Glycoconj J (2011) 28:427–435
DOI 10.1007/s10719-011-9342-5

Specificity analysis of the C-type lectin from rattlesnake venom, and its selectivity towards Gal- or GalNAc-terminated glycoproteins

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Received: 20 April 2011 / Revised: 21 June 2011 / Accepted: 12 July 2011 / Published online: 30 July 2011

Abstract The rattlesnake (*Crotalus atrox*) venom lectin is a readily-prepared decameric C-type lectin, specific for Gal and GalNAc. Glycan microarray analysis showed it reacted with a wide range of glycans, chiefly recognizing sets of compounds with Galβ1-4GlcNAc (LacNAc), α-Gal or α-GalNAc non-reducing termini. Its array profile was therefore distinctly different from those of four previously studied mammalian C-type lectins with the same Gal/GalNAc monosaccharide specificity, and it was more broadly reactive than several Gal- or GalNAc-specific plant lectins commonly used for glycan blotting. Though a general reactivity towards glycoproteins might be expected from the avidity conferred by its high valence, it showed a marked preference for glycoproteins with multiple glycans, terminated by Gal or GalNAc. Thus its ten closely-spaced sites each with a K_D for GalNAc of ~2 mM appeared to make RSVL more selective than the four more widely-spaced sites of soybean agglutinin, with a ten-fold better K_D for GalNAc.

Keywords Rattlesnake venom lectin · *Crotalus atrox* · C-type lectin · Glycan array · Lectin subsites · Avidity

Introduction

Despite their very simple interactions with monosaccharides, and the relatively weak binding these confer, glycan array studies by the Consortium for Functional Glycomics (CFG) [1, 2] of mammalian C-type lectins specific for Gal and/or GalNAc have shown that they have remarkable discriminatory powers among carbohydrates that bear these sugars [3]. Their specificities arise from several causes, such as additional subsites near the main calcium one, steric exclusion, and the geometric arrangement of the carbohydrate recognition domains (CRDs) caused by ancillary domains [4]. They also show a greater affinity for glycoproteins than free glycans [5].

Rattlesnake venom lectin (*Crotalus atrox* lectin, RSVL) shares the specificity for Gal [6] or GalNAc of these mammalian lectins, but it is very different in oligomeric structure. It is a member of the Group VII subfamily of C-type animal lectins [7, 8]. As such, its CRD has no N- or C-terminal extensions to cause the formation of specifically clustered oligomers. Instead, it forms a decamer comprising a pentameric ring of disulphide-linked dimers, whose structure has been determined [9]. RSVL recognizes both Gal and GalNAc residues with similar affinity [9], and it binds them through interactions with key hydroxyl groups at C3 and C4 mediated by a calcium atom, as is characteristic of the C-type family. Discrimination of Gal from Man is achieved by the positioning of a Tyr residue by the neighboring side-chains of Gln and Lys residues, rather than the positioning of a Trp or Phe by a loop of Gly residues seen in mammalian Gal-specific C-type lectins [10]. The binding sites are arranged around the perimeter of the rings in a zigzag pattern with site spacings as close as 27Å. This is unlike the facial presentation of the sites in the structures of other pentameric lectins such as the pentraxin C-reactive protein [11].

RSVL is the prototype for a family of homologous lectins in the venoms of other snakes, chiefly from members of the Elapidae and Vipiridae families. Models of related lectins have been made on the basis of the RSVL

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monomer structure though their quaternary structures may not be decamers [12]. From their sequences, these lectins are also predicted to be Gal-specific, but additional Man-specific ones have been found, *e.g* in *Bungarus fasciatus* [13]. The same residue differences that confer specificity for Man instead of Gal or GalNAc in mammalian lectins are present in the *B. fasciatus* lectins, *i.e.* QPDXY/F becomes EPNXS [12, 13].

The RSVL structure determined with bound lactose [8] did not suggest any additional subsites in it. Nevertheless, we investigated its specificity by means of the CFG glycan array. The data for RSVL, the first obtained for a C-type lectin from a non-mammalian vertebrate, show it has discrete preferences for Galβ1-4GlcNAc (LacNAc) and GalNAc compounds, and a broader specificity than either mammalian C-type Gal/GalNAc lectins or the plant lectins commonly used as reagents for glycan structures. RSVL has a lower binding affinity than the plant lectins typically show, but a greater potential for avidity effects due to its decameric structure. We therefore examined RSVL's interaction with polyvalent glycans by surface plasmon resonance (SPR) and with glycoproteins by blotting experiments, to determine if this readily-prepared lectin could be useful as a reagent for the general detection of both N- and O-linked asialoglycoproteins.

Materials and methods

Carbohydrates

The blood group A trisaccharide octylglycoside was provided by Dr Monica Palcic and the α-methylcyclopropane glycoside of Galβ1-4GlcNAcβ1-2Man was provided by Drs Warren Wakarchuk and Dennis Whitfield. Fetuin from fetal calf serum, human serum transferrin, Galβ1-4GlcNAc and *p*-nitrophenyl glycosides were obtained from Sigma-Aldrich Canada Inc, Oakville ON. Asialotransferrin and asialofetuin were prepared by treatment of the glycoproteins with *Micromonospora viridans* sialidase, provided by Dr Michel Gilbert. Transferrin glycopeptides were prepared by pronase digestion of human serum transferrin (100 mg) in 0.1 M Tris buffer pH 8, with a 2% enzyme:substrate ratio for 24 h at 37°; after a second digestion with another aliquot of pronase, the glycopeptide product was then purified by gel filtration on BioGel P2 in 0.2 M ammonium bicarbonate. After repeated freeze-drying, ~4 mg of the glycopeptide were obtained and de-sialylated by treatment with 5 units of sialidase (Prozyme Inc., Hayward CA; sialidase T) in 1 ml of 0.1 M ammonium acetate buffer, pH 5.0, for 24 h at 37°. After repeated freeze-drying, the yield was 2.7 mg of asialoglycopeptide. Mass spectrometry indicated the major components were Asn-Lys and Asn-Ser-Gly bi-antennary glycopeptides, in accordance with the amino-acid sequences around the two glycosylation sites in transferrin. *N*-linked glycans were released from reduced and alkylated fetuin by digestion with PNGase F (provided by Dr Michel Gilbert) and separated from the protein by filtration on a 30,000 Mwt cutoff filter (Amicon Ultra, Millipore Corp., Billerica MA). Asialoglycans were produced by treatment with sialidase and purification by gel-filtration as above. Mass spectrometry showed that the predominant glycan was the triantennary form without fucose. *Campylobacter jejuni* glycoproteins were purified from a cell extract of a *pglI* mutant of NCTC11168 [14], provided by Dr Christine Szymanski, by affinity chromatography on soybean agglutinin (SBA) -agarose gel (EY Laboratories Inc., San Mateo CA) [15]. The cluster compound, Lac-Daisy, in which ten lactose termini are displayed symmetrically on a highly branched structure, was provided by Drs David Bundle and Pavel Kitov [16].

Glycan array assays

The glycan array data were collected by the CFG Core H staff using RSVL labeled with Alexa Fluor 488 (Invitrogen) and version 2 of the CFG glycan array; these data can be viewed in full on the CFG website (CFG 2010).

SDS-PAGE lectin blotting

RSVL in 0.1 M $NaHCO_3$ was labeled with biotin by reaction with aminohexanoyl-biotin-*N*-hydroxysuccinimide ester, according to the manufacturer's direction (Zymed; Invitrogen Corporation, Carlsbad CA). Proteins were separated by SDS-PAGE on 12.5% homogeneous Mini Protean II (BioRad Laboratories, Mississauga ON) slab gels. The gels were electroblotted onto PVDF membrane at 50 V for 2 h in 10 mM 3-(cyclohexylamino)-1-propane sulfonic acid buffer, pH 11, containing 10% methanol. The membrane was washed with de-ionised water, stained with G-250 Coomassie blue, or for subsequent lectin probing the membrane was blocked in Tris-buffered saline (100 mM NaCl, 50 mM Tris, pH 7.5) and 2% blocking buffer (Roche Diagnostics Corp., Laval QC) for 2 h at room temperature. Following blockage, blots were further incubated with either an SBA-alkaline phosphatase conjugate (EY Laboratories Inc., San Mateo CA) or biotinylated RSVL at a concentration of 10 μg/ml in the above blocking solution for 1 h at room temperature, followed by three washes in Tris-buffered saline containing 0.05% Tween-20. Blots probed with biotinylated-RSVL were further incubated with a 1:1,000 dilution of a streptavidin-alkaline phosphatase conjugate (R&D Systems, Minneapolis, MN) for 1 h at room temperature and washed three times as described above. Blots were developed using nitro blue tetrazolium

chloride/5-bromo-4-chloro-3-indoyl phosphate in 0.1 M NaCl, 0.1 M Tris pH 9.5, with 50 mM $MgCl_2$.

Surface plasmon resonance

The interactions of sugar ligands with immobilized RSVL were measured by SPR using a BIACORE 3000 instrument (GE Healthcare). Immobilizations were carried out on research grade CM5-sensorchips (GE Healthcare) at protein concentrations of 50 μg/ml in 10 mM sodium acetate buffer pH 4.5, using the amine coupling kit supplied by the manufacturer. The amount of immobilized protein ranged from 5,954 to 10,137 resonance units (RU) for RSVL and 5,420–9,922 RU for bovine serum albumin as a reference protein. Except for the fetuin asialoglycopeptide, all binding analyses experiments were performed at 25°C in 10 mM Hepes buffer, pH 7.4 containing 150 mM NaCl and 0.005% surfactant P20, at a flow rate of 20 μl/min.

Between analyses, the surfaces were washed with running buffer only, *i.e.* without regeneration solution. The binding analysis experiments with fetuin asialoglycopeptide were performed at 25°C in the above buffer, at a flow rate of 10 μl/min; to regenerate the RSVL surface, a 15 min wash with 3 mM EDTA was used, then a 15 min wash with 1 mM $CaCl_2$ and equilibration with the Hepes buffer. All data were analyzed with the BIAevaluation 4.1 software.

Results

The glycan array results are presented graphically in Fig. 1, and the compounds are listed in order of apparent affinity in Table 1, scaled to the most strongly reactive one. The apparent affinities of binding for weaker ligands can be affected by ligand density and protein concentration [17], but the rank order is relatively robust. Broadly speaking, the list can be divided into three subsets of glycans with either Galβ1-4GlcNAcβ (LacNAc), GalNAcα or Galα at the non-reducing end, and the top five compounds include examples of each of these subsets. Previously, SPR experiments using an inhibition format had showed that RSVL had similar affinities for the β-Gal and α-GalNAc compounds, *i.e.* lactose and *p*-nitrophenyl α-GalNAc [9] and all but one of the reactive compounds on the array have these terminal groups. The exception is #157, which has an O6 substituent on the Gal. However, there are many other Gal compounds on the array with much lower reactivity. Larger glycans with one or two terminal LacNAc structures were considerably better ligands than the disaccharide itself (compound #153). RSVL bound to several compounds related to the blood group A trisaccharide on the array, through the terminal GalNAc, but remarkably not to blood group B compounds, such as compound #99, which is the α-Gal equivalent of compound #84. RSVL discriminates Gal from Man by a slightly different structural means than most other C-type lectins [9], but this feature does not appear relevant for its fine specificity.

To assess the effects of the decavalence of RSVL on its interactions with glycoproteins, transferrin and fetuin samples were treated with a bacterial neuraminidase to uncover Gal termini. The proteins were run on SDS-PAGE, and blotted with biotin-labeled RSVL followed by an avidin-alkaline phosphatase conjugate. The removal of sialic acid increased the reactivity of fetuin considerably more than the reactivity of transferrin (Fig. 2a). To test the reactivity of RSVL with GalNAc-terminated glycoproteins, a mixture of GalNAc glycoproteins were extracted from a cell lysate of a *pglI* mutant strain of *Campylobacter jejuni,* by affinity chromatography on an SBA column. This mutation leads to glycoproteins with linear $GalNAc_5Bac$ glycans [14], *i.e.* they lack the usual Glc side-chain [15]. Their blotting characteristics with RSVL and SBA were very different (Fig. 2b). Notably, a higher molecular weight glycoprotein was much more reactive with RSVL than with SBA, while the bands in the central region, which arise from the most abundant glycoprotein Cj1670 (CpgA), were relatively weakly stained. The reactive glycoprotein was identified by mass spectrometry as Cj0843c, a trans-glycosylase. Its sequence contains four of the D/E-X-N-X-S/T sequons required for N-glycosylation in *C. jejuni* [18], as does the sequence of Cj1670, but only a single band was seen with Cj0843c, suggesting uniform modification with the glycan.

To explore the array and gel results further, direct SPR measurements of affinity were made with glycans related to the active compounds on the array and with glycoproteins (Table 2, Figs. 3 and 4). The order of the affinities obtained for the blood group A trisaccharide glycoside, the transferrin asialoglycopeptide and Galβ1-4GlcNAc was the same as for their counterpart compounds #81, #52 and #153 on the array, but the β-lactose compound #154 had much lower affinity than all the other compounds in Table 1. SPR experiments were also performed with the two asialoglycoproteins used in the blotting experiments and glycopeptides obtained from them by pronase digestion. These glycopeptides had similar affinities to the monosaccharides, indicating that bi- or tri-antennary structures do not form multiple attachments to individual RSVL molecules. The asialotransferrin was bound an order of magnitude more strongly than its glycopeptide, an effect that has been previously reported with other C-type lectins [5]. The two anomeric forms of *p*-nitrophenyl GalNAc had similar association constants, indicating that the lack of β-GalNAc compounds in the array results (Table 2) may be due to the scarcity of them in the ligand set rather than lower affinities for RSVL.

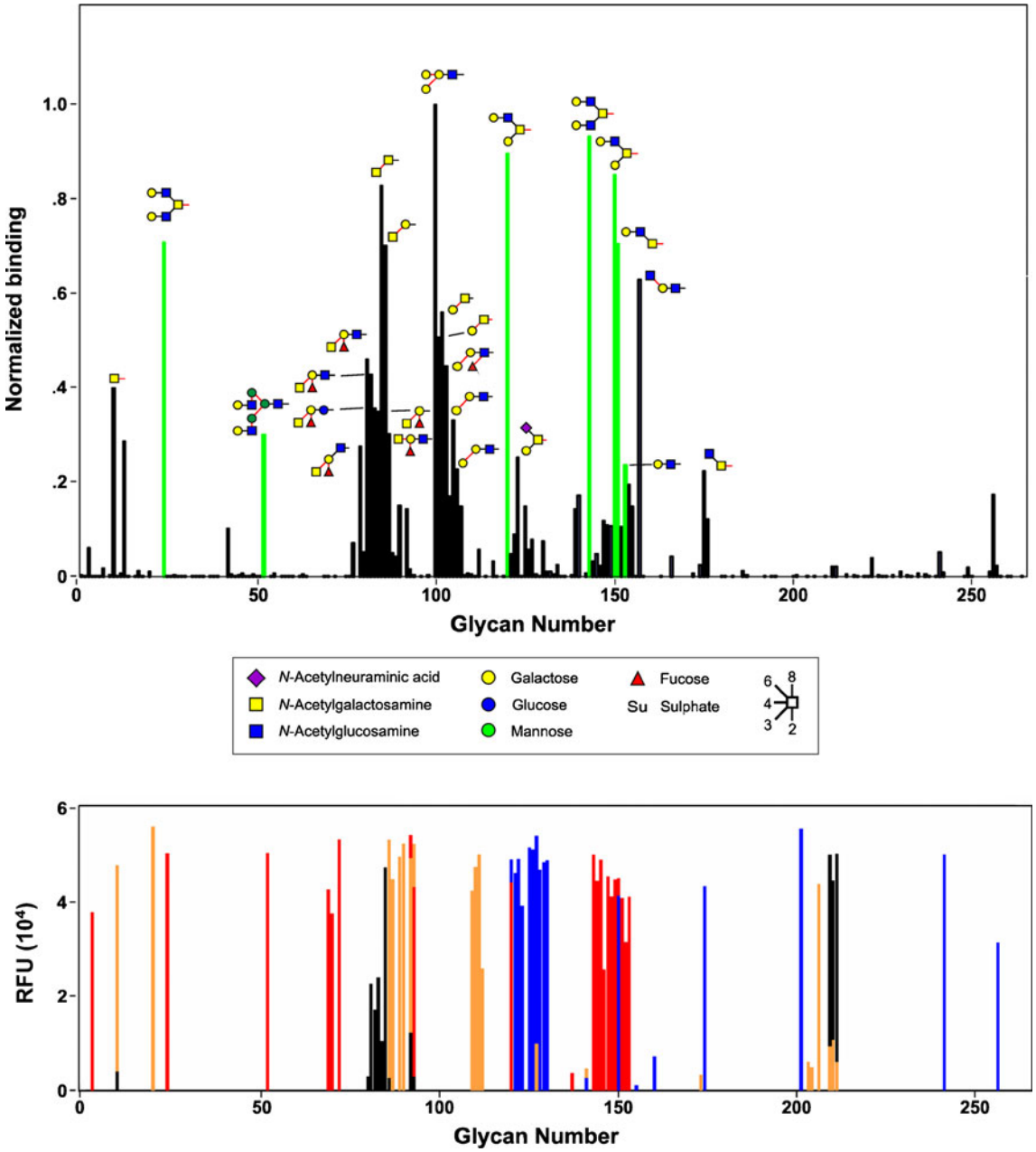

Fig. 1 Glycan array data for RSVL. Green bars indicate compounds with terminal Galβ1-4GlcNAcβ groups. Symbols for the sugars are given below, and α-glycosidic bonds are shown in red. Binding activities are scaled to that of the compound with the highest binding (fluorescence). The diagram is based on the CFG spreadsheet which can be accessed at: http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_PA_v2_233_01202006 The bottom panel shows CFG data obtained with the same v2 array as RSVL for four biotinylated plant lectins (only the most reactive ligands are shown): ECL, LacNAc-specific (*red*); DBA, blood group A-specific (*black*); PNA, Galβ1-3GalNAc-specific (*blue*); and SBA, α-Gal/α-GalNAc-specific (*tan*)

Two more complex ligands were bound with far higher avidities, but gave binding data that could not be fitted to simple models throughout the concentration ranges (Fig. 4). In contrast to asialotransferrin, asialofetuin was far more strongly bound than its asialoglycopeptides, and had notably slower on-rates than the simple ligands. Plotting the data from the end of the binding phase, which was not at equilibrium, gave a highly-curved Scatchard plot, with the lower concentration data indicating apparent K_D values ~50 nM, and the higher concentrations ~400 nM. Even stronger binding was indicated by kinetic analyses, with data fitting to a 1:1 model for the three lowest ligand concentrations giving a k_a of 3.6×10^4 M^{-1} s^{-1}, a k_d of 1×10^{-4} s^{-1} and an apparent K_D of 2.8 nM. To investigate the avidity of RSVL towards a more defined species with similarly high numbers of Gal termini, a synthetic penta-

Table 1 The most active ligands from the microarray experiments

Glycan #	Compound	Fluorescence (%)[a]
100	Galα1-3(Galα1-4)Galβ1-4GlcNAcβ	100
143	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6) GalNAcα	93.2
120	Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα	90.0
150	Galβ1-4GlcNAcβ1-6(Galβ1-3)GalNAcα	85.1
85	GalNAcα1-3 GalNAcβ	82.8
24	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6) GalNAcα	70.9
151	Galβ1-4GlcNAcβ1-6GalNAcα	70.6
86	GalNAcα1-3Galβ	70.2
157	GlcNAcα1-6Galβ1-4GlcNAcβ	63.0
102	Galα1-3GalNAcβ	56.1
101	Galα1-3GalNAcα	50.7
81[b]	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ	46.0
103	Galα1-3Galβ1-4(Fucα1-3)GlcNAcβ	44.6
82	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ	42.8
10	GalNAcα	40.0
83 [b]	GalNAcα1-3(Fucα1-2)Galβ1-4Glcβ	35.6
84	GalNAcα1-3(Fucα1-2)Galβ	34.9
105	Galα1-3Galβ1-4GlcNAcβ	33.0
87	GalNAcα1-4(Fucα1-2)Galβ1-4GlcNAcβ	30.2
52	Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)-Manβ1-4GlcNAcβ1-4GlcNAcβ	30.0
13	Rhaα	28.6
79 [b]	GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ	27.6
123	Galβ1-3(Neu5Acβ2-6)GalNAcα	25.3
153	Galβ1-4GlcNAcβ	23.6

[a] Expressed as a percentage of the strongest ligand, whose fluorescence reading was 49535

[b] Compounds with a shorter linker, Sp0 compared to the Sp8 of the others [1]

antennary glycan with ten Gal termini, Lac-Daisy [17], was tested. In this compound, the antennae consist of two lactose residues each connected by an 11-atom linker to a branch-point, which in turn is connected by a 28-atom linker to one of the five hydroxyls of a central hexose. The Lac-Daisy binding was too complex for reduction to a simple binding model, particularly in the dissociation phase (Fig. 4). Estimates of affinities from a Scatchard plot suggested apparent K_D values between 15 nM and 1 μM. A 1:1 model fitted the experimental data for the three lowest ligand concentrations reasonably well, giving a k_a of 7×10^6 M^{-1} s^{-1}, a k_d of 5.8×10^{-2} s^{-1} and an apparent K_D of 8.4 nM. A bivalent analyte model fitted the experimental data for the dissociation phase somewhat better, but physical interpretation of this model is not straightforward.

Discussion

The CFG glycan microarray has been a powerful tool to discriminate among C-type lectins that have specificities for Gal and/or GalNAc, and the profile of RSVL clearly distinguishes it from all four of the mammalian C-type lectins compared by Coombs *et al.* [3], both in the number of active compounds and its fine specificity. For example, the macrophage galactose lectin and the scavenger receptor lectin preferred structures with Fuc residues adjacent to the Gal such as Lewis[a] and Lewis[x], which RSVL did not recognize, yet blood group A or B structures were not bound by them. The preference of RSVL for LacNAc termini and blood group A structures also sets it apart from the hepatic lectin and the Kupfer cell receptor [3]. Only the macrophage galactose lectin recognized biantennary LacNAc compounds, but similar GalNAcβ1-4GlcNAc structures were recognized by three of the lectins. The latter structure does not appear in the set of RSVL compounds, and the GalNAc compounds in it all had α-linkages.

The Gal in ligands such as LacNAc is positioned in the binding site by the interaction of its C3 and C4 hydroxyls with the calcium atom and by hydrophobic interaction with the ring of Tyr100 [9]. Oligosaccharides with β-linked substituents on the Gal, such as GlcNAc, extend into a groove on the protein surface, formed chiefly by Lys94, Asn95 and Leu112 (Fig. 5). The LacNAc preference of RSVL may arise from an interaction of the acetyl group with the sidechain of Lys94. In GlcNAcβ1-2Man compounds, the acetyl group of the GlcNAc is close to the Man ring. Modeling of the trisaccharide Galβ1-4GlcNAcβ1-2Man, using PDB coordinates from the Glycam site of Dr R.J. Woods (http://glycam.ccrc.uga.edu/ccrc/) with the RSVL-lactose structure, suggests the acetyl group would be positioned between the side chain of Lys94 and the Man ring (Fig. 5). This trisaccharide occurs in nearly all *N*-linked glycans and RSVL's preference for LacNAc termini suggests its biological receptor, probably on platelets [19], may be a structure of this type.

In SPR analysis, the affinity of RSVL for a LacNAc compound, the transferrin biantennary asialoglycopeptide, was not much greater than its affinity for simple sugars. This is in marked contrast to codakine, which is the only other non-mammalian C-type lectin that has been characterized by both crystallography and glycan array analysis [20]. Codakine, from the bivalve *Codakia orbicularis*, also occurs as disulfide-linked dimers, however this crosslink is at a different position in the C-type lectin fold, and higher oligomers are not formed. It recognizes mannose with a K_D of 0.27 mM, but it is able to bind biantennary glycans with a very high affinity, a K_D of 0.43 μM. This affinity is due to additional interactions near the primary site with other parts of the glycan, not by forming bivalent complexes [20].

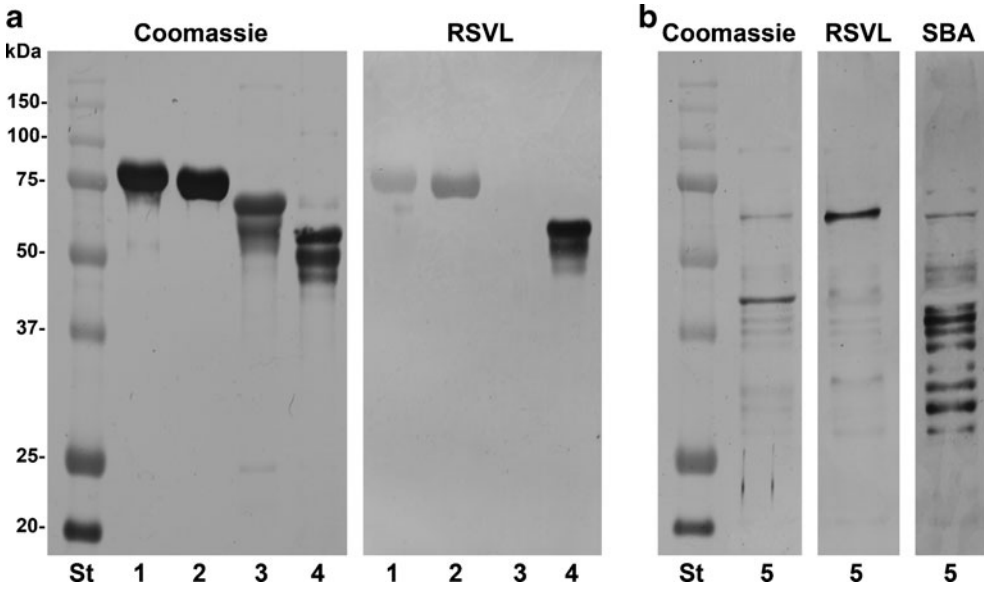

Fig. 2 Interactions of RSVL with glycoproteins. **a** Reaction with serum glycoproteins: lanes 1 transferrin, lanes 2 asialotransferrin, lanes 3 fetuin, lanes 4 asialofetuin. The left portion shows a blot stained with Coomassie blue and the right panel shows staining with biotin-RSVL followed by an avidin-akaline phosphatase conjugate. **b** Reactions with glycoproteins from *C. jejuni* with GalNAc termini, lanes 5. The panels are respectively blots stained with Coomassie blue, with biotin-RSVL followed by an avidin-akaline phosphatase conjugate and with an SBA-akaline phosphatase conjugate

It is instructive to compare the glycan array profile of RSVL with the profiles of some plant lectins that are commonly used as reagents for glycans with Gal or GalNAc termini. Many of the compounds on the array are grouped into clusters of related structures, hence the specificities of these lectins lead to readily seen characteristic binding patterns [2]. The major regions of reactivity (Fig. 1) can be summarized as follows: compounds #143–152 for the LacNAc-specific lectin from *Erythrina crystagalli* (ECL); #120–130 for the Galβ1-3GalNAc specific lectin from *Arachis hypogaea* (PNA); #85–93 and #110–112 for the α-Gal/α-GalNAc specific lectin SBA; #75–80 and #209–211 for the blood group A specific lectin from *Dolichos biflorus* (DBA). The specificity of RSVL clearly overlaps those of the four plant lectins, though its affinity for these compounds will be lower.

This comparison suggested testing RSVL as a general reagent for Gal or GalNAc-terminated glycans, *i.e. N*- or *O*-linked glycopeptides, to be followed by individual lectins to determine the reactive structure more specifically. C-type lectins have not been previously considered for use as reagents for glycoconjugates for several reasons. Their affinities for monosaccharides are low, compared to other families such as plant lectins, their extraction from natural sources is difficult and the recombinant forms of C-type lectins have been mostly designed to be monomers. However, RSVL has considerable advantages over other C-type lectins, being easily made from venom by affinity chromatography on unmodified agarose beads and having high avidity due to its relatively simple decameric form. Compared to plant lectins, the distances between binding sites in RSVL are smaller and more compatible with the spacing of terminal groups on biantennary glycopeptides. The minimum distance between sites on opposite pentamers is only 27Å [9], compared to ~50Å in legume lectins such as peanut agglutinin. However, the SPR experiments did not indicate any bivalent binding was occurring with the two asialoglycopeptides we tested.

When tested as a detection agent with a small set of glycoproteins, RSVL showed unexpected selectivity. Its reaction with asialotransferrin, which has two biantennary *N*-linked glycans, was only an order of magnitude greater than with the isolated glycopeptides, an increase similar to that previously found for glycoproteins *vs* their glycopeptides [5]. It also reacted minimally with *C. jejuni* glycoproteins that had only a few GalNAc-terminated glycans, further confirming the importance of the multivalency of the ligand. In contrast, RSVL reacted well with asialofetuin, which has three triantennary *N*-glycans and two *O*-glycans, the bacterial glycoprotein Cj0843c, which has four glycosylation sites, and with the synthetic decavalent compound Lac-Daisy. Thus RSVL reactivity requires the glycoproteins to have clusters of suitably positioned glycans, rather than single multi-antennary glycopeptides. For this reason, its usefulness as a reagent would be relatively limited. It is noteworthy that its ten closely-spaced sites each with 2 mM K_D for GalNAc somehow render it more selective for glycoproteins than SBA, which has four sites ~50Å apart with 0.17 mM K_D [21].

The SPR experiments with Lac-Daisy and asialofetuin showed complex binding behavior that could not be fitted to a simple model throughout the concentration ranges used. The two also had very different kinetics, with 100-fold differences in both the on and off rates, but had similar apparent K_D values. Lac-Daisy has ten Gal termini, with five biantennary lactose structures each attached to a hydroxyl of a central hexose [16], and asialofetuin has a total of nine Gal termini, from its three triantennary N-

Table 2 Binding constants determined by SPR

Ligand	K_D (mM)
Galβ1-4Glc (lactose)[a]	2.6
Galβ1-4GlcNAc (LacNAc)	1.3
α-methylcyclopropane Galβ1-4GlcNAcβ1-2Man	2.4
Blood group A trisaccharide—octylglycoside	1.7
p-Nitrophenyl α-GalNAc [a]	2.9
p-Nitrophenyl β-GalNAc	1.6
Transferrin asialoglycopeptide	3.4
Asialotransferrin	0.12
Fetuin asialoglycopeptide	1.7

[a] Values from Walker *et al*. [9]

linked glycans. From the three lowest concentrations for each ligand, estimates of apparent K_D values in the low nM range were obtained *i.e.* 8.4 nM and 2.8 nM, respectively. The data at higher concentrations could not be analysed to obtain Kd values but lower affinities were apparent when the data are converted to Scatchard plots (Fig. 4), *i.e.* negative cooperativity was occurring. These nM apparent affinities are ~10^5 fold higher than those of the simple ligands in Table 2, which might be taken as an indication of di- or trivalent binding by RSVL. However, the work of Dam, Brewer and their associates on the thermodynamics of lectin-glycoprotein systems [22–24] suggests other mechanisms are probably operating as well. For example, there was a 50 to 80-fold difference between the binding by a monomeric galectin of LacNAc and asialofetuin, in measurements made by isothermal titration microcalorim-

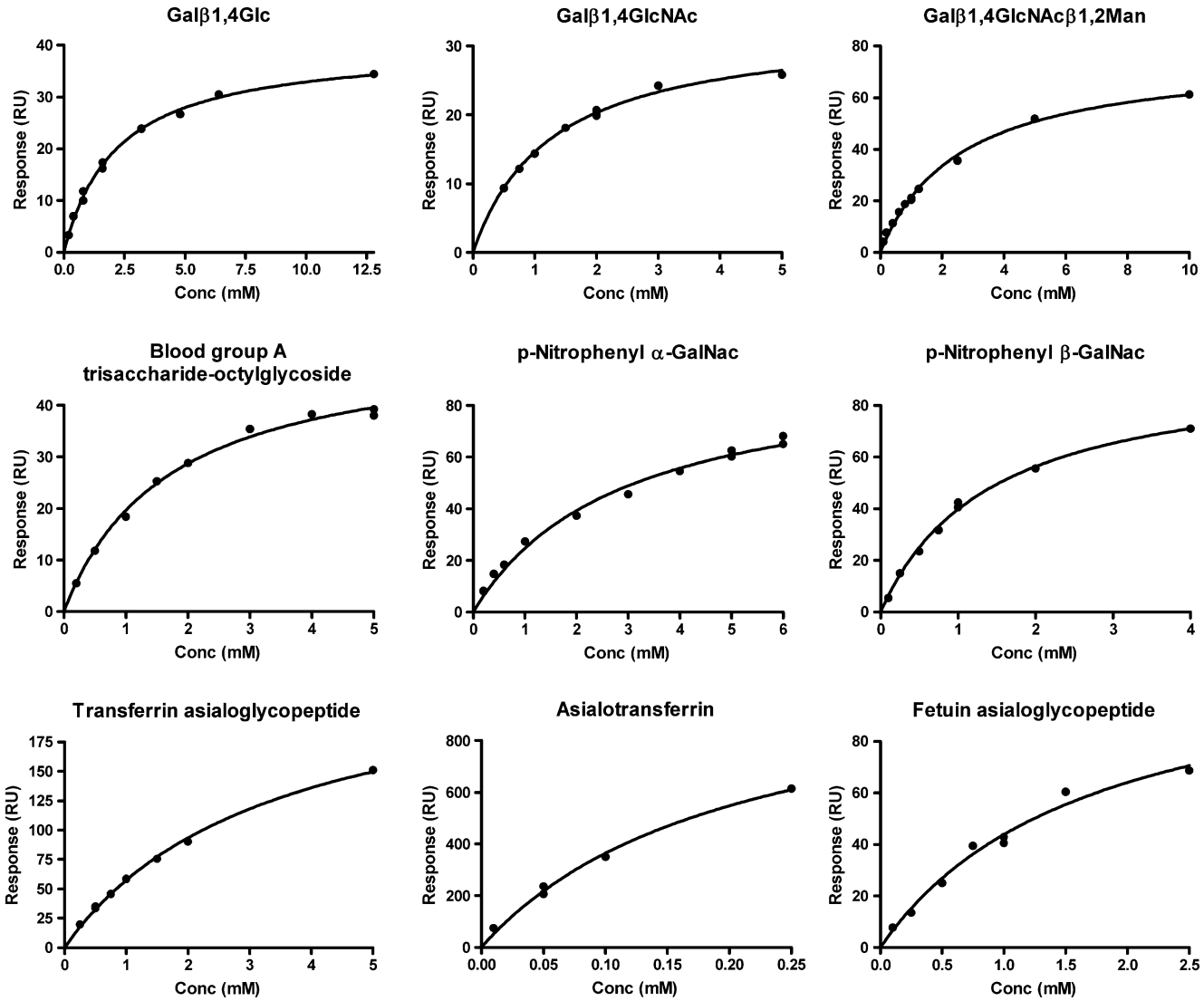

Fig. 3 Fitting of SPR equilibrium data for monovalent ligands to a steady state affinity model. The K_D values obtained from the fitted curves are given in Table 2

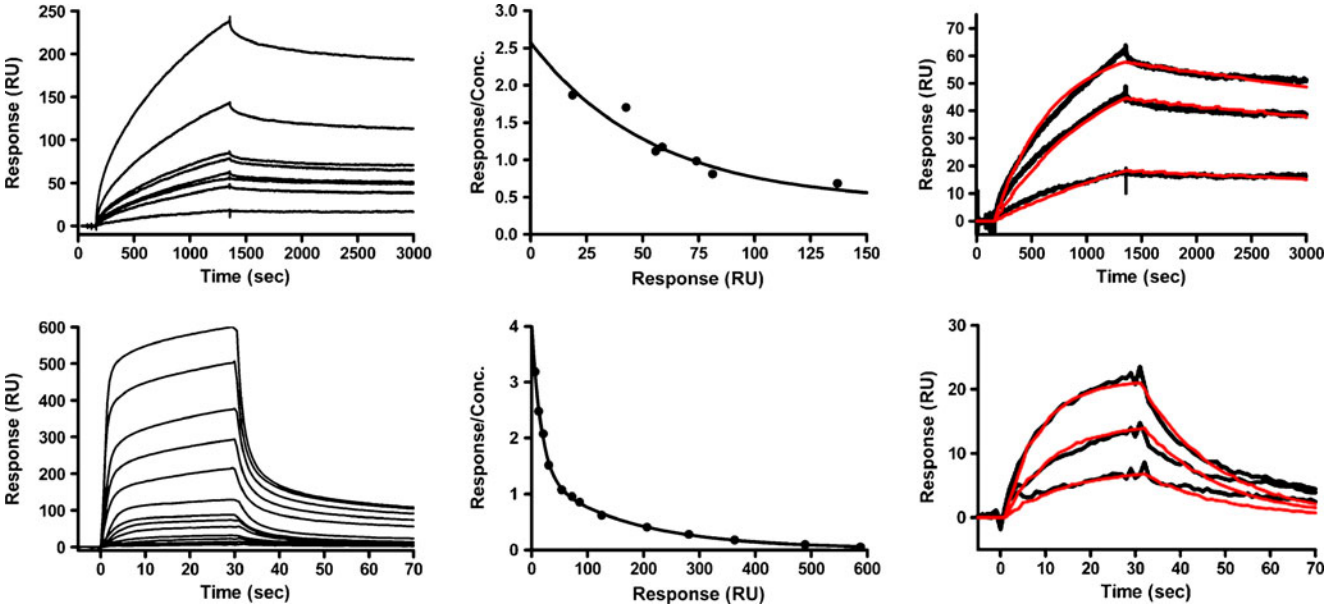

Fig. 4 SPR binding curves and analyses for Lac-Daisy and asialofetuin. *Upper row*: Asialofetuin binding data, Scatchard analysis of the average response during the 1,140–1,170 s time period, and kinetic analysis of the data from the three lowest ligand concentrations with a monovalent analyte model (*fitted curves in red*). *Lower row*: Lac-Daisy binding data, Scatchard analysis of the average response during the 20–27 s time period, and kinetic analysis of the data from the three lowest ligand concentrations with a monovalent analyte model

etry [21]. The galectin binding also showed strong negative cooperativity, with the first galectin monomer having ~6,000-fold higher affinity than the last monomer, and an estimated K_D of 10 nM for the first monomer. While similar behavior could well occur with the first molecule of RSVL, steric crowding must play a larger role in interactions with additional RSVL molecules at higher concentrations, than would be the case with a galectin, due to the decamer's greater size. A more elaborate form of this mechanism, which may be more pertinent to RSVL, was developed from studies of tetrameric lectins, such as SBA, binding to mucins [22]. In this "bind-and-jump" model, a lectin at low concentration dissociates and then rebinds to a nearby epitope on the same glycoprotein through a second binding site, leading to a lower off-rate and thus a higher K_D. In the present case, the lower number of epitopes on asialofetuin and Lac-Daisy than on a mucin may be offset by the higher valence of RSVL compared to SBA. The large difference in the kinetic rates of the two ligands suggests their geometries are critical to the binding process, though their K_D values are similar.

The biological target of this family of venom lectins has not been identified, but it is presumably a platelet glycoprotein

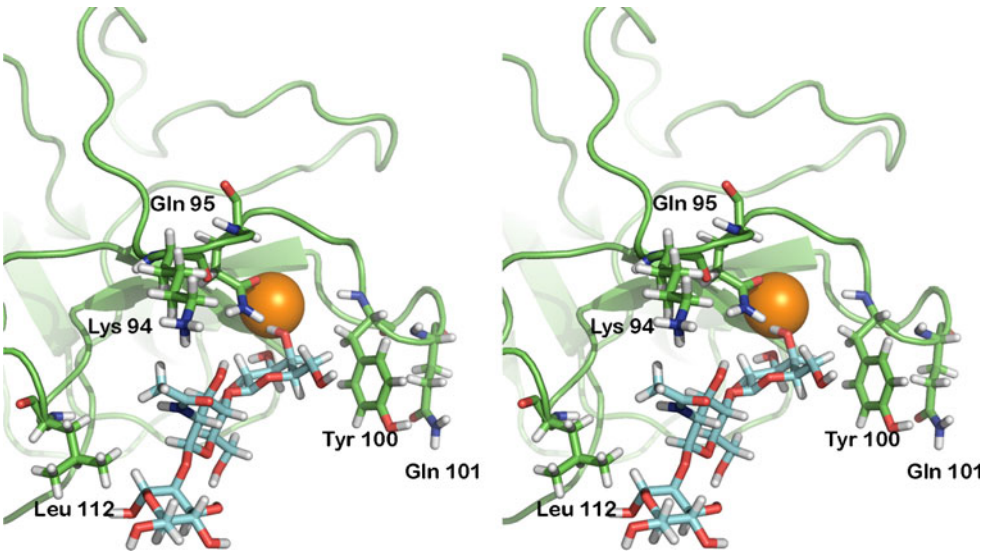

Fig. 5 A model for the binding of Galβ1,4GlcNAcβ1,2Manβ to RSVL, based on the structure of the lactose complex, PDB entry 1JZN [9]. Selected residues around the ligand and the calcium ion are shown

[19]. Our findings with these model glycoproteins suggest a cluster of glycans, either on a highly glycosylated protein or possibly a glycolipid patch, will be critical for this interaction. Its preference for multimeric glycans would help to overcome competition from other Gal- or GalNAc-terminated structures that this family of lectins might encounter within the tissues of the snake's victim. RSVL thus provides a notable example of the geometric arrangement of subunits in the C-type lectins being as important as the fine specificities of the binding-sites in creating their biological properties, and of the key role of epitope density in the biology of lectin-glycan systems [22, 23].

Acknowledgements We thank Dr Richard Alvarez, University of Oklahoma, and Dr David F. Smith, Emory University, for the glycan array analyses at the Core H facilities of the CFG (funded by NIGMS grant GM62116). We also thank the colleagues who provided materials, Tomoko Hirama for preliminary SPR measurements, Dr Jian-Jun Li and Anna Robotham for mass spectrometry, and Dr Warren Wakarchuk, Nam Huan Khieu and Tom Devecseri for assistance with the figures.

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