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Exquisite binding specificity of *Sclerotium rolfsii* lectin toward TF-related O-linked mucin-type glycans

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Abstract *Sclerotium rolfsii* lectin (SRL), a secretory protein from the soil borne phytopathogenic fungus *Sclerotium rolfsii,* has shown in our previous studies to bind strongly to the oncofetal Thomson-Friedenreich carbohydrate (Galβ1-3GalNAc-ser/thr, T or TF) antigen. TF antigen is widely expressed in many types of human cancers and the strong binding of SRL toward such a cancer-associated carbohydrate structure led us to characterize the carbohydrate binding specificity of SRL. Glycan array analysis, which included 285 glycans, shows exclusive binding of SRL to the O-linked mucin type but not N-linked glycans and amongst the mucin type O-glycans, lectin recognizes only mucin core 1, core 2 and weakly core 8 but not to other mucin core structures. It binds with high specificity to "α-anomers" but not the "β-anomers" of the TF structure. The axial C4-OH group of GalNAc and C2-OH group of Gal is both essential for SRL interaction with TF disaccharide, and substitution on C3 of galactose by sulfate or sialic acid or *N*-acetylglucosamine, significantly enhances the avidity of the lectin. SRL differs in its binding to TF structures compared to other known TF-binding lectins such as the *Arachis hypogea* (peanut) agglutinin, *Agaricus bisporus* (mushroom) lectin, Jackfruit, *Artocarpus integrifolia* (jacalin) and *Amaranthus caudatus* (Amaranthin) lectin. Thus, SRL has unique carbohydrate-binding specificity toward TF-related O-linked carbohydrate structures. Such a binding specificity will make this lectin a very useful tool in future structural as well as functional analysis of the cellular glycans in cancer studies.

Keywords *Sclerotium rolfsii* lectin · Mucins · Glycan array · Thomson Friedenreich antigen · *O*- and *N*-glycans · Ceruloplasmin

Abbreviations

ABL	*Agaricus bisporus* lectin
ACA	*Amaranthus caudatus* agglutinin
AGP	α-1 Acid glycoprotein
ELLA	Enzyme-Linked Lectinosorbent assay
GalNAc	*N*-acetylgalactosamine
GBP	Glycan Binding Proteins
GIPC	Glycosyl Inositol Phosphoryl Ceramide
GlcNAc	*N*-acetylglucosamine
G	Glycan
GM1	Ganglioside GM1
LacNAc	*N*-acetyllactosamine
Neu5Ac	*N*-acetylneuraminic acid
PNA	Peanut agglutinin
RFU	Relative Fluorescence Units
SRL	*Sclerotium rolfsii* lectin
TF	Thomson Friedenreich
core 1	Galβ1-3GalNAcα
core 2	Galβ1-3 (GlcNAcβ1-6) GalNAcα
core 3	GlcNAcβ1-3GalNAcα
core 4	GlcNAcβ1-3 (GlcNAcβ1-6) GalNAcα
core 6	GlcNAcβ1-6GalNAcα
core 8	Galα 1-3GalNAcα

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Introduction

Lectins are carbohydrate binding proteins and are valuable tools in decoding the information of cellular glycans on glycoproteins and glycolipids. One of the common features of lectins is that each lectin is often seemed to recognize multiple carbohydrate structures though with different specificities and affinities [1]. Understanding the carbohydrate binding specificity and affinity of each lectin will help for its effective application as useful analytic tool for studying carbohydrate structures. Thus it becomes imperative to determine the binding of a lectin with wide range of glycans and in the recent years, glycan microarray screening for glycan binding proteins has become a powerful technique [2].

Our earlier studies have shown that the *Sclerotium rolfsii* lectin (SRL), a protein secreted by the soil borne phytopathogenic fungus *Sclerotium rolfsii,* binds strongly to the oncofetal Thomsen-Friedenreich (Galβ1-3GalNAc-*O*-ser/thr, or TF) antigen [3, 4]. X-ray crystal structure of SRL in free form and in complex with GalNAc and GlcNAc determined at 1.1Å, 2.0Å and 1.7Å, respectively, revealed the presence of two distinct carbohydrate-binding sites, a primary site exclusively binding to GalNAc and the secondary site binding to GlcNAc only [5]. We have shown that the interaction between SRL and its putative receptor, glycosyl inositol phosphoryl ceramide (GIPC), play key role in the development and morphogenesis of *Sclerotium rolfsii* [6]. Since the oncofetal TF antigen is a pan-carcinoma antigen and increasingly expressed in all types of human cancer cells [7], the strong binding of SRL to this cancer-associated carbohydrate structure prompted us to further characterize the carbohydrate binding property of this lectin.

Materials and methods

Materials

Carboxy methyl cellulose and *N*-hydroxysuccinimidobiotin were obtained from Sigma Chemical Co., St. Louis, USA. Superdex G-75 was purchased from GE Healthcare Bio-Sciences, Sweden. Schott Nexterion H slides (Schott Cat. No. 1070936B) were printed at the Consortium for Functional Glycomics, Carbohydrate Synthesis/Protein Expression Core D at the Scripps Research Institute, USA.

Purification of SRL

Sclerotium rolfsii lectin (SRL) was purified from the sclerotial bodies of the fungus as described earlier [3]. In brief, SRL was extracted with 50 mM acetate buffer containing 100 mM NaCl (pH 4.3) and subjected to 30% methanol precipitation, then passed through CM-cellulose column equilibrated with extraction buffer. The bound proteins were eluted with salt gradient from 100 to 600 mM of NaCl in the equilibration buffer. The protein peak fractions with hemagglutinating activity were pooled, dialyzed against 25 mM ethylene diamine-acetate buffer (pH 4.5) and further the lectin was purified on Superdex G-75 gel filtration column equilibrated with 50 mM Tris-HCl, pH 7.2.

Biotinylation of SRL

Biotinylation of SRL was carried out essentially according to the procedure described by Duk *et al.* [8]. In brief, the lectin (2 mg/ml in 50 mM phosphate buffered saline; PBS) was mixed with 20 fold molar excess of *N*-hydroxysuccinimidobiotin prepared in DMSO and incubated for 1 h at room temperature. The biotinylated lectin was dialyzed for 2-3 h against distilled water and then dialyzed extensively against tris buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.2) at 4°C. After dialysis, the protein concentration was estimated by the method of Lowry *et al.* [9] and used for glycan array analysis.

Glycan array screening

The carbohydrate binding specificity of SRL by Glycan micro array analysis was performed on printed glycan array slides using a panel of 285 glycans including six glycoproteins at the Consortium for Functional Glycomics [www.functionalglycomics.org].

Slide conditioning

Preprinted slides (Glycan concentration: 100 μM) were soaked in deionized water for 5 min at room temperature and dried under a stream of nitrogen.

Assay Buffers: TSM Buffer (20 mM Tris-HCl or MOPS, pH 7.4 containing 150 mM NaCl, 2 mM $CaCl_2$ 2 mM $MgCl_2$), TSM Wash Buffer (TSM buffer + 0.05% Tween 20) and TSM Binding Buffer (TSM buffer + 0.05% Tween 20+ 1% BSA).

Binding assay

Biotin labeled SRL was screened on the Consortium printed array as described by Blixt *et al.* [2]. Briefly, the biotinylated SRL (0.5 μg/ml) was diluted in binding buffer and 50 μl was applied to the conditioned pre-printed surface of the slide and cover-slipped. The slide is incubated in a humidified chamber for 1 h at room temperature, protected from light. Slides were removed and washed after removing cover slip for 10 s each in washing buffer, washing buffer lacking Tween 20 and deionized water. The slides were dried under a stream of

nitrogen. The binding image (Relative florescent units; RFU) was read in a Perkin Elmer Microscan array XL4000 scanner and TIFF file of image was stored. Finally, data were plotted by using Microsoft EXCEL software.

Results

A complete list of glycans and the RFU values obtained for the SRL binding, along with statistical analysis are available at http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_840.

Interaction of SRL with TF disaccharide and its substituted forms

The binding affinities of SRL with substituted TF disaccharides are listed in Table 1, and to compare the affinity of SRL to various glycans, a RFU value ≥27000 (50% of highest binding affinity shown for G# 32) is arbitrarily considered as significant binding. SRL showed very strong binding affinity to TF disaccharide (Galβ1-3GalNAcα-Sp8) and oligosaccharides containing TF disaccharide when compared to other glycans, which lack this moiety. Binding of SRL toward TF disaccharides was increased 1.40, 1.38 and 1.26-fold, respectively when the C3 position of galactose was substituted by sulfate (G# 32; *[3OSO3]* Galβ1-3GalNAcα–Sp8), *N*-acetylneuraminic acid (G# 202; *Neu5Acα2-3*Galβ1-3GalNAcα-Sp8) or *N*-acetylglucosamine (G# 163; *GlcNAcβ1-3*Galβ1-3GalNAcα-Sp8) as compared to TF disaccharide (1.0 fold).

SRL also showed increased binding to TF disaccharide when the C6 of GalNAcα was substituted by *N*-acetylglucosamine (G# 121; Galβ1-3 *(GlcNAcβ1-6)* GalNAcα-Sp8; 1.13-fold increase), *N*-acetylneuraminic acid (G# 122; Galβ1-3*(Neu5Acα2-6)*GalNAcα-Sp8; 1.13-fold increase), disaccharide, Galβ1-4GlcNAc (G# 120; Galβ1-3*(Galβ1-4GlcNAcβ1-6)*GalNAcα-Sp8; 1.20-fold increase) and Neu5Acα2-3Galβ1-4GlcNAc (G# 277; Galβ1-3*(Neu5Acα2-3Galβ1-4GlcNAcβ1-6)* GalNAc-T; 1.16-fold increase).

In contrast, substitution of TF disaccharide on C2 position of galactose by fucose (G# 58; *Fucα1-2*Galβ1-3GalNAcα–Sp8; 0.40) or *N*-acetylglucosamine (G# 158; *GlcNAcβ1-2*Galβ1-3GalNAcα–Sp8; 0.06) drastically reduced the SRL binding. Also substitution on the C6 hydroxyl group of GalNAc by Neu5Ac through β 2-6 linkage (G# 256; *Neu5Acβ2-6* (Galβ1-3) GalNAcα–Sp8) abolished the binding, but substitution by Neu5Ac through α2-6 linkage (G# 241; *Neu5Acα2-6* (Galβ1-3) GalNAcα–Sp8) did not make much of difference in binding.

Similarly, substitution of TF disaccharide on C6 of GalNAc by Neu5Ac with β2-6 linkage (G# 123; Galβ1-3 *(Neu5Acβ2-6)* GalNAcα–Sp8) markedly reduced SRL binding while the substitution by Neu5Ac with α2-6 linkage (G# 122; Galβ1-3 *(Neu5Ac α2-6)* GalNAcα-Sp8) did not affect SRL binding. These findings indicated that the C2 hydroxyl group of the terminal galactose in TF

Table 1 Binding of SRL towards TF disaccharide and its variants

G#	Variants of TF disaccharides	RFU	Preferential binding[a]
32	[3OSO3]Galβ1-3GalNAcα–Sp8	53789	1.40
220	Neu5Acα2-3Galβ1-3 [6OSO3] GalNAcα-Sp8	52862	1.38
202	Neu5Acα2-3Galβ1-3GalNAcα-Sp8	51164	1.33
163	GlcNAcβ1-3Galβ1-3GalNAcα-Sp8	48442	1.26
120	Galβ1-3(Galβ1-4GlcNAcβ1-6) GalNAcα-Sp8	45889	1.20
277	Galβ1-3(Neu5Acα2-3Galβ1-4GlcNAβ1-6)GalNAc-T	44627	1.16
121	Galβ1-3(GlcNAcβ1-6)GalNAcα-Sp8	43476	1.13
122	Galβ1-3(Neu5Acα2-6)GalNAcα-Sp8	43402	1.13
221	Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAcα–Sp8	41598	1.08
150	Galβ1-4GlcNAcβ1-6(Galβ1-3) GalNAcα–Sp8	38990	1.02
125	Galβ1-3GalNAcα-Sp8(TF antigen)	38207	1.00
241	Neu5Acα2-6 (Galβ1-3)GalNAcα–Sp8	33000	0.86
275	Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAc-T	32707	0.85
276	Galβ1-3(GlcNAcβ1-6)GalNAc-T	29393	0.76
174	GlcNAcβ1-6 (Galβ1-3) GalNAcα–Sp8	23652	0.61
123	Galβ1-3 (Neu5Acβ2-6)GalNAcα-Sp8	15537	0.40
58	Fucα1-2Galβ1-3GalNAcα–Sp8	1603	0.40
256	Neu5Acβ2-6 (Galβ1-3)GalNAcα–Sp8	1266	0.30
158	GlcNAcβ1-2Galβ1-3GalNAcα–Sp8	239	0.06

Sp8 (-$CH_2CH_2CH_2NH_2$), T (Threonine)

[a] fold increase in binding compared to Galβ1-3GalNAcα-Sp8

Table 2 SRL binding towards different "anomers" of TF disaccharide

G#	"Anomers" of TF disaccharides	RFU
125	Galβ1-3GalNAcα-Sp8 (TFβα)	38207
101	Galα1-3GalNAcα-Sp8 (TFαα)	15097
126	Galβ1-3GalNAcβ–Sp8 (TFββ)	1009
102	Galα1-3GalNAcβ–Sp8 (TFαβ)	496

disaccharide play critical role in SRL-TF interaction where as free hydroxyl of C6 in Gal and GalNAc is not absolutely essential for binding of TF disaccharide but interestingly linkage specificity of Neu5Ac play important role.

Binding specificity of SRL to TF-related glycans

The binding affinities of SRL toward four different anomers of TF disaccharide (Table 2) were compared. TFβα and TFββ are naturally occurring glycans, while TFαα and TFαβ are synthetic glycans. It was found that SRL binding is highly specific for "α" anomer [TFβα] TF stucture (G# 125), which is predominantly found in glycoproteins. Compared with its binding to "α" anomer TF, SRL binding to β linked TF disaccharide (TFββ), which is exclusively found in glycolipids, was much weaker. The binding of SRL toward TF-containing structures is in the sequence of TFβα >>>TFαα >> TFββ > TFαβ. Thus, SRL binds in great preference to TF structures in glycoproteins than that in glycolipids.

Binding of SRL to core O-linked mucin-type glycans

SRL binds to core 1 (G# 125; Galβ1-3GalNAcα-Sp8) and its GlcNAc substituted core 2 (G# 121; Galβ1-3 (GlcNAcβ1-6) GalNAcα-Sp8) structures with similar ability (Table 3). SRL also showed weak binding to core 8 structure but negligible binding towards core 3, 4 and 6 structures (Binding of SRL to mucin core 5 and 7 were not determined).

Binding of SRL to glycoproteins

Amongst the six glycoproteins tested (Table 4), SRL showed strong binding to ceruloplasmin, a copper carrying serum protein and α-1 acid glycoprotein (AGP). SRL showed no binding to the ConA bound (AGP-B) or unbound (AGP-A) AGP fractions and also it did not show any binding to transferrin and fibrinogen.

Table 4 Affinity of SRL towards different glycoproteins

G#	Glycoproteins	RFU
4	Ceruloplasmin	42353
1	Alpha1-acid glycoprotein (AGP)	31541
2	AGP-A (AGP ConA flow through)	6608
6	Transferrin	1880
3	AGP-B (AGP ConA bound)	1605
5	Fibrinogen	846

Binding of SRL to N-glycans

Amongst the various N-linked oligosaccharides tested by the glycan array analysis, none of these oligos bind to SRL except one trisaccharide (G# 156, GlcNAcα1-3Galβ1-4GlcNAcβ-Sp8) (Table 5). These finding are in consistence with the observed binding specificity of SRL towards O-linked mucin-type glycans. Interestingly, although SRL binds to GlcNAcα1-3Galβ1-4GlcNAcβ-Sp8 (G# 156), it does not bind to a closely-related glycan, GlcNAcα1-6Galβ1-4GlcNAcβ-Sp8 (G# 157) where the terminal GlcNAc is α1–6 linked to the galactose (Table 5).

Binding of SRL to monosaccharides

SRL showed no significant binding to any of the monosaccharides tested in the glycan array including galactose and *N*-acetylgalactosamine (Table 6), components of TF disaccharide.

Comparison of SRL binding specificity with other known TF-binding lectins

The binding affinity of SRL toward some of the glycans was compared with several known TF binding lectins such as, ACA, ABL, PNA and Jacalin. Glycan array analysis data for

Table 3 Comparative binding of SRL towards mucin core structures

G#	Mucin core structures	Mucin cores	RFU
121	Galβ1-3(GlcNAcβ1-6) GalNAcα-Sp8	2	43476
125	Galβ1-3GalNAcα-Sp8	1	38207
101	Galα1-3GalNAcα-Sp8	8	15097
161	GlcNAcβ1-3GalNAcα–Sp8	3	2632
175	GlcNAcβ1-6GalNAcα–Sp8	6	1649
159	GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα–Sp8	4	367

Table 5 Binding of SRL to different N-linked oligosaccharides

G#	N-glycans	RFU
50	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Gly	1914
51	GlcNAcβ1-2Manα1-3(GlcNAcβ1-2Manα1-6)Manβ1-4 GlcNAcβ1-4 GlcNAcβ-Gly	189
52	Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4 GlcNAcβ1-4GlcNAcβ-Gly	157
53	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-Galβ1-4 GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Gly	125
54	Neu5Acα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Neu5Acα2-Galβ1-4 GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp8	94
65	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ-Sp0	201
79	GalNAcα1-3(Fucα1-2)Galβ1-3GlcNAcβ-Sp0	301
146	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ–Sp0	169
156	GlcNAcα1-3Galβ1-4GlcNAcβ-Sp8	39063
157	GlcNAcα1-6Galβ1-4GlcNAcβ-Sp8	352
160	GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ–Sp8	687
173	GlcNAcβ1-4GlcNAcβ1-4GlcNAcβ–Sp8	4677
188	Kdnα2-3Galβ1-4GlcNAcβ–Sp0	116
192	Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα2Manα 1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N	120
193	Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα2Manα2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ-N	103
208	Neu5Acα2-3(6OSO3)Galβ1-4(Fucα1-3) GlcNAcβ–Sp8	271
216	Neu5Acα2-3Galβ1-3(6OSO3)GlcNAcβ-Sp8	1956
230	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ–Sp0	152
233	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4 GlcNAcβ-Sp8	1923
247	Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ-Sp0	1638
255	Neu5Acβ2-6Galβ1-4GlcNAcβ-Sp8	433
285	Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-3GlcNAcβ-Sp0	156

Sp0 ($-CH_2CH_2NH_2$), Sp8 ($-CH_2CH_2CH_2NH_2$), N (Asparagine) and Gly (Glycine)

Table 6 Binding of SRL to different monosaccharides

G#	Monosaccharides	RFU
16	β-Neu5Ac-Sp8	904
8	α-D-Glc–Sp8	609
23	β-D-GlcN (Gc)-Sp8	551
10	α-D-GalNAc–Sp8	501
14	α-Neu5Ac–Sp8	441
11	α-L-Fuc–Sp8	420
9	α-D-Man–Sp8	352
13	α-L-Rhα–Sp8	234
22	β-GlcNAc–Sp8	231
18	β-D-Glc–Sp8	223
17	β-D-Gal–Sp8	205
12	α-L-Fuc–Sp9	199
21	β-D-GlcNAc–Sp0	172
20	β-D-GalNAc–Sp8	154
7	α-D-Gal–Sp8	139
15	α-Neu5Ac–Sp11	63
19	β-D-Man–Sp8	63

Sp0 ($-CH_2CH_2NH_2$), Sp8 ($-CH_2CH_2CH_2NH_2$), Sp9 ($-CH_2CH_2CH_2CH_2CH_2NH_2$), Sp11 ($-OCH_2C_6H_4$-*p*- $NHCOCH_2NH$)

these lectins available from the Glycoconsortium data base was used for the comparison (ABL -PA_v2_351_10102005, PNA-PA_v1_366_08312005, ACA -PA_v1_110_06302005 and Jacalin-PA_v2_360_11042005). SRL showed differences in its binding with all these TF-binding lectins. Notably, it binds more strongly to the sulfate substituted TF structure at terminal galactose than any of the other TF-binding lectins.

Various spacer arms; Sp0 ($-CH_2CH_2NH_2$), Sp8 ($-CH_2CH_2CH_2NH_2$), Sp9 ($-CH_2CH_2CH_2CH_2CH_2NH_2$), Sp10 ($-NHCOCH_2NH$), Sp11 ($-OCH_2C_6H_4$-*p*-$NHCOCH_2NH$), T (Threonine), N (Asparagine) and Gly (Glycine) attached to glycans in the assay, did not show any significant influence on SRL binding.

Discussion

This study shows that SRL binds exclusively to O-linked but not to N-linked glycans. SRL shows strong binding to the TF-related O-linked carbohydrate structures in particular the core 1 and core 2 structures. Amongst the various TF glycans tested, SRL shows specific binding to only α-anomers found in glycoprotein but not to the β-anomers

occurring in glycolipids. The axial C4-OH group of GalNAc and C2-OH group of Gal in the TF structure is essential for the SRL interaction with TF. Substitution on C3 of Gal by sulphate, sialic acid or *N*-acetylglucosamine, significantly enhances the avidity of SRL binding.

Our earlier studies on SRL carbohydrate binding by hemagglutination and enzyme linked lectinosorbent assays (ELLA) have revealed binding of SRL to O-linked glycans and many glycoproteins containing them [3, 4]. The present study by the more powerful glycan array analysis further confirms the binding preference of SRL toward O-linked glycans but not N-linked sugar chains and also revealed the structural requirements for the binding. Property of SRL to distinguish O- and N-linked glycans makes it a valuable tool in carbohydrate structural studies.

It is interesting to note high binding affinity of SRL towards ceruloplasmin, a copper containing glycoprotein with ferroxidase activity known to carry several Asn linked bi and tri antennary N-glycans [10, 11]. However, there are no reports on the occurrence of O-linked glycans in ceruloplasmin so far. Considering the high specificity of SRL to O-linked glycans and its strong binding to ceruloplasmin suggests that ceruloplasmin is most likely to contain O-linked glycans. SRL did not bind well with transferrin and fibrinogen as they predominantly contain N-linked oligosaccharides [12]. Lectin also showed weak binding to ConA bound and unbound acid glycoprotein fractions. The bound fraction is known to contain only bi-antennary and the unbound fraction contain tri as well tetra-antennary *N-glycans*. However it is intriguing to note higher affinity of SRL to unfractionated acid glycoprotein (AGP).

Present study reveals that substitution on C3 of terminal galactose by negatively charged groups (SO_3 and Neu5Ac) markedly influences on SRL binding compared to the neutral group (GlcNAc). Also, various substitutions on C6 of core GalNAc favor the SRL binding. Considering the presence of His70 and Arg105 at the primary active site of SRL revealed by X-ray crystal structure [5], it may be concluded that these two positively charged residues reinforce the binding apart from H-bonds formed by Ala28, Thr49, Val69, Trp75, Tyr96 and Glu106.

Four other well characterized lectins known to recognize the TF structures are ACA, ABL, PNA and Jacalin [13–17]. In comparison with these TF binding lectins, SRL shows differences in its carbohydrate binding properties and the observed differences are summarized in Table 7. SRL binds to core 1 and core 2 mucin type glycans with high affinity compared to other core structures (core 3, 4, 6 and 8). Unlike SRL, ACA, ABL and PNA recognize core 1 and core 2 structures with equal affinities [http://www.functionalglycomics.org/glycomics/publicdata]. Also these lectins show binding to core 3, whereas Jacalin recognizes only core 1 and core 3 structures [17]. Enhanced binding property of SRL towards TF disaccharide with $3OSO_3$ substitution on C3 of galactose is similar to that of ACA and Jacalin, but ABL show weak binding, whereas PNA does not bind to [$3OSO_3$] Galβ1-3GalNAcα–Sp8 [13]. Increased affinity shown by SRL for the TF disaccharide with substitution on C6 of GalNAc by $3OSO_3$ or sialic acid or *N*-acetylglucosamine is similar to that of ACA and Jacalin, but different from PNA and ABL.

Recently, it has been demonstrated that human galectin-1 [18] and galectin-3 [19] also recognize the TF structure although they are specific to Galβ1-4GlcNAc. Sulfation of TF disaccharide enhances the binding of galectin-1 [20], galectin-3 and galectin-4 [19, 21, 22] and also the binding of many siglecs such as siglec-1, siglec-4 and siglec-8 [23]. Sulfation of TF is an important event and its role in cell adhesion, bacterial binding and regulation of biosynthetic pathways has been reported [24, 25]. Addition of $3OSO_3$ to

Table 7 Comparative binding of SRL with other TF antigen binding lectins

G#	Glycans	SRL	ABL	PNA	ACA	JAC
278	Galβ1-3GalNAcα (TFα)	+++	+++	+++	+++	+++
126	Galβ1-3GalNAcβ (TFβ)	–	++	++	–	–
202	Neu5Acα2-3Galβ1-3GalNAcα	+++	+++	–	+++	+++
122	Galβ1-3(Neu5Acα2-6)GalNAcα	++++	–	+++	+++	NK
32	[3OSO3] Galβ1-3GalNAcα	+++++	+++	–	NK	NK
134	Galβ1-3GlcNAc	–	–	+++	–	NK
153	Galβ1-4GlcNAc	–	++	+++	–	–
125	Galβ1-3GalNAcα-Sp8	++	+++	++	+++	+++
276	Galβ1-3(GlcNAcβ1-6)GalNAcα	+++	++	+++	NK	–
161	GlcNAcβ1-3GalNAcα	–	–	++	–	++
17	Galactose	–	++	+++	++	++
20	*N*-acetylgalactosamine	–	++	++	++	++

NK Not known

the terminal galactose of core 1 structure prevents the branching reaction to form core 2 structure. Thus, the very strong binding affinity exhibited by SRL toward sulfated TF structure makes SRL an important tool in studying such kind of oligosaccharides.

Although SRL recognizes TF disaccharide (Galβ1-3GalNAc) and its substituted forms, it does not recognize disaccharides like Lacto-*N*-biose (G# 133; Galβ1-3GlcNAc) and LacNAc (G# 152; Galβ1-4GlcNAc), which are conformationally related disaccharides. This is in contrast to the binding of ABL [14], Jacalin [16] and ACA [13]. On the other hand PNA recognizes both the disaccharides; Lacto-*N*-biose and LacNAc [15]. This binding property of SRL demonstrates the crucial requirement of axially oriented C-4 hydroxyl group of the GalNAc residue in Galβ1-3GalNAc for SRL binding. This is possibly due to the inversion of C-4 hydroxyl group in lacto-*N*-biose (Galβ1-3GlcNAc). It also suggests that the major stabilizing force for the binding of Galβl-3GalNAc to the SRL is provided by the C-4 hydroxyl group of the GalNAc.

SRL does not recognize GlcNAcβ1-2Galβ1-3GalNAcα–Sp8 (G# 158) and Fucα1-2Galβ1-3GalNAcα–Sp8 (G# 58) but binds to GalNAcβ1-3GalNAcα–Sp8 (G# 88) with moderate affinity. This suggests that C2 substitution on galactose by a bulkier group is not favorable for SRL binding possibly due to steric hindrance. In contrast, substitution on C3 of galactose by SO_3, sialic acid and GlcNAc significantly favored the SRL binding. Unlike that of SRL, the substitution on C2 of galactose does not impair the binding of ACA and Jacalin, whereas the binding of ABL and PNA show considerable intolerance for C2 substitution on TF disaccharide.

In sharp contrast to ABL and PNA but similar to Jacalin and ACA, SRL recognizes only the "α anomer" of TFD (Galβ1-3GalNAcα-Sp8; TFβα) but not the corresponding "β anomer" (Galβ1-3GalNAcβ–Sp8; TFββ) [13, 17]. SRL, like Jacalin, also does not recognize asialo-GM1 (G# 129; Galβ1-3GalNAcβ1-4Galβ1-4Glcβ–Sp8) [16], although this oligosaccharide contains TFD in β-linkage whilst ABL binds to this tetrasaccharide [14]. Thus SRL resembles Jacalin and ACA more closely than PNA and ABL in binding to TF antigen.

SRL does not show binding to any of the monosaccharides including galactose and *N*-acetylgalactosamine. This property is very different from the other known TF antigen binding lectins. For example, PNA [26] binds to galactose while ACA [13], ABL [14] and Jacalin [16] bind to GalNAc. In the light of these observed differences in binding specificities with other known TF binding lectins SRL could be a valuable reagent in the future and warrant for more elaborate investigations.

As SRL does not bind to any glycans with GlcNAc as the core residue, it is intriguing to note the binding of SRL to GlcNAcα1-3Galβ1-4GlcNAcβ-Sp8 (G# 156). This may be related to the secondary binding site of SRL, which has shown in our earlier study to be exclusive to GlcNAc [5]. Similar binding was reported for ABL which recognizes galactosylated GlcNAc of N-glycans [27].

Thus, SRL showed unique carbohydrate-binding specificity toward TF-related O-linked carbohydrate structures. Such a binding specificity of SRL makes this lectin a very useful tool for future structural as well as functional analysis of cellular glycans in cancer studies.

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