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Differential affinities of *Erythrina cristagalli* lectin (ECL) toward monosaccharides and polyvalent mammalian structural units

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Abstract Previous studies on the carbohydrate specificities of *Erythrina cristagalli* lectin (ECL) were mainly limited to analyzing the binding of oligo-antennary Galβ1→4GlcNAc (**II**). In this report, a wider range of recognition factors of ECL toward known mammalian ligands and glycans were examined by enzyme-linked lectinosorbent and inhibition assays, using natural polyvalent glycotopes, and a glycan array assay. From the results, it is shown that GalNAc was an active ligand, but its polyvalent structural units, in contrast to those of Gal, were poor inhibitors. Among soluble natural glycans tested for 50% molecular mass inhibition, *Streptococcus pneumoniae* type 14 capsular polysaccharide of polyvalent **II** was the most potent inhibitor; it was 2.1×10^4, 3.9×10^3 and 2.4×10^3 more active than Gal, tri-antennary **II** and monomeric **II**, respectively. Most type **II**-containing glycoproteins were also potent inhibitors, indicating that special polyvalent **II** and Galβ1-related structures play critically important roles in lectin binding. Mapping all information available, it can be concluded that: [a] Galβ1→4GlcNAc (**II**) and some Galβ1-related oligosaccharides, rather than GalNAc-related oligosaccharides, are the core structures for lectin binding; [b] their polyvalent **II** forms within macromolecules are a potent recognition force for ECL, while **II** monomer and oligo-antennary **II** forms play only a limited role in binding; [c] the shape of the lectin binding domains may correspond to a cavity type with Galβ1→4GlcNAc as the core binding site with additional one to four sugars subsites, and is most complementary to a linear trisaccharide, Galβ1→4GlcNAcβ1→6Gal. These analyses should facilitate the understanding of the binding function of ECL.

Keywords Carbohydrate specificities · ECL · Glycoprotein binding · Lectins · Polyvalency

Abbreviations

ASG	Armadillo salivary glycoprotein
BSM	bovine submandibular mucin/glycoprotein
ECL	*Erythrina cristagalli* lectin
ELLSA	Enzyme-linked lectinosorbent assay
gp	Glycoprotein
HOC	human ovarian cyst fluid
OSM	ovine submandibular mucin/glycoprotein
ps	Polysaccharide
PSM	Porcine salivary mucin/glycoprotein
THGP	Tamm-Horsfall glycoprotein
II	Galβ1→4GlcNAc, human blood group type II precursor sequence

Written in bold letters are the mammalian carbohydrate structural units

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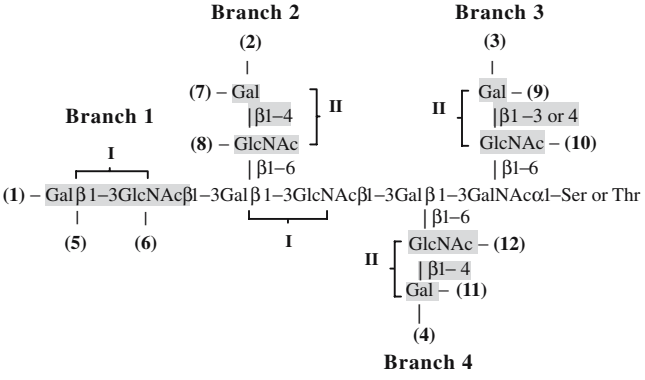
Introduction

Erythrina cristagalli lectin (ECL) is a Galβ1→/GalNAc specific lectin isolated from the seeds of *Erythrina cristagalli*. It is a glycoprotein (4.5% carbohydrate) of 56.8 kDa consisting of two subunits of 28 and 26 kDa [1, 2], arranged back-to-back, forming a handshake motif [3, 4]. Although ECL can agglutinate human erythrocytes of A, B, O and AB types, its function in the legume is still unknown. ECL has been reported to be mitogenic to human peripheral T lymphocytes [1, 2], and has been used to isolate human natural killer (NK) cells. In previous reports, knowledge of the binding properties of ECL and of the homologous *Erythrina corallodendron* lectin (ECorL) that possesses an essentially identical specificity was limited to oligo-antennary Galβ1→4GlcNAc (**II**) as the most active ligand, and it reacted more strongly with fucosylα1→2 lactose and fucosylα1→2-*N*-acetyllactosamine than with *N*-acetyllactosamine lactose, *N*-acetylgalactosamine and galactose [5–11]. However, the affinity of ECL for many mammalian sugar structural units and the effects of polyvalent glycotopes on their binding to the lectin have not been thoroughly investigated [12, 13]. Therefore, the glycotope specificities of ECL, using our collection of ligands and polyvalent natural glycans with known glycotopes, using a sensitive enzyme-linked lectinosorbent assay (ELLSA) [14, 15] were analyzed. From the results, it is concluded that: binding of ECL with GalNAc, Gal and their related ligands is different; Galβ1→4GlcNAc and other Galβ1-related oligosaccharides, in contrast to those of GalNAc, are essential factors for binding; based on molecular mass 50% inhibition, polyvalent **II** forms within macromolecules are the most potent recognition factors reacting with ECL, while oligo-antennary **II** forms play only a limited role in binding; the shape of ECL combining site may form a cavity, recognizing Galβ1→4GlcNAc as the major binding motif with additional one to four sugars as subsites; thus, its binding site may be more extended than revealed by X-ray crystallography [3] and as large as a tetra- to hexasaccharide of the β-anomer of Gal at the non-reducing end, in which Galβ1→4GlcNAcβ1→3Galβ1→4Glc (**II**β1→3**L**) and Galβ1→4GlcNAcβ1→6Gal (human blood group I Ma trisaccharide) are the best linear-form inhibitors. Although GalNAc binds stronger to the lectin than Gal, most of its oligovalent forms do not exhibit enhanced binding to the lectin. In this report, the hierarchy of potencies toward mammalian sugar structural units, expressed as nanomoles of 50% inhibition, is presented (Table 3). Differential binding properties of **II** reactive lectins were demonstrated. Variations in binding to Gal, GalNAc and their derivatives among ECL, RCA_1 and ricin were compared (Table 4).

Materials and methods

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, precipitation with increasing concentrations of ethanol [16–19], and extraction of the dried ethanol precipitates with 90% phenol. The insoluble fraction is named after the blood group substance (*e.g.* cyst Tighe phenol insoluble, where "Tighe" denotes the HOC sample code). A similar procedure was applied to other HOC collections (*e.g.* Beach, Mcdon, MSS, Tighe, and N-1, etc). The supernatant was fractionally precipitated by addition of 50% ethanol in 90% phenol to the indicated concentrations [16]. 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 carried out (*e.g.* 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 reducing ends to

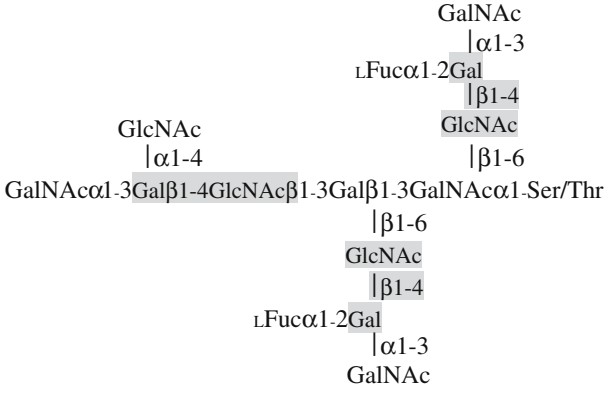


Structure 1 Proposed representative carbohydrate side chains of blood group active glycoproteins, prepared from human ovarian cyst fluid. This structure represents the internal portion of carbohydrate chains to which various human blood group determinants are attached. The four-branched structure (**1** to **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 [19] and can be prepared by Smith degradation of A, B, H active glycoproteins, purified from human ovarian cyst fluids [17–19, 21]. Numbers in parentheses indicate the site of attachment for the human blood group A, B, H, Le^a, and Le^b determinants. These determinants as well as the structural units at the nonreducing end are the sources of lectin reactive **A**/$\mathbf{A_h}$, **B**, **I**/**II**, **T**, and **Tn** determinants [27]. This megalo-saccharide of twenty-four sugars has not been isolated. However, most of the carbohydrate chains isolated are parts of this structure. Shaded areas are proposed to be the reactive glycotopes for ECL

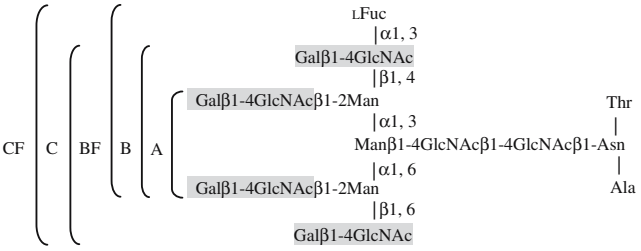
serine or threonine residues of the polypeptide backbone [17–20] (Structure 1). In general, the "P-1" fractions (*e.g.* cyst Beach P-1, cyst Mcdon P-1 and cyst Tighe P-1) represent the nondialyzable portion of the blood group substances after mild hydrolysis at pH 1.5–2.0, 100°C for 2 h which removes most of the sialic acids, L-fucopyranosyl end groups (LFucα1→2, 3 and 4), as well as some blood group A and B active oligosaccharide side-chains (GalNAcα1→3 and Galα1→3) [21–23]. 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". The 1st Smith-degraded products of blood group A active substances (MSS 10% 2×), in which almost all of the sugar groups at the nonreducing ends are removed, were prepared as described earlier [24, 25].

Hog gastric mucin no. 4, a blood group A+H substance, was derived from crude hog stomach mucin as described previously [26] (Structure 2). Treatment of mucin no. 4 with HCl (pH 2, 100°C, 90 min) yields hog gastric mucin no. 9, while acid hydrolysis (pH 1.5, 100°C, 2 and 5 h) gives hog gastric mucins no. 14 and 21, respectively. Extensive hydrolysis leads to destruction of blood group activities [27].

Human and bovine α_1-acid gp were purchased from Sigma. Human α_1-acid gp contains tetra-, tri- and di-antennary complex type glycans in the ratio of 2:2:1 [28, 29] (Structure 3). Fetuin (Gibco Laboratories, Grand Island, NY, USA) is the major gp in fetal calf serum and has six oligosaccharide side chains per molecule, three *O*-glycosidically-linked to Ser/Thr and three *N*-glycosidically-linked to Asn [30] and contains tri- and di-antennary complex type glycans in the ratio of 1:2.



Structure 2 Proposed structure of carbohydrate side chains of hog A + H stomach glycoproteins [26]. Shaded areas (**II**) are proposed to be the reactive glycotopes for ECL

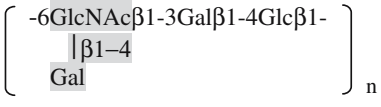


Structure 3 Structure of the carbohydrate of human serum α_1-acid glycoprotein. The primary structure of classes A, B, BF, C and CF carbohydrate units of the glycosylation site in human serum α_1-acid glycoprotein [28] is indicated in the above structure for asialo α_1-acid glycoprotein. The carbohydrate 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 ECL

Tamm-Horsfall gp, which was kindly provided by the late Dr. W.M. Watkins, was isolated with 0.58 M NaCl from the urine of a single donor (W.T.J.M.) with the Sd (a^+) blood group by the method of Tamm and Horsfall [31, 32].

Mucus gp (or native bird-nest gp), the so-called nest-cementing substance [33] from the salivary gland of Chinese swiftlets (genus *Collocalia*), was extracted with distilled water at 60°C for 20 min from commercial bird-nest substance (Kim Hing Co., Singapore).

Ovine, bovine, armadillo and porcine submandibular gps were purified according to the method of Tettamanti and Pigman (1968) with modifications [34, 35]. About two-third of the carbohydrate side chains of armadillo submandibular gland mucin (ASG-A) are GalNAcα1→Ser/Thr (**Tn**) and one-third are Neu5Acα2→6GalNAcα1-*O*-Ser/Thr (sialyl **Tn**) [35–37]. Native ASG-A [38], a salivary gp of the nine-banded armadillo (*Dasypus novemcinctus mexicanus*) containing only **Tn** (GalNAcα1→Ser/Thr) as carbohydrate side chains, was isolated from a 0.01 M PBS, pH 6.8 gland extract after removal of ASG-A, which is one of the sialoglycoproteins in armadillo glands [36]. Asialo PSM contains Galβ1→3GalNAcα1→ ($\mathbf{T}_\alpha$) together with **Tn** and GalNAcα1→3Gal (**A**) sequences, as most of the outer fucosyl residues and sialic acids are cleaved by mild acid hydrolysis.



Structure 4 Repeating unit of the *Pneumococcus* type 14 capsular polysaccharide. Shaded area (**II**, Galβ1-4GlcNAc) is proposed to be the reactive glycotope for ECL

The anti-freeze gp from the Antarctic fish (*Trematomus borchgrevinki*) which contains only $\mathbf{T}_{\alpha}$ carbohydrate chains [39] was provided by Dr. R. E. Feeney (Department 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).

The *pneumococcus* type 14 polysaccharide (Structure 4), isolated from *Streptococcus pneumoniae* capsule [40], was a generous gift from the late Dr. E.A. Kabat.

Yeast high-mannose type glycan (mannan), poly-2,8-*N*-acetylneuraminic acid capsular polysaccharide (colominic acid) from *E. coli* and pectins from apple and citrus fruits were purchased from Sigma.

Lectin

Biotinylated *Erythrina cristagalli* lectin (ECL) was purchased from Vector Laboratories (Burlingame, CA, USA).

Sugars used for inhibition studies

Mono-, di- and oligosaccharides were purchased from or prepared by Dextra (Berkshire, UK) and Sigma. Triantennary **II** glycopeptides (Tri-**II**) were prepared from asialofetuin by pronase digestion and repeatedly fractionated by BioGel P-4 400 mesh column chromatography [41]. The **Tn** clusters used for this study were mixtures of **Tn** containing glycopeptides from OSM in the filterable fraction (Molecular mass cut-off <3,000) [42].

The microtiter plate lectin-enzyme binding assay

Enzyme-linked lectinosorbent binding assay (ELLSA) was performed according to the procedures described [14, 15]. The volume of each reagent applied to the plate was 50 μl/well, and all incubations, except for coating, were performed at room temperature (20°C). The reagents, if not indicated otherwise, were diluted with TBS containing 0.05% Tween 20 (TBS-T). TBS or 0.15 M NaCl containing 0.05% Tween 20 was used for washing the plate between incubations.

Ninety six-well microtiter plates (Nunc, MaxiSorp, Vienna, Austria) were coated with gps in 0.05 M carbonate buffer, pH 9.6, and incubated overnight at 4°C. After washing the plate, biotinylated lectins (10 ng) were added to each well and incubated for 30 min. The plates were washed to remove unbound lectin and ExtrAvidin/alkaline phosphatase solution (diluted 1:10,000, Sigma) 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 4 h incubation with the substrate.

For inhibition studies, serially diluted inhibitor samples were mixed with an equal volume of lectin solution containing a fixed amount of lectin. The control lectin sample was diluted twofold with TBS-T. After 30 min incubation at 20°C, samples were tested in the binding assay, as described above. 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 value 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 experiments, the control wells, where coating or addition of biotinylated lectin was omitted, gave low absorbance values (below 0.1). It showed that blocking the wells before lectin addition was not necessary when Tween 20 was present in the TBS.

Glycan array screening

Labelled ECL was screened in binding buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.05% Tween 20 and 1% BSA). The lectin was screened on the streptavidin/biotin array (EA V3) as described previously [43]. Biotinylated glycosides [44] were bound to streptavidin-coated microtiter plates in replicates of n=4. Pre-coated plates were washed three times with 100 μl of wash buffer (binding buffer without BSA) before incubation. A stock solution of ECL-Alexa Fluor® 488 was added to each well and incubated at room temperature for 1 h. The plates were washed and read in 25 μl of wash buffer on a Victor-2™ 1420 Multi-label Counter (PerkinElmer Life Sciences) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

To analyze the results on the streptavidin/biotin array, all glycans were ranked according to their S/N (signal to noise ratio) by dividing their mean relative fluorescence units by the mean background generated in control wells that lacked glycosides. This value was compared with the average S/N for all wells in the array, and the results were then ranked as high affinity (>3×mean S/N), medium affinity (>2×mean S/N) and low affinity (>mean S/N).

Results

ECL-glycan interaction

The binding profiles of ECL with glycoproteins, as studied by a microtiter plate enzyme-linked lectinosorbent assay (ELLSA), are shown in Table 1 and the interaction patterns for glycoproteins are illustrated in Fig. 1 in Supplementary Section. Among the glycoproteins tested, ECL reacted best with several high-density Galβ1→4/

Table 1 Binding of ECL to human blood group A, B, H, P_1, Le^a and Le^b active glycoproteins (gps), sialo- and asialo glycoproteins as determined by ELLSA[a]

Activity order/bar no. in Fig. 1 in Supplementary Section	Glycoprotein (lectin determinants[b]; blood group specificity)	1.5 (A_{405}) unit (ng)	Maximum A_{405} absorbance reading
Blood group precursor equivalent gps			
1	Cyst Tighe P-1 (**I**, **II**, **T**, **Tn**)	10.0	4.6
5	Cyst OG 10% 2× ppt (**I/II**)	22.0	4.4
8	Cyst Beach P-1 (**I**, **II**, **T**, **Tn**)	160.0	3.0
9	Cyst Mcdon P-1 (**I**, **II**, **T**, **Tn**)	400.0	3.0
11	Cyst MSS 1st Smith degraded (**I**, **II**, **T**, **Tn**)	400.0	2.0
12	Hog gastric mucin no. 14 (**I/II**)	–	1.2
15	Hog gastric mucin no. 21 (**I/II**)	–	1.0
16	Cyst Tij 20% of 2nd 10% 2× (**I/II**, **B**)	–	1.0
Oligo-antennary Galβ1→4GlcNAc (**II**) in *N*-linked gps			
3	Asialo human α_1-acid gp (m**II**)	7.0	4.5
6	Asialofetuin (m**II**, **T**)	46.0	4.4
10	Asialo bovine α_1-acid gp (m**II**)	280.0	2.6
23	Asialo human lactoferrin (**II**)	–	0.5
Poly **II** containing polysaccharide			
20	*Pneumococcus* type 14 ps (**II**)	–	0.9
GalNAcβ1→4Galβ1→4GlcNAc-containing gp			
7	Asialo THGP Sd. (a^+) W. M. (**II**, **S**)	31.0	4.4
Galα1→4Galβ1→4Galβ1→4GlcNAc-containing gp			
2	Asialo bird nest gp (**II**, **E**, $\mathbf{T}_\alpha$, $\mathbf{F}_\alpha$)	12.0	4.6
GalNAcα1→Ser/Thr (**Tn**)/Galβ1→3GalNAcα1→Ser/Thr (**T**)-containing gps			
24	Asialo HSM (**Tn**)	–	0.5
26	Asialo BSM (**Tn**, GlcNAcβ1→3**Tn**)	–	0.4
27	Asialo ASG (**Tn**)	–	0.4
29	Asialo OSM (**Tn**)	–	0.2
36	Asialo PSM (**T**, **Tn**, $\mathbf{A}_h$, **H**)	–	0.04
37	Active antifreeze gp ($\mathbf{T}_\alpha$)	–	0.03
40	Inactive antifreeze gp ($\mathbf{T}_\alpha$)	–	0.01
Sialylated (crypto) **II**, **T**/**Tn**-containing gps			
17	THGP Sd. (a^+) W. M. (**II**, **S**)	–	1.0
21	Bird nest gp (sialyl **II**, **E**, $\mathbf{T}_\alpha$, $\mathbf{F}_\alpha$)	–	0.7
30	Fetuin (sialyl **II**, **T**)	–	0.2
31	BSM (sialyl **Tn**, sialyl GlcNAcβ1→3**Tn**)	–	0.1
32	HSM (sialyl **Tn**)	–	0.1
33	Human α_1-acid gp (sialyl m**II**)	–	0.1
38	OSM (sialyl **Tn**)	–	0.03
39	Human lactoferrin (sialyl **II**)	–	0.03
41	ASG-A (sialyl **Tn**)	–	0.01
42	PSM (sialyl **T**, **Tn**)	–	0.01
Blood group **ABH** and Lewis-containing gps			
4	Cyst N-1 Le^a 20% 2× (**Le^a**, **I/II**)	43.0	4.5
13	Hog gastric mucin no. 9 ($\mathbf{A}_h$, **H**)	–	1.2
14	Cyst 19 (**A**, **B**)	–	1.1
18	Cyst Beach phenol insoluble (**B**)	–	1.0
19	Hog gastric mucin no. 4 ($\mathbf{A}_h$, **H**)	–	1.0
22	Cyst Mcdon ($\mathbf{A}_h$)	–	0.6
25	Cyst JS phenol insoluble (**H**)	–	0.5
28	Cyst Tighe phenol insoluble (**H**, **Le^b**)	–	0.4
34	Cyst MSS 10% 2× ($\mathbf{A}_h$)	–	0.09
35	Cyst 9 ($\mathbf{A}_h$)	–	0.07

[a] 10 ng of biotinylated ECL was added to various glycoprotein concentrations ranging from 1 ng to 2.5 μg/50 μl

[b] The symbol in parentheses indicates the human blood group activity and/or lectin determinants [13]; Expressed in bold are: **I/II** (Galβ1→3/4GlcNAc); **T** (Galβ1→3GalNAc); **Tn** (GalNAcα1→Ser/Thr); **S** (GalNAcβ1→4Gal); **E** (Galα1→4Gal); **F** (GalNAcα1→3GalNAc); $\mathbf{A}_h$ (GalNAcα1→3[LFucα1→2]Gal); **B** (Galα1→3Gal); **H** (LFucα1→2Gal); m (multivalent)

3GlcNAc (**II**/**I**) containing gps. These include asialo human α1-acid gp (bar 3 in Table 1 and Fig. 1 in Supplementary Section; Structure 3) and blood group ABH(O) precursor equivalent gps prepared from human ovarian cyst fluid (cyst Tighe P-1, bar 1, cyst OG 10% 2× ppt, bar 5; Structure 1). Less than 22 ng of these gps were required to reach 1.5 absorbance units at A_{405}. ECL also bound strongly with many structure **II** or **II**/**I**-containing *N*-linked and *O*-linked gps (asialofetuin, bar 6 and asialo Tamm-Horsfall Sd. (a^+) urinary glycoprotein, bar 7), asialo bird nest gp (bar 2) and cyst N-1 Le^a 20% 2× (bar 4). The lectin reacted moderately or slightly with some human blood group **ABH** precursor equivalent gps (cyst Beach P-1, bar 8, cyst Mcdon P-1, bar 9, cyst MSS 1st Smith degraded, bar 11, and cyst Tij 20% of 2nd 10% 2×, bar 16), human blood group **ABH** active gps (Cyst 19, bar 14 and cyst Beach phenol insoluble, bar 18) and mild-acid treated hog mucin nos. 9, 14 and 21 (bars 13, 12 and 15). ECL reacted weakly or was inactive with **Tn**-containing gps (asialo HSM, asialo BSM, asialo ASG, asialo OSM and asialo PSM, bars 24, 26, 27, 29 and 36, Table 1 and Fig. 1 in Supplementary Section) and sialic acid containing glycoproteins, such as human α_1-acid (bar 33), fetuin (bar 30), bird nest gp (bar 21) and cryptic **Tn**-containing gps (BSM, HSM, OSM, ASG and PSM, bars 31, 32, 38, 41 and 42, Table 1 and Fig. 1 in Supplementary Section), mannan, colominic acid and pectins (Table 1).

Inhibition of ECL-glycan interaction by various polyvalent glycotopes in glycoproteins and expressed by molecular mass 50% inhibition

The ability of various glycoproteins to inhibit the binding of ECL with an oligo-antennary Galβ1→4GlcNAc (**II**)-containing gp (asialo human α_1-acid glycoprotein) was also analyzed by ELLSA. The amounts of glycoprotein (nanogram) required for 50% inhibition are listed in Table 2 and the inhibition profile is shown in Fig. 2 in Supplementary Section.

Among the glycans tested for inhibition, *Pneumococcus* type 14 polysaccharide, which is composed of repeating units of **II** structural sequence as side chain (Structure 4), was the best inhibitor, requiring only 0.8 ng to inhibit 50% of the lectin-glycan binding. It was 2.1×10^4, 3.9×10^3 and 2.4×10^3 more active than Gal, tri-antennary **II** and monomeric **II**, respectively (bar 1 *vs* bars 32, 30 and 27, in Table 2 and Fig. 2 in Supplementary Section). Most of the other high-density **II**/**I**- or **II**-containing gps were also potent inhibitors. These included blood group precursor equivalent **II**/**I**-containing gps (bars 2–5, 7, 10 and 11), mild-acid-hydrolyzed hog gastric mucin A+H substances (hog gastric mucin nos. 14 and 21, bars 6 and 9) and *N*-linked multi-antennary **II**-containing gp (asialo THGP, bar 8). Their reactivities were between 1.7×10^3 to 8.5×10^3 times higher than that of monomeric Gal (bars 2–11 *vs* bar 32, Table 2 and Fig. 2 in Supplementary Section) and up to 1.6×10^3 and 9.5×10^2 times higher than that of Tri-**II** and monomeric **II** (bars 30 and 27, Table 2 and Fig. 2 in Supplementary Section), respectively. The reaction was also strongly inhibited by hog gastric mucin A+H substances (bars 12 and 13), human blood group **ABH** active gps (bars 14, 16, 17 and 19), asialo bird nest gp (bar 15) and poly oligo-antennary **II**-containing *N*-linked gps (bars 20 and 21). They were up to 9.4×10^2 times more reactive than Gal. Although monomeric GalNAc was active, even slightly more active than Gal (bar 31 *vs* bar 32), but most of its polyvalent forms didn't contribute to binding. These included **Tn**- or **T**-containing gps such as asialo HSM, asialo OSM, asialo PSM and active antifreeze gp (Table 2). Except for THGP and bird nest gp (bars 18 and 24), most sialic acid-containing gps (such as human α_1-acid, fetuin and OSM, Table 2) were either weakly active or inactive, indicating that sialic acid has a masking effect.

Inhibition of ECL-glycan interaction by mono- and oligo-saccharides

The ability of various sugars to inhibit the binding of ECL by an oligo-antennary Galβ1→4GlcNAc (**II**)-containing gp (asialo human α_1-acid gp) is shown in Fig. 3 in Supplementary Section and the amounts of molar ligands required for 50% inhibition of the lectin-glycan interaction are showed in Table 3. Among the **II**, **II**-related oligosaccharides and mammalian oligo-**II** antennary glycotopes tested, human blood group type II sequence (Galβ1→4GlcNAc, **II**) was 18 times more active than Gal, indicating that GlcNAcβ1→ added to carbon-4 of Gal plays a very important role in binding (bar 6 *vs* bar 34, Table 3 and Fig. 3 in Supplementary Section). Bi-antennary hexasaccharides **II**β1-6(**I**β1-3)**L** (lacto-*N*-neohexaose, LNnH, bar 1), tri-antennary Galβ1→4GlcNAc at the non-reducing end in *N*-linked glycopeptides (Tri-**II**, bar 2) and bi-antennary **II**β1-6(**II**β1-3)**L** (lacto-*N*-hexaose, LNH, bar 3) were up to 5 and 90 times more efficient than **II** disaccharide and Gal, respectively (bars 1 to 3 *vs* bars 6 and 34, Table 3 and Fig. 3 in Supplementary Section), suggesting that most bi- or oligo-antennary glycotopes enhance the reactivities strongly. When **II**β1-6 was added to Gal of **L** (Galβ1→4Glc) as in **II**β1-3**L** (type 2, bar 5), the reactivity of the hexasaccharide **II**β1-6 (**II**β1-3)**L** was 2.3 and 2.5 times more active than **II**β1-3**L** and **II** (bar 3 *vs* bars 5 and 6, Table 3 and Fig. 3 in Supplementary Section), respectively, confirming that the affinity of ECL for glycoforms can be increased by formation of bi-antennary **II**. Human blood group I Ma trisaccharide, **II**β1-3**L** (type 2) and **II** were equally active and about 13 times more active than Galβ1→3GlcNAc (**I**)

Table 2 Amount of different gps giving 50% inhibition of binding of ECL (5 ng/50 μl) to asialo human α_1-acid gp (200 ng/50 μl) binding[a]

Activity order/ bar no. in Fig. 2 in Supplementary Section	Glycoprotein/glycans	Quantity giving 50% inhibition (nanograms)	Relative potency[b]
1	*Pneumococcus* type 14 ps (**II**)	0.8	2.1×10^4
2	Cyst OG 10% 2x ppt (**I/II**)	2.0	8.5×10^3
3	Cyst Tighe P-1 (**I**, **II**, **T**, **Tn**)	2.0	8.5×10^3
4	Cyst MSS 1st Smith degraded (**I**, **II**, **T**, **Tn**)	4.0	4.3×10^3
5	Cyst N-1 Le[a] 20% 2x (**Le[a]**, **I/II**)	5.0	3.4×10^3
6	Hog gastric mucin no. 14 (**I/II**)	6.0	2.8×10^3
7	Cyst Mcdon P-1 (**I**, **II**, **T**, **Tn**)	6.0	2.8×10^3
8	Asialo THGP Sd. (a^+) W. M. (**II**, **S**)	8.0	2.1×10^3
9	Hog gastric mucin no. 21 (**I/II**)	9.0	1.9×10^3
10	Cyst Beach P-1 (**I**, **II**, **T**, **Tn**)	10.0	1.7×10^3
11	Cyst Tij 20% of 2nd 10% 2x (**I/II**, **B**)	10.0	1.7×10^3
12	Hog gastric mucin no. 9 ($\mathbf{A_h}$, **H**)	18.0	9.4×10^2
13	Hog gastric mucin no. 4 ($\mathbf{A_h}$, **H**)	22.0	7.7×10^2
14	Cyst Mcdon ($\mathbf{A_h}$)	30.0	5.7×10^2
15	Asialo bird nest gp (**II**, **E**, $\mathbf{T_\alpha}$, $\mathbf{F_\alpha}$)	40.0	4.3×10^2
16	Cyst Beach phenol insoluble (**B**)	40.0	4.3×10^2
17	Cyst JS phenol insoluble (**H**)	50.0	3.4×10^2
18	THGP Sd. (a^+) W. M. (**II**, **S**)	60.0	2.8×10^2
19	Cyst Tighe phenol insoluble (**H**, **Le[b]**)	65.0	2.6×10^2
20	Asialo human α_1-acid gp (m**II**)	120.0	1.4×10^2
21	Asialofetuin (m**II**, **T**)	120.0	1.4×10^2
22	Cyst MSS 10% 2x ($\mathbf{A_h}$)	200.0	85.0
23	Asialo BSM (**Tn**, GlcNAcβ1→3**Tn**)	300.0	56.7
24	Bird nest gp (sialyl **II**, **E**, $\mathbf{T_\alpha}$, $\mathbf{F_\alpha}$)	300.0	56.7
25	Cyst 9 ($\mathbf{A_h}$)	1000.0[c]	17.0
26	Asialo human lactoferrin (**II**)	1400.0	12.1
27	Galβ1→4GlcNAc (**II**)	1900.0	8.9
28	BSM (sialyl **Tn**, sialyl GlcNAcβ1→3**Tn**)	2000.0	8.5
29	Galβ1→4GlcNAc ↓β1-6 Galβ1→4Glc ↑β1-3 Galβ1→4GlcNAc Lacto-*N*-hexaose [LNH; Di-**II** or **II**β1-6(**II**β1-3)**L**]	2100.0[d]	8.1
30	Galβ1→4GlcNAcβ1→2Man ↓α1-6 Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAcβ1→4GlcNAcβ1-N-Asn ↑β1-4 Galβ1→4GlcNAc Tri-antennary Galβ1→4GlcNAc (Tri-**II**)	3100.0[d]	5.5
31	GalNAc	15000.0	1.1
32	Gal	17000.0	1.0
	Asialo HSM (**Tn**)	> 138.9 (28.9%)[e]	–
	Human lactoferrin (sialyl **II**)	> 555.6 (26.7%)	–
	HSM (sialyl **Tn**)	> 222.2 (24.6%)	–
	ASG-A (sialyl **Tn**)	> 555.6 (21.9%)	–
	Asialo OSM (**Tn**)	> 555.6 (20.8%)	–
	Active antifreeze gp ($\mathbf{T_\alpha}$; M.W. 1.0-2.1×10[4])	> 277.8 (18.7%)	–
	Human α_1-acid gp (sialyl m**II**)	> 555.6 (12.1%)	–
	Asialo PSM (**T**, **Tn**, $\mathbf{A_h}$, **H**)	> 555.6 (9.0%)	–
	OSM (sialyl **Tn**)	> 555.6 (8.2%)	–
	PSM (sialyl **T**, **Tn**)	> 555.6 (7.5%)	–
	Inactive antifreeze gp ($\mathbf{T_\alpha}$; M.W. 2.6-3.8×10[3])	> 277.8 (7.5%)	–
	Fetuin (sialyl **II, T**)	> 555.6 (4.4%)	–

[a] The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor giving 50% inhibition; Total volume was 50 μl. [b] Relative potency = Quantity of Gal (bar 32) required for 50% inhibition is taken as 1.0 / Quantity of sample required for 50% inhibition. [c] Extrapolation. [d] Based on carbohydrate content. [e] The inhibitory potency of inactive glycoproteins are expressed as the maximum amount of gps tested that yield inhibition (in parenthesis) below 50%; Other gps in which 278 ng were used for inhibition of ECL-gp binding but did not reach 50% inhibition: mannan; colominic acid; pectin-A and pectin-C.

Table 3 Amount of various saccharides giving 50% inhibition of binding of ECL (5 ng/50 μl) to asialo human α_1-acid gp (200 ng/50 μl)[a]

Activity order/ bar no. in Fig. 3 in Supplementary Section	Saccharides	Quantity giving 50% inhibition (nanomoles)	Relative potency[b]
Galβ1→4/3GlcNAcβ→ (**II**/**I**)/Galβ1→4Glcβ-related ligands			
1	Galβ1→4GlcNAc ↓β1-6 Galβ1→4Glc ↑β1-3 Galβ1→3GlcNAc Lacto-*N*-neohexaose [LNnH; **II**β1-6(**I**β1-3)**L**]	1.0	90.0
2	Galβ1→4GlcNAcβ1→2Man ↓ α1-6 Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAcβ1→4GlcNAcβ1-N-Asn ↑ β1-4 Galβ1→4GlcNAc Tri-antennary Galβ1→4GlcNAc (Tri-**II**)	1.5	60.0
3	Galβ1→4GlcNAc ↓β1-6 Galβ1→4Glc ↑β1-3 Galβ1→4GlcNAc Lacto-*N*-hexaose [LNH; Di-**II** or **II**β1-6(**II**β1-3)**L**]	2.0	45.0
4	Galβ1→4GlcNAcβ1→6Gal (Human blood group I Ma trisaccharide)	3.3	27.0
5	Galβ1→4GlcNAcβ1→3Galβ1→4Glc (Type 2, **II**β1→3**L**)	4.5	20.0
6	Galβ1→4GlcNAc (**II**)	5.0	18.0
12	Galβ1→4Glc (**L**; Lactose)	25.0	3.6
17	Galβ1→3GlcNAcβ1→3Galβ1→4Glc (Type 1, **I**β1→3**L**; Lacto-*N*-tetraose)	50.0	1.8
25	Galβ1→3GlcNAc (**I**)	65.0	1.4
Mammalian carbohydrate structural units			
14	GalNAcα1→Ser/Thr (**Tn**)	30.0	3.0
18	Fucα1→2Gal (**H** disaccharide)	50.0	1.8
19	Galα1→4Gal (**E**)	50.0	1.8
20	**Tn**-containing glycopeptides from ovine salivary glycoprotein (M.W. < 3.0×10^3)	50.0	1.8
21	Galα1→3Gal (**B**)	60.0	1.5
22	Galα1→3Galβ1→4GlcNAc (**B** active **II**)	60.0[c]	1.5
23	GalNAcβ1→3Gal (**P**)	60.0[c]	1.5
30	GalNAcβ1→4Gal (**S**)	90.0[c]	1.0
31	GalNAcα1→3Gal (**A**)	90.0[c]	1.0
32	GalNAcα1→3GalNAc (**F**)	90.0[c]	1.0
35	Galβ1→3GalNAcα1→benzyl ($\mathbf{T}_\alpha$)	100.0	0.9
36	Galβ1→4(Fucα1→3)GlcNAc ($\mathbf{Le}^x$)	150.0	0.6
38	Galβ1→3GalNAc (**T**)	200.0	0.5
	Galβ1→3(Fucα1→4)GlcNAc ($\mathbf{Le}^a$)	> 63.3 (17.3%) [d]	–
Monosaccharides, their derivatives and other ligands			
7	*p*-NO_2 phenyl βGal	9.0	10.0
8	*p*-NO_2 phenyl βGalNAc	15.0	6.0
9	*p*-NO_2 phenyl αGal	15.0	6.0
10	*p*-NO_2 phenyl αGalNAc	15.0	6.0
11	Fucα1→2Galβ1→4Glc (2'-fucosyllactose)	20.0	4.5
13	Galβ1→4Man	25.0	3.6
15	Methyl αGal	40.0	2.3
16	Methyl αGalNAc	40.0	2.3
24	Methyl βGalNAc	60.0	1.5
26	Galα1→6Glc (Melibiose)	70.0	1.3
27	Galα1→6Glcβ1→2Fruf (Raffinose)	75.0	1.2
28	Galα1→6Galα1→6Glcβ1→2Fruf (Stachyose)	80.0	1.1
29	GalNAc	80.0	1.1
33	Methyl βGal	90.0	1.0
34	Gal	90.0	1.0
37	Galβ1→6GlcNAc	150.0	0.6

Table 3 (continued)

Activity order/ bar no. in Fig. 3 in Supplementary Section	Saccharides	Quantity giving 50% inhibition (nanomoles)	Relative potency [b]
39	GalN	200.0	0.5
40	DFuc	250.0	0.4
	LAra	600.0	0.2
	LRhamnose	1500.0	0.06
	Man	60000.0	0.002
	Glc	60000.0	0.002
	GlcNAc	100000.0	0.0009
	Galβ1→4(Fucα1→3)Glc (3-fucosyllactose)	> 68.3 (16.7%)	–
	p-NO_2 phenyl αMan	> 65.6 (20.6%)	–
	p-NO_2 phenyl βMan	> 85.6 (17.5%)	–
	DAra	> 1375.6 (17.4%)	–
	LFuc	> 7742.5 (15.9%)	–

[a] The inhibitory activity was estimated from the inhibition curve and is expressed as the amount of inhibitor giving 50 % inhibition. Total volume was 50 µl. [b] Relative potency = Quantity of Gal (bar 34) required for 50% inhibition is taken as 1.0 / Quantity of sample required for 50% inhibition. [c] Extrapolation value. [d] The inhibitory potency of inactive saccharides are expressed as the maximum amount of sugars tested that yield inhibition (in parenthesis) below 50%.

(bars 4 to 6 *vs* bar 25, Table 3 and Fig. 3 in Supplementary Section), suggesting the major combining sites of ECL should be **II**. Galβ1→4Glc (**L**) and Galβ1→4Man (bars 12 and 13) had the same degree of activity. They were 2.6 and 6 times more active than Galβ1→3GlcNAc (**I**) and Galβ1→6GlcNAc, respectively (bars 12 and 13 *vs* bars 25 and 37); the preference of ECL to the linkage of penultimate sugar in decreasing order as: Galβ1–4 > Galβ1–3 > Galβ1–6. The affinity of ECL for Galβ1→ 4GlcNAc (**II**) is 5 times greater than for Galβ1→4Glc (**L**) (bar 6 *vs* bar 12), demonstrating that the binding force is enhanced by substitution of hydroxyl group with acetamido group at C-2 of Glc.

The effect of various substitutions on Galβ1→4GlcNAc (**II**) and Galβ1→4Glc (**L**) containing compounds on the binding is also shown in Table 3. Galβ1→4(Fucα1→3) GlcNAc (**Le^x**) was 30 times less active than Galβ1→4Glc-NAc (**II**) and Galβ1→4(Fucα1→3)Glc (3-fucosyllactose) was also a poor inhibitor as it did not reach 50% inhibition, indicating that adding LFucα1→3 to subterminal GlcNAc of Galβ1→4GlcNAc (**II**) and Galβ1→4Glc (**L**) blocks interaction; Fucα1→2Galβ1→4Glc (2'-fucosyllactose) was 1.25 times more active than Galβ1→4Glc (**L**) (bar 11 *vs* bar 12), implying that Fucα at the 2-position of the Gal of Galβ1→4Glc (**L**) slightly promotes the reactivity. Adding Galα1→3 to Gal of **II** as Galα1→3Galβ1→4Glc-NAc (**B** active **II**) was 12 times less active than Galβ1→4GlcNAc (**II**) (bar 22 *vs* bar 6).

All mammalian GalNAcα/β-disaccharide structural units-**P**, GalNAcβ1→3Gal (bar 23); **S**, GalNAcβ1→4Gal (bar 30); **A**, GalNAcα1→3Gal (bar 31); **F**, GalNAcα1→3-GalNAc (bar 32) and **Tn**, GalNAcα1→Ser/Thr (bar 14), were poor inhibitors. Of the Gal and GalNAc derivatives examined, *p*-nitrophenyl βGal was the best inhibitor, and was 1.7 and 10 times more active than the α-anomer and Gal, respectively (bar 7 *vs* bars 9 and 34). For methyl Gal glycosides, the α-anomer was a 2.3 times better inhibitor than the β-anomer (bar 15 *vs* bar 33). *P*-nitrophenyl α- and β-Gal were 2.7 and 10 times more active than methyl α- and β-Gal, respectively (bar 9 *vs* bar 15, and bar 7 *vs* bar 33), indicating that hydrophobic forces may participate in ECL-carbohydrate interaction. The results show that Gal-NAc was 1.1 and 2.5 times more active than Gal and GalN, respectively (bar 29 *vs* bars 34 and 39), and that Gal was 2.2 times more active than GalN (bar 34 *vs* bar 39), which implies that the carbonyl group of the acetamido radical enhances the reactivity and overcomes the blocking effect of the amido radical in the ECL-glycotope interaction. DFuc was 2.8 times less active than Gal (bar 40 *vs* bar 34), while LAra, which has the same configuration as Gal but lacks C-6, was 6.7 times less potent than Gal (Table 3), indicating that the CH_2OH of C-6 is significantly involved in submolecular recognition. Glc and Man showed 1/667 of Gal activity, demonstrating that the conformation of carbon-4 and carbon-2 in Gal is essential for binding.

Verification of ELLSA results by glycan array screening

The affinities of ECL for different glycotopes were determined further using the glycan array available through the Consortium for Functional Glycomics (http://www.functionalglycomics.org/fg/). ECL showed high affinity for glycan no. 133 and 165, known as biantennary Galβ1→4GlcNAc (**II**) (12.34 S/N binding) and biantennary Galβ1→4GlcNAc (**II**) core α6Fuc (9.98 S/N binding), respectively (Fig. 1). Other glycans, such as glycan no. 112

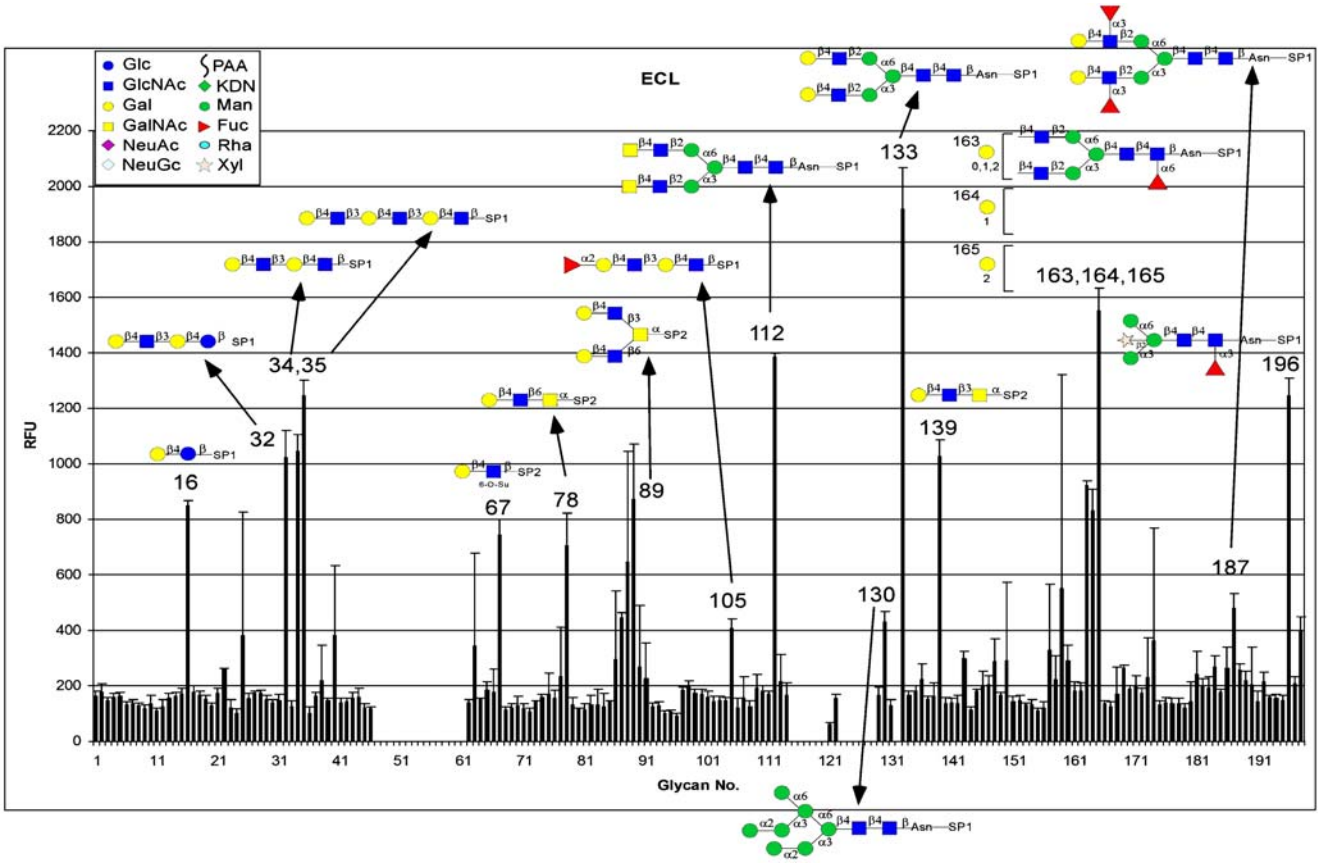


Fig. 1 Results from the ECL-glycan binding array analysis. A total of 198 glycans were screened for binding along with positive and negative controls, as described under "Materials and methods"

(Bi-LDN), 196 (HRP mixture), 35 ([Galβ1→4GlcNAc]$_3$), 34 ([Galβ1→4GlcNAc]$_2$), 139 (Galβ1→4GlcNAcβ1→3-GalNAc), 32 (Galβ1→4GlcNAcβ1→3Galβ1→4Glc), 163 (mix-biantennary Gal$_{(0,1,2)}$ core α6Fuc), 89 ([Galβ1→4GlcNAcβ]$_2$-3,6-GalNAc), 16 (Lactose), and 164 (mix-biantennary Gal$_{(1)}$ core α6Fuc) also showed high affinity, but their S/Ns were 1.4–2.3 times less than that of biantennary Galβ1→4GlcNAc (**II**) (Fig. 1).

Discussion

During the past two decades, several reports on binding properties of ECL have been published. Previously, the binding characteristics of this lectin and its homologous ECorL were analyzed by quantitative precipitin (QPA) and precipitin inhibition assays (QPIA) [11], hapten inhibition of hemagglutination [1, 2], spectrofluorimetric titration [10], chromatogram binding assay [5], solid-phase binding assays and molecular modeling [7], enzyme linked lectin assay (ELLA) [8] and microcalorimetry [6, 9]. However, the effects of polyvalency on the binding activities of many mammalian sugar structural units toward ECL have not been well characterized. In the present study, the recognition factors of ECL were examined, using our collection of ligands and polyvalent glycotopes of natural glycans, by enzyme-linked lectinosorbent assay [14, 15], which is a sensitive and time/reagent-saving method. To avoid potential problems caused by differences in adsorption of glycoproteins to the microplate wells, the glycoproteins used in Table 1 (Fig. 1 in Supplementary Section) were tested for their ability to inhibit lectin-glycoprotein binding (Table 2 and Fig. 2 in Supplementary Section). Overall, the inhibitory profile, expressed as the amount of glycans (in nanogram) required to inhibit 50% of the ECL-glycoprotein interaction, is in agreement with the interaction profile (Table 1 *vs* Table 2). Since it is quite difficult to obtain accurate numbers of glycotopes present in complex-*O*-linked glycoproteins [19, 35], the data expressed on a nanogram basis should be more conservative and more reliable than the nanomoles system as amino acid contents is included in the amount of molecular mass required. As shown in Table 1 and Fig. 1 in Supplementary Section, the strength of ECL-glycan interactions showed some variations among the **II** containing glycans. This may be due to difference in microplate adsorption of various glycans, especially in the case of *Streptococcus pneumoniae* type 14 polysaccharide. Therefore, our interpretations of binding

reactivities of glycans in this report are based on the data of molecular mass inhibition assay (Table 2).

Among soluble natural glycans tested, *Streptococcus pneumoniae* type 14 polysaccharide, composed of repeating units with **II** (Galβ1→4GlcNAc) as side chain (Structure 4), was the most potent inhibitor. It was 2.1×10^4, 3.9×10^3 and 2.4×10^3 times more active than Gal, tri-antennary **II** (Tri-**II**) and **II**, respectively. Most type **II**-containing glycoproteins (gps), especially human blood group precursor gps and hog gastric mucin, were also powerful inhibitors. These included many polyvalent **I**/**II**-containing gps from human ovarian cyst (cyst OG 10% 2× ppt, cyst Tighe P-1, cyst MSS 1st Smith degraded, cyst Mcdon and Beach P-1 and Cyst Tij 20% of 2nd 10% 2×, bars 2–4, 7, 10 and 11, Table 2 and Fig. 2 in Supplementary Section). They were 1.9×10^2 to 9.6×10^2 and 3.1×10^2 to 1.5×10^3 times more active inhibitors than monomeric Galβ1→4GlcNAc (**II**) and Tri-**II**, respectively (bars 27 and 30, Table 2 and Fig. 2 in Supplementary Section). The tremendous increase in inhibitory potency observed from free monomeric sugar to poly-glycotopes present on gps suggests that polyvalent **II** glycotopes play a critical role in ECL binding. Furthermore, the ability of ECL to recognize many human blood-group **ABH** active cyst gps (cyst N-1 Le^a 20% 2×, cyst Mcdon, cyst Beach phenol insoluble, cyst JS phenol insoluble, cyst Tighe phenol insoluble, cyst MSS 10% 2×; bars 5, 14, 16, 17, 19 and 22, Fig. 2 in Supplementary Section), demonstrated that incomplete carbohydrate chains of exposed precursor **I**/**II** residues at the nonreducing end exist in these cyst gps [27, 45] and that ECL can tolerate Fucα2 residues without change in binding strength [5–7]. Except for bird nest and Tamm-Horsfall gps (THGP), ECL reacted weakly or not at all with gps containing sialylated multivalent **II**/**I** determinants (Tables 1 and 2). The poor or negative reactivities of ECL with sialylated gps can be ascribed to the effect of the bulkiness and/or charge on sialic acid on binding, or because sialic acid sterically prevents the access of the determinant sugars to the lectin combining site, or both. Certainly, density of sialic acid should be an important factor involved in masking lectin-carbohydrate interactions. The unusual affinity of bird nest gp and THGP for ECL may be either due to some non-sialylated residues within their structures or to non-specific interactions which remain to be clarified.

In this study, the effect of a wide range of oligosaccharide ligands on the binding profile of ECL has been examined. Our results are generally, in agreement with inhibitory profiles described in the reports mentioned. The data of glycan array obtained from Consortium for Functional Glycomics support part of the conclusions (Fig. 1; entire glycan array version is available at http://www.functionalglycomics.org/glycomics/publicdata/selectedScreens.jsp). The overall reactivity of ECL on a nanomole basis can be shown in decreasing order to be as follows: **II**β1-6(**I**β1-3)**L** > Tri-**II** > Di-**II** > Galβ1→4GlcNAcβ1→6Gal > **II**β1-3**L** > Galβ1→4GlcNAc (**II**) > *p*-NO_2 phenyl βGal > *p*-NO_2 phenyl βGalNAc, *p*-NO_2 phenyl αGal, *p*-NO_2 phenyl αGalNAc > Fucα1→2Galβ1→4Glc > Galβ1→4Glc (**L**; Lactose), Galβ1→4Man > GalNAcα1→Ser/Thr (**Tn**) > Methyl αGal, Methyl αGalNAc > LFucα1→2Gal (**H** disaccharide), Galα1→4Gal (**E**), **I**β1-3**L** > Galα1→3Gal (**B**), Galα1→3Galβ1→4GlcNAc (**B** active **II**), GalNAcβ1→3Gal (**P**), Methyl βGalNAc > Galβ1→3GlcNAc (**I**) > Galα1→6Glc > GalNAc > Methyl βGal, Gal, GalNAcβ1→4Gal (**S**), GalNAcα1→3Gal (**A**), GalNAcα1→3GalNAc (**F**) > Galβ1→3GalNAcα1-benzyl ($\mathbf{T}_\alpha$) > Galβ1→4(Fucα1→3)GlcNAc ($\mathbf{Le}^x$), Galβ1→6GlcNAc > Galβ1→3GalNAc (**T**), GalN > DFuc > LAra etc. (Table 3). Thus, the relationship among **II**, **T**, **Tn** and other mammalian sugar structural units has been better clarified. Although monomeric GalNAc is active, even slightly more active than Gal monomer, most of its polyvalent forms do not enhance binding (Tables 1 and 2).

It is of interest to compare the binding properties of the three **II** reactive lectins -RCA_1, ricin and ECL). (1) They were all Galβ1→4GlcNAc (**II**) specific, but the inhibitory profiles of these lectins toward the monosaccharides were

Table 4 Variations in binding to monosaccharides and their glycosides among ECL, RCA_1 and ricin[a] [46, 48]

Lectin	RP[b] of Gal/GalNAc	Sugar anomerism in: (RP of β- and α-anomers)				Hydrophobicity in: (RP of *p*-nitrophenyl and methyl glycosides)			
		p-NO_2 phenyl Gal	Methyl Gal	*p*-NO_2 phenyl GalNAc	Methyl GalNAc	Gal α-anomer	Gal β-anomer	GalNAc α-anomer	GalNAc β-anomer
ECL	0.9	1.7	0.4	1.0	0.7	2.7	10.0	2.6	4.0
RCA_1	83.3	2.0	2.4	Inactive	Inactive	4.5	3.8	Inactive	Inactive
Ricin	2.0	2.0	0.9	1.0	1.0	1.0	2.2	1.3	1.3

[a] Values are calculated from nanomoles required for inhibition
[b] Expressed as reciprocal of relative potency (RP)

different. As shown in Table 4, it was Gal ≤ GalNAc (relative potency [RP] of Gal/GalNAc was 0.9) for ECL; Gal > GalNAc (RP: 2.0) for ricin; and Gal >> GalNAc (RP: 83.3) for RCA_1 [46]; (2) the RP of Tri-**II**/Gal varied with lectins; ECL, ricin and RCA_1 bound strongly with poly oligo-antennary **II** (Galβ1→4GlcNAc) structures in *N*-linked gps (asialo human α_1-acid gp, asialofetuin); (3) RCA_1 showed high affinity for *Pneumococcus* type 14 polysaccharide, but both ECL and ricin showed poor reactivity which can be attributed to the poor adsorption of most polysaccharides on microplate wells; (4) although GalNAc was slightly or as active as Gal for ECL, its reactivity was not be enhanced by polyvalent effects. The reactivity of lectins toward polyvalent **Tn**/**T** gps can be ranked as ricin >> RCA_1=ECL; (5) in ECL and RCA_1, the hydrophobicity surrounding α/β-anomeric Gal may enhance binding, whereas in ricin, its contribution is limited to β-anomeric Gal (Table 4).

In summary, it is concluded that: (1) although GalNAc is slightly more active than or as active as Gal for ECL, its polyvalent forms are poor enhancing factors; (2) Galβ1→4GlcNAc and other Galβ1-related oligosaccharides, rather than GalNAc-related oligosaccharides, are theme elements for binding (Table 3); (3) their polyvalent form in glycoproteins is the most important binding factor. From the results of molecular mass 50% inhibition assay (Table 2), the contribution of the recognition factors can be expressed in decreasing order to be as follows: polyvalent **II** and its related oligomers >> oligo-antennary **II** and monomeric **II** > Gal and GalNAc. The importance of the polyvalence in binding is also found in other plant and animal lectins, such as *Agaricus bisporus* Agglutinin (ABA) [47], *Ricinus communis* agglutinin 1 (RCA_1) [46], *Ricinus communis* toxic protein (ricin) [48], *Vicia villosa* B_4 ($VVL\text{-}B_4$) [49], *Maclura pomifera* agglutinin (MPA) [50], galectin-4 from rat gastrointestinal tract [51]; (4) based on the results of molecular mass 50% inhibition (Table 2), the contribution of oligo-antennary **II** to binding is weak. However, polyvalent oligo-antennary **II** form as in asialo human α_1-acid gp increases binding strongly; (5) from the molar 50% inhibition profile, the shape of the lectin binding domains may correspond to a cavity type in which Galβ1→4GlcNAc is the core binding site with additional contacts with one to four sugars; (6) it is most complementary to Galβ1→4GlcNAcβ1→6Gal (human blood group I Ma trisaccharide, bar 4, Table 3) and Galβ1→4GlcNAcβ1→3Galβ1→4Glc (**II**β1→3**L**, bar 5, Table 3) for a linear sequence, and Galβ1→4GlcNAcβ1→6(Galβ1→3GlcNAcβ1→3)Galβ1 →4Glc (lacto-*N*-neohexaose, LNnH, **II**β1-6[**I**β1-3]**L**, bar 1, Table 3) for a branch form; (7) ECL has a preference for the β-anomer of Gal at the non-reducing end of oligosaccharides with a Galβ1→4 linkage > Galβ1→3 > Galβ1→6; (8) since the ratio of Galβ-anomer of *p*-nitrophenyl glycosides per methyl glycosides is 10.0 (Table 4)], hydrophobic interaction of Galβ may also contribute as a binding factor; (9) **II** sugar residue is about 5 times more active than the **L** residue (bar 6 *vs* bar 12, Table 3), indicating that the *N*-acetyl group at subterminal Gal is also an important binding factor; (10) Fucα1→2Galβ1→4Glc is slightly more active than Galβ1→ 4Glc (**L**), suggesting that Fucα linked to the C2-position of terminal Gal contributes to binding to the lectin (bar 11 *vs* bar 12, Table 3); (11) The carbohydrate specificity of ECL for mammalian carbohydrate structural units can be mapped as: Tri-**II** > Di-**II** > I Ma > **II** > **L**, **Tn**, **I**, **H**, **E**, **B**, **P**, **S**, **A**, **F**, $\mathbf{T}_\alpha$, $\mathbf{Le^x}$, **T**. These results will be helpful establishing the functional role of ECL and in characterizing polyvalent glycotopes of mammalian glycans.

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