Different affinity of galectins for human serum glycoproteins: Galectin-3 binds many protease inhibitors and acute phase proteins

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Here we report the first survey of galectins binding to glycoproteins of human serum. Serum was subjected to affinity chromatography using immobilized galectins, and the bound glycoproteins were analyzed by electrophoresis, Western blotting, and mass spectrometry. Galectins-3, -8, and -9 bound a much broader range of ligands in serum than previously known, galectin-1 bound less, and galectins-2, -4, and -7 bound only traces or no serum ligands. Galectin-3 bound most major glycoproteins, including alpha-2-macroglobulin and acute phase proteins such as haptoglobin. It bound only a selected minor fraction of transferrin, and bound none or little of IgG. Galectins-8 and -9 bound a similar range of glycoproteins as galectin-3, but in lower amounts, and galectin-8 had a relative preference for IgA. Galectin-1 bound mainly a fraction of alpha-2-macroglobulin and only traces of other glycoproteins. The binding of galectin-3 to serum glycoproteins requires affinity for LacNac, since a mutant (R186S), which has lost this affinity, did not bind any serum glycoproteins. The average affinity of galectin-3 for serum glycoproteins was estimated to correspond to $K_d \sim 1–5 \mu M$ by modeling of the affinity chromatography and a fluorescence anisotropy assay. Since galectins are expressed on endothelial cells and other cells exposed to serum components, this report gives new insight into function of galectins and the role of their different fine specificity giving differential binding to the serum glycoproteins.

Introduction

The galectins are small soluble proteins, defined by a carbohydrate recognition domain (CRD) with affinity for $\beta$-galactosides and a conserved sequence motif (Leffler et al. 2004). There are about 15 mammalian galectins with one or two CRDs. Some, such as galectins-1, -3, -8, and -9, are widely distributed in many cell types, whereas others like galectins-2, -4, and -7 have a much more restricted distribution (Poirier 2002; Hirashima et al. 2004; Huflejt and Leffler 2004; Lahm et al. 2004). Galectins play regulatory roles in inflammation, immunity, and cancer (Almkvist and Karlsson 2004; Ideo et al. 2005; Dumic et al. 2006; Rabinovich et al. 2007) via effects on cells such as modulation of cell adhesion, and induction of apoptosis and other signals. Even if galectins are synthesized as typical cytosolic proteins, they reach the cell surface or extracellular space (Brewer 2002; Nieminen et al. 2007) by a nonclassical secretory pathway (Baptiste et al. 2007), and the mentioned effects on cells are supposed to be mediated by their binding to $\beta$-galactoside containing glycoconjugates. Therefore, the binding specificity of their CRD, both for model saccharides and natural ligands, is of prime functional interest.

The galectin CRD has about 130 amino acids folded as a slightly bent $\beta$-sandwich forming a groove on the concave side. This groove forms the galectin carbohydrate recognition site with five subsites (A–E) (Leffler et al. 2004). Subsite C is the defining conserved binding site of the galectin CRDs and gives them their shared specificity for $\beta$-galactose residues having positions 6 and 4 free. The other subsites determine the variable fine specificity for larger saccharides. There is only little knowledge, however, on how the galectin fine specificity determines binding to natural ligands and the consequent biological effects (Leppanen et al. 2005; Cabrera et al. 2006; Fry et al. 2006; Hernandez et al. 2006; Patnaik et al. 2006; Stillman et al. 2006; Stowell et al. 2008).

As a way of expanding this knowledge, we have studied the interaction between different galectins and human serum glycoproteins. As a model system, serum provides a well-characterized mixture of glycoproteins, which can be used to test the role of galectin fine specificity for natural ligand binding. The N-linked glycan structures found in serum are also found in glycoproteins in many tissues and cells, sometimes in similar proportions, sometimes in others. A few galectin ligands have been identified in serum as minor components (Grassadonia et al. 2004), but there has been no systematic study of galectin interaction with human serum glycoprotein ligands.

As a biological system, serum contains glycoproteins with which galectins are very likely to interact in vivo, even if the concentration of galectins in serum itself is very low (Grassadonia et al. 2004; Saussez et al. 2008). Most cells expressing and externalizing galectins are likely to be exposed to serum components, the most obvious being endothelial cells (Thijssen, Hulsmans, et al. 2007; Thijssen, Poirier, et al. 2007; Yu et al. 2007); other cells of the body (outside the CNS) are also surrounded by a high concentration of serum proteins that are...
Galectin-binding glycoproteins

**Results**

**Affinity chromatography of serum on immobilized galectin-3: specificity and yield**

Galectin-3 was immobilized on 1 mL of NHS-activated Sepharose in a prepacked column. Normal human serum (0.1 mL) was diluted to 2 mL in PBS, and circulated on the column for 30 min. The column was washed with 32 mL of PBS and then bound glycoproteins were eluted with PBS containing lactose. The unbound fraction was subjected to a second round of affinity chromatography with new lactose eluted (E-II) and a new unbound fraction (UB-II), that was subjected to affinity chromatography a third time (not shown). The left blot was overdeveloped to reveal any traces of alpha-2-macroglobulin in the unbound or second time eluted fractions.
150 mM lactose (Figure 1A). The loaded sample contained about 7.2 mg protein, and 0.7 mg protein was recovered in the main lactose-eluted fractions (peak after arrowhead in the upper chromatogram of Figure 1A). The remainder could be accounted for by the unbound protein, as measured in the volume left in the loading test tube (2 mL) and in the earliest wash fractions (1–4 mL in Figure 1A), coming from the free solution on the column and in connecting tubes. SDS–PAGE showed various glycoproteins in the bound, lactose-eluted fractions (lanes E1 and E2 in Figure 1B), and albumin and other proteins in the unbound fractions (lane UB). The identifications of the bound proteins are described below; the major one was alpha-2-macroglobulin.

To further examine the specificity of the interaction between galectin-3 and serum glycoproteins, affinity chromatography was performed under the same conditions using an immobilized galectin-3 R186S mutant. This mutant has an altered subsite D no longer binding GlcNAc but still binding Glc, which results in selectively lost affinity for LacNAc as is found in glycoproteins; the affinity for lactose is only slightly lowered giving evidence that the general folding stability of the mutant is preserved, also confirmed by scanning calorimetry (Cumpstey et al. 2007). Affinity chromatography on the R186S mutant gave no (or very little) bound and lactose-eluted proteins (Figure 1A and lane E in Figure 1B), and the unbound fraction (lane UB) resembled the initial sample (lane S). No alpha-2-macroglobulin, the major wild type (wt) galectin-3 ligand as described above, was detected in the eluted fractions after immunoblotting (Figure S11). This shows that the binding of wt galectin-3 to serum glycoproteins depends on its affinity for LacNAc.

The sum of the bound and unbound proteins in the experiments described above equaled the amount loaded within the margin of measurement error, leaving little possibility of significant amounts left on the column. To examine if smaller amounts of bound proteins remained on the columns after lactose elution, they were opened after the chromatography, and 100 mg of column material (equivalent to ~0.1 mL) was taken from the top and boiled in a 50 µL SDS–PAGE sample buffer. Electrophoresis of 2.5 µL of this material (lane CM in Figure 1B), which is an equivalent fraction of the total compared to the amount applied of other fractions, revealed only galectin-3 at ~31 kDa as confirmed by Western blot (not shown). Electrophoresis of 10 times more revealed some additional bands corresponding to major serum proteins from the galectin-3 wt column but no more bands from the galectin-3 R186S column (Figure S2). This shows that there is no or very little interaction between serum proteins and the galectin-3 columns that does not depend on the carbohydrate recognition site. The galectin-3 in the CM lanes cannot originate from serum where the known amount (~100 ng/mL) would be too low to be detected even if all of it bound; instead it must be from the immobilized material, of which it represents ~0.2%.

Comparison of affinity chromatography of serum on different immobilized galectins

Galectins-1, -2, -4, -7, -8, and 9 were immobilized on 1 or 5 mL prepacked columns under conditions where they have been shown to remain active previously (Hirabayashi et al. 2002), and the columns were used for affinity chromatography of human serum in the same way as described above for the galectin-3 column. The properties of the columns are given in Table S1. They contained on average 220 nmol bound galectin (range 110–590) per mL bed volume. If most of the immobilized galectin remains active, it is enough to bind the available glycans in the applied serum sample (0.1 mL/mL column); this contains about 80 nmol of N-glycans as calculated from the quantitation by Kita et al. (2007), and of these 15 nmol potentially bind galectins (see Discussion).

A summary of the results with the different galectin columns is shown in Table I. Columns with immobilized galectin-1, galectin-3, galectin -8 and either CRD of galectin-9 bound clearly detectable levels of serum proteins, whereas columns with galectins-2, -4, and -7 did not. The serum ligand binding was compared to the ability of the columns to bind monovalent fluorescent saccharide probes, under similar chromatography conditions (Table S1). For probes with known solution $K_d < 1 \mu M$, over 90% bound their respective immobilized galectins-3, -4, -8, or 9N, and also remained after washing with 20 times the column volume. For probes with known solution $K_d > 25 \mu M$, 10–50% bound initially (to galectins-7 and 9C), but did not remain after washing. For the probes with $K_d > 25 \mu M$, 10–50% bound initially (to galectins-1 and -2) but none remained after washing. This suggests that an affinity of a ligand for the immobilized galectin should be $K_d > 1–5 \mu M$ or better to be found in the lactose-eluted fraction under the present conditions. Mathematical modeling of the column performance also supported this conclusion, and agreed with the results of Table S1.

**Galectin–serum interaction in solution measured by fluorescence anisotropy**

To compare the column binding data with interactions between serum glycoproteins and galectins in solution, the potency of serum to inhibit interaction with fluorescein-tagged saccharide probes was examined with a fluorescence anisotropy assay, which was used extensively by us before for small molecule

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1 Figures S1–S3 and Tables SI–SIII are found in supplementary material.

<table>
<thead>
<tr>
<th>Galectin</th>
<th>Serum protein bound to immobilized galectin (mg/mL serum)</th>
<th>Relative interaction of serum with galectin in solution</th>
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<tbody>
<tr>
<td>Galectin-1</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Galectin-2</td>
<td>&lt;0.01</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Galectin-3</td>
<td>0.02</td>
<td>1</td>
</tr>
<tr>
<td>Galectin-3 R186S</td>
<td>0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Galectin-4</td>
<td>&lt;0.01</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Galectin-4C</td>
<td>&lt;0.01</td>
<td>Not tested</td>
</tr>
<tr>
<td>Galectin-7</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Galectin-8S</td>
<td>2</td>
<td>Not tested</td>
</tr>
<tr>
<td>Galectin-8N</td>
<td>3</td>
<td>Not tested</td>
</tr>
<tr>
<td>Galectin-9N</td>
<td>1.6</td>
<td>Not tested</td>
</tr>
<tr>
<td>Galectin-9C</td>
<td>2.8</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

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*a Serum protein recovered by lactose elution from chromatography on immobilized galectin given as the typical amount of recovered protein corresponding to 1 mL of original serum.

*b Relative potency of serum to inhibit binding of different galectins to fluorescein-tagged saccharide probes, as measured by a fluorescence anisotropy assay. The potency to inhibit galectin-3 was arbitrarily set to 1, and was equivalent that of 150 µM of an inhibitor with a $K_d$ of 5 µM, or to 2.5 mM lactose with a $K_d$ of 150 µM.
Galectin-binding glycoproteins

Fig. 2. Galectin-3 binding serum proteins separated by 2D-gel electrophoresis. (A) Galectin-3 binding proteins from human serum (purified as in Figure 1A) were separated by isoelectric focusing (17 cm strip, 3–10 nonlinear) followed by 12.5% SDS–PAGE. Protein spots were stained with Sypro Ruby. Numbered spots were cut out, digested, and identified with mass spectrometry (see Table II and SII). (B) Galectin-3 binding proteins were separated on a 12% SDS–PAGE gel and numbered bands (right) were identified by mass spectrometry (see Table II and SII). The mobilities of molecular weight markers are indicated to the left.

inhibitors (Sorme et al. 2004; Cumpstey et al. 2007) (Table I). Galectin-3 was clearly inhibited by serum components, whereas the R186S mutant was, as expected, not significantly inhibited (Figure S3). The potency of serum to inhibit galectin-3 was, for example, equivalent to that of 60 µM of an inhibitor with $K_d$ 1 µM, 150 µM of an inhibitor with $K_d$ 5 µM, or 2.5 mM lactose with a $K_d$ of 150 µM. The potency of serum to inhibit galectin-1 was about 10-fold less than that for galectin-3, which agrees with the significant but lower binding of serum proteins to the galectin-1 column. Galectins-2, -4 and -7 were not inhibited, but bound their respective probes equally well in the presence or absence of serum, showing that they retain their activity in serum but that their affinity for serum ligands is low, hence confirming the column binding data.

Identification of galectin-3 binding and nonbinding proteins in human serum

The galectin-3 ligands in serum were analyzed in detail (Figures 1 and 2, Figure S1 B–C, Tables II and SII) using 1D (SDS–PAGE) and 2D electrophoresis followed by identification by mass spectrometry (MALDI-TOF) of tryptic fragments from selected bands and spots, and confirmation by Western blotting. The major galectin-3 ligand of human serum was alpha-2-macroglobulin but a number of other glycoproteins also bound, including haptoglobin and a fraction of transferrin. As mentioned above, the galectin-3 mutant R186S did not bind any glycoproteins.

The pattern of galectin-3-binding serum glycoproteins was similar in 10 individual sera (Figure 1C, right panel), and different quantity of the haptoglobin beta chain (band at 45 kDa) due to genetic polymorphism (Wassell 2000).

Alpha-2-macroglobulin was found to be completely absorbed on the galectin-3 column (Figure 1D, left panel). Even when the blot was overexposed in regard to the signal from bound fractions (E-I), no signal was found in the unbound fraction (UB). Consistent with this, rechromatography of the unbound fraction did not result in any more alpha-2-macroglobulin in the bound fraction (E-II). The closed form of alpha-2-macroglobulin, induced by methylamine (Gunnarsson and Jenssen 1998), bound equally well to the column (not shown).

Most of the serum glycoprotein IgG did not bind galectin-3. In Figure 1B the IgG heavy and light chains are clearly visible in the unbound fraction (UB). Quantitation of IgG using turbidometry showed that most of it did not bind galectin-3. In contrast, a major part of IgM, IgA, and IgE bound galectin-3 (not shown). The complement factors C3 and C4, which are fairly abundant, were not detected in the bound fractions.

A part of transferrin bound galectin-3 (Figure 1D, right panel) estimated as ~10% by examining dilution series of bound and unbound fractions on Western blots (not shown). When purified human transferrin was subjected to affinity chromatography under the same conditions, only ~5% was bound and eluted with lactose, and when the unbound fraction was rechromatographed, only traces (<0.5%) bound to the column (Figure S4). Thus, a small fraction of transferrin has high affinity for galectin-3 whereas the major part does not.

To examine the mechanism for the partial binding of transferrin, the N-glycan structures of the bound and unbound fractions were compared. Tryptic peptides were analyzed by HPLC-FTICR mass spectrometry, and the two N-glycosylated
ones identified, one of which is shown in Figure S5. The saccharide structures in each were identified by the glycopeptide mass and by sequencing using MS/MS and MS3. The predominant signal in all fractions was from a disialylated bi-antennary N-glycan, which constitutes ~95% of the total N-glycans in normal human transferrin (Yamashita et al. 1989; Satomi et al. 2004; Wada et al. 2007). In the bound fraction, in addition, partially sialylated bi- and triantennary N-glycans were clearly detected and together gave 20% signal intensity of the main peak. These were not detected (<1%) in the unbound fraction. Thus, galectin-3 selectively binds a small subfraction of transferrin specifically enriched in partially sialylated bi- and triantennary N-glycans. These could be more than 20% in the bound fraction, since the mass spectrometry signal intensity does not correlate linearly with the glycan amount. This was also indicated by the comparison of the bound and unbound fractions by isoelectric focusing (not shown).

Low amounts of galectin-1 binding proteins in human serum
Galectin-1 bound much less protein from serum than galectin-3, as stated above and confirmed by the fluorescence anisotropy assay (Table I). Alpha-2-macroglobulin was the only protein identified (Figure 3, first lane), and only traces of other proteins bound from a normal serum. When the unbound fraction from the galectin-1 column was chromatographed on a galectin-3 column, the proteins bound and eluted with lactose were similar to the galectin-3 binding proteins shown in Figures 1B and 2B, except for the reduction of alpha-2-macroglobulin by about the amount bound by galectin-1. This confirms the clear difference in selectivity between these two galectins.

Galectins-8 and -9 binding proteins in human serum
Immobilized galectins-8 and 9 bound significant amounts of serum proteins but less than galectin-3 did (Table I). As for galectin-3, alpha-2-macroglobulin and haptoglobin were major components (Figure 3), but there were some notable differences. Galectin-8 bound relatively more IgA but did not bind detectable amounts of transferrin and alpha-1-antitrypsin. The two CRDs of galectin-9 bound more of higher molecular weight glycoproteins. Galectin-9C also bound relatively more IgM, plasma protease C1 inhibitor, and apolipoprotein E, but not alpha-1-antitrypsin or transferrin. Galectin-9N bound less serum proteins than galectin-9C with a similar pattern of protein binding but with differences in the relative quantities.

The isolation of less serum proteins on the galectins-8 and -9 columns compared to galectin-3 may be caused by some glycoforms of the proteins that do not bind or bind with lower affinity. To assess this possibility, the unbound fraction from a galectin-9N column was applied to a galectin-3 column. Galectin-3 binding proteins could not be eluted after washing (not shown), in contrast to the case with the galectin-1 unbound fraction described above. This suggests that the “missing” glycoproteins had high enough affinity to initially bind the galectin-9N column, but not high enough to remain after washing, which is consistent with what is found with saccharide probes of affinity $K_d \sim 5 \mu M$. 

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### Table II. MS identification of serum glycoproteins bound by galectins

<table>
<thead>
<tr>
<th>Name of protein</th>
<th>Gal-3 spots, Figure 2A</th>
<th>Gal-3 bands, Figure 2B</th>
<th>Gal-1 bands, Figure 3</th>
<th>Gal-8S bands, Figure 3</th>
<th>Gal-9N bands, Figure 3</th>
<th>Gal-9C bands, Figure 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein B-100 (516 kDa)</td>
<td>1</td>
<td></td>
<td></td>
<td>*</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Fibronectin 1 preprotein (262 kDa)</td>
<td>2</td>
<td></td>
<td></td>
<td>*</td>
<td>2</td>
<td></td>
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<tr>
<td>Alpha-2-macroglobulin (163 kDa)</td>
<td>3, 4, 5</td>
<td>1</td>
<td>1</td>
<td>*</td>
<td>3, 4</td>
<td></td>
</tr>
<tr>
<td>Inter-alpha-trypsin inhibitor (heavy chain H4, 101 kDa)</td>
<td>4</td>
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<td></td>
<td></td>
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<tr>
<td>Transferrin (77 kDa)</td>
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<tr>
<td>Alpha-1B-glycoprotein (55 kDa)</td>
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<td>Plasma protease C1 inhibitor (55 kDa)</td>
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<td>IgA constant heavy chain 1 (53 kDa)</td>
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<td>Haptoglobin (31 kDa)</td>
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<tr>
<td>Ig kappa chain V-III (12 kDa)</td>
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<td></td>
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<td>7</td>
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</table>

*aNumbers indicate the gel band for the respective galectin and figure, where the protein was identified. *Marks galectin-9C ligands tentatively assigned based on the same size as identified ligands for galectin-9N. Molecular weights given after protein names are of fragment identified in data base search.
*The protein was identified as a nonsignificant match but identity could be confirmed by Western blot.*
Galectin-binding glycoproteins

Lack of galectin-2, -4, -4C, and -7 binding serum ligands

Galectin-2, -4, -4C, and -7 typically bound less than 10 μg protein per mL of serum (Table I), which is at least 500-fold less compared to galectin-3. After the concentration of galectin-4 bound fractions from one serum, a faint visible band could be detected on SDS–PAGE (not shown) but was identified by MS as albumin, which is probably a nonspecific contaminant. In another case (one out of six), galectin-4C bound ~40 μg/mL, identified as mainly alpha-2-macroglobulin.

Discussion

This report is the first survey of galectin binding to human serum glycoproteins. We show that galectins-3, -8, and -9 bind a wide range of serum glycoproteins in contrast to galectin-I that binds lower levels of a limited range and galectins-2, -4, and -7 that bind barely detectable amounts. This provides insight into how galectin fine specificities translate into binding when confronted with a complex set of natural glycoproteins, as found in serum. Most of the ligands found here have not been reported to bind galectins before. They are major serum glycoproteins and include protease inhibitors, apo-lipoproteins and immunoglobulins, and 44% of the identified proteins also act in the acute phase response being either up or down regulated. The biological significance of the galectin interaction with these ligands will depend on their affinities and their concentrations in cellular environments where galectins are present and thought to function.

The ligand affinities for galectins were first estimated from modeling of the affinity chromatography and the behavior of fluorescent probes on the same columns. This showed that binding required an affinity of Kd 1–5 μM or better, which is similar to estimates of galectins-1 and -3 affinity for cell surface ligands (Hernandez et al. 2006; Stowell et al. 2008). In solution, the potency of serum to inhibit binding of galectin-3 to a fluorescent saccharide probe, measured by fluorescence anisotropy, was equivalent to inhibitors with rescent saccharide probe, measured by fluorescence anisotropy, and (Hernandez et al. 2006; Stowell et al. 2008). In solution, to estimates of galectins-1 and -3 affinity for cell surface ligands that bind barely detectable amounts. This provides insight into how galectin fine specificities translate into binding when confronted with a complex set of natural glycoproteins, as found in serum. Most of the ligands found here have not been reported to bind galectins before. They are major serum glycoproteins and include protease inhibitors, apo-lipoproteins and immunoglobulins, and 44% of the identified proteins also act in the acute phase response being either up or down regulated. The biological significance of the galectin interaction with these ligands will depend on their affinities and their concentrations in cellular environments where galectins are present and thought to function.

The discussed above suggests that galectin-3 selects glycoproteins in serum based on at least one available LacNAc residue (free or 2–3 sialylated), even when found as a minor component among mainly nonbinding glycoproteins carried by the same glycoprotein. As expected from this, the profile of the N-glycans of galectin-3 bound glycoproteins was very similar to that of unfractionated serum (Cederfur, Radcliffe, Rudd, Leffler, unpublished), where nonbinding glycans predominate (Table III). This model for binding to serum glycoproteins, where binding correlates with the likelihood of finding an available LacNAc residue for binding, may also act in cellular systems. A larger number and more branching of N-glycans were shown to increase galectin-3 binding, which, in turn, was proposed to determine cell surface residence time of various growth factor receptors (Lau et al. 2007).
Galectin-1 bound only a fraction of alpha-2-macroglobulin but not most other human serum glycoproteins. The galectin-1 CRDs bind more weakly to single N-glycans ($K_d \sim 50 \, \mu M$) compared to galectin-3 (Hirabayashi et al. 2002), and higher affinity may require divalent binding to two glycans at the same time (Leppanen et al. 2005). Two properly spaced N-glycans with available galactose residues would be statistically unusual and perhaps only present in a multimeric highly glycosylated protein such as alpha-2-macroglobulin. In earlier studies, affinity chromatography of N-glycans or glycoproteins on immobilized galectin-1 purified only those carrying poly-N-acetyl-lactosaminoglycans (Merkle and Cummings 1988; Zhou and Cummings 1993). However, poly-N-acetyl-lactosamines are not typically present on serum glycoproteins (Kita et al. 2007).

The different selectivity of galectins-1 and -3 may be similar in cellular systems. Th2-lymphocytes, in contrast to Th1 and Th17 cells, bind reduced amounts of galectin-1 and are resistant to its apoptotic effect (Toscano et al. 2007). This resistance was in part due to increased 2–6 sialylation and could be reversed by desialylation with neuraminidase. In contrast, galectin-3 bound and induced apoptosis in the Th2 cells despite the increased 2–6 sialylation. The binding to the remaining nonsialylated galactosides may have been enough for galectin-3 but not for galectin-1, just as found here for serum glycoproteins.

Galectins-8 and -9 bound similar, but not identical, arrays of serum glycoproteins as galectin-3, but total amounts were lower (Table I). Although further studies are necessary, some differences are worthwhile noting already. Galectin-8 bound relatively more IgA (Figure 3) compared to galectin-3, probably because the relative amounts of other glycoproteins were less. The preference for IgA may be due to the particularly high affinity of galectin-8N for 2–3-sialylated galactosides (Carlsson et al. 2007), which is unique among galectins. Moreover, it especially prefers galactosides linked to the 3 position of GlcNAc or GalNAc (found in T-antigen), where the latter is found in, e.g., O-linked glycans of IgA (Mattu et al. 1998). Interestingly, deficient galactosylation of IgA O-glycans, which is associated with IgA nephropathy (Smith et al. 2006), may then alter binding of galectin-8. Galectin-8 binds nonsialylated LacNAc residues in N-glycans with much lower affinity compared to galectin-3 (Hirabayashi et al. 2002). However, 3-sialylated galactosides which occur on one of the antennae in a fraction of tri- or tetra-antennary N-glycans in serum (Kita et al. 2007) could perhaps explain binding of a fraction of alpha-2-macroglobulin and other glycoproteins also to galectin-8. Moreover, simultaneous low affinity binding of the two CRDs may combine to high enough affinity in certain cases (Carlsson et al. 2007). Galectin-9 does not prefer 3-sialylated galactosides like galectin-8, but binds many nonsialylated N-glycans much better although the affinities reported in Hirabayashi et al. (2002) differ significantly from those in a more recent publication (Nagae et al. 2008).

The interaction of galectins with serum glycoproteins is functionally relevant because, as mentioned in the Introduction, endothelial cells (Thijssen, Hulsmans, et al. 2007; Thijssen, Poirier, et al. 2007; Yu et al. 2007) and most other cells outside CNS express galectins and are likely to be exposed to high concentrations of serum components (Rippe and Haraldsson 1994). Here we show that serum diluted to 50% (the estimated steady-state concentration of many serum proteins in a tissue (Rippe and Haraldsson 1994)) can almost completely compete 2 µM galectin-3 off a ligand with sub-micromolar affinity (Figure 3).

These numbers are similar to the active galectin-3 concentration required and estimated cell surface affinity commonly found in experimental assays in the cell culture. Therefore, any extracellular or cell surface activity of galectin-3 in vivo, outside the CNS, will most likely involve serum glycoproteins, either as inhibitors or as functional partners. Indeed, besides their function in serum many of the glycoproteins also function by interaction with tissue cells, e.g. alpha-2-macroglobulin (Bonacci et al. 2007), and haptoglobin (Larsen et al. 2006).

Galectins-2, -4, and -7 bound at most trace amounts (at least 500 times less compared to galectin-3) of serum glycoproteins on the columns and did not interact measurably in solution. They have a much more narrow tissue-specific expression than the galectins mentioned above (Poirier 2002), and may be adapted to bind glycoproteins from these tissues having other glycan structures. For example, galectin-4 is expressed in gastrointestinal epithelium and binds glycoproteins from intestinal epithelial cells (Ideo et al. 2005; Danielsen and Hansen 2006), which carry glycans that are quite different from those on serum glycoproteins (Finne et al. 1989). These galectins may also be adapted for low binding to serum glycoproteins, in order not to be inhibited when they act extracellularly. They may, thus, gain increased potency compared to galectins-3, -8, and -9 in the presence of serum.

A few studies describe use of galectin-3 to detect glycoproteins (Bresalier et al. 2004; Grassadonia et al. 2004) associated with pathological conditions. The present results provide a basis for further development of this. Galectins not binding particular normal serum proteins may be especially useful to pick up glycoproteins with altered glycosylation either as induced indirectly or because they were derived from a pathological tissue. For example, galectin-4 is known to bind carcinoembryonic antigen from colon (Ideo et al. 2005), and transferrin from serum of hepatoma patients had dramatically increased amounts of tri-antennary N-glycans (Yamashita et al. 1989), which may cause binding to galectin-3 as indicated here.

Materials and methods

**Serum samples**

Blood samples from healthy volunteers were collected in Vacutainer tubes (silicon treated with coagulation activator, Becton Dickinson, Plymouth, UK), kept at room temperature for 1 h to permit coagulation and sedimentation, and centrifuged at 290 × g for 15 min, after which the serum supernatants were harvested and stored at −20°C until used. To convert alpha-2-macroglobulin to its closed form, 100 µL serum was mixed with 100 µL 0.8 M methylamine in PBS and incubated for 1 h at room temperature (Gunnarsson and Jenssen 1998).

**Galectins**

All galectins were produced in *Escherichia coli* BL21 Star (Invitrogen, San Diego, CA) and purified by chromatography on lactosyl-Sepharose as described earlier, with rat galectin-1, and human galectins-2, -3, -4, -4C as the native proteins (Sorme et al. 2004), human galectins-8S, -8N, -9N, and -9C as thioredoxin fusion proteins (Salameh et al. 2006; Carlsson et al. 2007), and mouse galectin-7 as a GST-fusion protein (Salameh et al. 2006). A galectin-3 R186S mutant was also included (Cumpstey et al. 2007; Delacour et al. 2007).
Galectin affinity columns

Galectins were coupled to 1 or 5 mL NHS-activated Hi-Trap affinity columns (GE Healthcare, Uppsala, Sweden) as described by the manufacturer, using, per mL column, 5–8 mg galectin in a 2 mL coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). To test galectin activity on the columns, we examined their ability to bind monovalent fluorescein-tagged saccharide probes (Sorme et al. 2004; Carlsson et al. 2007), 2 µM in PBS (118 mM NaCl, 67 mM Na/K-phosphate, pH 7.2). The probe solution, 0.2 mL per mL column, was kept on ice in a test tube and circulated on the column by pumping from the bottom of the test tube and readding the column effluent at the top, for 30 min, at 0.6 mL/min or 4 mL/min for the 1 and 5 mL columns, respectively. The column was sealed for 30 min to enhance the probe–galectin binding. The column was then washed with 20 column volumes of PBS and fractions were collected. Bound probe was eluted with 150 mM lactose in PBS. The percentage of initially bound probe was calculated from the difference in fluorescence, measured (0.1 mL aliquots using a PolarStar instrument, BMG Lab Technologies, Offenburg, Germany), in the sample before and after circulation, and correcting for the even distribution of the remaining free probe also in the soluble phase of the column and tubing used for the circulation. The percentage of bound probe after washing was obtained by subtracting measured fluorescence in the wash fraction from the initial percentage.

Serum ligand isolation

While kept on ice, 0.1 mL serum in 1.9 mL PBS or 0.5 mL human serum diluted with 9.5 mL PBS was circulated for 30 min at 0.6 or 4 mL/min on a 1 mL or 5 mL galectin column, respectively. The column was sealed for 30 min to enhance the ligand–galectin binding. The unbound fraction (flow through) was saved and the column was washed with at least 20 column volumes of PBS. Galectin-binding proteins were eluted with 150 mM lactose in PBS, in fractions of 0.2, 0.5, or 0.7 mL, until three to four column volumes. Protein concentrations were determined with the Bio-Rad protein assay (BioRad, Hercules, CA) and fractions with the highest protein concentrations were kept.

In some cases serum samples were applied repeatedly to the same column to ensure and demonstrate the complete removal of bound ligands. For this a sample was first applied to a galectin column as described above, and the unbound fraction kept. The column was then washed with lactose in PBS to elute any bound ligands, re-equilibrated with 200 mL PBS, and then the first unbound fraction was applied again. The unbound fraction from this second run was used for further analysis, e.g. applied to a third round of chromatography on the same column or applied to another galectin column.

In some cases, the possible retention of protein on columns after lactose elution was examined. For this, the columns were opened, and 100 mg sepharose was removed from the top and boiled for 1 min in a 50 µL sample buffer (62.5 mM Tris–HCl, 10% glycerol, 2% SDS, 0.025% bromophenol blue, 5% mercaptoethanol); the boiled samples were centrifuged at 20,800 × g for ~5 min, and the supernatant was separated by 4–20% SDS–PAGE.

Modeling of affinity chromatography

A computer program in the scripting language PHP3 was written to model the affinity chromatography and to relate the yield of eluted ligands to a likely range of affinities (Kₜₐₜ) of the bound galectin for soluble ligands. This was based on a simple plate model of the column as follows. The theoretical plates of the column (segments) were represented by elements of two arrays, one giving the concentration in each segment of bound ligand and the other the concentrations in each of free ligand. An additional element at the beginning of each array described the concentration of free ligand in the solution entering the column, e.g. equal to the sample concentration during initial loading and zero during washing. An element added to the end of the array describes the concentration coming out and was used to construct a chromatogram. At a given moment (step) the concentration of free and bound ligand was calculated for each column segment from the equation of mass action:

\[
K_d = \frac{[\text{Galectin}_\text{Free}][\text{Ligand}_\text{Free}]}{[\text{Ligand}_\text{Bound}]} \quad \text{giving}
\]

\[
[Ligand_{Bound}] = \frac{T}{2} - \sqrt{(T/2) - Galectin_{tot}^2/Ligand_{tot}}
\]

\[
T = K_d + \text{Galectin}_{tot} + \text{Ligand}_{tot}
\]

\[
[Ligand_{Free}] = [\text{Ligand}_{tot}] - [\text{Ligand}_{Bound}].
\]

Then the solution phase was assumed to move by the same volume as one segment and a new equilibrium between bound and free ligand re-established. New concentrations of free and bound ligand were calculated using [Ligand_{me}] as the sum of [Ligand_{bound}] left from before and [Ligand_{free}] carried from the previous column segment. For each segment, the new values of free and bound ligand concentration replaced the old to form new arrays, which in turn formed the basis for next round of calculations. This process of moving the solution down the column in steps of one segment volume was iterated as many times as needed to account for the experimental sample loading volume, time of circulation, wash volume, and elution volume, and with appropriate changes in the value of the first array element representing ligand concentrations entering the column. During elution, affinity was assumed to drop to make fraction bound ligand in each plate 10%, which resulted in quick elution of remaining bound ligand, and its percentage of total loaded ligand was calculated. The following parameters were needed in the model, and a range of values were tested for each. The concentration of bound active galectins exposed to the solution phase was estimated from the measured amount of bound galectin per mL of the column bed volume, assuming that the solution phase of the column was 70% of total bed volume, and that 10–100% of the galectin was active, as found by others (Hirabayashi et al. 2002). This did not alter the results much, since the bound galectin would be well above the likely ligand concentration in most cases. The number of segments (theoretical plates) in the column was varied between 1 and 10, but this also did not alter the results significantly. The ligand concentration (µM) in the loaded sample was estimated from the experimentally determined amount of isolated ligand and assumed yields ranging from 10 to 90%. The Kₜₐₜ of the galectin–ligand interaction was based on known solution phase affinities and was varied between 0.1 and 100 µM.
Measurement of serum–galectin interaction by fluorescence anisotropy

Average serum affinity for each galectin was estimated from its ability to inhibit interaction with fluorescein-tagged saccharide probes measured by fluorescence anisotropy as described (Sorme et al. 2004; Carlsson et al. 2007). The measurements had to be done in the presence of 2 µM probe instead of 0.1 to overcome the serum autofluorescence compared to the probe fluorescence. Different concentrations of galectin were mixed with 2 µM of the optimal probe for each (Tables I), and either serum to a final dilution of 1/2 (50%), or PBS. The percentage bound probe was calculated from the measured anisotropy value, the value for probe only (A0), which was higher in serum, and the estimated maximum anisotropy representing all probe bound (Amax). Binding curves, with percentage binding on the X-axis and log galectin concentration on the Y-axis, were constructed and compared for the case with serum and with PBS. A shift of the curve for the sample with serum to the right would indicate inhibition by serum. An average affinity (Kd) of serum for galectin was also calculated for each data point as described in Sorme et al. (2004) and Carlsson et al. (2007) using different estimates of glycan concentration in serum based on Kita et al. (2007) as “inhibitor concentration” and the binding in PBS as the value in the absence of an inhibitor. The relative inhibitory potency of serum for different galectins was calculated from this, which was now independent of the assumed glycan concentration.

One-dimensional gel electrophoresis and Western blotting

Samples containing approximately 5–10 µg of total protein were separated on one-dimensional 12% SDS–PAGE gels. The gels were stained using Bio-Safe Coomassie (BioRad) after washing in deionized water. Bands of interest were excised and treated for mass spectrometry as described below. For Western blotting, proteins were transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked overnight using 5% non-fat dry milk (Bio-Rad) in 0.1% tween in PBS before incubation with primary antibodies diluted ~1/4000 (Sigma-Aldrich, Stockholm, Sweden) and horseradish peroxidase-conjugated anti-goat (Jackson Immunoresearch, West Grove, PA) secondary antibody, diluted 1/15,000. Immune complexes were detected using the ECL plus system (GE healthcare, Little Chalfont, Buckinghamshire, UK) and scanned using a GelDoc imager (BioRad).

Two-dimensional gel electrophoresis

Each sample (350 µL) was dialyzed in 1 L of dialysis buffer (8 M urea, 2% CHAPS, 15 mM DTT, with 2 mM EDTA and 100 µM PMSF) with one change of the buffer and then dialyzed overnight. Before isoelectric focusing (IEF), strips were rehydrated in a 300 µL rehydration solution (8 M urea, 15 mM dithiothreitol, 0.5% (w/v) CHAPS and 1% IPG buffer 3–10) containing 300–700 µg protein. Rehydration was performed overnight under a layer of mineral oil at 20 °C and 50 V in a Protean IEF Cell (BioRad). Focusing was carried out at 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 1000–8000 V for 30 min, and 8000 V for 35,000 Vh. Strips were equilibrated for 15 min in 6 M urea, 30% (w/v) glycerol and 2% (w/v) SDS in a 0.05 M Tris–HCl buffer, pH 8.8 with 65 mM dithiothreitol, and then in 6 M urea, 30% (w/v) glycerol and 2% (w/v) SDS in a 0.05 M Tris–HCl buffer, pH 8.8 with 260 mM iodoacetamide. Second dimension runs were performed in an Ettan Daltix12 electrophoresis system (GE Healthcare) or Ettan DaltTwelve electrophoresis system. Strips, covered with 0.5% agarose at the top of second dimension gels, were run overnight until the tracking dye reached the anodic end. Gels were fixed in 40% ethanol and 10% acetic acid for 3 h, stained overnight with SYPRO® Ruby protein gel stain (BioRad), washed in 10% methanol and 7% acetic acid for 30–60 min, in deionized water, and scanned with an FLA 3000 gel scanner (Fujifilm Life Science, Stanford, CT).

Mass spectrometry and protein identification

Mass spectrometric analysis was performed at the Swegene core facility, Lund University, Sweden. Gel spots were excised manually with a scalpel and transferred to Eppendorf tubes, or excised using an Ettan Spot workhandling station (GE Healthcare). Gel pieces were digested with 50% acetonitrile, 25 mM NH4HCO3, dried using a speed vac concentrator, and digested overnight at 37°C using 12.5 µg/mL trypsin (Promega, Madison, WI) in 50 mM NH4HCO3 and then 5% trifluoroacetic acid in 75% acetonitrile to terminate the reaction. 0.5 µL of sample was eluted onto 0.5 µL of dried matrix solution (20 mg/mL alfa-cyano-4-hydroxyccinnamic acid (CHCA) in 50% acetonitrile and 0.05% trifluoroacetic acid) on the standard 96-well stainless steel MALDI target plate ( Waters, Manchester, UK). One-dimensional gel spots were, before trypsination, additionally reduced/alkylated in 10 mM dithiothreitol (DTT)/50 mM NH4HCO3 (1 h at 56°C)/55 mM iodoacetamide/50 mM NH4HCO3 (45 min), and then washed in 100 mM NH4HCO3 and acetonitrile.

Mass spectrometry was performed using a MALDI-TOF instrument (MALDI LR HT, Waters); each spectrum represented up to 200 laser shots. Database searching was performed against the comprehensive, nonredundant IPI human database using the Plums software, and multiple searches were done using the automated Plums (Levander et al. 2004). The software Mascot 2.0.0 was used for additional database searching. Carbamidomethylation was set as a fixed modification and methionine oxidation as a variable one; one missed cleavage was allowed. The peptide tolerance was set to 50–200 ppm. The protein identifications were considered to be confident when the expectancy score was below 0.001.

Affinity chromatography of transferrin and glycan analysis

Human lyophilized transferrin (catalogue no. T3309, Sigma-Aldrich) was dissolved at 1 mg/mL in PBS and subjected to affinity chromatography on a 1 mL column with immobilized galectin-3 as described for chromatography of serum. Transferrin samples (20 µg unfractionated transferrin, 2.5 µg bound fraction, and 20 µg of unbound fraction) were dialyzed against 50 mM NH4HCO3 using Slide-A-Lyser Mini Dialysis Units, 10,000 molecular weight cutoff (Pierce, Rockford, IL), reduced/alkylated using 10 mM dithiothreitol (DTT) for 60 min at 37°C followed by 50 mM iodoacetamide (Sigma) at room temperature for 30 min, and then dialyzed again against 50 mM NH4HCO3. The reduced/alkylated samples were digested with 1 µg sequence grade-modified porcine trypsin (Promega) for 8 h at 37°C, and then lyophilized and stored at −20°C until mass spectrometric analysis. The tryptic peptides were redissolved in 0.1% formic acid, and separated by reversed-phase HPLC on an Ettan MDLC system.
(GE Healthcare) using a 150 × 0.075 mm fused silica column (Zorbax 300-SB-C18, Agilent Technologies, Kista, Sweden) with a 50-min gradient from 0 to 50% acetonitrile, 0.1% formic acid at a flow rate of 200–300 nL/min. The chromatographic system was connected online through a nanoelectrospray ion source to a hybrid linear ion trap FTICR mass spectrometer equipped with a 7T ICR magnet (LTQ-FT; Thermo Electron, Bremen, Germany). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS, MS/MS, and MS3 acquisition. The survey MS spectra (from m/z 300 to 2000) were acquired in the FTICR cell with resolution 100,000 at m/z 400 (after accumulation to a target value of 1,000,000 in the linear ion trap). The most intense ion from the survey scan was isolated and fragmented in the linear ion trap using collision-induced dissociation at a target value of 2000. For each MS/MS spectrum data-dependent settings were chosen to trigger MS3 scans for the five most intense fragment ions. Former target ions selected for MS/MS were dynamically excluded for 60 s. The general mass spectrometric conditions were spray voltage, 1.3 kV; ion transfer tube temperature, 200 ◦C. Ion selection thresholds were 500 counts, normalized collision energy 30%, activation q = 0.25, and activation time 30 ms was applied in MS/MS and MS3 acquisitions.

Analysis of immunoglobulins

Levels of immunoglobulins were determined in a galectin-3 bound and unbound serum fraction as well as in serum before passing the column (diluted 1/20), using standard assays employed at the Clinical Immunology Laboratory of Lund University Hospital. Total IgG and IgM were measured turbidometrically using a Cobas Pentra instrument and Immunkemi kit (Triolab, M¨olndal, Sweden), and IgA levels were determined using rocket immuno electrophoresis (Laurell 1966). Total IgE levels were determined by a fluoroenzymeimmuno assay using an ImmunoCap 1000 instrument and ImmunoCap kit (Phadia, Uppsala, Sweden).

Supplementary Data

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

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Conflict of interest statement.

None declared.

Abbreviations

CRD, carbohydrate recognition domain; IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry; PBS, phosphate-buffered saline; wt, wild type.

References


