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Carbohydrate Specificity of Chicken and Human Tandem-Repeat-Type Galectins-8 in Composition of Cells

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Abstract—The network of adhesion/growth-regulatory galectins in chicken (chicken galectin, CG) has only one tandem-repeat-type protein, CG8. Using a cell-based assay and probing galectin reactivity with a panel of fluorescent neoglycoconjugates (glycoprobes), its glycan-binding profile was determined. For internal validation, human galectin-8 (HG8) was tested. In comparison to HG8, CG8 showed a rather similar specificity: both galectins displayed high affinity to blood group ABH antigens as well as to 3′-sialylated and 3′-sulfated lactosamine chains. The most remarkable difference was found to be an ability of HG8 (but not CG8) to bind the disaccharide Galβ1-3GlcNAc (Le^c) as well as branched and linear oligolactosamines. The glycan-binding profile was shown to be influenced by glycocalix of the cell, where the galectin is anchored. Particularly, glycosidase treatment of galectin-loaded cells led to the change of the profile. Thus, we suppose the involvement of *cis*-glycans in the interaction of cell-anchored galectins with external glycoconjugates.

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Key words: ABH blood group antigens, galectin, cell glycoconjugates

The glycan chains of cellular glycoconjugates are a versatile biochemical platform to store biological information that can be translated into physiological effects by tissue lectins [1]. Among them, the adhesion/growth-regulatory galectins target distinct glycoconjugates to trigger signaling with integrins or receptor kinases [2-6]. From this group, galectin-8 (G8) is currently receiving special attention since: i) it is a potent inducer of apoptosis on tumor cells and in inflammation on synovial fluid cells (here interacting with $\alpha_3/\alpha_6\beta_1$-integrins or the CD44*v*RA variant, respectively); ii) it is a bimodal activator of $CD4^+$ T cells, and iii) a modulator of lymphatic endothelial functions [7-12]. Finally, galectin-8 is the only tandem-repeat-type protein in the galectin network of the chicken [13], indicating a distinct functionality for this type of display of the CRD (carbohydrate recognition domain), as also supported by its distinct expression profile in adult organs [13-15]. Tandem-repeat-type galectins have two homological but not identical CRD (N- and C-CRD) [7]. Due to asymmetry, the lectin potentially can expose on cells surface one or another CRD, thus re-switching ability of the cell to recognize external glyco-molecules; no doubt, this feature of tandem galectin should complicate interpretation of data on binding of external ligands.

Knowledge about specificity of galectins associated with cell membrane is limited. Artificial test systems sometimes do not reflect the real situation when galectin is anchored on a cell surface due to interaction with complementary ligands. We have suggested a cell model that allowed us to study the carbohydrate-binding profile of galectins situated on a cell surface [16]: galectins were loaded on a cell surface followed their probing with a variety of fluorescent-labeled glycoconjugates. The results were compared with data published previously [17-20]. Besides, experiments with chicken galectin loaded on different cells lines, namely Raji, MDCC-MSB1, MDCK, and MDCK6ST, reveal competition of cell glycans (*cis*-ligands) with glycoprobes (*trans*-ligands).

Abbreviations: BSA, bovine serum albumin; CG8, chicken galectin-8; CRD, carbohydrate recognition domain; FITC, fluorescein isothiocyanate; Glyc-PAA, conjugate of glycan with polyacrylamide; HG8, human galectin-8; PBA, phosphate-buffered saline (pH 7.2) containing 0.2% BSA; PBS, phosphate-buffered saline (pH 7.2).

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MATERIALS AND METHODS

Materials. The following reagents were used in the study: bovine serum albumin (BSA) (Sigma, USA); culture media RPMI-1640 and DMEM-F12, L-glutamine, antibiotic-antimycotic (Invitrogen, GB); fetal calf serum (Biohit, Germany); conjugates of anti-rabbit IgG and streptavidin with fluorescein isothiocyanate (FITC), respectively (Sigma, USA). Fluorescein-labeled glycoconjugates Glyc-PAA-fluo (M_w = 30 kDa), where PAA is polyacrylamide, Glyc is carbohydrate residue (20 mol %), and fluo is fluorescein residue (1 mol %), were obtained from Lectinity (Russia). Glycoconjugate structures (38 probes) are given in Table 1. The avian (CG8) and human (HG8) galectins-8 and specific rabbit polyclonal antibodies against them were prepared as described previously [10, 13, 21]. Biotinylated plant lectins UEA-1 (*Ulex europaeus*), LTL (*Lotus tetragonolobus*), DSA (*Datura stramonium*), and GSL-I (*Griffonia simplicifolia*) were obtained from Vector Laboratories (USA); the digoxigenin-labeled lectins SNA (*Sambucus nigra*), MAA (*Maackia amurensis*), and PNA (*Arachis hypogea*) and the conjugate of anti-digoxigenin with FITC were from Roche (Germany). Neuraminidase from *Vibrio cholerae* and β-galactosidase from *Aspergillus oryzae* were from Sigma (USA). Glycan structures recognized by these lectins are given in Table 2. All other reagents were from Reakhim (Russia).

Cells. Raji (human B-lymphocytes origin; ATCC No. CCL86), MDCC-MSB1 (chicken lymphoblastoma), and MDCK (canine kidney) were obtained from the Institute of Cytology, Russian Academy of Sciences; MDCK cells transfected with cDNA for 6-sialyltransferase (MDCK6ST) were kindly donated by Dr. Matrosovich (Marburg, Germany).

Galectin loading onto cells. Raji cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37°C in an atmosphere containing 5% CO_2; MDCC-MSB1 and MDCK cells were cultured in DMEM-F12 medium with the same additives. The cells were washed three times with PBA (phosphate-buffered saline (pH 7.2) containing 0.2% BSA) and centrifuged at 800 rpm. All following steps were performed at 4°C. Aliquots of the cells suspension ($2 \cdot 10^5$ cells in 100 µl PBA) were incubated with 50 µl of galectin-containing solution (0.4 mg/ml in PBA) for 30 min at 4°C under gentle agitation on a shaker. Then the cells were washed by centrifugation under the same conditions to remove unbound galectin.

Control of loading. Cells loaded with galectin were incubated with 50 µl of solution containing galectin-type-specific antibodies (0.01 mg/ml) for 30 min at 4°C under gentle agitation on a shaker. To measure extent of cell fluorescence a conjugate of anti-rabbit IgG labeled with FITC was added to the cell suspension (dilution 1 : 50 in PBA) followed incubation for 30 min at 4°C. Thereafter, cells were washed three times with PBA, transferred into tubes for flow cytometry analysis, and mixed with 2 ml PBS (phosphate-buffered saline (pH 7.2)). Flow cytometry was performed using a FACScan instrument (Becton-Dickinson, USA) (at 488 nm and room temperature) equipped with WinMDI 2.8 software. In each sample a total of 3000 cells were analyzed.

Probing of galectin-loaded cells with Glyc-PAA-fluo. *Direct binding.* The cells loaded with galectin ($1 \cdot 10^5$ per well in 50 µl) were incubated with 50 µl of Glyc-PAA-fluo solution in PBA (100 µM) for 40 min at 4°C under gentle agitation. Finally, the cells were washed three times with PBA to remove the unbound probe (800 rpm). Binding of Glyc-PAA-fluo was quantitated as described above.

Probing after enzyme treatment. The MDCC-MSB1 cells loaded with avian G8 were sequentially incubated with neuraminidase (4 U/ml) and β-galactosidase (2 U/ml) in DMEM-F12 medium for 3 h at 37°C, following centrifugation and then probing with LN-PAA-fluo, $(LN)_3$-PAA-fluo, or A (type 2)-PAA-fluo, as described above.

Glycophenotyping of cells. Adherent MDCK and MDCK6ST cells were harvested with Versene solution. Cell suspensions were washed three times with PBA including centrifugation at 800 rpm. Then cells ($2 \cdot 10^5$ in 100 µl) were incubated with 50 µl of lectin-containing solution, with digoxigenin (1 µg/ml) or biotin (20 µg/ml) as label, for 30 min at 4°C followed by washing with PBA and incubation with the corresponding FITC-labeled conjugates of antibodies against digoxigenin (dilution 1 : 10 in PBA) or of streptavidin (dilution 1 : 50 in PBA) for 30 min under the same conditions. Cells were then washed including centrifugation at 800 rpm and resuspended in 2 ml PBS. Binding of lectins to cells was quantitated by flow cytometry as described above.

RESULTS

Loading of galectins on cells. The Raji and MDCC-MSB1 cell lines were selected because: i) the Raji cells were negative in galectin screening by RT-PCR [22], and ii) MDCC-MSB1 cells did not interact with a panel of antibodies against galectins (not shown). Neither cell line bound neoglycoconjugates in the absence of loaded galectin, extent of the loading being controlled by the specific antibodies (Fig. 1, a-c), and this probing also ensured that no loss of bound lectin occurred by washing.

Probing of CG8 and HG8 loaded on Raji cells with Glyc-PAA-fluo. Reactivity of neoglycoconjugates was systematically assessed for both galectins presented on Raji cells (Table 1). Because of various affinities of antibodies to galectins, it is not possible to compare *quantitatively* the loading of human *vs.* avian galectins; however, we can compare their glycan-binding profiles. In particular, both CG8 and HC8 displayed affinity to $T_{\beta\beta}$, whereas bound

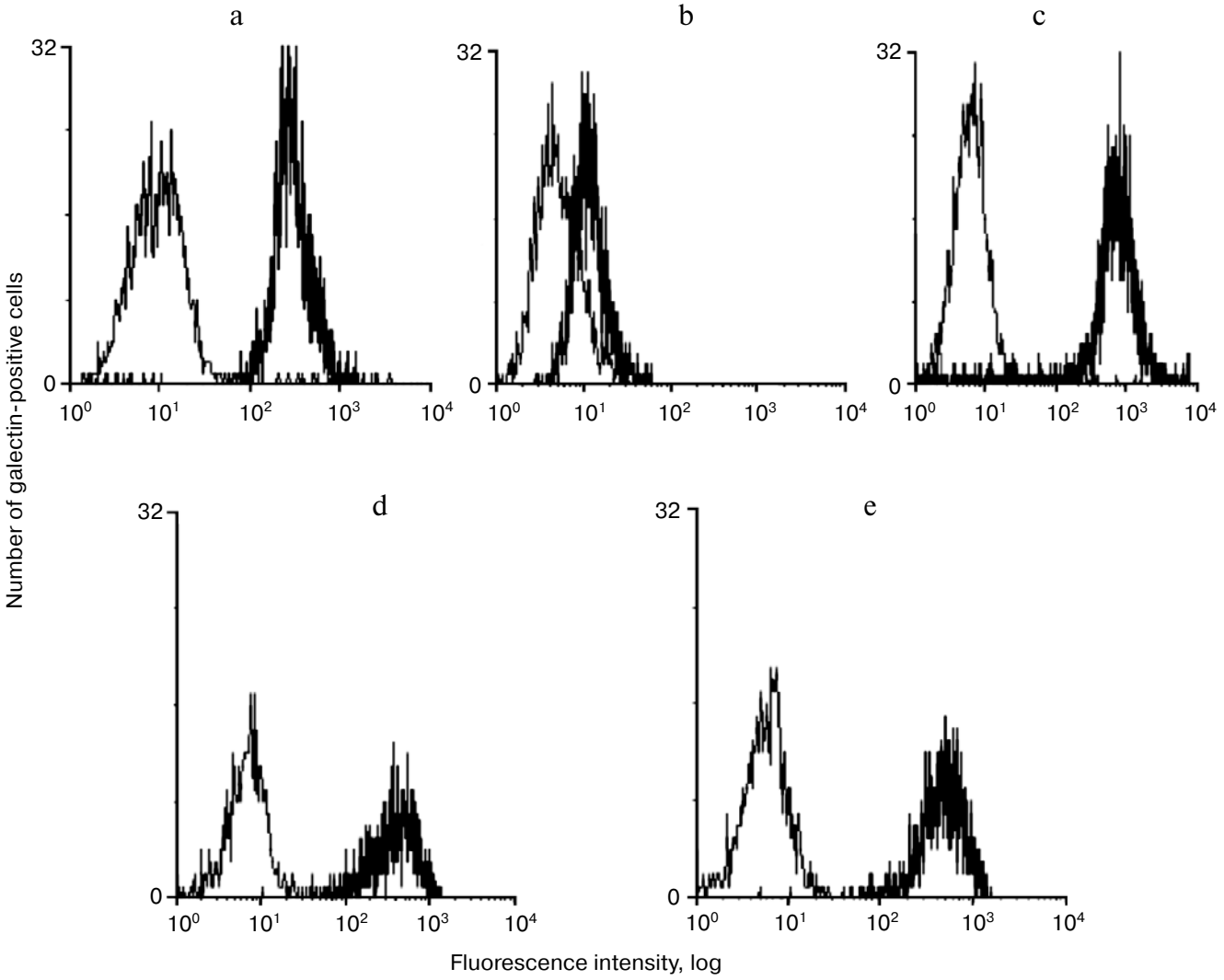


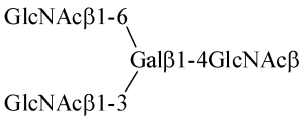
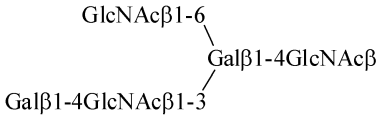
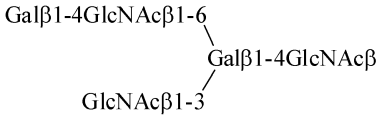
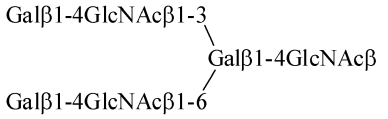
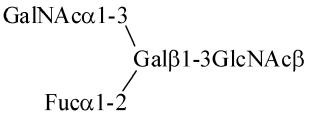
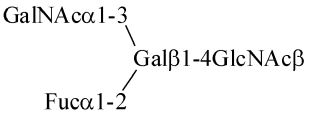
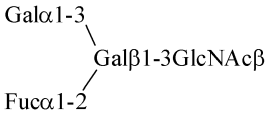
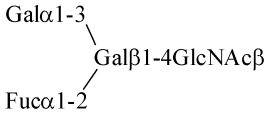
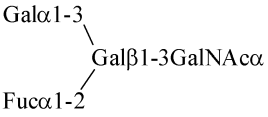
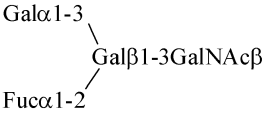
Fig. 1. Control for quantitation of galectin-8 loading onto cells by antibodies: CG8 and Raji (a), MDCC-MSB1 (c), MDCK (d), and MDCK6ST (e) cells; b) HG8 and Raji cells. Galectins were incubated with cells followed by addition of corresponding antibodies as described in "Materials and Methods". Binding of FITC-labeled anti-rabbit IgG in the absence of the incubation steps with lectin/anti-lectin antibody is the background control.

weakly to canonical disaccharide motif, LN (Fig. 2). Increase of LN repeats in the composition of the oligo-LN chain provides better binding. No binding to Le^c disaccharide was found in case of avian protein. Similarly to other galectins, binding of CG8 is tolerant to 3′-O- or 6-O-substitution of N-acetyllactosamine (Table 1, Nos. 5, 6, 8, 10, 11), but is not to substitution at C-4 or C-6 of galactose residue (Table 1, Nos. 7 and 9). The highest affinity ligands of CG8 and HG8 were A (type 2) and B (type 2) tetrasaccharides. It should be noted that homology between N- and C-CRDs is 74 and 81%, correspondingly, that provides similarity in carbohydrate-binding profiles of two proteins.

Glyc-PAA-fluo probing of CG8-loaded MDCC-MSB1 cells. We next proceeded to map the reactivity profile of CG8 loaded on the avian cells. Glycan binding patterns of CG8-loaded Raji and MDCC-MSB1 cells are similar, though the level of the binding in case of MDCC-MSB1 cells was higher (Fig. 3). Remarkable difference was observed only in the case of Le^c (Fig. 3), the binding being observed only in the case of MDCC-MSB1 cells, whereas loaded Raji cells were silent.

Glycosidase treatment of galectin loaded cells. To reveal the expected influence of glycans (*cis*-glycans) situated on the same cell where the lectin is loaded, on its glycan-binding properties, the cells were enzymatically treated to sequentially reduce their sialylation and β-galactosylation. MDCC-MSB1 cells loaded with CG8 were incubated with neuraminidase followed by β-galactosidase. Staining with SNA and DSA (plant lectins sensing α2,6-sialylation and β-galactosides, respectively) of the glycosidase-treated cells was drastically reduced (not

Table 1. Structures of oligosaccharides in composition of Glyc-PAA-fluo conjugates

No.	Glyc	Abbreviation
	Disaccharides	
1	Galβ1–4GlcNAcβ	LN
2	Galβ1–3GalNAcα	TF
3	Galβ1–3GlcNAcβ	Le^c
4	Galβ1–3GalNAcβ	$T_{ββ}$
5	3-O-Su-Galβ1–4(6-O-Su)Glcβ	3',6OSu_2Lac
6	3-O-Su-Galβ1–4GlcNAcβ	3'OSuLN
7	6-O-Su-Galβ1–4GlcNAcβ	6'OSuLN
8	Galβ1–4(6-O-Su)GlcNAcβ	6OSuLN
9	4-O-Su-Galβ1–4GlcNAcβ	4'OSuLN
10	3-O-Su-Galβ1–3GalNAcα	3'OSuTF
11	3-O-Su-Galβ1–3GlcNAcβ	3'OSuLe^c
	Trisaccharides	
12	Galα1–3Galβ1–4GlcNAcβ	Galα3'LN
13	Fucα1–2Galβ1–3GlcNAcβ	H (type 1)
14	Fucα1–2Galβ1–4GlcNAcβ	H (type 2)
15	Fucα1–2Galβ1–3GalNAcα	H (type 3)
16	Fucα1–2Galβ1–3GalNAcβ	H (type 4)
17	GalNAcα1–3(Fucα1–2)Gaβ	A_{tri}
18	Galα1–3(Fucα1–2)Galβ	B_{tri}
19	Neu5Acα2–3Galβ1–4GlcNAcβ	3'SiaLN
20	Neu5Acα2–6Galβ1–4GlcNAcβ	6'SiaLN
	Tetra-, penta- and hexasaccharides	
21	Galβ1–3GlcNAcβ1–3Galβ1–4Glcβ	LNT
22	Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ	LNnT
23	Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ	LN3'LN
24	Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAcβ	LN6'LN
25	Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ	$(LN)_3$
26		$(GlcNAc)_2$3,6'SLN
27		LN3'(GlcNAc6')LN
28		LN6'(GlcNAc3')LN
29		$(LN)_2$3',6'LN
30	Galβ1–3GlcNAcβ1–3Galβ1–4GlcNAcβ	Le^c3'LN
31	Galβ1–3GlcNAcβ1–3Galβ1–3GlcNAcβ	Le^c3'Le^c
32	Galβ1–3GlcNAcβ1–6Galβ1–4GlcNAcβ	Le^c6'LN
33		A (type 1)
34		A (type 2)
35		B (type 1)
36		B (type 2)
37		B (type 3)
38		B (type 4)

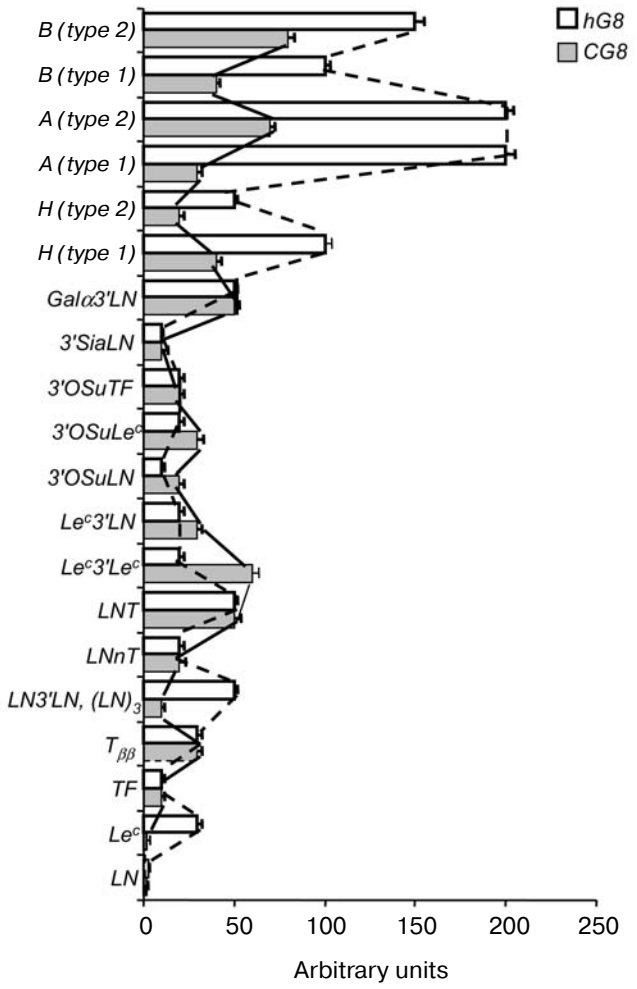


Fig. 2. Comparison of glycan-binding profiles for CG8/HG8, flow cytometry data. Galectins were loaded on the cells followed by probing with fluorescent glycoconjugates (see "Materials and Methods"). The data are presented in arbitrary units, normalized with data on TF-PAA-fluo binding due to its moderate binding.

shown). Gentle washing of cells after enzyme treatment did not lead to galectin removal (not shown). Desialylation of cells led to an increase of binding of the low-affinity probe LN-PAA-fluo as well as of moderate-affinity one, $(LN)_3$-PAA-fluo (Fig. 4). On the other hand, binding of high-affinity tetrasaccharide A (type 2) was found to be reduced by a factor of six (not shown). β-Degalactosylation did not affect binding of any of the three probes.

Probing of MDCK and MDCK6ST cells with A (type 2)-PAA-fluo. A different approach to manipulate the glycomics profile is the use of cell transfectants altered in expression of glycosyltransferases. We tested a line with engineered overexpression of α2,6-sialyltransferase. The binding of galectin-loaded MDCK6ST (and MDCC-MSB1) cells with A (type 2) probe was five times higher than MDCK cells (Fig. 5).

This difference is attributed to the stage of the glycoprobe binding rather than stage of galectin loading, because loading rates in the case of MDCK and their hypersialylated version MDCK6ST cells were comparable as evidenced in Fig. 1.

To confirm increased level of α2,6-sialylation (and other aspects of the glycophenotype), plant lectins were tested as probes.

Glycophenotyping of Raji, MDCC-MSB1, MDCK, and MDCK6ST cells. Glycans, potential "anchors" of galectins, were revealed by means of plant lectins with well-known specificity. All the four cell lines were stained with lectins SNA and MAA (Table 2): binding of Raji, MDCC-MSB1, and MDCK6ST cells with MAA (Sia2-3Gal specific) was weaker than MDCK; probing with Sia2-6-specific SNA was found to have an opposite mode. Only MDCK cells interacted with UEA-1 (recognizing Fucα1-2Gal fragment of ABH antigens), whereas neither of the cell lines bound another fucose-specific lectin, LTL. Besides, cells did not bind lectin GSL-I, rec-

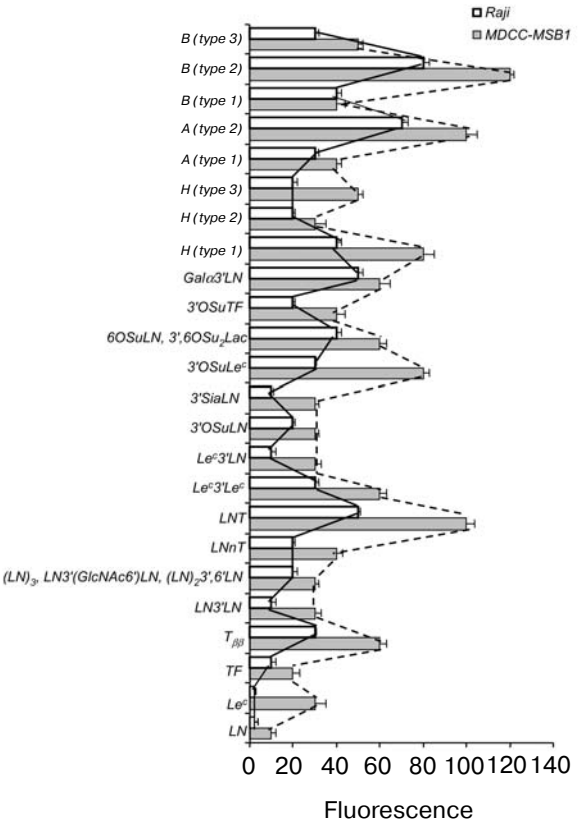


Fig. 3. Glycan-binding profiles of CG8 loaded onto Raji and MDCC-MSB1 cells. Galectin was incubated with the cells followed by staining with glycoconjugates. Fluorescence was calculated as $[(F_i/F_0) \times 100] - 100\%$, where F_i is fluorescence intensity of cells loaded with galectins and F_0 is fluorescence intensity of galectin-free cells after incubation with the fluorescent probes only, as background control.

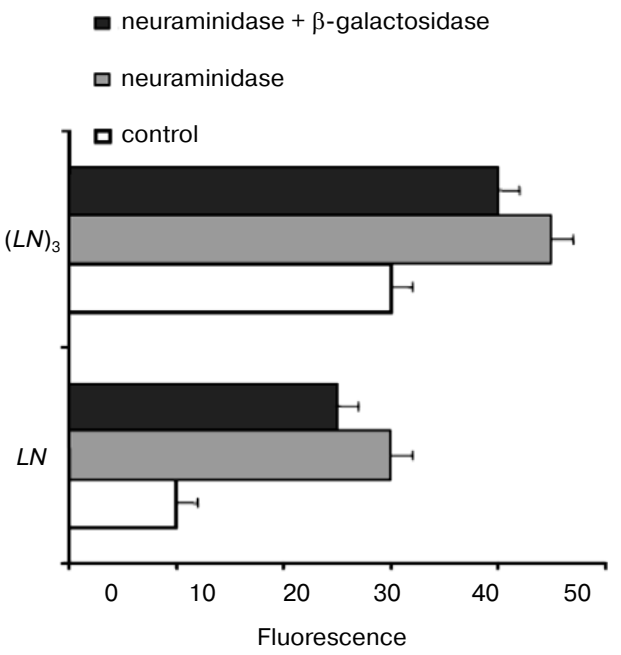


Fig. 4. Influence of glycosidase activity on glycan-binding potency of CG8 loaded onto MDCC-MSB1 cells, probing with LN-PAA-fluo and $(LN)_3$-PAA-fluo. Galectin was incubated with the cells followed by treatment with neuraminidase followed by β-galactosidase as described in "Materials and Methods"; finally, cells were stained with LN-PAA-fluo or $(LN)_3$-PAA-fluo. Fluorescence was calculated as $[(F_i/F_0) \times 100] - 100\%$, where F_i is fluorescence intensity of cells after incubation with Glyc-PAA-fluo; F_0 is fluorescence intensity of cells after incubation with FITC-labeled antibodies against digoxigenin or streptavidin as background control.

ognizing terminal Galα of blood group B glycans. Presence of β-galactosides was ascertained by positivity with DSA/PNA (Table 2). In sum, unsubstituted as well as sialylated β-galactosides are present on the surfaces of all the tested cells.

DISCUSSION

Our study uses binding of galectins that maintain reactivity to neoglycoconjugates to cells as a platform for testing. The presented data thus extend our previous experience with galectins-1 and -3 [16]. As internal validation, the experimental series with HG8 report on strong binding to histo-blood group epitopes and 3′-sialylated/sulfated cores, in accord with previous reports using e.g. microarrays and surface plasmon resonance measurements [17-20]. Running these experiments with CG8 resolves two pertinent questions. First, the reactivity profile to glycans is mostly rather similar to HG8, reflecting conservation of carbohydrate selectivity between these two tandem-repeat-type galectins and supporting their designation as orthologs. Difference was found only in respect of one glycan, Galβ1-3Gal (Le^c) disaccharide. Second, in spite of absence of blood group ABH carbohydrate chains in chickens, the highest affinity ligands for CG8 was shown to be this family of glycans; the preferential binding to histo-blood group determinants sets the profile apart from that of CG1A [23, 24], whereas human analog, HG8, demonstrates more pronounced preference towards ABH determinants com-

Table 2. Staining of Raji, MDCC-MSB1, MDCK, and MDCK6ST cell surface with plant lectins

Lectin	Specificity of lectin	Intensity of staining*			
		Raji	MDCC-MSB1	MDCK	MDCK6ST
SNA	Neu5Acα2–6Gal-	++	++++	+	++
MAA	Neu5Acα2–3Gal-	+	++	+++	++
UEA-1	Fucα1–2Gal-	–	–	++	+/–
LTL	Fucα-, Le^y-	–	–	–	–
DSA	Galβ1–4GlcNAc-	+++	+++	+++	++
GSL-I	Galα-	–	–	–	–
PNA	Galβ1–3GalNAc-	++	+/–	++	++

* Binding of plant lectins was analyzed by flow cytometry. Negative value was accepted as mean of fluorescence intensity <20, designated as "–"; 20-50, "+"; 50-300, "++"; 300-500, "+++"; >500, "++++". Fluorescence was calculated as $[(F_i/F_0) \times 100] - 100\%$, where F_i is fluorescence intensity of cells after incubation with lectin, F_0 is fluorescence intensity of cells after incubation with conjugate of FITC-labeled antibodies against digoxigenin or streptavidin.

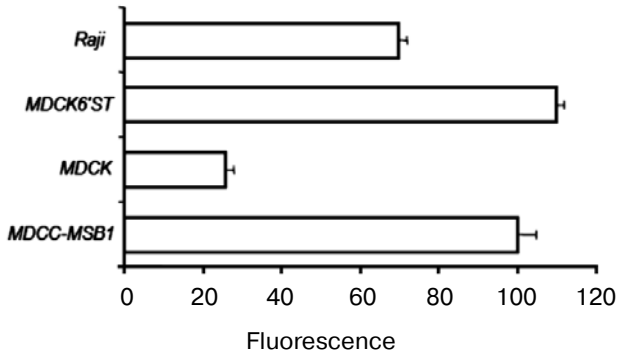


Fig. 5. Glycan-binding potency of CG8 loaded onto different cells: MDCC-MSB1, MDCK, and MDCK6ST, probing with the A (type 2)-PAA-fluo. Fluorescence was calculated as $[(F_i/F_0) \times 100] - 100\%$, where F_i is fluorescence intensity of cells loaded with galectins after incubation with A (type 2)-PAA-fluo, and F_0 is fluorescence intensity of galectin-free cells after incubation with the probe as background control.

pared to avian galectin. It is logical to suppose that CG8 has yet unknown cognate high-affinity ligand(s), possibly fucosylated, on chicken cells, whereas the observed elevated affinity to ABH glycans seems to be a coincidence, not reflecting any real functionality. Third, glycan-binding potency of cell-anchored CG8 is cell type dependent. The most demonstrative experiment in this respect appears to be the comparison of regular MDCK cells (with predominant Neu5Acα2-3Gal phenotype) with MDCK6ST version, i.e. cells that lost predominant Neu5Acα2-3Gal phenotype due to transfection with 6-sialyltransferase. In spite of equally binding to cells (according to quantitation with antibodies) lectin demonstrates modulation of affinity, namely CG8 loaded on MDCK cells possessing more Neu5Acα2-3Gal motifs displayed poor binding to high-affinity ligand compared to other tested cells. Besides, desialylation of preloaded galectin causes significant increase of binding of the glycoprobes. This means that low-affinity Neu5Acα2-3Gal terminated ligands seem to play a crucial role as a regulatory factor in interaction of anchored galectin with external ligands – because of their high abundance as well as close proximity to exposed second CRD of the tandem galectin.

Earlier, we have shown that *cis*-masking does abolish interaction of human galectins-1 and -3 with "weak" but not with high-affinity *trans*-ligands [16]. Here we demonstrated that *cis*-ligands under certain conditions (particularly their favorable situation to galectin) affect galectin interaction with exogenous ligand even if the latter is of high affinity ones.

A question which of the two non-identical CRDs of galectin-8 is responsible for the anchoring, and which CRD of anchored protein interacts with external ligands, remains to be unanswered; we focus on this issue in our next publication.

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