Carbohydrate specificity of a galectin from chicken liver (CG-16)

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Owing to the expression of more than one type of galectin in animal tissues, the delineation of the functions of individual members of this lectin family requires the precise definition of their carbohydrate specificities. Thus, the binding properties of chicken liver galectin (CG-16) to glycoproteins (gps) and *Streptococcus pneumoniae* type 14 polysaccharide were studied by the biotin/avidin-mediated microtitre-plate lectin-binding assay and by the inhibition of lectin–glycan interactions with sugar ligands. Among 33 glycans tested for lectin binding, CG-16 reacted best with human blood group ABO (H) precursor gps and their equivalent gps, which contain a high density of D-galactopyranose(β1–4)2-acetamido-2-deoxy-D-glucopyranose [Gal(β1–4)GlcNAc] and Gal(β1–3)GlcNAc residues at the non-reducing end, but this lectin reacted weakly or not at all with A-, H-type and sialylated gps. Among the oligosaccharides tested by the inhibition assay, the tri-antennary Gal(β1–4)GlcNAc (Tri-II) was the best. It was 2.1×10^3 nM and 3.0 times more potent than Gal and Gal(β1–4)GlcNAc (II)/Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc (lacto-N-tetraose) respectively. CG-16 has a preference for the β-anomer of Gal at the non-reducing end of oligosaccharides with a Gal(β1–4) linkage $>$ Gal(β1–3) $\geqslant$ Gal(β1–6). From the results, it can be concluded that the combining site of this agglutinin should be a cavity type, and that a hydrophobic interaction in the vicinity of the binding site for sugar accommodation increases the affinity. The binding site of CG-16 is as large as a tetrasaccharide of the β-anomer of Gal, and is most complementary to lacto-N-tetraose and Gal(β1–4)GlcNAc related sequences.

Key words: agglutinin, carbohydrate selection, glycoproteins, lectin, protein–carbohydrate interaction.

INTRODUCTION

Animal lectins serve multifarious functions including glycoconjugate routing, cell adhesion and growth modulation [1]. Within the families of lectins, currently categorized based on binding site topology, duplication and sequence divergence of ancestral genes is most likely the origin for closely related members of a family. This diversification raises the question as to whether these related molecules may fulfill overlapping or distinct activities. Since the recognition of a carbohydrate ligand is the primary event to initiate ensuing post-binding activities, it is pertinent to thoroughly map the binding profile of glycans for such related agglutinins. In order to take the geometrical parameters of ligand presentation adequately into account, it is reasonable to employ natural glycans as assay substances in addition to a panel of sugar derivatives.

Vertebrate species express not only one distinct β-galactoside-binding protein, but a family of related Ca^{2+}-independent lectins with a jelly-roll-like folding pattern and invariant amino acids in the carbohydrate-recognition domain [1]. Whereas the number of these galectins has reached eleven for mammals, chickens are apparently restricted to the expression of two members of this family which have independent developmental regulation [2–4]. A putative third galectin cDNA has previously been cloned from chondrocytes [5] and is waiting for confirmation of its assumed sugar-binding capacity at the protein level. On the basis of their electrophoretic mobilities under denaturing conditions, these lectins are referred to as chicken galectin (CG)-14, abundantly present in adult chicken intestine, and CG-16, found in large amounts in adult chicken liver and pancreas [2]. They seem to be involved in the regulation of differentiation, because their contents can change significantly during development in different organs [2–4]. For example, contributions by CG-16 to the morphogenesis of cartilage [6] and by CG-14 in the formation of embryonic skin have been suggested [7,8].

In addition to their sequences, the two galectins are known to differ in their propensity to form dimers. Purified CG-16 is usually present as a dimer, and has a higher haemagglutination activity than CG-14 [7]. This property, influencing the extent of formation of cross-linked aggregates [10] and readily visible in the crystallographic structure harbouring an interface area of 563 Å^2 for the CG-16 homodimer [11], may be of relevance for the differential potency to initiate signalling [9]. As already referred to, the complexity of galectin expression in mammalian cells such as splenocytes hampers the comparative analysis with functional implications. In this respect, the avian system presents

Abbreviations used: CG, chicken galectin; Gal, D-galactopyranose; Glc, D-glucopyranose; Man, D-mannopyranose; LFuc, L-fucopyranose; DFuc, D-fucopyranose; Fruf, fructofuranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; gp, glycoprotein; PSM, porcine salivary gp; HSM, hamster submaxillary gp; OSM-major, major fraction of ovine submandibular gp; BSM-major, major fraction of bovine submandibular gp; RSL, rat sublingual gp; TBS, Tris/HCl-buffered saline; TBS-T, TBS containing 0.05% (v/v) Tween 20; ELLSA, enzyme-linked lectinosorbent assay. The bioactive carbohydrates and blood group active sugar sequences used are: A, human blood group A specific disaccharide, GalNAc(α1–3)Gal; A_h, GalNAc(α1–3)[LFuc(α1–2)]Gal, blood group A specific trisaccharide containing crypto H determinant; H, Fuc(α1–2)Gal; Tn, GalNAc(α1–)Ser/Thr; I, human blood type I precursor sequence, Gal(β1–3)GlcNAc; II, human blood type II precursor sequence, Gal(β1–4)GlcNAc; T, the mucin type sugar sequence on the human erythrocyte membrane, Gal(β1–3)GalNAc; B, blood group B specific disaccharide, Gal(α1–3)Gal; B_h, Gal(α1–3)[LFuc(α1–2)]Gal; E, galabiose, Gal(α1–4)Gal sequence, a receptor of the uropathogenic *Escherichia coli* ligand; Le^a, Gal(β1–3)[Fuc(α1–4)]GlcNAc; Le^b, Fuc(α1–2)Gal(β1–3)[Fuc(α1–4)]GlcNAc; Le^x, Gal(β1–4)[Fuc(α1–3)]GlcNAc; Le^y, LFuc(α1–2)Gal(β1–4)[Fuc(α1–3)]GlcNAc.

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a simple model for in-depth study of the functions and binding properties of the two galectins. They apparently share the importance of the 4′- and 6′-hydroxyl groups on galactose for docking as revealed with engineered ligand derivatives [12], but may display fine-specificity differences to natural ligands which are relevant for elucidating their functions. Knowledge of the carbohydrate specificity of CG-16 is, therefore, an important step to understand the functional roles of this lectin. Moreover, this information may initiate applications in glycoconjugate analysis or purification. For this purpose, it is of vital importance to delineate the binding properties of this galectin for natural glycans. Thus, the binding properties of chicken hepatic galectin, CG-16, to glycoproteins (gps) and polysaccharides were studied by the biotin/avidin-mediated microtitre-plate lectin-binding assay, and by the inhibition of agglutinin–glycan interactions with sugar ligands. The results indicate that CG-16 reacted best with human blood group ABH(O) precursor gps and their equivalent gps which contain a large number of multivalent Gal(β1–4)GlcNAc or Gal(β1–3)GlcNAc oligosaccharides. The CG-16 lectin reacted weakly, or not at all, with A- and H-active gps or sialylated gps. The combining (sugar-binding) site of the agglutinin should be a cavity, and its binding size might be as large as a tetrasaccharide of β-anomeric Gal and is most complementary to Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc (lacto-N-tetraose) and Gal(β1–4)GlcNAc. Multivalent Gal(β1–4)GlcNAc clustering and/or elongation of Gal(β1–3)GlcNAc enhances the reactivity. The results of this study also provide an essential reference for studying the differential binding properties of the other related galectins.

EXPERIMENTAL

CG-16 purification and biotinylation

The predominant galectin of chicken liver was purified from liver extracts by successive steps including affinity chromatography on lactose–Sepharose 4B, obtained by ligand coupling to divinylsulphone-activated resin [13], and anion-exchange chromatography on a Mono Q column (1 ml column resin; Amersham Pharmacia Biotech, Freiburg, Germany), as described previously [9]. Removal of the minor contamination with CG-14 was ascertained by two-dimensional PAGE analysis as described previously [9]. For biotinylation, the lectin was stabilized by treatment with iodoacetamide, under activity-preserving conditions with the *N*-hydroxysuccinimide ester derivative of biotin (Sigma) as described in detail previously [14].

Gps and polysaccharides

The purified blood group substances used [Table 1 and Figure 1 (Structure I)] were prepared from human ovarian cyst fluid and from animal stomach [15–21]. The blood group substances were purified from the human ovarian cyst fluid by digestion with pepsin and precipitation with increasing concentrations of ethanol; the dried ethanol precipitates were extracted with 90 % phenol and the insoluble fraction named after the blood group substance (e.g. Cyst Beach phenol insoluble). The supernatant was fractionally precipitated by addition of 50 % ethanol in 90 % phenol to the indicated concentrations [15]. The designation 10 % or 20 % (ppt) denotes a fraction precipitated from phenol at an ethanol concentration of 10 % or 20 %; 2 × signifies that a second phenol extraction and ethanol precipitation were carried out (e.g. Cyst OG 20 % 2 ×).

The P-1 fractions, in general, 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 groups A- and B-active oligosaccharide side chains [15,22]. The 1st-Smith-degraded products of blood group A active substance (MSS 10 % 2 ×, Structure I), in which almost all of the sugar groups at the non-reducing ends were removed [23,24], was prepared as described previously [17,23,24]. Both P-1 and 1st-Smith-degraded gps have the same reactivities as blood group precursor substances such as cyst OG in Table 1 and are defined as precursor equivalent gps (Structure I) [22–26].

Table 1 Identification of the curves from Structure I*

Curve in Figure 6	Blood group active gp purified from human ovarian cyst fluid	Bioactive determinants or human blood-group specificity present	Sugar added to Structure I	Site of addition to Structure I
c	Cyst OG 10% 2 × ppt	I/II		
a	1st Smith cyst MSS†	Pneumococcus type 14		
c	Beach phenol P-1†	polysaccharide antigenic		
b	Mcdon P-1†	determinant (II)	None	
d	Tighe P-1†			
d	Asialo HOC 350†‡ Tighe phenol insoluble	H active glycotopes	LFuc(α1-2)	(5), (7), (9), (11)
d	Cyst N-1 Le^a 20% 2 ×	Le^a, Le^b	LFuc(α1-4)	(6)
e		Le^x and Le^y	LFuc(α1-3)	(8), (10), (12)
a	MSS, native		GalNAc(α1-3)	(1), (2), (3) and/or (4)
a	MSM 10% ppt	A_h	and as in H, and Le^b	(5), (7), (9) and (12)
b	Cyst 14			
a	Cyst 9			
b	Cyst Mcdon			
c	Beach phenol insoluble	B_h	Gal(α1-3) and as in H and Le^b	(1), (2), (3) and/or (4), (5), (7), (9), (6), (8) and (12)
c	Cyst 19	B_h		
c	Cyst Tij 20% of 2 × 10%	B_h		

* Cited from [25].

† These are human precursor equivalent gps, which are defined as A, B, H active gps after removal of A, B, H, Le^a, Le^b, Le^x and Le^y active key sugars by mild acid hydrolysis, Smith degradation or glycosidases.

‡ Cited from [30].

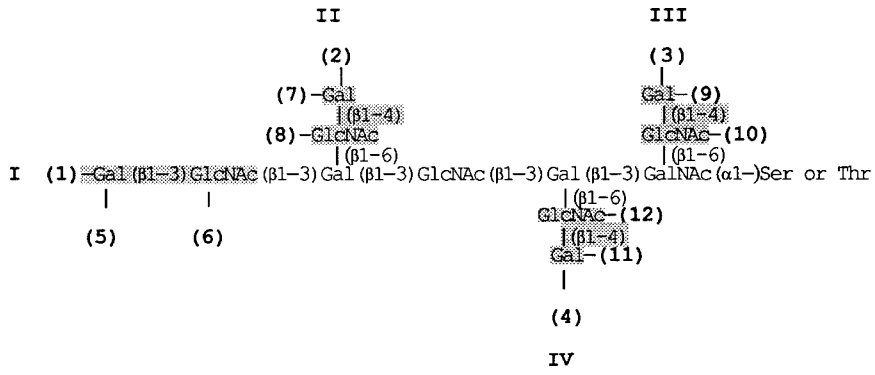

Figure 1 Structure I: Proposed representative carbohydrate side chains of blood group active gps, prepared from human ovarian cyst fluid

This structure represents the internal portion of carbohydrate chains to which various human blood group determinants are attached. The four-branched structure (I–IV) shown is the representative internal portion of the carbohydrate moiety of blood group substances to which the residues responsible for the activities of A, B, H, Le^a and Le^b are attached. This structure represents precursor blood group active gps [19,25] and can be prepared by Smith degradation of active A, B and H gps, purified from human ovarian cyst fluids [22–26]. Numbers in parentheses indicate the site of attachment for the human blood group A, B, H, Le^a and Le^b determinants (Table I). These determinants, as well as the structural units at the nonreducing end, are the sources of the bioactive (lectin) reactive A/A_h, B/B_h, I/II, T, and Tn determinants [27,28]. A megalosaccharide of 24 sugars has not yet been isolated. However, most of the carbohydrate chains isolated are parts of this structure. Shaded areas are proposed to be glycotopes of CG-16.

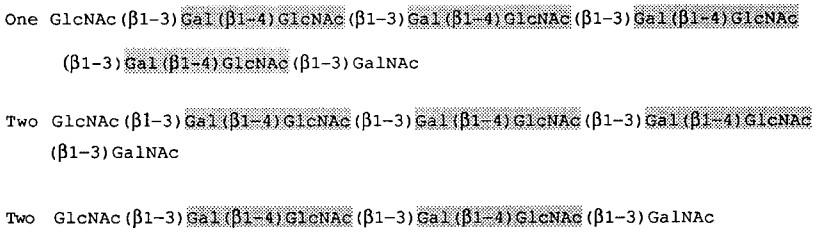

Figure 2 Structure II: Established structure of the carbohydrate moiety of asialo RSL contains chains of three different lengths

Most of the carbohydrate chains are parts of the asialo RSL structure [32,33]. Furthermore, variations in this ratio and in chain lengths are expected to occur in different mucin preparations [33]. Shaded areas are proposed to be glycotopes of CG-16.

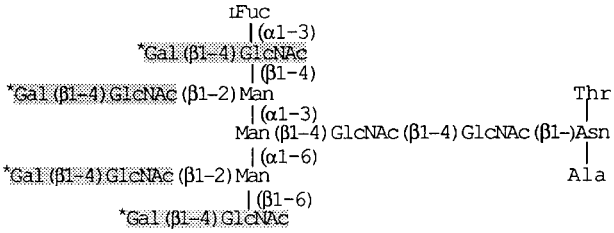

Figure 3 Structure III: Carbohydrate units of the asialo-human α_1-acid gp (orosomucoid) [34]

The sugars shown are the proposed sialylation on Gal*. Shaded areas indicate the possible active structural units, and are proposed to be the glycotopes of CG-16. Due to the limited number of active sugars in the molecules, it is a poor inhibitor.

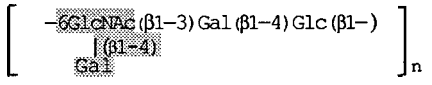

Figure 4 Structure IV: Type 14 Pneumococcal polysaccharide repeating unit

Shaded area [Gal(β1–4)GlcNAc] is proposed to be glycotope of CG-16 [36].

The HOC 350 from human ovarian cyst fluid [29,30], which is a sialic acid-rich and Le^a-active gp, was kindly provided by Dr. W. M. Watkins (University of London, Royal Postgraduate Medical School, Hammersmith Hospital, London, U.K.). The rat sublingual gp (RSL) [Figure 2 (Structure II)] was prepared by the method of Moschera and Pigman [31]. Human α1-acid gp [Figure 3 (Structure III)] [34], fetuin [35] and porcine thyroglobulin, which also contain multivalent Gal(β1–4)GlcNAc sequences, were purchased from Sigma. The Pneumococcus type-14 polysaccharide [Figure 4 (Structure IV)] [36,37], consisting of the repeat unit of Gal(β1–4)GlcNAc as branched chain, was a gift from the late Dr. E. A. Kabat (Department of Microbiology, Columbia University, New York, NY, U.S.A.).

The major fractions of ovine and bovine submandibular gps (OSM-major and BSM-major respectively) and porcine salivary mucin (PSM) were purified according to the method of Tettamanti and Pigman [38] with modifications [39,40], and hamster submaxillary gp (HSM) by the method of Downs and Herps [41]. Their chemical compositions were as described previously [38–42].

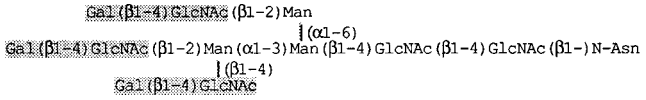

Figure 5 Structure V: Carbohydrate moiety of tri-antennary Gal(β1–4)GlcNAc (tri-II) glycopeptides

Shaded area, Gal(β1–4)GlcNAc, is proposed to be the glycotope of CG-16.

Desialylation of sialic acid containing gps was performed by mild acid hydrolysis with 0.01 M HCl at 80 °C for 90 min, and dialysis against distilled water for 2 days to remove small fragments [38,42].

Native hog gastric mucin-4 [28,43] was derived from crude hog stomach mucin which was dissolved in water and centrifuged at 3.01×10^4 ***g*** for 3 h, followed by exhaustive dialysis (molecular-mass cut-off at 8.0 kDa) against distilled water. Treatment of mucin-4 with HCl, pH 2, for 90 min at 100 °C yielded hog gastric mucin-9, while hog gastric mucin-14 and -21 were obtained from mucin-4 by acid hydrolysis, pH 1.5, at 100 °C for 2 and 5 h, respectively. Each hydrolysis step was followed by extensive dialysis.

Inhibiting sugars

D-Gal, D-Fuc, L-Fuc, D-Man, GalNAc, GlcNAc, L-Ara, melibiose, raffinose, stachyose, methyl α-D-Gal, methyl β-D-Gal, *p*-nitrophenyl α-D-Gal, *p*-nitrophenyl β-D-Gal, *p*-nitrophenyl α-D-GalNAc, *p*-nitrophenyl β-D-GalNAc, Lac, Lacto-N-tetraose, Gal(α1–4)Gal, Gal(α1–3)Gal, Gal(β1–4)Man, Gal(α1–3)Gal(α1–)*O*-methyl, Gal(β1–3)GalNAc, Gal(β1–3)GalNAcα1-*O*-benzyl, Gal(β1–3)GlcNAc, GlcNAc(β1–4)GlcNAc, and Gal(β1–4)GlcNAc, Gal(β1–6)GlcNAc, Gal(α1–3)Gal(α1–)*O*-methyl, phenyl β-D-Gal, and Gal(β1–3)Ara were purchased from Sigma.

Tri-antennary Gal(β1–4)GlcNAc gp [Figure 5 (Structure V)] was prepared as described previously [44], in which asialofetuin was digested by pronase and repeatedly fractionated by BioGel P-4 (400 mesh) column chromatography (Bio-Rad). Peak IIa was used in this study [44].

The microtitre plate lectin–enzyme binding assay

The test was performed according to Duk et al. [45] and Lisowska et al. [46]. Plates were coated with gps and the binding of biotinylated CG-16 was determined. The volume of each reagent applied to the plate was 50 μl/well, and all incubations, except for coating, were performed at 20 °C. The reagents, if not indicated otherwise, were diluted with Tris/HCl-buffered saline (TBS) containing 0.05% (v/v) Tween 20 (TBS-T). The TBS buffer or 0.15 M NaCl containing 0.05% Tween 20 were used for washing the plates between incubations.

For the inhibition studies, the 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 2-fold with TBS-T. After 30 min at 20 °C, the 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 (nmol/well) giving 50% inhibition of the control lectin binding.

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

RESULTS

Lectin–glycan interaction

The avidity of CG-16 for gps and Pneumococcus type 14 polysaccharide as studied by a microtitre plate enzyme-linked lectinosorbent assay (ELLSA) is summarized in Table 1, based on the interaction profiles shown in Figure 6. CG-16 reacted strongly with high density Gal(β1–3/4)GlcNAc containing glycans. These include five human blood group precursor equivalent gps (Table 1 and Structure I: 1st Smith degraded cyst MSS gp, Figure 6a, cyst Mcdon P-1, Figure 6b; cyst Beach P-1 and cyst OG 10% 2× ppt, Figure 6c; cyst Tighe P-1, Figure 6d), a Le^a active gp, cyst N-1 gp (Figure 6e), and asialo RSL (Structure II, Figure 6f), in which less than 140 ng of the gps coated was required to interact with 250 ng of lectin to yield an A_{405} of 1.5 within 4 h. Although the percentage of the gps immobilized on the microtitre plate has not been determined, the amount of gp required to give 1.5 units (A_{405}) with this lectin has to be equal to or less than 140 ng. This lectin also bound well an asialo blood group Le^a gp from human ovarian cyst fluid (asialo HOC 350 in Figure 6d), asialo human α_1-acid gp (Figure 6f) and asialofetuin (Figure 6e) and Pneumococcus type 14 polysaccharide (Structure IV and Figure 6f), which contain different amounts of Gal(β1–3/4)GlcNAc residues. To ascertain that carbohydrate binding is responsible for the signal quantity, the CG-16–glycan interaction was interrupted by various glycoforms as described below (see Figure 7 and Table 2).

The blood group A, B, H or Le^b substances (Structure I and Table 1) and mammalian gps containing Gal(β1–4)GlcNAc and Gal(β1–3)GalNAc masked by sialic acids were either weakly active or inactive (Figure 6 and Table 2). Except for asialo RSL, neither salivary native gps nor their asialo products containing mainly crypto GalNAc(α1-)Ser/Thr (Tn) and exposed Tn determinants reacted with CG-16.

Inhibition of CG-16–glycoform interaction by various glycans

The ability of various sugars to inhibit the binding of CG-16 with a Gal(β1–3/4)GlcNAc containing gp (1st Smith degraded cyst MSS gp; Table 1 and Structure I) was analysed by ELLSA (Figure 7 and Table 2). Among the glycans tested for inhibition of this interaction, a human blood group precursor (Figure 7; curve 1, cyst Mcdon P-1) was the best, requiring less than 16 ng to inhibit the interaction by 50%. Six other human blood group (precursor) equivalent gps (curves 2, 3, 5 and 8–10 in Figure 7; Table 2) and Pneumococcus type-14 polysaccharide (Figure 7, curve 4; Table 2) were also potent inhibitors. They were much more active inhibitors than monomeric Gal(β1–4)GlcNAc, Gal(β1–4)Glc and Gal (Figure 7, curves 7, 11 and 14). The precursor equivalent gps and asialo-gps were much more active than their more heavily glycosylated or sialylated (native) compounds. Despite several exceptions, the inhibitory reactivities of glycoforms toward CG-16 broadly agree with the maximum absorbances recorded in the binding assay (Figure 6 and Table 2). Three Gal(β1–4)GlcNAc containing N-linked gps (asialofetuin, asialo human α1-acid gp and asialo porcine thyroglobulin), which reacted well with CG-16, were poor inhibitors.

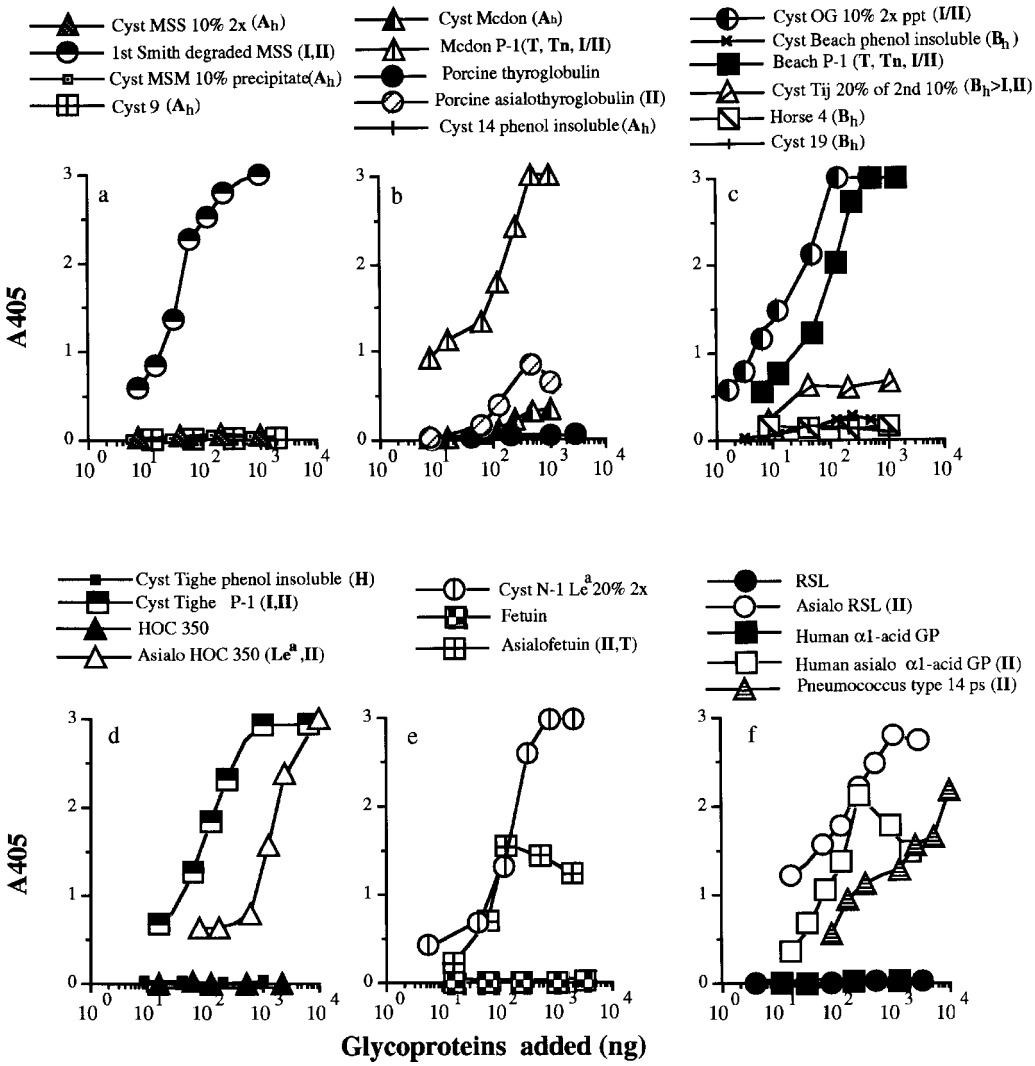

Figure 6 Binding of CG-16 to microtitre plates coated with serially diluted human blood group A, B, H, Le^b and II/I active gps, sialo- and asialo-gps and polysaccharide

The amount of lectin used was 250 ng per well. Total volume of the assay was 50 μl. A_{405} was recorded after 4 h incubation. The following glycans did not bind with CG-16 ($A < 0.2$): Hog gastric mucin-4 ($A_h + H$), Ovine salivary gp (OSM), asialo OSM, Bovine salivary gp (BSM, sialyl Tn and GlcNAcβ-Tn), asialo BSM and HSM (sialyl Tn).

This can be explained by an insufficient number of Gal(β1–4) GlcNAc residues in these three N-glycans.

Inhibition of the lectin–glycan interaction by mono- and oligosaccharides

The ability of various sugars to inhibit the binding of CG-16 with a Gal(β1–3/4)GlcNAc containing gp (1st Smith degraded cyst MSS gp) is shown in Figure 8, and the amounts of ligands required for 50% inhibition of the lectin–glycan interaction are listed in Table 3. Among the oligo- and monosaccharides tested, tri-antennary Gal(β1–4)GlcNAc was the best, which was three times more active than monomeric Gal(β1–4)GlcNAc (curve 1 versus curve 3 in Figure 8, and Table 3); it was slightly more active than di-antennary Gal(β1–4)GlcNAc (Di-II) and was about 2.1×10^3 times more active than Gal (Figure 8; curve 1 versus curve 21), and 61 times more active than Gal(β1–3) GlcNAc (I) (Figure 8; curve 1 versus curve 10).

Gal(β1–4)Glc and Gal(β1–4)Man (Figure 8; curves 5 and 6) had the same degree of activity. They were about half as active as Gal(β1–4)GlcNAc (II) (curve 4), but 10 times more active than Gal(β1–3)GlcNAc (I) and Gal(β1–6)GlcNAc (Figure 8; curves 5 and 6 versus curves 10 and 11). These results show that CG-16 has a preference in the order: Gal(β1–4) > Gal(β1–3) $\geqslant$ Gal(β1–6).

Stachyose [Gal(α1–6)Gal(α1–6)Glc(β1–2)DFruf], raffinose [Gal(α1–6)Gal(β1–2)DFruf] and melibiose (Figure 8; curves 16–18) were about equally active and 600 times less active than tri-II, suggesting that Gal(β1–4) clusters are the most important ligands for CG-16.

Of the other monosaccharides studied, *p*-nitrophenyl α-DGal (Figure 8a; curve 9) was the best inhibitor, it was 57.6 times less active than tri-II (Figure 8b; curve 1) and 36.8 and 7.4 times more active than Gal (Figure 8; curve 21), and *p*-nitrophenyl β-DGal (Figure 8; curve 12) respectively. As shown in Table 3, *p*-nitrophenyl α-DGal was about 10 times better than the methyl-α derivative (Figure 8a; curve 9 versus curve 15), and only a 2-or 4.6-fold difference was observed between the *p*-nitrophenyl,

Table 2 Binding of CG-16 lectin to human blood group A, B, H, P_1 and Le^b active gps, sialo- and asialo-gps analysed by ELLSA[1], and the amount of various gps giving 50% inhibition of the binding of CG-16 (125 ng/50 μl) by a Gal(β1–) containing gp (1st Smith degraded cyst MSS gp, 50 ng/50 μl)[2]

Notes: [1]Biotinylated lectin (250 ng) was added to various concentrations of gp ranging from 8–1×10^3 ng. [2]The inhibitory activity was estimated from the inhibition curve in Figure 7, and is expressed as the amount of inhibitor giving 50% inhibition. The total volume was 50 μl. Gal(β1–4)GlcNAc (curve 7, Figure 7) is 383.4 ng and equal to 1.0 nmoles; Lac. (curve 11, Figure 7) is 684.6 ng and equal to 2.0 nmoles; Gal (curve 14, Figure 7) is 1.26×10^5 ng and equal to 700.0 nmoles. [3]The symbol in parentheses indicates the human blood group activity, and/or the bioactive (lectin) determinants [27,28] are expressed in **bold:** A_h, GalNAc(α1-3)[LFuc(α1-2)]Gal; B, Gal(α1-3)Gal; B_h, Gal(α1-3)[LFuc(α1-2)]Gal; E, Gal(α1-4)Gal; I/II, Gal(1-3/4)GlcNAc. [4]Glycans were tested up to 2000 ng and did not reach 1.5 (A_{405}). [5]Glycans were tested from 277.8–555.6 ng and did not reach 50% inhibition. [6]The results were interpreted according to the spectrophotometric absorbance value at 405 nm after 4 h incubation as follows: +++++, $A \geqslant 2.5$; ++++, $A = 2.0$–2.5; +++, $A = 1.5$–2.0; ++, $A = 0.75$–1.5; +, $A = 0.75$–0.2; −, $A < 0.2$.

Activity order	Figure 6(a–f)	Glycoprotein Lectin determinants[3] (Blood group specificity)	Quantity of glycan (ng)	Quantity giving 50% inhibition[2] (ng)	Maximum A_{405}	
					A_{405}	Binding intensity[6]
1	c	Cyst OG 10% 2× ppt (**I/II**)	17.0	70.0	3.0	+++++
2	a	First Smith degraded cyst MSS (**I**, **II**)	34.0	30.0	3.0	+++++
3	f	Asialo RSL (**II**)	50.0	110.0	2.8	+++++
4	b	Cyst Mcdon P-1 (**I**, **II**)	70.0	16.0	3.0	+++++
5	c	Cyst Beach P-1 (**I**, **II**)	80.0	42.0	3.0	+++++
6	d	Cyst Tighe P-1 (**I**, **II**)	80.0	400.0	2.9	+++++
7	e	Asialofetuin (**II**, **T**)	120	–[5]	1.6	+++
8	e	Cyst N-1 Le^a 20% 2× (Le^a)	140	310.0	2.9	+++++
9	f	Asialo human α_1-acid gp (**II**)	150	–[5]	2.1	++++
10	d	Asialo HOC 350 (**II** > Le^a)	500	350.0	3.0	+++++
11	f	Pneumococcus type 14 ps (**II**)	2.2×10^3	60.0	2.2	++++
12	b	Asialo porcine thyrogobulin (**II**)	–[4]	–[5]	0.9	++
13	c	Cyst Tij 20% of 2nd 10% (**B_h** > **I/II**)	–[4]	710.0	0.7	+
14	b	Cyst Mcdon (**A_h**)	–[4]	–[5]	0.4	+
15	c	Cyst Beach phenol insoluble (**B_h**)	–[4]	–[5]	0.3	+
16	a	Cyst MSS 10% 2× (**A_h**)	–[4]	–[5]	0.1	−
17	d	Cyst Tighe phenol insoluble (**H**, Le^b)	–[4]	–[5]	0	−
18	d	HOC 350 (sialyl Le^a)	–[4]	–[5]	0	−
19	f	Rat sublingual major-gp (RSL) (Sialyl **II**)	–[4]	–[5]	0	−

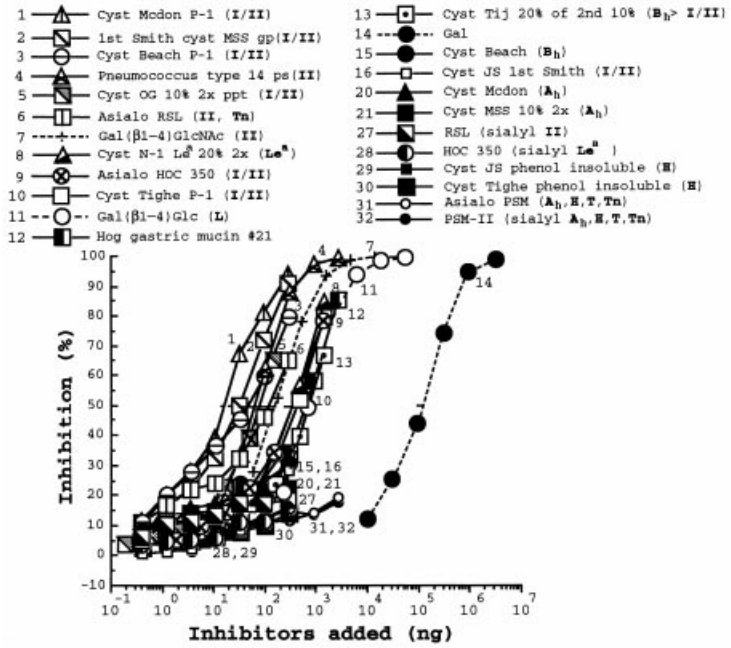

Figure 7 Inhibition of CG-16 binding to a Gal(β1–) containing gp (1st Smith degraded cyst MSS gp)-coated ELLSA plates with various gps

The quantity of gp in the coating solution was 50 ng per well. The quantity of lectin used for the inhibition assay was 125 ng per well. The total volume was 50 μl. A_{405} was recorded after a 4 h incubation. The amount (ng) of gp required to induce 50% inhibition was determined. Two different amounts of the gps were used for inhibition of CG-16–gp interaction. The following gps, in addition to the results presented in this Figure, did not reach 50% inhibition with 277.8 ng gp as inhibitors: Cyst Beach phenol insoluble (B_h); Asialo BSM (Tn & GlcNAcβ-Tn); Bovine asialo α_1-acid gp; Cyst Mcdon (A_h); Hog gastric mucin 4 (A_h + H); Hog gastric mucin 9 (A_h + H); Hog gastric mucin 14 (A_h); Fetuin; Human α_1-acid gp; BSM; RSL; HOC 350; Cyst Tighe phenol insoluble (H, Le^b); Bovine α_1-acid gp. With 2778 ng gp as inhibitors: Horse 4 (B_h); Human asialo α_1-acid gp (sialyl II); Cyst 14 phenol insoluble (A_h); Cyst 19 (B_h); Porcine thyroglobulin; Porcine asialothyroglobulin; Asialo OSM (Tn); OSM; Asialo PSM and PSM; HSM (sialyl Tn).

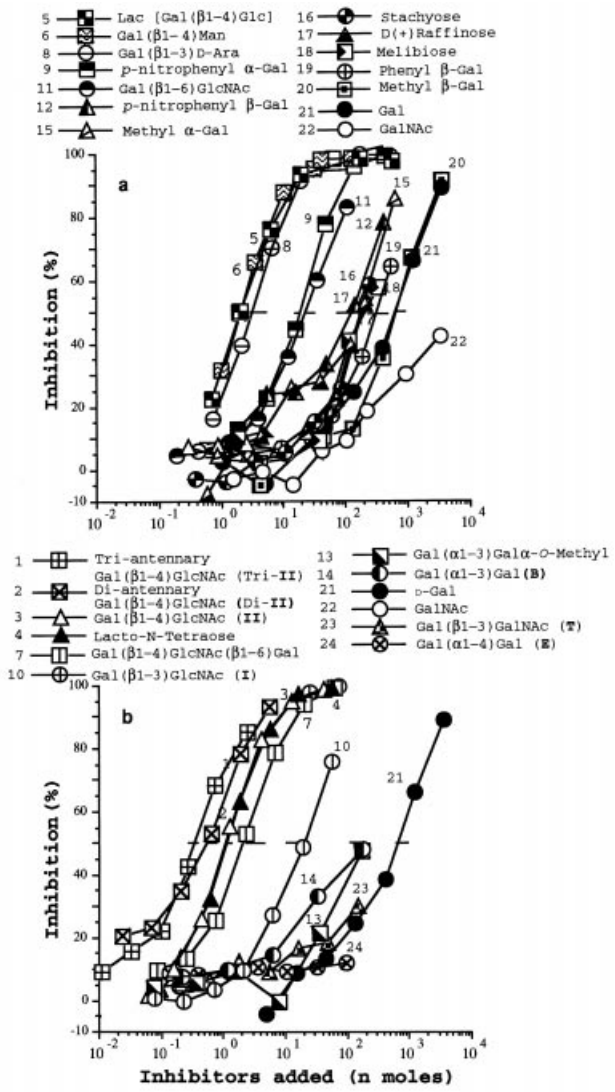

Figure 8 Inhibition of CG-16 binding to a Gal(β1–) containing gp (1st Smith degraded cyst MSS gp)-coated ELLSA plates by various saccharides

The amount of gps in the coating solution was 50 ng per well. The lectin (250 ng per well) was pre-incubated with an equal volume of serially diluted inhibitor. The final CG-16 content was 125 ng per well. The total volume was 50 μl. A_{405} was recorded after a 4 h incubation. The following sugar inhibitors were tested from 95–5000 nmol and found to be inactive, *p*-nitrophenyl α-DGalNAc, *p*-nitrophenyl β-DGalNAc, L-Ara, Man, DFuc, LFuc and GlcNAc.

and methyl derivatives of β-DGal (Figure 8; curves 12 and 19 versus curve 20), indicating that a hydrophobic interaction with an aglycon is more important with the α-anomer of Gal than its β-D-anomer.

GalNAc was three times less active than Gal (Figure 8; curve 21 versus 22). L-Ara, D-Man, D-Fuc, D-GlcNAc and L-Fuc were tested at concentrations ranging from 1110–5150 nmol, but no inhibition was observed (Table 4).

DISCUSSION

Owing to the expression of often more than one type of galectin in animal tissues [1], the delineation of the functions of individual members of this lectin family requires the precise definition of their carbohydrate specificities. Fine differences may translate into distinct spectra of bound glycans, presumably connecting the presence of certain lectins with expression of characteristic cognate oligosaccharides [47]. Besides employing oligosaccharides as inhibitors of ligand binding, determination of their avidity towards glycotopes (carbohydrate epitopes) in natural glycans is useful to map the profile of their reactive structures. In this study, the carbohydrate-binding activity of CG-16 was examined by using an ELLSA method [45,46] in which the lectin is biotinylated and its binding detected with alkaline phosphatase-conjugated avidin. The great advantage of this method is that the lectin amount required for the assay is about 1/10 to 1/3500 of that required for the quantitative precipitin and quantitative precipitin-inhibition assays [28,47,48]. Furthermore, a wide range of special gp and oligosaccharide preparations had been used to characterize the binding site of this lectin [28,47]. Inhibition of the lectin–glycoform binding using a variety of defined oligosaccharides confirmed the preliminary results on the carbohydrate specificity of this lectin, and enabled its detailed mapping [47,48]. Since it is difficult to quantitate precisely the amounts of the gps adsorbed on to the microwells [48], the interactions of CG-16 with various glycoforms were examined by three parameters: (1) amount of the gps added to wells that gave 1.5 units at A_{405} (Table 1), (2) the maximum A_{405} value for each gp after 4 h incubation (Figure 6 and Table 1), and (3) the amount of gps required to give 50 % inhibition (Figure 7 and Table 2). It is obvious that some conclusions can be drawn when the interplay of the three parameters are compared (Table 2).

The most important observation was the very high affinity of this lectin for a group of human blood group precursor-equivalent gps [Structure I, Cyst OG 10 % 2 × ppt, and Beach P-1 (Figures 6c and 7); 1st Smith degraded cyst MSS gp (Figures 6a and 7); Cyst Mcdon P-1 (Figures 6b and 7); Cyst Tighe P-1 (Figures 6d and 7); Cyst N-1 Le^a 20 % 2 × (Figures 6e and 7)] and a strong reaction with asialo RSL [Structure II, Figures 6f and 7 (curve 6)]. The weak or negative reactivities of CG-16 with the human blood group A, B and H active gps [Cyst Mcdon and Cyst MSS (Figures 6a and 6b), Cyst Beach phenol insoluble (Figure 6c), Cyst Tighe phenol insoluble (Figure 6d)] (Table 1) or sialylated gps [human α1–acid gp (Figure 6f) and RSL (Figure 6f)] could be ascribed to the masking effects of LFuc(α1–), GalNAc(α1–) and sialic acid at the non-reducing terminal of Gal(β1–) and/or poor adsorption of the glycoform on to the microwell plate. As a means to address this issue, parallel inhibition assays are essential to infer actual ligand properties independent of the coating factor. These inhibition assays also present the sugars free in solution, without any spatial interference by the protein or plate surface, which might shift conformational equilibria and affect ligand affinity. Indeed, CG-16 can perform differential conformation selections, which is yet another reason to combine the two independent techniques [49].

Mild acid hydrolysis (pH 1.5 at 100 °C for 2 h) removes most of the L-Fuc(α1–) linked and some blood group A- and B-active oligosaccharide side chains [15,22], while Smith degradation removes almost all non-reducing terminal sugars (Tables 1 and 2, and Figures 6 and 7) [23]. Use of the respective asialo gps should significantly increase their interactions with this lectin [Figure 7 (curves 6 and 9) and Table 2].

From the results of the binding assay, Pneumococcus type-14 polysaccharide (Structure IV) is shown to be a reactive glycan (Figure 6f and activity order 9 in Table 1), and CG-16 required over 2.2×10^3 ng of this polysaccharide to reach 1.5 units (A_{405}) which is equivalent to a 129 times greater amount than that of 1st Smith degraded MSS cyst gp required to obtain 1.5 units (A_{405}). As shown in Figure 7 and Table 2, this polysaccharide is one of

Table 3 Amount of various saccharides giving 50% inhibition of binding of CG–16 (125 ng/50 μl) by a Gal(β1–) containing glycoprotein (1st Smith degraded MSS gp, 50 ng/50 μl)

The inhibitory activity was estimated from the inhibition curve in Figure 8, and is expressed as the amount of inhibitor giving 50% inhibition. The total volume was 50 μl. Abbreviation: L, Gal(β1–4)Glc.

Curve number	Figure 8(a) or (b)	Inhibitor	Quantity giving 50% inhibition (nmol)	Reciprocal of relative potency*
1	b	Tri-antennary Gal(β1–4)GlcNAc (Tri-II, Structure V)	0.33	2121.3
2	b	Di-antennary Gal(β1–4)GlcNAc (Di-II)	0.56	1250.0
3	b	Gal(β1–4)GlcNAc (II)	1.0	700.0
4	b	Gal(β1–3)GlcNAc(β1–3)Gal(β1–4) Glc (Lacto-N-Tetraose)	1.0	700.0
5	a	Gal(β1–4)Glc (L, Lac)	2.0	350.0
6	a	Gal(β1–4)Man	2.0	350.0
7	b	Gal(β1–4)GlcNAc(β1–6)Gal	2.1	333.3
8	a	Gal(β1–3)DAra	3.0	233.3
9	a	*p*-Nitrophenyl α-DGal	19.0	36.8
10	b	Gal(β1–3)GlcNAc (I)	20.0	35.0
11	a	Gal(β1–6)GlcNAc	22.0	31.8
12	a	*p*-Nitrophenyl β-DGal	140.0	5.0
13	b	Gal(α1–3)Galα1–*O*–Methyl (Bα*O*–Methyl)	150.0	4.7
14	b	Gal(α1–3)Gal (B)	150.0	4.7
15	a	Methyl α-DGal	190.0	3.7
16	a	Stachyose [Gal(α1–6)Gal(α1–6)Glc(β1–2)DFru*f*]	200.0	3.5
17	a	Raffinose [Gal(α1–6)Glc(β1–2)DFru*f*]	200.0	3.5
18	a	Melibiose [Galα(1–6)Glc]	210.0	3.3
19	a	Phenyl β-DGal	320.0	2.2
20	a	Methyl β-DGal	650.0	1.1
21	a, b	Gal	700.0	1.0

* Reciprocal of relative potency of sugars when Gal was taken as 1.0.

Table 4 Maximal quantities of various saccharides giving negligible or weak inhibition of CG-16 with a Gal(β1–) containing glycoprotein (1st Smith degraded cyst MSS gp*)

CG-16 (125 ng) plus 1st Smith degraded MSS gp (50 ng) (Structure I) were used. The total volume was 50 μl.

Curve number	Figure 8(a) or (b)	Inhibitor	Maximum amount of inhibitor used (nmol)	Percentage inhibition (%)
		p-nitrophenyl α-GalNAc	120	39.2
		p-nitrophenyl β-GalNAc	95	17.6
22	a	GalNAc	1500	41.0
23	b	Gal(β1–3)GalNAc (T)	130	30.2
24	b	Gal(α1–4)Gal (E)	101	13.3
		LAra	1110	2.6
		Man	2700	12.0
		DFuc	1020	1.3
		LFuc	5150	5.1
		GlcNAc	5000	5.0

the best ligands to inhibit CG-16–gp binding (Figure 7; curve 4). The difference in activity observed between the two different assays (activity order 11 in Table 2 versus curve 4 in Figure 7 and Table 2 of 50% inhibition) can be explained by the problem of polysaccharide adsorption on to microplates (Figure 6f). This problem has been solved by the inhibition assay (Figure 7 and Table 2).

Among the mono-, oligosaccharides and N-glycan chains from asialofetuin tested for inhibition of lectin–glycoform interaction, the tri-antennary chain with terminal Gal(β1–4) GlcNAc was the best; being 2.1×10^3 times more active than Gal (Figure 8; curve 1 versus curve 21) and about three times more active than human blood group type II precursor sequence [Gal(β1–4)GlcNAc] (Figure 8; curve 1 versus curve 3). The latter was 20 times more active than Gal(β1–3)GlcNAc (I) (Figure 8; curve 3 versus curve 10), corroborating the differences in free enthalpy between these isomers and lactose measured by isothermal titration calorimetry [50], while Gal(β1–3)GalNAc (T) and Gal(α1–4)Gal (E) tested up to 130 nmol were inactive.

With the present results, it is possible to extend our systematic analysis to the properties of the related chicken galectin (CG-14) which has reduced propensity for dimerization. In this respect, functional differences, e.g. in mediation of inhibitory effects on activated T-cells [9], can reside either in a disparate profile of binding partners or in differential capacity to trigger post-binding signalling. In aggregate, the carbohydrate specificity of CG-16 can be defined as: (1) CG-16 reacted best with human blood group ABH(O) precursor gps and their equivalent gps; (2) glycosylation of Gal(β1–) of the CG-16-reactive gps at non-reducing end by ABH determinants [GalNAc(α1–), Gal(α1–)

and/or L-Fuc(α1–)] or sialylation appears to strongly interfere with its binding; (3) Gal(β1–4)GlcNAc clustering and elongation of Gal(β1–3)GlcNAc enhances the reactivity; (4) from the inhibitory profile (curves 3–8 in Figure 7 and 50 % inhibition in Table 2), the combining site of agglutinin should be a cavity type rather than a groove type. Its binding size is as large as a tetrasaccharide of β-anomeric Gal and is most complementary to Gal(β1–4)GlcNAc / Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc (lacto-N-tetraose); (5) the carbohydrate specificity of CG-16 can be defined in decreasing order as Tri-antennary Gal(β1–4)GlcNAc (Tri-II) $\geqslant$ Di-antennary Gal(β1–4)GlcNAc (Di-II) > monomeric Gal(β1–4)GlcNAc (II) > monomeric Gal(β1–3)GlcNAc (I) > Gal(α1–6)Glc > Gal > GalNAc (inactive). These results should provide essential information to explain the delineation of the functions of individual members of this lectin family.

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