Comparative study of the glycan specificities of cell-bound human tandem-repeat-type galectin-4, -8 and -9

Olga AVokhmyanina2, Eugenia M Rapoport2, Sabine Andrè3, Vyacheslav V Severov2, Ivan Ryzhov2, Galina V Pazynina2, Elena Korchagina2, Hans-J Gabius3, and Nicolai V Bovin1,2

1Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS, 117997, Miklukho-Maklaya 16/10, Moscow, Russia and 2Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Ludwig-Maximilians-University, Veterinärstr. 13, D-80539 Munich, Germany

Received on December 29, 2011; revised on April 24, 2012; accepted on April 25, 2012

Adhesion/growth-regulatory galectins (gals) exert their functionality by the cis/trans-cross-linking of distinct glycans after initial one-point binding. In order to define the specificity of ensuing association events leading to cross-linking, we recently established a cell-based assay using fluorescent glycoconjugates as flow cytometry probes and tested it on two human gals (gal-1 and -3). Here we present a systematic study of tandem-repeat-type gal-4, -8 and -9 loaded on Raji cells resulting in the following key insights: (i) all three gals bound to oligolactosamines; (ii) binding to ligands with Galβ1-3GlcNAc or Galβ1-3GalNac as basic motifs was commonly better than that to canonical Galβ1-4GlcNAc; (iii) all three gals bound to 3′-O-sulfated and 3′-sialylated disaccharides mentioned above better than that to parental neutral forms and (iv) histo-blood group ABH antigens were the highest affinity ligands in both the cell and the solid-phase assay. Fine spatial differences were revealed as follows: (i) gal-8 and -9, but not gal-4, bound to disaccharide Galβ1-3GlcNAc; (ii) increase in binding due to negatively charged substituents was marked only in the case of gal-4 and (iii) gal-4 and -8 bound preferably to histo-blood group A glycans, whereas gal-9 targeted B-type glycans. Experiments with single carbohydrate recognition domains (CRDs) of gal-4 showed that the C-CRD preferably bound to ABH glycans, whereas the N-CRD associated with oligolactosamines. In summary, the comparative analysis disclosed the characteristic profiles of glycan reactivity for the accessible CRD of cell-bound gals. These results indicate the distinct sets of functionality for these three members of the same subgroup of human gals.

Introduction

Diversification within lectin families is a major route to ensure versatility for translating the sugar-encoded information of cellular glycans into physiological activities (Gabius et al. 2011). In principle, two key mechanisms (and a combination thereof) can drive the establishment of a toolbox of such effectors: the sequence of an ancestral carbohydrate recognition domain (CRD) can diverge and the topology of CRD presentation can vary from monomers to different modes of clustering and modularity, as traced in detail for C-type lectins and galectins (gals) (Cooper 2002; Houzelstein et al. 2004; Greedy and Zelensky 2009). Looking more closely at gals, their β-sandwich fold with a common sequence signature, characterized especially by the presence of a Trp residue central for ligand contact, is encountered in three types of spatial CRD display, i.e. proto-type (mono- or homodimeric), chimera-type (one domain associated with two other modules) and tandem-repeat-type (two different domains linked by a peptide) (Kasai and Hirabayashi 1996; Cooper 2002).

Phylogenetically, a marked difference in degree of diversification has been revealed for the latter group (Cooper 2002; Kaltner and Gabius 2012). Whereas the chicken genome harbors only one gene for a gal with two different CRDs connected by a linker peptide, termed chicken gal-8 (Kaltner et al. 2009), mammals express several proteins of this class. This implies the possibility for the development of distinct functionality. Indeed, current information on cellular counter-receptors for human gal-4, -8 and -9 appears to support this view. To provide graphic examples, dipeptidyl peptidase IV, carcinoembryonic antigen and mucin-like membrane MUC1 were identified as binding partners for gal-4 (Huflejt and Leffler 2004; Morelle et al. 2009; Stechly et al. 2009), ααββ-integrin, the CD11b subunit, promatrix metalloproteinase-9, podoplanin and a distinct variant of CD44 (termed CD44vRA) for gal-8 (Hadari et al. 2000; Nishi et al. 2003; Sebban et al. 2007; Cueni and Detmar 2009) and Tim-3, a T11-specific type 1 membrane glycoprotein, for gal-9 (Zhu et al. 2005). These data call for an analysis of glycan specificity of the three mentioned proteins under identical conditions. Toward this

Keywords: blood group antigens / cell adhesion / galectins / glycan / glycomics

© The Author 2012. Published by Oxford University Press. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com
end, a recently developed cell-based assay is suited to probe into the reactivity profile essential for cis/trans-cross-linking (Rapoport et al. 2008; Vokhmyanina et al. 2011).

This assay combines the physiological context of analyzing the cell-bound lectin with the versatility of fluorescent glycoconjugates, Glyc-PAA-fluo, to act as flow cytometry probes (Scheme 1).

Of note, the CRD accessible to the sugar headgroup of a neoglycoconjugate, which is known to visualize lectin presence on cells and in tissue (Gabius et al. 1993; Kayser et al. 1994; Kurmyshkina et al. 2010), would engage in cis/trans-interactions with cognate glycans en route to eliciting lectin-dependent effects (adhesion or signaling). The strength of gal anchoring proved sufficient for cell surface manipulations (for instance, enzymatic deglycosylation) without loss of the loaded gals. As added benefits, the assay also avoids labeling of the lectin and lectin exposure on an artificial surface. Thus, the comparative mapping of glycan binding of gal-4, -8 and -9 is reported, using a panel of 46 synthetic glycoconjugates. The probes cover the structural changes of N-acetyllactosamine (LN) termini including 3′-substitution and the local density of LN epitopes by branching/repetition and extension to histo-blood group epitopes. Since the linker peptide is an integral part of tandem-repeat-type gal functionality (Levy et al. 2006; André et al. 2008; Bi et al. 2008) and, even more intriguing, its shortening appears to affect the hydrodynamic behavior significantly with potential impact on ligand binding (Göhler et al. 2010), we initiated the analysis of binding for a truncated version in the case of gal-4. In this case, we also added the monitoring of the two separate domains by a solid-phase assay (Scheme 2) to extend an initial study, which detected nearly 5-fold differences in affinity to the histo-blood group A (type 2) tetrasaccharide for rat gal-4 (Oda et al. 1993). In summary, our study addresses the issues of inter-gal comparison for human gal-4, -8 and -9, extending initial evidence for gal-8 (Vokhmyanina et al. 2011), as well as of relevance of linker length and inter-CRD comparison in the case of human gal-4.

Results

Loading of gals onto cell surface

To set a common standard, we took the advantage of the recently reported reactivity of Raji cells with human gal-8 (Vokhmyanina et al. 2011).

Gal-4, -8 and -9 were incubated with Raji cells, the binding was detected with the use of the corresponding antibodies (Figure 1A–C). (Because the avidity of anti-gal antibodies can vary, a direct quantitative comparison of gal loading is precluded.) The specificity of the accessible CRD of cell-bound gals was inferred by the panel of fluorescent glycoconjugates, Glyc-PAA-fluo (for the summary of glycan structures, see Table I).

Probing of gal-4 with Glyc-PAA-fluo. Cell-loaded gal-4 did not bind the disaccharides LN, Lec and TF, whereas a moderate level of binding was observed for Tββ disaccharide (Figure 2A and Supplementary data, Figure S1A), which was comparable with reactivity with linear and branched LN-containing glycans (Figure 2A and Supplementary data, Table S1). Regarding glycans containing the Galβ1-3GlcNAc motif (i.e. Leα), this lectin associated with LNT (i.e. Lec1-3′Lac) and, somewhat weaker, with Leα3′Lec and Leα3′LN. Binding of sulfated and sialylated glycans, as illustrated in Figure 2A and Supplementary data, Figure S1A, was much higher than the binding of the unsubstituted glycans; the maximum level of reactivity was observed for 3′,6OSu2Lac. The most potent ligands of gal-4 were tetrasaccharides A (type 2) and A (type 1), whereas B-type tetrasaccharides and the H trisaccharide (type 1) bound to a lesser extent (Figure 2A and Supplementary data, Figure S1A). Binding to the H (type 2) was weaker, whereas no binding to the H (type 3) and H (type 4) trisaccharides was measured (Supplementary data, Table S1). When considering the aspect of inter-gal comparison already at this stage, a rather low level of reactivity...
was discernible also for gal-8 and -9 for the H (type 4) trisaccharide, whereas the type 3 isomer (differing only by the anomeric configuration of the reducing end) shown conspicuously increased reactivity.

Probing of gal-8 with Glyc-PAA-fluo. As reported previously in detail (Vokhmyanina et al. 2011) and presented here for comparison, gal-8 bound weakly the disaccharide probe LN, whereas binding to TF was 4-fold higher, the binding to Le\(^\ddagger\) and T\(_{ββ}\) was 1 order of magnitude higher (Figure 2B and Supplementary data, Table S1). The extent of binding to linear LN3′LN (Supplementary data, Figure S1B) and LN3 and to LNNnT was 1 order of magnitude higher than that to the LN disaccharide and that of the gal to branched oligo-LN3,6LN was 2-fold weaker than that to the linear structure, whereas no binding was observed for LN6′ (GlcNAc3′)LN and (GlcNAc)2,6′LN (Supplementary data, Table S1). Gal-8 interacted with tetrascarides containing the Le\(^\ddagger\) motif weaker than with the corresponding glycans terminated with its isomer LN. For instance, affinity to Le3′LN was 6-fold lower than that to LN3′LN, whereas, in the case of H (type 1) vs H (type 2), this tendency appears to be weaker (Figure 2B and Supplementary data, Table S1).

Regarding negatively charged glycans, 3′-sialylated and 3′-O-sulfated derivatives of disaccharides LN, Le\(^\ddagger\) and TF, i.e. 3′OSuLe\(^\ddagger\), 3′OSuLN, 3′,6OSu2Lac, 3′SiaLN, 3′SiaLe\(^\ddagger\), 3′ SiaTF and 3′OSuTF, were strong ligands; much weaker was 6OSuLN (Figure 2B and Supplementary data, Figure S1B). It should be noted that the affinity of gal-8 to 3′OSuLe\(^\ddagger\) and 3′ SiaLe\(^\ddagger\) was lower than that to non-substituted Le\(^\ddagger\), whereas the affinity to 3′OSuTF and 3′SiaTF was higher than that to TF (Figure 2B and Supplementary data, Table S1). Gal-8 did not bind other sulfated LNs, i.e. 4′OSuLN, 6′OSuLN and 6′ SiaLN. It displayed the highest affinity to A tetrascarides (types 1 and 2) and the H trisaccharide (type 1), than to H tetrascarides (types 2 and 3). As for B tetrascarides of types 1, 2, 3 and 4, the binding was considerably reduced compared with reactivity levels of listed A- and H-type glycans (Supplementary data, Figure S1B).

Probing of gal-9 with Glyc-PAA-fluo. Gal-9 exhibited weak binding to LN, whereas reactivity to other disaccharides, here Le\(^\ddagger\), T\(_{ββ}\) and TF, was two or even more times higher (Figure 2C and Supplementary data, Figure S1C). High affinity was observed to linear and branched oligo-LNs, i.e. LN3′LN, (LN)3, LNNnT, LN3′(GlcNAc6′)LN and (LN)3,6′LN (Supplementary data, Table S1). With respect to β1,3/4-selectivity, the binding to Le3′Lac (LNT) was notably higher than that to Le3′LN, and the binding to Le3′Le\(^\ddagger\) was lower than that to LN3′LN. LN6′LN, LN6′ (GlcNAc3′)LN and Le6′LN were ligands, too, though weaker than corresponding 3′-isomers. To solidify this observation, we have tested the level of inhibition with the probes (LN)3 and LN6′LN. Both proved to be inhibitors at the 50-μM level (Supplementary data, Figure S1).

The ability of gal-9 to bind glycans of the LN6′LN type had been revealed earlier in a cell-free system (http://www.functionalglycomics.org). This lectin displayed high affinity to blood group ABH glycans and to Galα3′LN (Figure 2C and Supplementary data, Figure S2). Notably, B (type 1), B (type 2), H (type 1) and H (type 2) were the most potent glycan ligands for gal-9, whereas the binding to B tetrascarides (types 3 and 4) was 1 order of magnitude lower. Similarly, the binding to H trisaccharide (type 3) was weaker than that to H (types 1 and 2) glycans, and no binding was observed to the H (type 4) glycans (Figure 2C and Supplementary data, Table S1). The binding to all A-type tetrascarides and to Galα3′LN was ~5-fold weaker than that to respective B-type glycans (Supplementary data, Table S1).

Concerning negatively charged glycans, gal-9 displayed affinity to several (Figure 2C). The reactivity, however, was ~1 order of magnitude lower than that to ABH glycans (Supplementary data, Table S1). It should be noted that gal-9 bound 3′OSuLe\(^\ddagger\) and 3′OSuTF, as it did the non-sulfated analogs, whereas complex formation with 3′OSuLN, 6OSuLN and 3′SiaLN was stronger than with the corresponding neutral glycans.

Besides, binding to high-affinity glycans is dose-dependent as indicated for gal-4 and -8 (Supplementary data, Figure S3A and B).
Table I. List of oligosaccharides presented by the Glyc-PAA-fluo probes

<table>
<thead>
<tr>
<th>No</th>
<th>Structure Glyc</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Galβ1-4GlcNAcβ</td>
<td>LN</td>
</tr>
<tr>
<td>2</td>
<td>Galβ1-3GlcNAcα</td>
<td>Leαβ</td>
</tr>
<tr>
<td>3</td>
<td>Galβ1-3GlcNAcα</td>
<td>TF</td>
</tr>
<tr>
<td>4</td>
<td>Galβ1-3GlcNAcα</td>
<td>Tβ1</td>
</tr>
<tr>
<td>5</td>
<td>GalNAcα1-3GalNAcβ</td>
<td>Fα2</td>
</tr>
<tr>
<td>6</td>
<td>GalNAcβ1-4GlcNAcβ</td>
<td>LacdiNAc</td>
</tr>
<tr>
<td>7</td>
<td>GalNAcβ1-4(6-O-Su)GlcNAcβ</td>
<td>6OSulNacNac</td>
</tr>
<tr>
<td>8</td>
<td>4-O-Sa-Galβ1-4GlcNAcβ</td>
<td>4OSulN</td>
</tr>
<tr>
<td>9</td>
<td>6-O-Sa-Galβ1-4GlcNAcβ</td>
<td>6OSulN</td>
</tr>
<tr>
<td>10</td>
<td>Galβ1-4(6-O-Su)GlcNAcβ</td>
<td>6OSulN</td>
</tr>
<tr>
<td>11</td>
<td>3-O-Sa-Galβ1-4(6-O-Su)GlcNAcβ</td>
<td>3'6OSulLac</td>
</tr>
<tr>
<td>12</td>
<td>3-O-Sa-Galβ1-3GlcNAcβ</td>
<td>3OSulLac</td>
</tr>
<tr>
<td>13</td>
<td>3-O-Sa-Galβ1-3GlcNAcβ</td>
<td>3OSulTF</td>
</tr>
<tr>
<td>14</td>
<td>3-O-Sa-Galβ1-3GlcNAcβ</td>
<td>3'SialLN</td>
</tr>
<tr>
<td>15</td>
<td>Neu5Acβ2-6Galβ1-4GlcNAcβ</td>
<td>6'SialLN</td>
</tr>
<tr>
<td>16</td>
<td>Neu5Acβ2-6Galβ1-4GlcNAcβ</td>
<td>6'SialLeαβ</td>
</tr>
<tr>
<td>17</td>
<td>Neu5Acβ2-6Galβ1-3GlcNAcβ</td>
<td>3'SialLeαβ</td>
</tr>
<tr>
<td>18</td>
<td>Neu5Acβ2-6Galβ1-3GlcNAcβ</td>
<td>3'SialTF</td>
</tr>
<tr>
<td>19</td>
<td>Galα1-3Galβ1-4GlcNAcβ</td>
<td>Galα3LN</td>
</tr>
<tr>
<td>20</td>
<td>Fucα1-2Galβ1-3GlcNAcβ</td>
<td>H (type 1)</td>
</tr>
<tr>
<td>21</td>
<td>Fucα1-2Galβ1-4GlcNAcβ</td>
<td>H (type 2)</td>
</tr>
<tr>
<td>22</td>
<td>Fucα1-2Galβ1-3GlcNAcβ</td>
<td>H (type 3)</td>
</tr>
<tr>
<td>23</td>
<td>Fucα1-2Galβ1-3GlcNAcβ</td>
<td>H (type 4)</td>
</tr>
<tr>
<td>24</td>
<td>Galα1-3(Fucα1-2)Galβ1</td>
<td>Bα1</td>
</tr>
<tr>
<td>25</td>
<td>GalNAcα1-3(Fucα1-2)Galβ1</td>
<td>Aα1</td>
</tr>
<tr>
<td>26</td>
<td>GlcNAcβ1-6</td>
<td>Core 2</td>
</tr>
</tbody>
</table>

Effect of linker length on glycan-binding properties of a tandem-repeat-type gal

To initiate the analysis of the impact of the linker on the carbohydrate reactivity of the tandem-repeat-type proteins, we tested an engineered variant of gal-4 (gal-4v). According to the data on antibody binding, both lectins were loaded on Raji cells to rather similar extents (results not shown). Overall, the variant presented by the cells interacted with glycoconjugates in rather similar manner as seen for the full-length protein (Figure 3 and Supplementary data, Table S1), except for ABH glycans. Here, the extent of binding of the engineered protein was much lower.

Binding of gals after enzymatic treatment of cells

In order to provide insights on the nature of glycans involved in cell-surface anchoring gals and an impact of N- and C-domains on this event, the cells, prior to loading, were processed with neuraminidase and β-galactosidase. Thereafter, the cells were loaded with a full-length protein or the N- and C-RDs of gal-4; protein loading was ascertained using the respective antibody. As a control for enzymatic desialylation or degalactosylation, the reactivity for the plant lectins Sambucus nigra (SNA) and Erythrina cristagalli (ECA) (recognizing terminal α2,6-sialogalactosides and β-galactosides, respectively) was decreased, at least in part (Figure 4A). Thus, the density of sialylation in neuraminidase-treated cells and of β1,4-linked galactose is reduced. Gal-4 and its domains benefited from desialylation (Figure 4B). In contrast, cell surface desialylation led to a decrease in anchoring for both versions of full-length gal-8 (Figure 4C). As seen for gal-9, the binding of cells for the full-length gal was increased (Figure 4D). As noted for the control with ECA, a reduction in cell reactivity for all tested gals was seen, when additional β-galactosidase treatment was performed that followed the
desialylation (Figure 4B–D). These assays also serve as a specificity control. Notably, after desialylation, the binding of gal-4 and -4v to cells increased 3–4 times, indicating the unmasking of affinity ligands resistant to the β-galactosidase action; the molecular mechanism of this phenomenon remains unclear.

Fig. 2. Comparison of glycan-binding profiles for gal-4 (A), -8 (B) and -9 (C) flow cytometry data. Gals were loaded onto the cells followed by probing with fluorescent neoglycoconjugates (see Materials and methods). Fluorescence was calculated as $[(F_i/F_0) \times 100] - 100$, where $F_i$ and $F_0$ are the fluorescence intensity of gal-loaded and gal-free cells probed with Glyc-PAA-fluo, respectively. Results shown include data from four experiments; error bars represent the standard deviation. The mean of fluorescence $<20$ was as a negative value. The complete list of glycans with the measured value of fluorescence intensity is given in Supplementary data, Table S1.
Evidently, ligands can be made cryptic by sialylation for gal-4 and -9, but not gal-8. In contrast to SNA/ECA, Raji cells were less sensitive to other probes used for glycophenotyping (Supplementary data, Figure S4).

**Solid-phase assay for N- and C-CRDs**

In order to further probe into the specificities of two separate domains for a tandem-repeat-type protein, we proceeded to compare the N/C-CRDs of gal-4 in a solid-phase assay (Scheme 2). Both domains did not bind to LN or Le^c, the C-terminal domain to disaccharides T_{ββ} and TF (Figure 5). Although both proteins bound to negatively charged glycans, namely 3′-OSuTF, 3′OSuLe^c, 3′,6OSu_{2}Lac, 6OSuLN and 3′ SiaLN, the affinity of the N-CRD was 1 order of magnitude higher than that of C-CRD. Also, the N-CRD bound to oligo-LN and glycans containing Le^c disaccharides. Of note, only the C-CRD displayed affinity to ABH-type glycans.

**Comparison of the affinity of gal-4 domains in the cell assay**

According to the Solid-phase assay for N- and C-CRDs, the affinity of the N-domain to the ABH glycans is lower than that of the C-domain. In order to confirm this result in the cell assay, we loaded cells with gal-4 domains, then incubated the cells with the A (type 1)-bearing fluorescent neoglycoconjugates in the standard flow-cytometry conditions, and finally quantitated eluted material using antibodies in a dot-blot assay. Without the probe, no elution takes place as expected, whereas, in its presence, a certain degree of release is observed (Supplementary data, Figure S5). In the case of gal-4 and the N-CRD, the release appears to be minor, confirming the validity of our assay especially for the bivalency of a gal and revealing sufficient affinity to surface ligands even for a monovalent protein, whereas ~25% of the cell-loaded C-domain was identified in the culture medium.

**Discussion**

Physiological cis/trans-interactions of tandem-repeat-type gals will depend on the glycan reactivity of the accessible CRD that is the N- or C-domain. Thus, the analysis of this property using cell-bound lectin is of conspicuous biorelevance. To address this issue, we have previously established an assay, as graphically summarized in Scheme 1 and tested its validity using gal-1 and -3 (Rapoport et al. 2008). Following this initial work, we proceeded with the analysis of human gal-8 (Vokhmynina et al. 2011), leading to this report with its comparison of gal-4, -8 and -9 in this manner, i.e. when gals are anchored to the cell surface. In order to visualize the relative binding properties, Figure 6 graphically presents respective profiles for all five human gals.

They teach the following lessons:

(i) there are obvious shifts in binding preferences between the groups and in the tandem-repeat-type category;

(ii) selectivity for Galβ1-3HexNAc is disclosed for tandem-repeat-type proteins, most prominently for gal-8 and -9, whereas binding to single Galβ1-4GlcNAc (LN) found to be weak;

(iii) LN repetition is favorable for gal-1, -3, -8 and -9, as also reported by using frontal affinity chromatography (Hirabayashi et al. 2002; Nagae et al. 2009) and arrays (Carlsson et al. 2007; Stowell, Arthur, Mehta, et al. 2008; Stowell, Arthur, Slanina, et al. 2008);

(iv) ABH-type oligosaccharides have exceptionally strong affinity for the tandem-repeat-type gals in cell system, in accord with previous data using frontal affinity chromatography, glycoprotein binding and array analysis (Hirabayashi et al. 2002; Wu et al. 2004; Krzeminski et al. 2011; www.functionalglycomics.org); gal-9 showed a strong preference for the B-type oligosaccharide, whereas gal-4 and -8 for A-type tetrasaccharide; taking into consideration that ABH glycans are alloantigens, the preference of tandem-repeat-type gals to them appears to be an intriguing fact;

(v) if substituted by an anionic group, 3′-sialylation enhances binding affinity for gal-4 and -8 (but not for gal-9), especially on core 1 O-glycan and LN, as does 3′-O-sulfation on LN, and other core assay solid-phase and chromatographic screening revealed the reactivity of gal-3 with 3′-O-sulfated galactose.

**Fig. 3.** Profiling of the linker-truncated gal-4 vs full-length gal-4, both loaded onto Raji cells. Binding to Glyc-PAA-fluo, flow cytometry data. As a negative control, gal-free cells were incubated with the same set of probes. Fluorescence was calculated as \([F_i/F_o] \times 100\) − 100, where \(F_i\) and \(F_o\) are the fluorescence intensity of gal-loaded and gal-free cells probed with Glyc-PAA-fluo, respectively. Results shown include data from four experiments; error bars represent the standard deviation. The mean of fluorescence <20 was considered as a negative value. The complete list of glycans with the measured value of fluorescence intensity is given in Supplementary data, Table S1.
Fig. 4. Effect of enzymatic treatment of cells on gal anchoring. Cells were treated with neuraminidase followed by β-galactosidase, then incubated with gals, and stained with anti-gal antibodies (B–D). In a negative control, cells were stained with secondary antibodies only. To test the effect of enzyme presence, the cells were stained with SNA or ECA (A). Fluorescence was calculated as \([\left(\frac{F_i}{F_0}\right) \times 100] - 100\), where \(F_i\) the fluorescence intensity of cells after incubation with digoxigenin or biotin-labeled lectins (A) or antibodies (B–D) and \(F_0\) the fluorescence intensity of cells stained with secondary antibodies or FITC streptavidin. Results shown include data from three experiments; error bars represent the standard deviation.
(Ideo et al. 2002; Ideo et al. 2003; Rapoport et al. 2008) and sulfatide is a known physiological ligand of human gal-4 (Delacour et al. 2005); trimming the linker of gal-4 reduced avidity for the core 2 O-glycan and most ABH-type epitopes, indicating the potential functional relevance of the linker length with respect to ligand binding, initially inferred for the cell binding of human neuroblastoma cells (Kopitz et al. 2012).

Of course, it should be kept in mind that the data rest on the glycan profile of this cell system, i.e. Raji cells. Our previous report indicated that alterations of the glycomic profile have a bearing on quantitative aspects of neoglycoconjugates reactivity of chicken gal-8 (Vokhmyanina et al. 2011). As fittingly noted in the introduction, distinct glycoproteins of certain cell types can have preferential target specificity to a certain gal. Thus, for the purpose of comparison, it is mandatory to keep conditions constant. This done, it has become obvious, as illustrated in Figure 6, that each gal appears to have developed its characteristic fingerprint when tested with neoglycoconjugates on the cell surface. Slight changes in the glycome will thus account for switching the cross-linking potential for gals.

Literature and our data on tandem-repeat-type gal specificity as well as on Raji cell glycome allow us to assert that N-CRD is responsible for anchoring gal via binding to highly abundant on the cell-surface lactosamine-type glycans, whereas C-CRD is exposed for interaction with the external ligands. In order to pursue this line of research experiments to systematically map, the binding properties of each domain are in progress with engineered tandem-repeat-type variants constituted by a single type of domain.

### Materials and methods

#### Reagents

Soluble 30-kDa polyacrylamide glycoconjugates, the neoglycoconjugates and their fluorescent derivatives (1% mol of fluorescein and 20% mol of glycan) were obtained from Lectinity (Moscow, Russia). Carbohydrate-free bovine serum albumin (BSA) and Tris HCl were from Merck (Darmstadt, Germany), and *Escherichia coli* β-galactosidase and digoxigenin-labeled agglutinin from SNA and FITC-labeled anti-digoxigenin antibodies were purchased from Roche (Mannheim, Germany); biotinylated agglutinin from ECA, *Ulex europaeus*, *Lotus tetragonolobus*, *Glycine max* (SBA) and *Griffonia simplicifolia* were from Vector (Burlingame, CA); mouse antibodies recognizing A- and B-type trisaccharides were obtained from Hematology Research Center (Moscow, Russia); 3,3′-diaminobenzidine, 4-chloronaphthol, FITC-labeled streptavidin, asialofetuin, neuraminidase from *Vibrio cholerae*, goat anti-rabbit and anti-mouse IgG labeled
with FITC and o-phenylenediamine were from Sigma (St Louis, MO). RPMI-1640 and glutamine were from Invitrogen (Paisley, UK). Goat anti-rabbit IgG conjugated with peroxidase were from the Gamaleya Research Institute of Experimental Microbiology (Moscow, Russia). All the other reagents were purchased from Reachim (Moscow, Russia).

**Gals and gal-specific antibodies**

Recombinant human gal-4, -8 and -9 and the two domains of gal-4 were prepared as described previously (Lensch et al. 2006; André et al. 2008; Solis et al. 2010) and then purified by affinity chromatography on lactosylated Sepharose 4B (Gabius, 1990; Lensch et al. 2006; Beer et al. 2008). Linker truncation for gal-4 encompassed 26 amino acids of the 42mer peptide, maintaining the phylogenetically highly conserved 3/13mer peptide stretches at each side (André et al. 2008; Kopitz et al. 2012). Homogeneity was ascertained by gel filtration and one- and two-dimensional gel electrophoresis. Polyclonal antibodies against gals were raised in rabbits under constant control of the titer, and the IgG fractions were isolated by affinity chromatography using protein-A Sepharose 4B (Pharmacia, Freiburg, Germany) and checked for lack of cross-reactivity against other members of this lectin family by western blotting and enzyme-linked immunosorbent assay. In cases of the positivity removal of such fractions was accomplished by affinity chromatography on gal-presenting resin (Lensch et al. 2006).

**Loading of cells with gals**

Raji cells (human B-lymphocyte origin, ATCC No. CCL86) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C in an atmosphere of 5% CO₂, and the assay was performed as described previously (Rapoport et al. 2008) to enable comparison. In detail, cells were washed three times with phosphate-buffered saline containing 0.2% BSA (PBA) using centrifugation at 450 g (Jouan CR3, rotor T20, France) and at 4°C. Aliquots of the cell suspension (1 × 10⁵ cells in 50 μL) were incubated with 50 μL of gal (0.4 mg/mL) for 30 min at 4°C under gentle agitation on a shaker. The concentration of gals added to cells previously defined as optimal when we studied the specificity of gal-1 and -3 loaded on Raji cells (Rapoport et al. 2008); at this concentration, we did not observe the agglutination of Raji cells (Supplementary data, Figure S6). To enable comparison between studies, this parameter was thus kept constant. Control of the loading was performed with the gal-type-specific antibodies and the corresponding fluorescent second-step reagent used in incubation steps lasting 20 min at 4°C. Flow cytometry was performed using a FACSscan instrument (Becton-Dickinson, San Jose, CA) equipped with the software WiNMDI 2.8.

**Probing the gal-loaded cells with Glyc-PAA-fluo**

Cells loaded with a gal were centrifuged at 800 rpm. The cells (1 × 10⁵ per well in 50 μL) were carefully resuspended in PBA and incubated with 50 μL of the Glyc-PAA-fluo probe in PBA.
(final concentration 100 μM) for 40 min at 4°C under gentle agitation. The cells were washed twice with PBA and analyzed by flow cytometry as given above. Mock-treated cells were used as a negative control. In inhibitory assay, gal-loaded cells were incubated with Glyc-PAA (100 μM) for 40 min at 37°C under gentle agitation, followed by centrifugation and incubation with Glyc-PAA-fluo (50 μM) as described in section Loading of cells with gals.}

Detection of gal presence in culture medium

Gal-4, 4N or 4C were loaded on Raji cells followed by incubation with A (type 2)-PAA-fluo as described above. After the sample was centrifuged, the supernatant was transferred into plate covered with nitrocellulose membrane (Millipore Billerica, MA); PBA was used as a negative control. The membrane was allowed to air-dry for 30 min. Non-specific sites were blocked with PBS containing 2% BSA for 1 h at room temperature followed by incubation with anti-gal-4 antibodies at 4°C in PBA overnight, three times washing with PBS containing 0.05% Tween-20 (P-Tw) and incubation with anti-rabbit IgG-peroxidase conjugate in PBA overnight, three times washing with PBS containing 2% BSA for 1 h at room temperature. The cells were washed thrice with PBA and analyzed by flow cytometry as given above. Gal was loaded on the cells; binding was analyzed by gal-reactive antibodies as described in section Loading of cells with gals.

Enzymatic deglycosylation of cells

Cells were treated with neuraminidase (4 U/mL) for 1.5 h at 30°C followed by β-galactosidase (2 U/mL) under the same conditions. Control for desialylation and degalactosylation was performed by using labeled plant lectins with affinity for α2,6-linked sialic acid (SNA) or for β-galactosides (ECA). Briefly, the cells (1 × 10^5 per well in 50 μL) were carefully resuspended in PBA and incubated with 50 μL of lectin in PBA (final concentration 20 μg/mL) following incubation with an FITC-labeled second reagent, anti-digoxigenin antibodies or streptavidin for 30 min at 4°C under gentle agitation. The cells were washed thrice with PBA and analyzed by flow cytometry as given above. Gal was loaded on the cells; binding was analyzed by gal-reactive antibodies as described in section Loading of cells with gals.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Funding

This work was supported in part by grants of the Russian Foundation for Basic Research (07-04-00969 and 10-04-00959), the grant of the Presidium of the Russian Academy of Sciences Program “Molecular and Cell Biology” and the generous funding from the EC Seventh Framework Program (FP7/2007-2013) under grant agreement no. 2602600 (GlycoHIT).

Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; CRD, carbohydrate recognition domain; ECA, Erythrina cristagalli; fluo, fluorescein residue; gal, galectin; Glyc, glycana; LN, N-acetyllactosamine; PAA, polyacrylamide; PBA, PBS containing 0.2% BSA; PBS, phosphate-buffered saline; P-Tw, PBS containing 0.05% Tween-20; PO, peroxidase; SNA, Sambucus nigra.

References


galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. *Glycobiology* 17:663–676.


