Fine specificity of domain-I of recombinant tandem-repeat-type galectin-4 from rat gastrointestinal tract (G4-N)

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Galectins, a family of β-galactoside-specific endogenous lectins, are involved in regulating diverse activities such as proliferation/apoptosis, cell–cell (matrix) interaction and cell migration. It is presently unclear to what extent the carbohydrate fine specificities of the combining sites of mammalian galectins overlap. To address this issue, we performed an analysis of the carbohydrate-recognition domain (CRD-I) near the N-terminus of recombinant rat galectin-4 (G4-N) by the biotin/avidin-mediated microtitre plate lectin-binding assay with natural glycoproteins (gps)/polysaccharide and by the inhibition of galectin–glycan interactions with a panel of glycosubstances. Among the 35 glycans tested for lectin binding, G4-N reacted best with human blood group ABH precursor gps, and asialo porcine salivary gps, which contain high densities of the blood group Ii determinants Galβ1-3GalNAc (the mucin-type sugar sequence on the human erythrocyte membrane) and/or GalNAcα1-Ser/Thr (**Tn**), whereas this lectin domain reacted weakly or not at all with most sialylated gps. Among the oligosaccharides tested by the inhibition assay, Galβ1-3GlcNAcβ1-3Galβ1-4Glc was the best. It was 666.7 and 33.3 times more potent than Gal and Galβ1-3GlcNAc, respectively. G4-N has a preference for the β-anomer of Gal at the non-reducing ends of oligosaccharides with a Galβ1-3 linkage, over Galβ1-4 and Galβ1-6. The fraction of **Tn** glycopeptide from asialo ovine submandibular glycoprotein was 8.3 times more active than Galβ1-3GlcNAc. The overall carbohydrate specificity of G4-N can be defined as Galβ1-3GlcNAcβ1-3Galβ1-4Glc (lacto-*N*-tetraose) > Galβ1-4GlcNAcβ1-3Galβ1-4Glc (lacto-*N*-neo-tetraose) and **Tn** clusters > Galβ1-4Glc and GalNAcβ1-3Gal > Galβ1-3GalNAc > Galβ1-3GlcNAc > Galβ1-4GlcNAc > GalNAc > Gal. The definition of this binding profile provides the basis to detect differential binding properties relative to the other galectins with ensuing implications for functional analysis.

Key words: enterocyte, lacto-*N*-tetraose, lectin-combining sites, metastasis, raft.

INTRODUCTION

Animal lectins serve multifarious functions including glycoconjugate routing, cell adhesion and growth modulation [1,2]. 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 occurrence of diversification raises the question as to whether these related molecules may fulfil overlapping or distinct activities. Because 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 related agglutinins.

Vertebrate species express not only one distinct Ca^{2+}-independent β-galactoside-binding protein with the jelly-roll-like motif of β-sheets (galectin) but a family [1,3,4]. Among the 13 presently known mammalian galectins [4], galectin-4 belongs to the subgroup of tandem-repeat proteins containing two carbohydrate-recognition domains (CRD-I on the N-terminal and CRD-II on the C-terminal sides) connected by a linker peptide within one peptide chain. It is present in the gastrointestinal tract, where its localization in brush border membranes of enterocytes fits the profile of a marker for cholesterol-independent microdomains [5,6]. At this site, protease-solubilized brush border or pancreatic digestive enzymes to enhance the digestive and adsorptive capacity, and/or glycans at lateral cell membranes of the surface-lining epithelial cells to support epithelial integrity by cross-linking, have been reported to be ligands [7,8]. A high-density oligonucleotide array screening including monitoring of the activity of about 6800 genes in gastric cancer cells illustrated

Abbreviations used: CRD, carbohydrate-recognition domain; CRD-I, carbohydrate-recognition domain on the N-terminal side; CRD-II, carbohydrate-recognition domain on the C-terminal side; G4-N, carbohydrate-recognition domain of rat galectin-4 on the N-terminal side; CG-16, chicken galectin-16; gp, glycoprotein; PSM, porcine salivary glycoprotein; OSM, ovine submandibular glycoprotein; BSM, bovine submandibular glycoprotein; RSL, rat sublingual glycoprotein; ELLSA, enzyme-linked lectinosorbent assay; Le^a, Galβ1-3[L-Fucα1-4]GlcNAc; Le^b, L-Fucα1-2Galβ1-3[L-Fucα1-4]GlcNAc. The carbohydrate structural units in glycans used to define binding properties of G4-N are: **A**, GalNAcα1-3Gal, human blood group A-specific disaccharide; **A$_h$**, GalNAcα1-3(L-Fucα1-2)Gal, human blood group A-specific trisaccharide containing crypto H determinant; **Tn**, GalNAcα1-Ser/Thr; **F**, GalNAcα1-3GalNAc; **P**, GalNAcβ1-3Gal; **S**, GalNAcβ1-4Gal; **I**, Galβ1-3GlcNAc, human blood group type I precursor sequence; **II**, Galβ1-4GlcNAc, human blood group type II precursor sequence; **L**, Galβ1-4Glc; **T**, Galβ1-3GalNAc, the mucin-type sugar sequence on the human erythrocyte membrane; **B**, Galα1-3Gal, human blood group B-specific disaccharide; **E**, Galα1-4Gal, galabiose; **I**β1-3**L** (type I), Galβ1-3GlcNAcβ1-3Galβ1-4Glc, lacto-*N*-tetraose; **II**β1-3**L** (type II), Galβ1-4GlcNAcβ1-3Galβ1-4Glc, lacto-*N*-neo-tetraose.

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another interesting aspect of galectin-4. Its gene belonged to a small, preferentially up-regulated set in the tested six scirrhous gastric cancer cell lines prone to peritoneal dissemination, pointing to a role in tumour metastasis [9]. The differential expression of galectin-4 mRNA in colon cancer lines likewise intimates a role of this lectin in colorectal malignancy [10,11]. Defining the ligand spectrum of galectin-4 is an important step towards elucidating the functional roles of this lectin. Moreover, this information may also initiate applications in glycoconjugate analysis and purification.

Towards these aims, it is of vital importance to delineate the binding properties of this galectin for natural glycans. Consequently, the interaction of CRD-I with glycoproteins (gps) and polysaccharide was studied by the biotin/avidin-mediated microtitre plate lectin-binding assay and by the inhibition of agglutinin–glycan interactions with sugar ligands [12,13]. Separate recombinant expression of these two CRDs enabled us to obtain the individual lectin sites of the tandem-repeat-type galectin. Due to the inherently low solubility and stability of the CRD-II, the analysis had to focus on the properties of the N-terminal domain (CRD-I). The results indicate that (i) CRD-I of galectin-4 reacted best with human blood group ABH precursor (equivalent) gps, asialo porcine salivary gp, and native and mild-acid-hydrolysed pig gastric mucin, (ii) it reacted weakly or not at all with sialylated gps, (iii) the interaction of this CRD is notably strong with the linear tetrasaccharide Galβ1-3GlcNAcβ1-3Galβ1-4Glc (lacto-*N*-tetraose; **I**β1-3**L**; containing human blood group type I precursor sequence at the non-reducing end) and (iv) CRD-I bound to clusters of Galβ1-3GalNAc (structure **T**; the mucin-type sugar sequence on the human erythrocyte membrane) and GalNAcα1-Ser/Thr (structure **Tn**), a feature with the potential to (therapeutically) interfere with galectin-4 binding. The binding characteristics defined in this study, as compared with the fine specificity of chicken galectin-16 (CG-16), a galectin from chicken liver with a prototype dimeric CRD display, support the concept that each galectin harbours distinct properties.

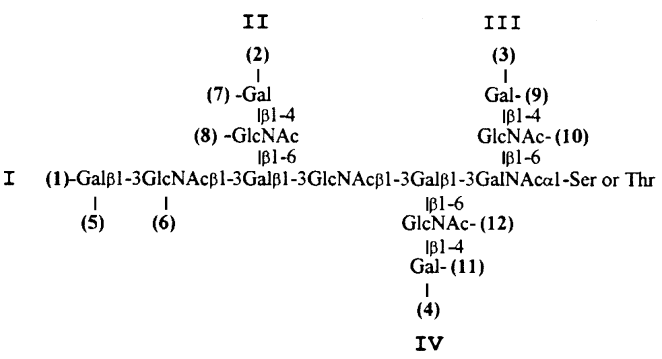

Figure 1 Proposed representative carbohydrate side chains of blood group-active gps, prepared from human ovarian cyst fluid

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 A, B, H, Le^a and Le^b activities are attached. It represents precursor blood group-active gps [19,20] and can be prepared by Smith degradation of A, B and H active gps, purified from human ovarian cyst fluids [17,19–21]. Numbers in parentheses indicate the sites of attachment for the human blood group A, B, H, Le^a and Le^b determinants (Table 1). These determinants as well as the structural units at the non-reducing end are the sources of lectin-reactive **A**/$\mathbf{A_h}$, **B**, **I**/**II**, **T** and **Tn** determinants [40,43,48] (for definitions and structures of these see the text). A megalosaccharide of 24 sugars has not been isolated. However, most of the carbohydrate chains isolated are part of this structure.

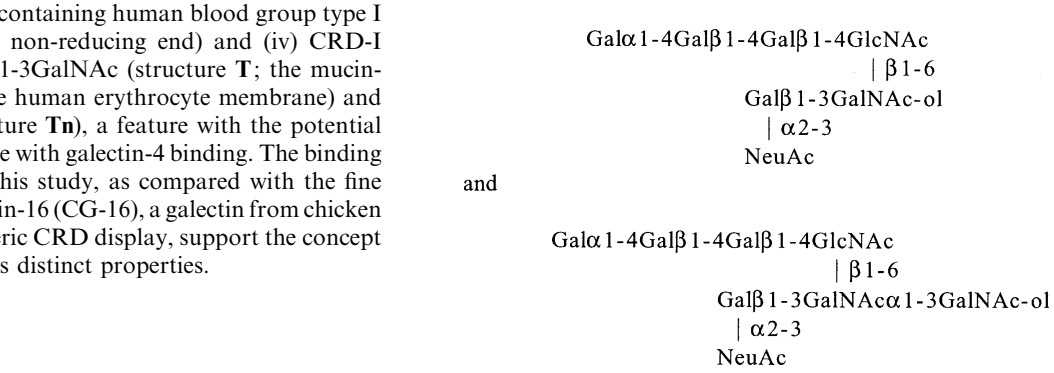

Figure 2 Mucus glycoproteins, so-called nest-cementing substances (bird-nest gp), from the salivary gland of Chinese swiftlets

These gps are constituted mainly of sialic acid-rich O-glycosylproteins [28]. The most complex representatives of the monosialyl fraction from *Collocalia* mucins are shown. Carbohydrate side chains of mild-acid-treated (desialized) product contain no NeuAc. The other compounds identified were partial structures thereof.

EXPERIMENTAL

The lectin

Recombinant tandem-repeat-type rat galectin-4 domains were purified from *Escherichia coli* BL21 (DE3)pLysS cells carrying the pGEX-2T expression vector as *Schistosoma japonicum* glutathione S-transferase ('GST') fusion proteins by affinity chromatography on lactosylated Sepharose 4B, derived from divinyl sulphone activation, as the crucial step [8,14]. Quality control of the products by one- and two-dimensional gel electrophoresis was performed routinely. The molecular mass of glutathione S-transferase–CRD was 43 kDa.

Gps and polysaccharides

The purified blood group substances used were prepared from human ovarian cyst fluid, and from animal tissues [15–17]. The blood group substances were purified from human ovarian cyst fluid by digestion with pepsin and precipitation with increasing concentrations of ethanol; the dried ethanol precipitates were extracted with 90% phenol, the insoluble fraction being named after the blood group substance (e.g. cyst Beach phenol insoluble). Codes such as OG, MSS, Tighe, Mcdon, Tij, JS and HOC 350 found throughout the text indicate different patients that the samples were taken from. 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 round of phenol extraction and ethanol precipitation was carried out (e.g. cyst OG 20% 2×). HOC 350, a sialic-acid-rich gp isolated from human ovarian cyst fluid [18], was kindly provided by Dr W. M. Watkins (Faculty of Medicine, Imperial College of Science, Technology and Medicine, London, U.K.). Regardless of their A, B, H or Le^b (L-Fucα1-2Galβ1-3[L-Fucα1-4]GlcNAc) activity, the purified water-soluble blood group substances from human ovarian cyst fluids have a similar precursor structure (Figure 1). They are polydispersed macromolecules (molecular mass $>$ 200000 Da) of similar composition (75–85% carbohydrate, 15–20% protein). They consist of multiple heterosaccharide branches attached by O-glycosidic linkages at their internal reducing ends to serine or threonine residues of the polypeptide backbone [16,19].

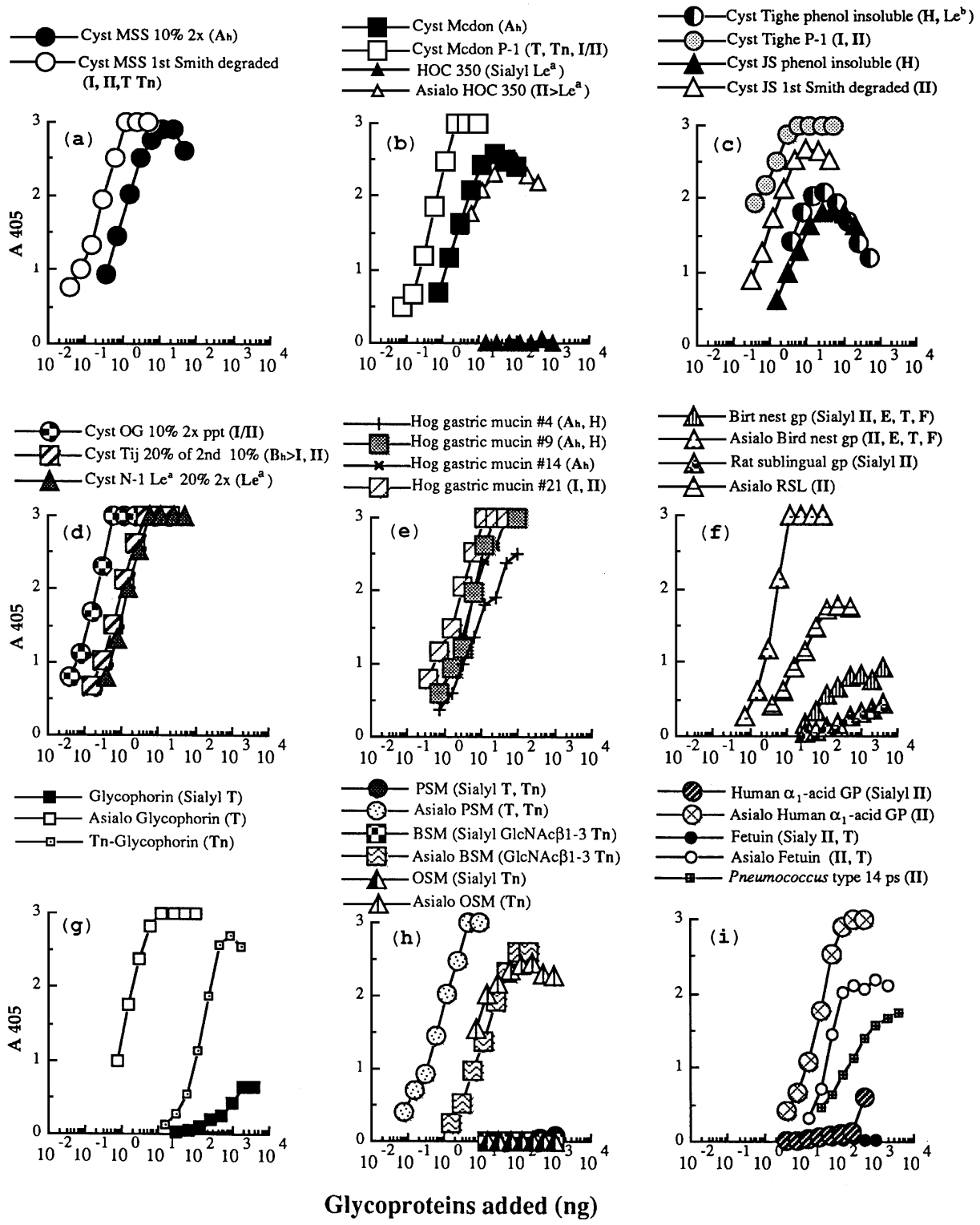

Figure 3 Binding of CRD-I of rat recombinant galectin-4 to microtitre plates coated with serially diluted human blood group A-, B-, H-, Le^b- and Ii-active gps, sialo- and asialoglycoproteins and *Pneumococcus* polysaccharide

The lectin was used at 50 ng/well. Total volume of the assay was 50 μl. A_{405} was recorded after 4 h of incubation.

In general, the P-1 fractions (e.g. cyst Mcdon P-1 or Tighe 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 [20–22]. The first Smith-degraded products of blood group A-

Table 1 Identification of the curves from Figure 3

Data are taken from [19]. The gps listed in the second column are human precursor equivalent gps, which are defined as A-, B- and 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 (pH 1.5, 100 °C for 2 h), Smith degradation or glycosidases. Le^a, Galβ1-3[L-Fucα1-4]GlcNAc.

Figure 3 panel	Blood group-active gp purified from human ovarian cyst fluid	Human blood group	Sugar added to structure in Figure 1	Site of addition to structure in Figure 1
3a	MSS first Smith	Ii		
3i		*Pneumococcus* type 14	None	
3b	Mcdon P-1	Polysaccharide		
3c	Tighe P-1	Antigenic determinant		
3d	Cyst OG 10% 2× ppt			
3b	Asialo HOC 350 [18]	Le^a	L-Fucα1-4	6 and 10
3d	Cyst N-1 Le^a 20% 2×			
3c	Tighe phenol insoluble	H and Le^b	L-Fucα1-2 and as in Le^a	5, 7, 9 and/or 11 6, 10
3a	MSS, native	A_1 or A_2	GalNAcα1-3	1, 2, 3 and/or 4
3b	Cyst Mcdon		and as in H and Le^b	5, 6, 7, 9, 10, 11
3d	Cyst Tij 20% of 2× 10%	B	Galα1-3	1, 2, 3 and/or 4
			and as in H and Le^b	5, 6, 7, 9, 10, 11

active substances (MSS 10% 2×; Figure 1), in which almost all of the sugar groups at the non-reducing ends were removed, were prepared as described earlier [23,24].

Human α_1-acid gp was purchased from Sigma (St. Louis, MO, U.S.A.). It is a N-glycan and the carbohydrate units of this asialoglycoprotein can be grouped into compounds with biantennary and triantennary structures with and without a fucose residue [25].

Fetuin (Gibco, Grand Island, NY, U.S.A.), which is the major gp in fetal calf serum and has a molecular mass of 48400 Da, is composed of 78% amino acids, 8.7% sialic acids, 6.3% hexosamines and 8.3% neutral sugars. It has six oligosaccharide side chains/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 N-glycans with terminal *N*-acetyl-lactosamine and a small amount of the Galβ1-3-linked isomer at the non-reducing terminal ends [26].

The *Pneumococcus* type 14 polysaccharide was a generous gift from the late Dr E. A. Kabat (Department of Microbiology, Columbia University, New York, NY, U.S.A.) [27].

Mucus gp (or native bird-nest gp), the so-called nest-cementing substance (Figure 2) [28] 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).

The rat sublingual gp was prepared by the method of Moschera and Pigman [29]. Its molecular mass is 2.2×10^6 Da and it is composed of 81% carbohydrates [29]. The carbohydrate side chains are O-glycosyl-linked to Ser or Thr residues of the protein core. The established structure has 9, 10, 12, 13 and 15 sugar residues with NeuNAcα2,6 linked to Gal and/or GalNAc at the reducing end, GlcNAc groups at non-reducing ends, and a repeating unit, Galβ1-4GlcNAcβ1-, in the carbohydrate core structure [30]. It also contains the **Tn** determinant [31].

The major fractions of ovine submandibular gp (OSM), bovine submandibular glycoprotein (BSM) and porcine salivary gp (PSM) were purified according to the method of Tettamanti and Pigman [32] with some modifications [33,34].

Desialylation of sialoglycoproteins was performed by mild acid hydrolysis in 0.01 M HCl at 80 °C for 90 min, and dialysis against distilled water for 2 days to remove small fragments [32,35].

Native hog gastric mucin no. 4 [36] was derived from crude hog stomach mucin that was dissolved in water and centrifuged at 1.53×10^4 ***g*** for 3 h, followed by exhaustive dialysis (molecular mass cut-off, 8.0×10^3 Da) against distilled water. Treatment of mucin no. 4 with HCl at pH 2 for 90 min at 100 °C yielded hog gastric mucin no. 9, while hog gastric mucins nos. 14 and 21 were obtained from mucin no. 4 by acid hydrolysis at pH 1.5 at 100 °C for 2 and 5 h, respectively. Each hydrolysis step was followed by extensive dialysis.

Glycophorin A was prepared from the membranes of outdated human blood group O erythrocytes by phenol/saline extraction and was purified by gel filtration in the presence of SDS [37]. Asialoglycoprotein was prepared by mild acid hydrolysis [35]. **Tn**-type glycophorin (**Tn**-glycophorin) was obtained by removing galactose residues from asialoglycophorin by periodate oxidation and mild acid hydrolysis (Smith degradation) [12,23].

Inhibiting sugars

Iβ1-3**L** (type I), **II**β1-3**L** (lacto-*N*-neo-hexaose; Galβ1-4GlcNAcβ1-3[Galβ1-4GlcNAcβ1-6]Galβ1-4Glc; containing human blood group type II precursor sequence at the non-reducing end), GalNAcα1-3(L-Fucα1-2)Gal (structure $\mathbf{A_h}$; human blood group A-specific trisaccharide containing crypto H determinant) and Galα1-3Gal (structure **B**; human blood group B-specific disaccharide) were purchased from Dextra (Reading, Berkshire, U.K.). GalNAcα1-3GalNAc (structure **F**), GalNAcβ1-3Gal (structure **P**), GalNAcα1-3Gal (structure **A**; human blood group A-specific disaccharide) and GalNAcβ1-4Gal (structure **S**) were prepared by Dextra.

D-Gal, D-Fuc, L-Fuc, D-Man, D-Glc, methyl-αGlc, methyl-βGlc, methyl-αGlcNAc, methyl-βGlcNAc, GalNAc, GlcNAc, L-Ara, melibiose, raffinose (Galα1-6Glcβ1-2D-fructose), stachyose (Galα1-6Galα1-6Glcβ1-2D-fructose), methyl-αGal, methyl-βGal, *p*-nitrophenyl-αGal, *p*-nitrophenyl-βGal, *p*-nitrophenyl-αGalNAc, *p*-nitrophenyl-βGalNAc, Lac, Galα1-4Gal (**E**; galabiose, a ligand of the uropathogenic *Escherichia coli* receptor), Galβ1-4Man, Galβ1-4Gal, **T**, Galβ1-3GlcNAc (**I**; the human blood group type I precursor sequence), Galβ1-3D-Ara, Galβ1-4ManNAc, GlcNAcβ1-3Gal-O-Me, GlcNAcβ1-6Galβ1-4Glc, *N*,*N*′-diacetyl-chitobiose, *N*,*N*′,*N*″-triacetyl-chitotriose, Galβ1-

(i) GalNAcα1-Ser/Thr (**Tn**)

(ii) Galβ1-3GalNAcα1-Ser/Thr (**T**)

(iii) GalNAcα1-3Galβ1-3GalNAcα1-Ser/Thr (**A**)

(iv) LFucα1,2Galβ1-3GalNAcα1-Ser/Thr (**H**)

(v) GalNAcα1-3Galβ1-3GalNAcα1-Ser/Thr (A_h)
|α1,2
LFuc

Figure 4 Porcine salivary gp with a molecular mass of 6.3×10^5 Da is composed of 35% protein, 15% sialic acid, 7.3% L-Fuc, 10% Gal and 26% HexN [33,42]

The carbohydrate side chains of this gp are also O-glycosidically linked through GalNAc at the reduced end of the carbohydrate side chain to Ser or Thr in the protein core. Twelve kinds of carbohydrate side chain have been isolated. They are composed of one of five sugar residues with Gal1-3GalNAc-1-Thr or Ser as the carbohydrate core region. Substitution by NeuNAc, NeuNGc, L-Fuc or GalNAc exhibits blood group A and H activities. The mild-acid-treated product of porcine salivary gp contains mainly mixtures of the above sequences. **H**, L-Fucα1-2Gal.

4GlcNAc (human blood group type II precursor sequence; **II**) and Galβ1-6GlcNAc were purchased from Sigma.

The **Tn** glycopeptides (**Tn** clusters) were prepared from asialo-OSM as described previously [38]. The **Tn** clusters used for this study were mixtures of **Tn**-containing glycopeptides in the filterable fraction (molecular mass cut-off, < 3000 Da). The size distributions of **Tn**-containing glycopeptides were examined by Bio-Gel P-2 (400 mesh) column chromatography using double-distilled water as eluant and dextran, raffinose and glucose as references.

Tri-antennary **II** glycopeptides were prepared from asialo fetuin by pronase digestion and repeatedly fractionated by BioGel P-4 (400 mesh) column chromatography. Peak II was used for this assay [39].

The microtitre plate lectin-enzyme binding assay

The test was performed according to the procedures described by Duk et al. [12] and Lisowska et al. [13]. The volume of each

Table 2 Binding of CRD-I of galectin-4 lectin to human blood group A-, B-, H-, P_1- and Le^b-active gps, sialo- and asialoglycoproteins analysed by ELLSA

Biotinylated lectin (50 ng) was added to various concentrations of gps ranging from 0.008 ng to 1 μg. The inhibitory activity was estimated from the inhibition curve in Figure 5 and is expressed as the amount (ng) of inhibitor giving 50% inhibition. The total volume was 50 μl. Lac (curve 32, Figure 5) is 7872.9 ng and equal to 23 nmol; Gal (curve 33, Figure 5) is 3.6×10^5 ng and equal to 2000 nmol. The symbols in parentheses indicate the human blood group activity and/or lectin determinants [40,49], which are shown in bold: **F**, GalNAcα1-3GalNAc; **H**, L-Fucα1-2Gal; **A**, GalNAcα1-3Gal; $\mathbf{A_h}$, GalNAcα1-3[L-Fucα1-2]Gal; **B**, Galα1-3Gal; $\mathbf{B_h}$, Galα1-3[L-Fucα1-2]Gal; **E**, Galα1-4Gal; **T**; **Tn** and **I**/**II** (Galβ1-3/4GlcNAc). The results of the binding intensity measurements were interpreted according to the spectrophotometric absorbance value at 405 nm after 4 h incubation as follows: +++++, $A \geqslant 2.5$; ++++, $A = 2.5$–2.0; +++, $A = 2.0$–1.5; ++, $A = 1.5$–1.0; +, $A = 1.0$–0.5; ±, $A = 0.5$–0.2; −, $A < 0.2$. Dashes indicate glycans that were tested up to 2000 ng and did not reach 1.5 (A_{405}). N.D., not determined.

Figure 3 panel	Figure 5 curve no.	Gp lectin determinant (blood group specificity)	Quantity required for 1.5 A_{405} units (ng)	Quantity giving 50% inhibition (ng)	Maximum absorbance (A_{405})	
					A_{405}	Binding intensity
3d	1	Cyst OG 10% 2× ppt (**I**/**II**)	0.13	0.16	3.0	+++++
3a	2	Cyst MSS first Smith degraded (**I**, **II**, **T**, **Tn**)	0.20	0.22	3.0	+++++
3c	3	Cyst Tighe P-1 (**I**, **II**)	< 0.30	1.90	3.0	+++++
3b	4	Cyst Mcdon P-1 (**T**, **Tn**, **I**/**II**)	0.40	0.28	3.0	+++++
3d	5	Cyst Tij 20% of second 10% ($\mathbf{B_h}$ > **I**/**II**)	0.60	0.18	3.0	+++++
3h	6	Asialo PSM (**T**, **Tn**)	0.65	3.50	3.0	+++++
3a	7	Cyst MSS 10% 2× ($\mathbf{A_h}$)	0.80	8.00	2.9	+++++
3c	8	Cyst JS first Smith degraded (**II**)	0.85	0.50	2.7	+++++
3d	9	Cyst N-1 Le^a 20% 2× (Le^a)	0.90	0.28	3.0	+++++
3g	10	Asialo glycophorin (**T**)	1.23	2.00	3.0	+++++
3e	11	Hog gastric mucin no. 21 (**I**, **II**)	1.60	19.00	3.0	+++++
3b	12	Asialo HOC 350 (**II** > Le^a)	2.00	1.70	2.5	+++++
3b	13	Cyst Mcdon ($\mathbf{A_h}$)	2.57	8.00	2.6	+++++
3e	14	Hog gastric mucin no. 14 ($\mathbf{A_h}$)	3.57	21.00	3.0	+++++
3e	15	Hog gastric mucin no. 9 ($\mathbf{A_h}$, **H**)	4.00	2.10	3.0	+++++
3f	16	Asialo bird nest gp (**II**, **E**, **T**, **F**)	4.00	0.55	3.0	+++++
3c	17	Cyst Tighe phenol insoluble (**H**, Le^b)	4.50	3.00	2.1	++++
3h	18	Asialo OSM (**Tn**)	7.00	1000.00	2.5	+++++
3e	19	Hog gastric mucin no. 4 ($\mathbf{A_h}$, **H**)	7.50	1.50	2.5	+++++
3c	20	Cyst JS phenol insoluble (**H**)	9.50	12.00	1.8	+++
3h	21	Asialo BSM (GlcNAcβ1-3, **Tn**)	14.61	1000.00	2.6	+++++
3i	22	Asialo human α_1-acid gp (**II**)	17.27	35.00	3.0	+++++
3i	23	Asialo fetuin (**II**, **T**)	63.33	13.00	2.2	++++
3f	24	Asialo RSL (**II**)	65.00	2000.00	1.8	+++
3g	25	Tn-glycophorin (**Tn**)	166.70	2100.00	2.7	+++++
3i	26	*Pneumococcus* type 14 ps (**II**)	700.00	150.00	1.8	+++
3f	27	Bird nest gp (Sialyl **II**, **E**, **T**, **F**)	—	100.00	0.9	+
3g	28	Glycophorin (Sialyl **T**)	—	800.00	0.6	+
3i	29	Human α_1-acid gp (Sialyl **II**)	—	> 277.80 (15.9% inhibition)	0.6	+
3f	30	Rat sublingual gp (RSL, sialyl **II**)	—	> 277.80 (12.8% inhibition)	0.4	±
3b	31	HOC 350 (sialyl Le^a)	—	> 2777.80 (45.6% inhibition)	0.06	−
3i		Fetuin (sialyl **II**, **T**)	—	ND	0.06	−
3h		PSM (sialyl **T**, **Tn**)	—	ND	0.01	−
3h		Ovine salivary gp (OSM, sialyl **Tn**)	—	ND	0.0	−
3h		BSM (sialyl GlcNAcβ1-3, **Tn**)	—	> 2777.80 (28.1% inhibition)	0.0	−

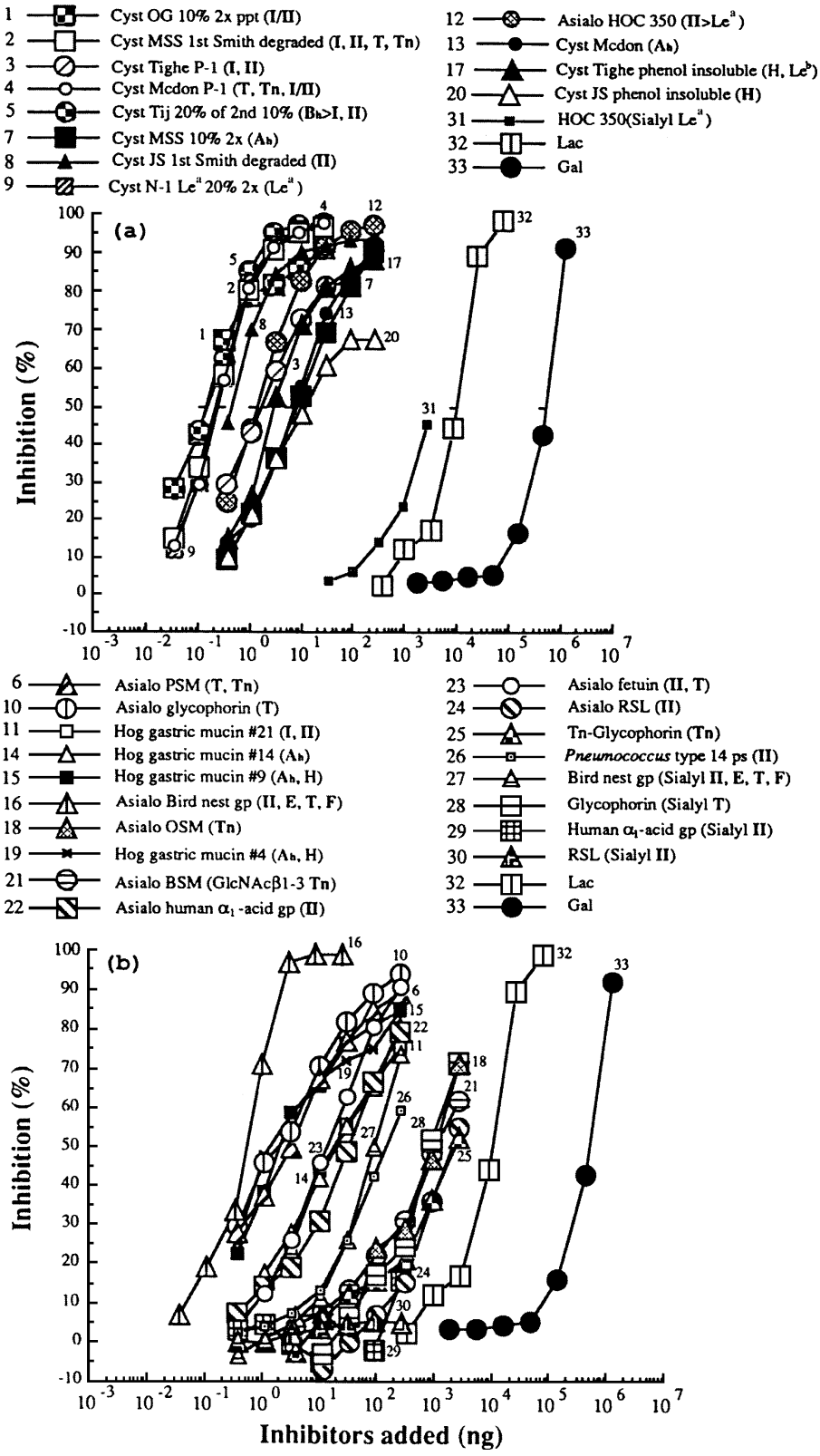

Figure 5 Inhibition of CRD-I of rat recombinant galectin-4 binding to a Galβ1-3/4GlcNAc-containing gp (first Smith-degraded MSS cyst gp)-coated ELLSA plates with various gps

The quantity of gp in the coating solution was 1 ng/well. The quantity of lectin used for inhibition assay was 50 ng/well. Total volume was 50 μl. A_{405} was recorded after 4 h incubation. The amount (ng) of gp required to induce 50% inhibition was determined. The following gps in which two different amounts of the gps were used for inhibition of CRD-I–gp interaction, in addition to the results presented in this figure, did not reach 50% inhibition: with 277.8 ng of gp as inhibitors, human α_1-acid gp and RSL; and 2777.8 ng gp as inhibitors, HOC 350 and BSM.

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-buffered saline containing 0.05 % Tween 20 (TBS-T). Tris-buffered saline or 0.15 M NaCl containing 0.05 % Tween 20 were used for washing the plates between incubations.

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 2-fold with TBS-T. After 30 min at 20 °C, the samples were tested in the binding assay, as described above. The inhibitory activities were estimated from the inhibition curve and are expressed as the amount of inhibitor (nmol/well) causing 50 % inhibition of control lectin binding.

All experiments were done in duplicate or triplicate and the data are mean values of the results. S.D. values did not exceed 10 % and in most experiments were 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 Tris-buffered saline.

RESULTS

Lectin–glycan interaction

The avidity of the CRD-I of galectin-4 for gps and *Pneumococcus* type 14 polysaccharide as studied by a microtitre plate enzyme-linked lectinosorbent assay (ELLSA) is summarized in Table 2 according to the interaction profiles shown in Figure 3. As can be seen, CRD-I reacted best with high-density Galβ1-3/4GlcNAc-containing glycans. These include six human blood group precursor (equivalent) gps (Table 1 and Figure 1): first Smith-degraded cyst MSS gp in Figure 3(a), cyst Mcdon P-1 in Figure 3(b), cyst Tighe P-1 and first Smith-degraded cyst JS gp in Figure 3(c), cyst OG 10 % 2 × ppt and cyst Tij 20 % of second 10 % in Figure 3(d), human blood group A- and Le^a (Galβ1-3[L-Fucα1-4]GlcNAc)-active gps (cyst MSS 10 % 2 × in Figure 3a and cyst N-1 Le^a 20 % 2 × in Figure 3d) and asialo porcine salivary gp (Figures 3h and 4), in which less than 1 ng of the gp coated was required to interact with 50 ng of lectin to yield an A_{405} value of 1.5 within 4 h. Although the percentage of the gps immobilized on the surface in the microtitre plate assay has not been assessed, the amount of gp required to give 1.5 absorbance units (A_{405}) with this lectin has to be equal to or less than 1 ng.

The CRD-I also strongly bound to human asialoglycophorin (Figure 3g), asialo blood group Le^a gp from human ovarian cyst fluid (asialo HOC 350 in Figure 3b), human blood group A-active cyst gp (cyst Mcdon in Figure 1 and Figure 3b), asialo bird-nest gp (Figures 2 and 3f), hog gastric mucin (hog gastric mucin no. 4 in Figure 3e) with its mild-acid-hydrolysed products (hog gastric mucin nos. 9, 14 and 21 in Figure 3e), and structure **II** containing N-linked gps (asialo human α_1-acid gp, Figure 3i). CRD-I reacted well with the other four **II**-containing gps or polysaccharide (asialo RSL in Figure 3f; asialofetuin, and *Pneumococcus* type 14 polysaccharide in Figure 3i), two **Tn**-containing gps (asialo bovine salivary mucin, asialo-OSM in Figure 3h, and human **Tn**-glycophorin in Figure 3g) and two human H-active gps (cyst Tighe phenol insoluble and cyst JS phenol insoluble in Figure 3c). To ascertain that carbohydrate binding is responsible for signal generation and to comparatively test the inhibitory potency of inhibitors, the CRD-I/glycan interaction was interfered with by various oligosaccharides as described below.

Regarding sialylation of the glycans, it is notable that most of sialic acid-containing gps (e.g. OSM, PSM and BSM in Figure 3h, RSL in Figure 3f, human α_1-acid gp and fetuin in Figure 3i, human glycophorin in Figure 3g, bird-nest gp in Figure 3f and HOC 350 in Figure 3b) were either weakly active or inactive (Figure 3 and Table 2), indicating a masking effect of sialic acids and/or poor adsorption of these glycoforms to the plate surface.

Inhibition of domain-I–glycoform interaction by various glycans

The ability of various glycans to inhibit the binding of the CRD-I of rat galectin-4 on the N-terminal side (G4-N) to a Galβ1-3/4GlcNAc-containing gp (first Smith-degraded cyst MSS gp; Table 1 and Figure 1) was analysed by ELLSA, as shown in Figure 5, with the representative panel of glycans given in Table 2. Among the glycans tested for inhibition of that interaction, four human blood group precursors (Figure 5a, curves 1, 2, 4 and 5) and a human blood group Le^a-active cyst gp (Figure 5a, curve 9; cyst N-1 Le^a 20 % 2 ×) were the best, requiring less than 0.3 ng to inhibit the interaction by 50 %. The other human blood group precursor equivalent gp (Figure 5a, curve 3), blood group A-active gps (Figure 5a, curves 7 and 13), Le^a active cyst gp (Figure 5a, curve 12), asialo bird-nest gp (Figure 5b, curve 16), hog gastric mucin and its mild-acid-hydrolysed products (Figure 5b, curves 11, 14, 15 and 19), Galβ1-3GalNAcα1-Ser/Thr (**T**-containing gp; Figure 5b, curves 6 and 10; Figure 4) and N-linked multi-antennary structure **II**-containing gp (Figure 5b, curve 23) were also potent inhibitors. They were much more active than monomeric lactose (Galβ1-4Glc; **L**) and Gal. The inhibitory reactivities of glycoforms towards CRD-I of galectin-4 in general square broadly with the maximum absorbances recorded in the binding assay (Figure 3 and Table 2).

Inhibition of lectin–glycan interaction by mono- and oligosaccharides

The ability of various sugars to inhibit the binding of CRD-I with a Galβ1-3/4GlcNAc-containing gp (first Smith-degraded cyst MSS gp) purified from human ovarian cyst fluid is shown in Figure 6 and the amounts of ligands required for 50 % inhibition of the lectin–glycan interaction are listed in Table 3. Among the oligosaccharides tested, **I**β1-3**L** (type I), which was 666.7, 333.4 and 4.0 times more active than Gal (Figure 6a, curve 1 versus curve 17), GalNAc (Figure 6a, curve 1 versus curve 15) and **II**β1-3**L** (type II; Figure 6a, curve 1 versus curve 2), respectively, was the best. It was about 7.7, 33.3 and 120 times more active than structures **L**, **I** and **II**, respectively (Figure 6a, curve 1 versus 6, 9 and 13), indicating that the *N*-acetylamino group of the subterminal GlcNAc residue at carbon-2 might interfere with optimal ligand accommodation and that the β1-3 linkage is better than the β1-4 linkage (Figure 6a, curve 9 versus curve 13). Although the amount of Galβ1-6GlcNAc tested was about 1.6 times higher than that required for **I** to reach 50 % inhibition, only 19 % of the interaction was inhibited. Thus, this domain has a general preference for the β-anomer as Galβ1-3 > Galβ1-4 and Galβ1-6. Besides, Galβ1-4Gal was tested up to 10 times more than the amount required to reach 50 % inhibition for **L** (Figure 6b, curve 25 versus Figure 6a, curve 6) but only achieved 8.7 % inhibition, implying that the configuration of C-4 of the subterminal sugar unit is important for binding.

The fraction of **Tn** glycopeptides from asialo-OSM was 4 times less active than **I**β1-3**L** (type I), but 8.3 times more active than **I**. **P** was about as active as **L** (Figure 6a, curve 5 versus curve 6). The other mammalian glycotopes, such as **A**, **A$_h$**, **F**, **S**, **B**, **E**,

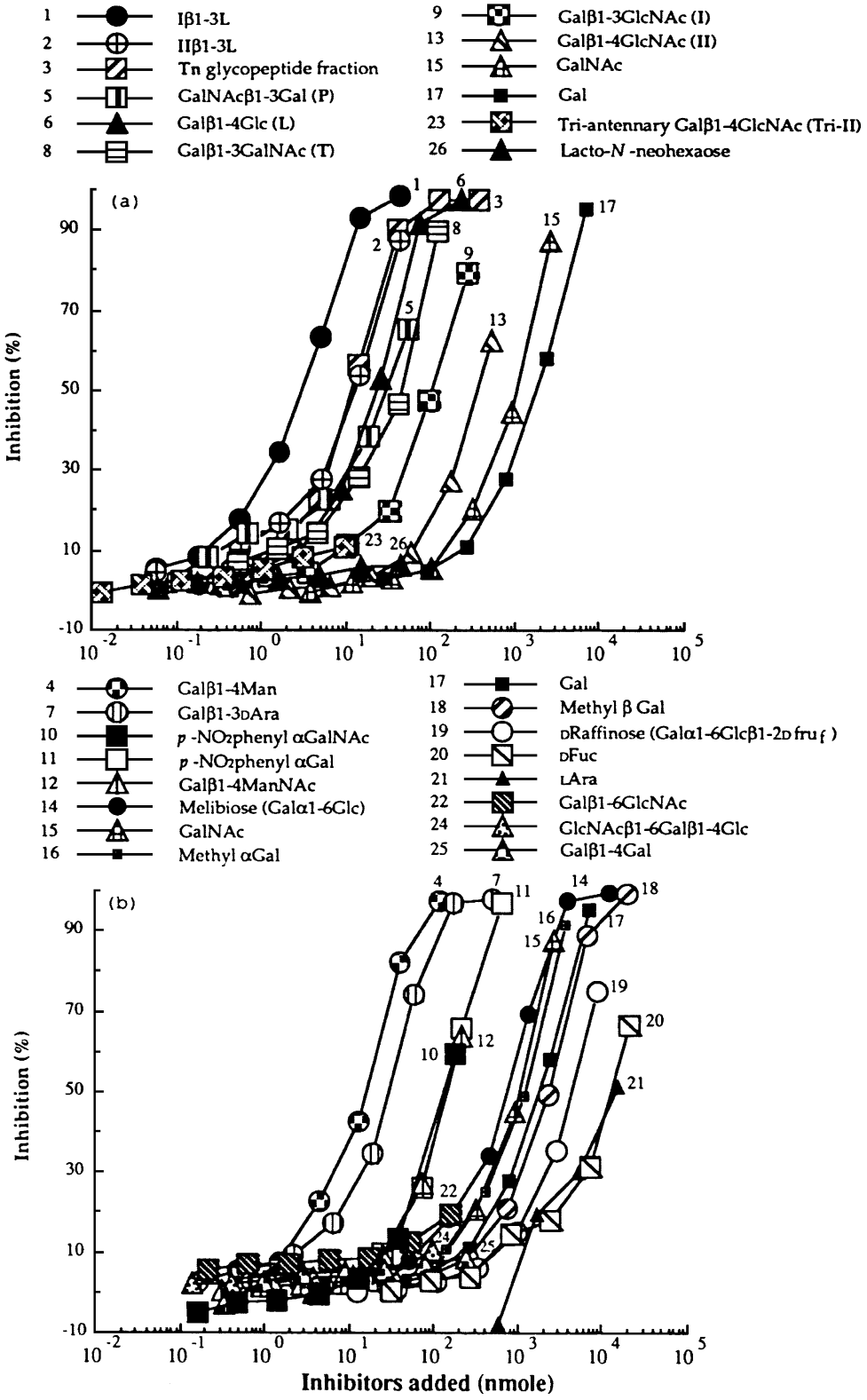

Figure 6 Inhibition of CRD-I of rat recombinant galectin-4 binding to Galβ1-3/4GlcNAc-containing gp (first Smith-degraded MSS cyst gp)-coated ELLSA plates by various saccharides

The amount of gps in the coating solution was 1 ng/well. The lectin (100 ng/well) was preincubated with an equal volume of serially diluted inhibitor. The final CRD-I of rat recombinant galectin-4 content was 50 ng/well. Total volume, 50 μl. A_{405} was recorded after 4 h incubation.

Table 3 Amounts of various saccharides giving 50% inhibition of binding of G4-N (50 ng/50 μl) by a Galβ1-containing gp (first Smith-degraded MSS gp, 1.0 ng/50 μl)

The inhibitory activity was estimated from the inhibition curve in Figure 6 and is expressed as the amount of inhibitor giving 50% inhibition of the control lectin binding. Total volume, 50 μl. Reciprocal of relative potency of sugars: Gal was taken as 1.0 [42].

Order of activity	Panel in Figure 6	Inhibitor	Quantity giving 50% inhibition (nmol)	Reciprocal of relative potency
1	6a	**I**β1-3**L** (type I)	3.0	666.7
2	6a	**II**β1-3**L** (type II)	12.0	166.7
26	6a	Lacto-*N*-neo-hexaose	> 45.8 (6.4% inhibition)	
23	6a	Tri-antennary **II**	> 10.0 (11% inhibition)	
3	6a	**Tn** gp fraction	12.0	166.7
4	6b	Galβ1-4Man	18.0	111.1
5	6a	**P**	21.0	95.2
6	6a	**L**	23.0	87.0
24	6b	GlcNAcβ1-6Galβ1-4Glc	> 101.1 (10.8% inhibition)	
25	6b	Galβ1-4Gal	> 243.3 (8.7% inhibition)	
7	6b	Galβ1-3D-Ara	30.0	66.7
8	6a	**T**	49.0	40.8
9	6a	**I**	100.0	20.0
22	6b	Galβ1-6GlcNAc	> 160.0 (19.7% inhibition)	
10	6b	*p*-Nitrophenyl-αGalNAc	150.0	13.3
11	6b	*p*-Nitrophenyl-αGal	150.0	13.3
12	6b	Galβ1-4ManNAc	170.0	11.8
13	6a	**II**	360.0	5.6
14	6b	Melibiose	800.0	2.5
15	6a, 6b	GalNAc	1000.0	2.0
16	6b	Methyl-αGal	1400.0	1.4
17	6a, 6b	D-Gal	2000.0	1.0
18	6b	Methyl-βGal	2500.0	0.8
19	6b	D-Raffinose	4500.0	0.4
20	6b	D-Fuc	1.4×10^4	0.14
21	6b	L-Ara	1.5×10^4	0.13

Table 4 Maximal quantities of various sugar inhibitors giving negligible or weak inhibition of G4-N with a Galβ1-containing gp

Domain-I of galectin-4 (50 ng) + 1.0 ng of Galβ1-containing gps (first Smith-degraded cyst MSS gp). Total volume, 50 μl.

Inhibitor	Maximum amount of inhibitor used (nmol)	Percentage inhibition (%)
A	58	6.5
$\mathbf{A_h}$	79	14.6
F	52	13.4
S	58	10.2
B	150	9.5
E	240	3.2
GlcNAcβ1-3Gal-O-Me	100	7.0
Stachyose	380	14.1
N,*N*′-Diacetyl-chitobiose	120	12.8
N,*N*′,*N*″-Triacetyl-chitotriose	78	7.9
Methyl-αGalNAc	140	6.7
Methyl-βGalNAc	140	10.8
p-Nitrophenyl-βGalNAc	170	36.4
p-Nitrophenyl-βGal	430	14.6
GlcNAc	1.4×10^4	8.9
Glc	2.1×10^4	14.6
L-Fuc	7.7×10^3	11.3
D-Man	2.8×10^4	15.2
Methyl-αGlc	2.1×10^4	7.4
Methyl-βGlc	2.0×10^4	12.4

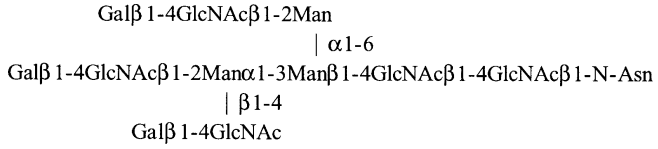

Figure 7 Carbohydrate moiety tri-antennary II gp [39]

This is a poor inhibitor for G4-N, but it is the most active ligand for CG-16 (Table 6) [48].

GlcNAcβ1-3Gal-O-Me (Table 4) and GlcNAcβ1-6Galβ1-4Glc (Table 3) were tested in a range from 10 to 30 times larger amounts than that required for **I**β1-3**L** (type I) to reach 50% inhibition, yet no 50% inhibition was found, underscoring the fact that **I**β1-3**L** (type I), **II**β1-3**L** (type II) and **L** are potent ligands for this domain (Figure 6a, curves 1, 2 and 6 and Table 3). Substitution at C-6 of Gal in **L** by GlcNAc reduces its potency significantly (Figure 6a, curve 1 versus curve 26 and Figure 6a, curve 6 versus Figure 6b, curve 24).

Tri-antennary **II** (Figure 7) and lacto-*N*-neo-hexaose were tested at up to 3.3 (Figure 6a, curve 23 versus curve 1) and 15 times (Figure 6a, curve 26 versus curve 1) more than the amount required for **I**β1-3**L** (type I) and only weak inhibition was obtained.

Melibiose (Galα1-6Glc) was slightly more active than Gal, 6.3 times more active than raffinose (Figure 6b, curve 14 versus curve 17 and curve 19 and Table 3), and significantly more active than stachyose (Table 4), demonstrating that the combined size of Gal for the α-anomer is smaller than two sugars.

Of the monosaccharides studied, *p*-nitrophenyl-αGalNAc was the best inhibitor, it was 6.7 times more active than GalNAc (Figure 6b, curve 10 versus curve 15), while *p*-nitrophenyl-αGalNAc and *p*-nitrophenyl-αGal (Figure 6b, curve 10 versus curve 11) were equally active. As shown in Table 3, *p*-nitrophenyl-

Table 5 Comparison of G4-N and CG-16 binding properties to human blood group A-, B-, H-, I/II-, Le^a- and Le^b-active gps, sialo- and asialoglycoproteins by ELLSA

Structures are described in the text. Data on CG-16 were obtained from [48]. Reading intensity results were interpreted according to the spectrophotometric absorbance value at 405 nm after 4 h incubation as detailed in the legend of Table 2. **B_h**, Galα1-3[L-Fucα1-2]Gal; **H**, L-Fucα1-2Gal.

	Maximum absorbance (A_{405})			
	Absorbance binding		Reading intensity	
Gp (Lectin determinant/bioactive structural units)	G4-N	CG-16	G4-N	CG-16
Cyst MSS 10% 2 × (**A_h**)	2.9	0.1	+++++	−
Cyst MSS first Smith-degraded (**I**, **II**, **T**, **Tn**)	3.0	3.0	+++++	+++++
Cyst Mcdon (**A_h**)	2.6	0.4	+++++	±
Cyst Mcdon P-1 (**T**, **Tn**, **I/II**)	3.0	3.0	+++++	+++++
Cyst Tij 20% of second 10% (**B_h** > **I/II**)	3.0	0.7	+++++	+
Cyst Tighe phenol insoluble (**H**, Le^b)	2.1	0.0	++++	−
Cyst Tighe P-1 (**I**, **II**)	3.0	2.9	+++++	+++++
Cyst JS phenol insoluble (**H**)	1.8	0.0	+++	−*
Cyst JS first Smith degraded (**II**)	2.7	0.3	+++++	±*
BSM (sialyl-GlcNAcβ1-3, **Tn**)	0.03	0.0	−	−
Asialo-BSM (GlcNAcβ1-3, **Tn**)	2.6	0.0	+++++	−
PSM (sialyl **T**, **Tn**)	0.2	0.04	±	−*
Asialo-PSM (**T**, **Tn**)	3.0	0.0	+++++	−*
RSL (sialyl **II**)	0.4	0.0	±	−
Asialo-RSL (**II**)	1.8	2.8	+++	+++++

* A.M. Wu, unpublished work.

Table 6 Differential binding of G4-N and CG-16

Reciprocal of relative potency of sugars: Gal was taken as 1.0.

	Quantity giving 50% inhibition (nmol)		Reciprocal of relative potency	
Inhibitor	G4-N*	CG-16†	G4-N*	CG-16†
Tri-antennary **II**	> 10.0	0.33	< 200.0	2121.3
Iβ1-3**L** (type I)	3.0	1.0	666.7	700.0
II	360.0	1.0	5.6	700.0
I	100.0	20.0	20.0	35.0
T	49.0	900.0‡	40.8	0.8‡
GalNAc	1000.0	6000.0‡	2.0	0.1‡
Gal	2000.0	700.0	1.0	1.0

* From Table 3.
† From [48].
‡ Extrapolation.

αGal was about 9.5 times better than the methyl-α derivative (Figure 6b, curve 11 versus curve 16), indicating that a hydrophobic interaction with an aglycon is important in the case of the α-anomer of Gal. GalNAc was more than twice as active as Gal (Figure 6a, curve 15 versus curve 17). D-Man, D-GlcNAc and L-Fuc were tested at amounts ranging from 7.7×10^3 to 2.8×10^4 nmol, but no inhibition was observed (Table 4).

DISCUSSION

Owing to the expression of often more than one type of galectin in animal tissues [1,4,11], delineation of the functions of individual members of this lectin family requires the precise definition of their carbohydrate specificities. Fine-specificity differences may well be translated into distinct spectra of bound glycans, as noted for plant lectins [40], presumably connecting the presence of a certain endogenous lectin with, at least in part, expression of a characteristic set of cognate oligosaccharides. Besides employing oligosaccharides as inhibitors of ligand binding, determination of their avidity towards determinants in natural glycans is useful to map the profile of their reactive structures (geometrical parameters). In this study, the carbohydrate-binding activity of domain-I of galectin-4 was examined by using a recently described ELLSA method [12,13] and a wide range of distinct gp and oligosaccharide preparations [19,41,42]. As it is difficult to quantitate precisely the amounts of gps adsorbed on to the microwells, the interactions of domain-I of galectin-4 with various glycoforms were examined using three parameters: (i) the amounts of gps added to wells that gave 1.5 A_{405} units (Figure 3 and Table 2), (ii) the maximum absorbance for each gp after 4 h of incubation (Figure 3 and Table 2) and (iii) the amount of gps required to give 50% inhibition (Figure 5 and Table 2). Although the structural details of the sugar chains of the mucins tested have so far not entirely been established, some conclusions can be drawn, when the interplay of the three parameters are analysed in detail (Table 2).

In addition, to react best with the human blood group precursor gps, one of the important findings is the high affinity of this lectin for human blood group ABH and Lewis active gps [Figure 1; cyst MSS 10% 2× ppt (Figures 3a and 5a); cyst N-1 Le^a 20% 2× (Figures 3d and 5a); hog gastric mucin no. 14 (Figures 3e and 5b); cyst Mcdon (Figures 3b and 5a); cyst Tighe P-1 (Figures 3c and 5a); and cyst JS phenol insoluble (Figures 3c and 5a)], indicating this lectin can recognize internal Galβ1-3/4GlcNAc with such substitutions. Because the α1,3-branching introduced by a fucose moiety to the Glc unit in **L** had a detrimental effect [5], the binding reactivity must be attributed to ABH epitopes. Interestingly, addition of β-linked GalNAc to a Gal unit also maintained binding activity [5], as we likewise observed for the A-determinant-presenting gps. Since these antigens found on erythrocytes are also expressed in large amounts on epithelial cell surfaces in the gastrointestinal tract [43], the preferential binding of CRD-I of galectin-4 to histo-

blood group determinants can have a bearing on its restricted tissue-specific distribution in the gastrointestinal tract [5]. Fittingly, brush border enzymes, ligands for galectin-4 at this site, have heavily glycosylated ectodomains, adding support to the concept of an intimate protein–carbohydrate interaction with the noted implications for the digestive and adsorptive capacity and/or the epithelial integrity [6–8]. Regarding other galectin family members, prototype galectin-1 has a clearly different localization pattern in the mammalian digestive tract, whereas the chimaera-type galectin-3 shares an epithelial expression profile [44]. Therefore, it will be essential to run this analysis with galectin-3 in order to elucidate whether and to what extent the two galectins with similarities in cellular expression can distinguish galactoside-harbouring glycans.

Among the mono- and oligosaccharides tested for inhibition of lectin–glycoform interaction, **I**β1-3**L** (type I) was the best. **L**, which is the disaccharide at the reducing end of **I**β1-3**L** (type I), was found to be 87 times more active than Gal, showing the importance of the β1-4-linked Glc at the reducing end. This finding is consonant with the results reported by Oda et al. [5] who tested 15 mono-, di- and oligosaccharides in a lactosyl-Sepharose binding assay. **I** was about 4 times less active than **L** (Figure 6a, curve 9 versus curve 6). However, the elongation by adding β1-3Galβ1-4Glc to its reducing end enhances the reactivity significantly. Thus the potency of these three related ligands can be expressed as **I**β1-3**L** (type I) > **L** > **I** (Figure 6a, curves 1, 6 and 9).

The three potent inhibitors of G4-N, **I**β1-3**L** (type I), **II**β1-3**L** (type II) and **L**, are respectively about 667, 133 and 87 times more active than Gal (Figure 6a, curves 1, 2 and 6 versus 17). They occur as a free form in human milk [45] and as conjugated forms in the glycan chains of glycosphingolipids [46]. In the free form, they may function as regulators to affect the lectin-receptor binding (i.e. to prevent pathogen attachment) and, in glycosphingolipids, they can be binding partners in *cis*- and/or *trans*-interactions: the cell-surface localization of galectin-4 gives the opportunity to be engaged in contacts on the surface of the same cell (*cis*-interaction), e.g. crosslinking of gps to form patches. Alternatively, galectin-4 can bind a glycan from another cell (*trans*-interaction) to mediate intercellular contact. Another active ligand, **P** (Figure 6a, curve 5 and Table 3), is also an important structural unit in the ganglio-series [46,47] and may also function as the docking site. The **Tn** clusters, which were as active as **II**β1-3**L**, were found to represent a ubiquitous epitope (type II; Figure 6a, curve 2 versus curve 3). This mucin-type glycosylation occurs in epithelial secretions or in the cell-surface membrane gps [33,34,37]. The **T** structure (Figure 6a, curve 8 and Table 3), which is about 25% as active as **Tn** clusters (Figure 6a, curve 3 and Table 3), is part of the glycan chain of ganglioside GM_1 at the non-reducing end or as a common core structure of O-glycans [46]. When Galβ1-3GalNAcα1-Ser/Thr residues occur in cluster forms, they can be as active as other potent ligands (Figure 5b, curves 6 and 10). The fact that G4-N binds well to carrier-immobilized lyso GM_1 supports this result (J. Kopitz and S. André, unpublished work). As indicated previously, lectin is found in the epithelial cells of the alimentary tract. It is intriguing that reactive glycotopes for this lectin are widely distributed in the mammalian digestive system [5,6]. The results of this study show that this galectin is able to preferentially interact with distinct sets of β-galactosides from the panel of natural glycotopes [40,47,49].

We have previously performed a similar analysis for CG-16, which is a developmentally regulated galectin from chicken liver with a prototype dimeric CRD display [48]. Due to the presence of defined amino acid differences within the homologous CRDs between these two galectins (CG-16 and G4-N), it is now possible to correlate them to subtle binding-specificity differences towards sugar ligands and glycans. As shown in Tables 5 and 6, when these two binding profiles are compared, conspicuous disparities become apparent, underscoring the occurrence of fine-specificity differences. Binding properties to human blood group ABH-active gps, T and Tn ligands, and also some tolerance of the N-acetyl group at the penultimate sugar moiety, are clearly non-uniform features. For example, structure **II**, a common sequence recognized specifically by the galectin family, is about 16 times less active than **L** in G4-N (Figure 6a, curve 6 versus curve 13 and Table 3). Thus it is evident that the individual galectin has its own characteristic binding preferences as documented for Gal/GalNAc-specific plant lectins [40,49].

From the present results, the following conclusions on the binding properties of the domain-I of galectin-4 can be made: (i) domain-I reacted best with human blood group ABH precursor gps and their equivalent gps, indicating that it can recognize crypto Galβ1-3/4GlcNAc covered by these determinants; (ii) sialylation, if not detrimental to immobilization on to a plate surface, appears to negatively affect its binding; (iii) this domain has a general preference for the β-anomer as Galβ1-3 > Galβ1-4 and Galβ1-6; (iv) **I**β1-3**L** (type I) is the most active linear ligand tested; (v) **Tn** clusters interact with G4-N; (vi) hydrophobic interaction with an aglycon is important with the α-anomer of Gal; (vii) ranking of the carbohydrate specificity of this domain can be defined as **I**β1-3**L** (type I) > **II**β1-3**L** (type II) and **Tn** clusters > **L** and **P** > **T** > **I** > **II** > GalNAc > Gal. In view of the reported association of up-regulated galectin-4 gene expression and gastric cancer dissemination [9] these results, especially the efficacy of **T** and **Tn** clusters, might be helpful for head-group design of potent adhesion inhibitors on a dendrimeric scaffold [50]. To avoid cross-reactivity with other galectins and to reach optimal selectivity for the galectin type, our results encourage us to analyse further mammalian galectins in the same systematic manner to relate structure differences to ligand selectivity and functional aspects.

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