Mannan	> 277.8 (18.3% inhibition)	
24	a	Arabinogalactan	> 277.8 (7.7% inhibition)	
25	a	Pectin A and pectin C	> 277.8 (< 7.7% inhibition)	
		Native and asialo THGP Sd. (a+) W.M. (II , S)	> 277.8 (< 0.7% inhibition)	
		Colominic acid	> 277.8 (0% inhibition)	
		Human glycophorin (sialyl T_α)	> 277.8 (—3.4% inhibition)	

* Relative potency (RP) = quantity of GalNAc (curve 22) required for 50% inhibition is taken as 1.0/quantity of sample required for 50% inhibition.

This result could help to explain the finding that IgA₁, which possesses additional O-linked T sugar chains besides N-linked II sequences, has a stronger affinity for ABA than the merely N-glycan carrier IgA₂ [40]. The differential binding of ABA to these two classes of glycan linkages may be valuable when applied as a structural probe.

In a previous report on the carbohydrate specificity of ABA [6], it was shown that the T disaccharide was the most active one and 2000-fold more potent than GalNAc. Gal β 1-6GlcNAc and L were approx. 8–10 times more active than GalNAc, whereas I was inactive. However, I in the present study was five times more active than GalNAc (Table 2), whereas L and Gal β 1-6GlcNAc (curves 8 and 9 in Figure 8) were inactive. These contrary results seem to warrant further investigation, but these differences can be considered as 'minor ones' since the binding power of the polyvalent glycotopes in glycans is much greater than that of their monomeric ligands.

The overall contributions from the present study are as follows. (a) In addition to T sequences, Tn glycotopes and I are also active ligands, whereas other mammalian carbohydrate structural units, such as A, B, P, F and L are inactive. (b) From the interaction and inhibition profiles of ABA–glycoprotein binding by glycans (Table 1 versus Table 3) in the present study, glycotope polyvalencies (high-density polyvalent glycoside effect), should be the most important factor for ABA binding. A similar phenomenon was also found in another fungal agglutinin, *Sclerotium rolfsii* lectin [41]. (c) Although II and triantennary II are poor haptens, their polyvalent II-containing glycans such as *Pneumococcus* type 14 polysaccharide, and asialofetuin (Table 3), are very potent ligands. (d) In most cases, ABA does recognize not only glycans exposing Gal β 1 and Tn glycotopes, but also their cryptofoms masked by blood group determinants or sialic acids. (e) Mapping the overall reactions, it is concluded that high densities of Tn, T α and I/II containing glycoproteins bind strongly to ABA (Figures 7 and 9; Tables 1 and 3); and its affinity for ligands can be ranked in decreasing order as follows: high densities of T α , Tn and I/II clusters >>>> monomeric T α and Tn > I > GalNAc >>> II, L and Gal (inactive). This information could not only help to explain the cell surface and/or intracellular receptor(s) of ABA, but also help us to understand its various biological activities with respect to lectin receptor recognition processes. These unique ABA-binding features also support the concept that every lectin has its own binding characteristics [36,42,43].

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REFERENCES

- Milton, J. D. and Rhodes, J. M. (1995) Dietary galactose-binding lectins and their effects on human colonic epithelial cells. In *Lectin: Biomedical Perspectives* (Pusztai, A. and Bardocz, S., eds.), pp. 225–234. Taylor and Francis, London.
- Ryder, S. D., Smith, J. A., Rhodes, E. G., Parker, N. and Rhodes, J. M. (1994) Proliferative responses of HT29 and Caco2 human colorectal cancer cells to a panel of lectins. *Gastroenterology* **106**, 85–93.
- Yu, L. G., Fernig, D. G., Smith, J. A., Milton, J. D. and Rhodes, J. M. (1993) Reversible inhibition of proliferation of epithelial cell lines by *Agaricus bisporus* (edible mushroom) lectin. *Cancer Res.* **53**, 4627–4632.
- Yu, L. G., Fernig, D. G., White, M. R., Spiller, D. G., Appleton, P., Evans, R. C., Grierson, I., Smith, J. A., Davies, H., Gerasimenko, O. V. et al. (1999) Edible mushroom (*Agaricus bisporus*) lectin, which reversibly inhibits epithelial cell proliferation, blocks nuclear localization sequence-dependent nuclear protein import. *J. Biol. Chem.* **274**, 4890–4899.
- Sueyoshi, S., Tsuji, T. and Osawa, T. (1985) Purification and characterization of four isolectins of mushroom (*Agaricus bisporus*). *Biol. Chem. Hoppe-Seyler* **366**, 213–221.
- Irazoqui, F. J., Vides, M. A. and Nores, G. A. (1999) Structural requirements of carbohydrates to bind *Agaricus bisporus* lectin. *Glycobiology* **9**, 59–64.
- Duk, M., Lisowska, E., Wu, J. H. and Wu, A. M. (1994) The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. *Anal. Biochem.* **221**, 266–272.
- Lisowska, E., Duk, M. and Wu, A. M. (1996) Preparation of biotinylated lectins and application in microtiter plate assays and Western blotting. *BioMethods* **7**, 115–129.
- Kabat, E. A. (1956) *Blood Group Substances: Their Chemistry and Immunology*, pp. 135–139. Academic Press, New York.
- Lloyd, K. O. and Kabat, E. A. (1968) Immunochemical studies on blood groups. XLI. Proposed structures for the carbohydrate portion of blood group A, B, H, Lewis^x and Lewis^y substances. *Proc. Natl. Acad. Sci. U.S.A.* **61**, 1470–1477.
- Maisonrouge-McAuliffe, F. and Kabat, E. A. (1976) Immunochemical studies on blood groups. Heterogeneity of oligosaccharides liberated by degradation with alkaline borohydride of two human ovarian cyst fractions differing in B, I, and i activities and in reactivity toward concanavalin A. *Arch. Biochem. Biophys.* **175**, 81–89.
- Wu, A. M. (1988) Structural concepts of the blood group A, B, H, Le^a, Le^b, I and i active glycoproteins purified from human ovarian cyst fluid. *Adv. Exp. Med. Biol.* **228**, 351–394.
- Kabat, E. A., Baer, H., Bezer, A. E. and Knaub, V. (1948) Immunochemical studies on blood groups VII. Chemical changes associated with destruction of blood group activity and enhancement of the type XIV cross reactivity by partial hydrolysis of hog and human blood group A, B, and O substances. *J. Exp. Med.* **88**, 43–57.
- Leskowitz, S. and Kabat, E. A. (1954) Immunochemical studies on blood groups XV. The effect of mild acid hydrolysis on the glucosamine and galactosamine in blood group substances. *J. Am. Chem. Soc.* **76**, 5060–5065.
- Allen, P. Z. and Kabat, E. A. (1959) Immunochemical studies on blood groups XXII. Immunochemical studies on the nondialyzable residue from partially hydrolyzed blood group A, B, and O (H) substances (P₁ fraction). *J. Immunol.* **82**, 340–357.
- Wu, A. M., Kabat, E. A., Pereira, M. E. A., Gruezo, F. G. and Liao, J. (1982) Immunochemical studies on blood groups: the internal structure and immunological properties of water-soluble human blood group A substance studied by Smith degradation, liberation and fractionation of oligosaccharides and reaction with lectins. *Arch. Biochem. Biophys.* **215**, 390–404.
- Wu, A. M., Kabat, E. A., Nilsson, B., Zopf, D. A., Gruezo, F. G. and Liao, J. (1984) Immunochemical studies on blood groups. Purification and characterization of radioactive ³H-reduced di- to hexasaccharides produced by alkaline β -elimination-borohydride ³H reduction of Smith degraded blood group A active glycoproteins. *J. Biol. Chem.* **259**, 7178–7186.
- De Vries, A. L., Komatsu, S. K. and Feeney, R. E. (1970) Chemical and physical properties of freezing point-depressing glycoproteins from antarctic fishes. *J. Biol. Chem.* **245**, 2901–2908.
- Lisowska, E., Messeter, L., Duk, M., Czerwinski, M. and Lundblad, A. (1987) A monoclonal anti-glycophorin A antibody recognizing the blood group M determinant: studies on the subspecificity. *Mol. Immunol.* **24**, 605–613.
- Tettamanti, G. and Pigman, W. (1968) Purification and characterization of bovine and ovine submaxillary mucins. *Arch. Biochem. Biophys.* **124**, 41–50.
- Wu, A. M. and Pigman, W. (1977) Preparation and characterization of armadillo submandibular glycoproteins. *Biochem. J.* **161**, 37–47.
- Herp, A., Borelli, C. and Wu, A. M. (1988) Biochemistry and lectin binding properties of mammalian salivary mucous glycoproteins. *Adv. Exp. Med. Biol.* **228**, 395–435.
- Herp, A., Wu, A. M. and Moschera, J. (1979) Current concepts of the structure and nature of mammalian salivary mucous glycoproteins. *Mol. Cell. Biochem.* **23**, 27–44.
- Lindberg, B., Lönngren, J. and Powell, D. A. (1977) Structural studies on the specific type-14 pneumococcal polysaccharide. *Carbohydr. Res.* **58**, 177–186.
- Nilsson, B., Norden, N. E. and Svensson, S. (1979) Structural studies on the carbohydrate portion of fetuin. *J. Biol. Chem.* **254**, 4545–4553.
- Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vlieghehart, J. F. G., Binette, J. P. and Schmid, K. (1978) Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma α_2 -acid glycoprotein by 360-MHz ¹H-NMR spectroscopy and permethylation analysis. *Biochemistry* **17**, 5206–5214.
- Donald, A. S., Soh, C. P., Watkins, W. M. and Morgan, W. T. (1982) N-Acetyl-D-galactosaminyl- β -(1-4)-D-galactose: a terminal non-reducing structure in human blood group Sda-active Tamm–Horsfall urinary glycoprotein. *Biochem. Biophys. Res. Commun.* **104**, 58–65.
- Tamm, I. and Horsfall, F. L. (1950) Characterization and separation of an inhibitor of viral hemagglutination present in urine. *Proc. Soc. Exp. Biol. Med.* **74**, 108–114.
- Wu, J. H., Song, S. C., Chen, Y. Y., Tsai, M. C., Kabat, E. A. and Wu, A. M. (1998) Multi-antennary Gal β 1-4GlcNAc and Gal β 1-3GalNAc clusters as important ligands for a lectin isolated from the sponge *Geodia cydonium*. *FEBS Lett.* **427**, 134–138.

- 30 Wu, A. M., Song, S. C., Chang, S. C., Wu, J. H., Chang, K. S. S. and Kabat, E. A. (1997) Further characterization of the binding properties of a GalNAc specific lectin from *Codium fragile* subspecies tomentosoids. *Glycobiology* **7**, 1061–1066
- 31 Lee, R. T. and Lee, Y. C. (2000) Affinity enhancement by multivalent lectin–carbohydrate interaction. *Glycoconj. J.* **17**, 543–551
- 32 Lee, Y. C. (1992) Biochemistry of carbohydrate–protein interaction. *FASEB J.* **6**, 3193–3200
- 33 Wu, A. M., Wu, J. H., Tsai, M. S., Kaltner, H. and Gabius, H.-J. (2001) Carbohydrate specificity of a galectin from chicken liver (CG-16). *Biochem. J.* **358**, 529–538
- 34 Weis, W. I. and Drickamer, K. (1996) Structural basis of lectin–carbohydrate recognition. *Annu. Rev. Biochem.* **65**, 441–473
- 35 Wu, A. M. and Sugii, S. (1988) Differential binding properties of GalNAc and/or Gal specific lectins. *Adv. Exp. Med. Biol.* **228**, 205–263
- 36 Wu, A. M. (2001) Expression of binding properties of Gal/GalNAc reactive lectins by mammalian glycotopes. *Adv. Exp. Med. Biol.* **491**, 55–64
- 37 Pereira, M. E., Kabat, E. A., Lotan, R. and Sharon, N. (1976) Immunochemical studies on the specificity of the peanut (*Arachis hypogaea*) agglutinin. *Carbohydr. Res.* **51**, 107–118
- 38 Wu, A. M., Lin, S. R., Chin, L. K., Chow, L. P. and Lin, J. Y. (1992) Defining the carbohydrate specificities of *Abrus precatorius* agglutinin as T (Gal β 1 $\rightarrow$ 3GalNAc) > I/II (Gal β 1 $\rightarrow$ 3/4GlcNAc). *J. Biol. Chem.* **267**, 19130–19139
- 39 Sanford, G. L. and Harris-Hooker, S. (1990) Stimulation of vascular cell proliferation by β -galactoside specific lectins. *FASEB J.* **4**, 2912–2918
- 40 Irazoqui, F. J., Zalazar, F. E., Nores, G. A. and Vides, M. A. (1997) *Agaricus bisporus* lectin binds mainly O-glycans but also N-glycans of human IgA subclasses. *Glycoconj. J.* **14**, 313–319
- 41 Wu, A. M., Wu, J. H., Tsai, M. S., Hegde, G. V., Inamdar, S. R., Swamy, B. M. and Herp, A. (2001) Carbohydrate specificity of a lectin isolated from the fungus *Sclerotium rolfsii*. *Life Sci.* **69**, 2039–2050
- 42 Wu, A. M., Wu, J. H., Chen, Y. Y., Song, S. C. and Kabat, E. A. (1999) Further characterization of the combining sites of *Bandeiraea (Griffonia) simplicifolia* lectin-I, isolectin A₄ (GSI-A₄). *Glycobiology* **9**, 1161–1170
- 43 Wu, A. M., Song, S. C., Chen, Y. Y. and Gilboa-Garber, N. (2000) Defining the carbohydrate specificities of *Aplysia* gonad lectin (AGL) exhibiting a D-galacturonic acid affinity. *J. Biol. Chem.* **275**, 14017–14024

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