Binding studies of adhesion/growth-regulatory galectins with glycoconjugates monitored by surface plasmon resonance and NMR spectroscopy

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Functionalized fluorescent glycans have the potential to act as tools to detect and analyze protein–carbohydrate interactions. We present here a facile strategy for immobilization of functionalized lactose as a model disaccharide. Bioactivity was tested with three members of the adhesion/growth-regulatory galectins family in different types of assay, *i.e.* matrix in surface plasmon resonance (SPR), free ligand in solution by STD/trNOESY and docking measurements. In all cases, the activity of the disaccharide was maintained. The attachment of this new fluorescent glycoconjugate to the surface results in a well-defined interface, enabling desired orientational flexibility and enhanced access of binding partners. The results indicate that this new glycoconjugate exhibits binding affinity to galectin-1, 3 and CG-16. Kinetic analysis of the interaction between these galectins and immobilized glycoconjugate by SPR yielded a K_D of 1.01 mM for galectin-1, 83.5 μM for galectin-3 and 0.28 mM for CG-16. No major contacts to the aglyconic part were detected, which might compromise the specificity of the binding process with other headgroups. Thus, testing these proteins offers the potential for medical applications to detect these endogenous effectors or further derivatives and characterize their carbohydrate specificity.

Introduction

The emerging insights into the functionality of glycans as bioactive signals in cell regulation, embodied by the concept of the sugar code, raise the interest in generating probes for interaction studies with their receptors, *i.e.* lectins.[1,2] To address this issue, we have previously prepared a new fluorescent lactose derivative functionalized with an amino group that can readily be immobilized on different surfaces.[3,4] These compounds are detectable by UV-visible or fluorescence spectroscopy and in addition, the free amino group enables its coupling to different surfaces functionalized with carboxylic groups. These molecules have been designed to obtain versatile systems to study their biological roles using different techniques. One of the main problems of fluorescent glycoconjugates is the non-specific binding of the aglycon to the proteins. Therefore, the aim of this study by SPR and NMR is to ascertain that the fluorescent tag does not impact the interaction therefore, the aglycon will not mask the results when other techniques based on fluorescent detection are used.

Having initially worked with a plant lectin as model compound, we herein broaden the scope of applicability of our synthetic compounds, also refining product yields, to animal lectins, *i.e.* the adhesion/growth-regulatory galectins. Thus, testing these homologous proteins offers the potential for medical applications to detect these endogenous effectors and characterize their carbohydrate specificity. The homodimeric galectin-1 and chimera-type galectin-3 are the most prominent members of this family, involved in tumor progression in diverse tumor types and in other diseases such as heart failure.[5-8] This status has prompted proof-of-principle studies with these two potent endogenous effectors that show disparate expression profiles.[9,10] As a further example, and considering sequence variations, we added an avian galectin to our study with mammalian proteins. It has formerly been called C-16 or CG-16, now CG-1A because of its structural relation to galectin-1.[11,12] The homology is shown by the conservation of the central Trp residue essential for stacking, while the neighboring residues can differ in sequence.[13,14] Thus, it was a challenge to not only ascertain the bioactivity of the fluorescently tagged glycans to the animal lectins but also to probe the potential of the aglyconic part for interactions with the lectin site. To meet this challenge we strategically combined SPR measurements with two NMR spectroscopy techniques to allow epitope contact mapping and rigid-body docking.

Results and discussion

A fluorescent glycoconjugate has been synthesised and used as a model to examine the influence of the linker unit on the binding to the three members of the adhesion/growth-regulatory galectin family (galectin-1, -3 and CG-1A).

The fluorescent naphthyl derivative was synthesized following the methodology previously developed in our laboratory[4] (Fig. 1). In this approach, peracetylated lactoside **1** was treated with

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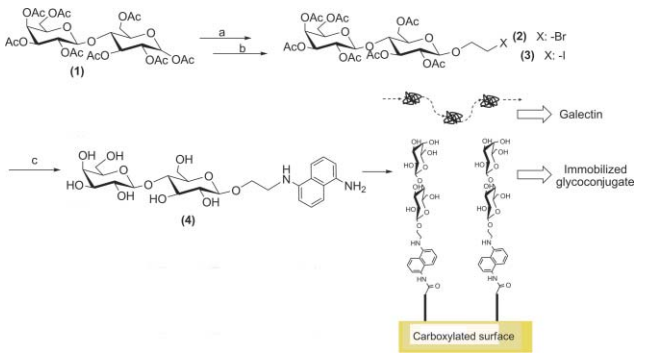

Fig. 1 Synthesis of LacDAN (**4**) and its immobilization on a carboxylated surface. (a) $BF_3{\cdot}Et_2O$, Br-ethanol, CH_2Cl_2 (40% yield). (b) NaI, acetone, reflux (100% yields). (c) DAN, toluene, reflux (70% yields).

2-bromoethanol to obtain compound **2** in 40% yield. Then, the bromine was exchanged by iodine using NaI in acetone at reflux temperature to afford compound **3** in quantitative yield. Finally compound **3** was treated with fluorescent probe 1,5-diaminonaphthalene DAN in toluene at 100 °C and the desired product **4** (LacDAN) was obtained in 70% yield (Fig. 1).

Then, the effects of the linker and the fluorescent tag in holding the model glycoconjugate on the surface of the sensor chip and any influence that chain may exert on the binding of the galectins were explored using SPR and saturation transfer difference (STD). Due to the fact that these techniques are not based on UV-fluorescence properties, the fluorescent residue effect on the carbohydrate–lectin interaction can be assessed.

For the SPR studies, the fluorescent derivative LacDAN was immobilized on a carboxymethylated dextran matrix CM5 chip through the free amino group. The intensity reached after the immobilization procedure was 660 RU, and the remaining activated residues were blocked with ethanolamine to avoid possible interferences in the study (Fig. 1).

The surface treated with the reagents for conjugation served as negative control to rule out carbohydrate-independent binding. Sensorgrams were recorded in this channel routinely to assess extent of signal.

Interaction studies of galectin-1 with LacDAN by SPR

Galectin-1 was injected using increasing concentrations (0.9–114.7 μM) and interactions with LacDAN led to an intense signal of 180 RU (Fig. 2). The interaction curves obtained for galectin-1 and LacDAN were fitted to a 1 : 1 Langmuir kinetic ($\chi^2 = 2.6$), with a fast association rate ($k_{on} = 192\ M^{-1}s^{-1}$) that reaches the steady state very fast, and also a rapid dissociation rate ($k_{off} = 2.1 \times 10^{-1}\ s^{-1}$). The apparent K_D value is 1.01 mM. Due to the fact that both association and dissociation phases occur so rapidly, we carried out a steady state affinity study and the resulting value of K_D obtained for that fitting was 0.55 mM (Table 1).

Table 1 Kinetic measurement of galectin-1 binding to immobilized LacDAN determined by SPR

Langmuir fitting				Steady state affinity
k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (mM)	χ^2	K_D (mM)
1.92×10^2	2.1×10^{-1}	1.01	2.6	0.55 ± 0.14

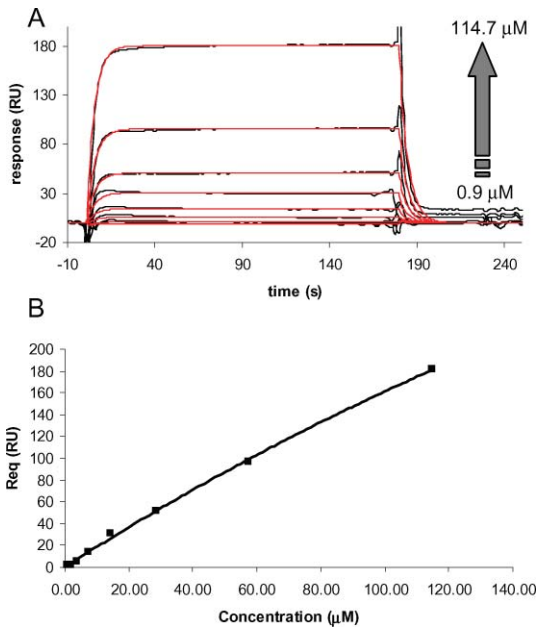

Fig. 2 **A**) Sensorgrams of galectin-1 binding to LacDAN showing the association phase and the dissociation phase. **B**) Steady state affinity study of the galectin-1–LacDAN interaction.

In addition, the k_{off} or K_D values obtained are in the same magnitude range to those described previously, using SPR with a neoglycoprotein or other techniques such as frontal affinity chromatography (FAC) or isothermal titration calorimetry (ITC).[15-20]

Different authors have reported the interaction of galectin-1 with different β-Gal and lactosamine (LacNAc, Galβ1→4GlcNAc) derivatives. Ahmad *et al.*[17] performed a thermodynamic study of the interaction of this galectin with different LacNAc by ITC. These authors reported a K_D value of 0.1 mM for the interaction with LacNAc, which is in good agreement with the results observed by SPR. Furthermore, additional studies of the interaction between galectin-1 and different pyridylaminated oligosaccharides has been measured by Hirabayashi *et al.*[16] by FAC. In this study, pyridylaminated lactosamine bind to galectin-1 in the same range.

Interaction studies of CG-1A with LacDAN by SPR

Following up the studies with mammalian galectin-1 to the next system, chicken galectin CG-1A was used as test analyte. It is related to galectin-1 in its carbohydrate recognition domain, but shows enough sequence deviations to be useful to broaden the scope of this study.[21]

The experiments with chicken galectin CG-1A were performed using the same settings as the ones with the mammalian galectin-1. Different concentrations of CG-1A (1.9 to 62.5 μM) were injected and the sensorgrams obtained were fitted a 1 : 1 Langmuir kinetic model ($\chi^2 = 3.2$) after subtracting the noise signal. As shown in Fig. 3, the association phase was very fast ($k_{on} = 2.5 \times 10^3\ M^{-1}s^{-1}$), reaching the steady state rapidly, and the dissociation phase was also very fast ($k_{off} = 2.1 \times 10^{-1}\ s^{-1}$). The apparent K_D value calculated for this interaction was 83.5 μM. When K_D was calculated from a general fitting to a steady state affinity model, the value was 28.8 μM (Table 2).

Table 2 Kinetic measurement of galectin-CG1A binding to immobilized LacDAN determined by SPR

Langmuir fitting				Steady state affinity
k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (μM)	χ^2	K_D (μM)
2.5×10^3	2.1×10^{-1}	83.5	3.2	28.8 ± 3.1

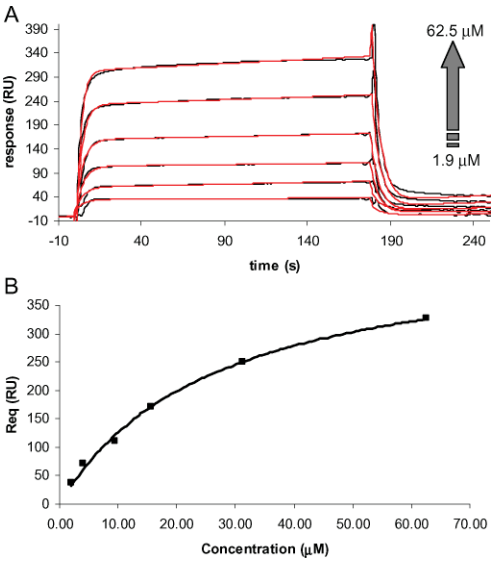

Fig. 3 **A**) Sensorgrams of galectin CG-1A binding to LacDAN showing the association phase and the dissociation phase. **B**) Steady state affinity study of the galectin CG-1A–LacDAN interaction.

The affinity and binding of the CG-1A to the LacDAN is stronger than its mammalian counterpart. The magnitude range significantly varies from a millimolar (as described above) to a micromolar range in this case. However, these values also confirmed what was previously by Hirabayashi *et al.*[16] These authors measured the interaction of CG-1A with pyridyl-LacNAc derivatives by frontal affinity chromatography (FAC) (K_D = 19 μM). The range of the binding, micromolar is in good agreement with our SPR measurements.

Interaction studies of galectin-3 with LacDAN by SPR

Different concentrations of murine galectin-3, in a concentration range between 0.5 to 11.8 μM, were injected over the LacDAN surface. After subtracting the noise signal, the maximum intensity reached was 440 RU (Fig. 4). The association and dissociation phases were very fast (k_{on} = 731 $M^{-1}s^{-1}$ and $k_{off} = 2.0 \times 10^{-1}$ s^{-1}). Consequently, the calculated K_D value was 0.28 mM according to a 1 : 1 Langmuir kinetic model with the "drifting baseline correction" as an ascending trend was observed in the experiments ($\chi^2 = 9.1$) (Table 3). In an analogous way to that described above

Table 3 Kinetic measurement of galectin-3 binding to immobilized LacDAN determined by SPR

Langmuir fitting				Steady state affinity
k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (mM)	χ^2	K_D (mM)
7.31×10^2	2.0×10^{-1}	0.28	9.1	0.80 ± 0.23

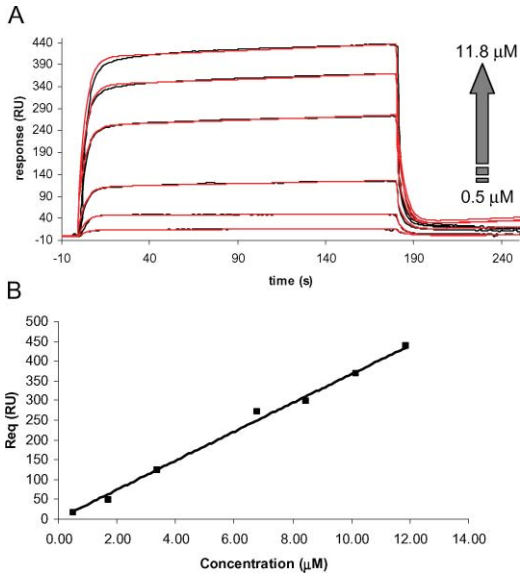

Fig. 4 **A**) Sensorgrams of galectin-3 binding to LacDAN showing the association phase and the dissociation phase. **B**) Steady state affinity study of the galectin-3–LacDAN interaction.

for the other galectins, we performed a steady state affinity curve fitting, yielding a value of K_D = 0.80 mM (Table 3).

Both kinetic and affinity studies provided results in millimolar ranges (0.28 and 0.80 mM). Compared to previous results for the interaction of galectin-3 with other saccharides, we found that the naphthyl derivative LacDAN behaves like similar compounds. For example, Hirabayashi *et al.*[16] studied the interaction of pyridylaminated lactosamine derivatives with galectin-3 by FAC. The affinity parameter calculated by FAC of these compounds with the galectin-3 (mM) lay in a similar range to our SPR values. Furthermore, a different study performed by Bachhawat-Sikder *et al.*[22] by ITC yielded similar results for the interaction of lactose and galectin-3 (K_D between 0.86 and 1.25 mM).

Molecular recognition studies by NMR and docking procedures

To further study the structural aspects of the interaction we proceeded to investigate binding in solution by NMR spectroscopy and computationally by molecular docking. Previous studies have documented that these derivatives do not aggregate in solution, adopt the low-energy conformation in solution for the saccharide part without deviation due to the presence of the bulky aromatic group and interact with the galactoside-binding plant lectin viscumin *via* the galactose headgroup.[3,4] With interest also in drug design due to *e. g.* galectin-1's pro-invasive features in glioblastoma or pancreatic cancer,[23-24] spatial aspects of ligand accommodation were mapped by STD NMR spectroscopy. This technique involves the transfer of saturation from the protein to the ligand and therefore provides information on the proximity of the individual protons of the ligand to the protein surface. Thus, contact mapping can be performed readily without isotope labelling.[25]

The STD experiments gave unambiguous confirmation that galectin-1 recognizes LacDAN in solution as a ligand. Clear STD signals for the sugar protons in the ligand were observed (Fig. 5). The maximum enhancements were observed for the protons at the Gal moiety, especially for Gal H4, with smaller but significant

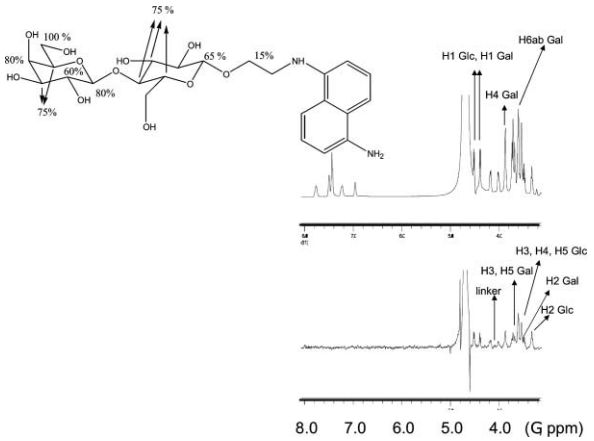

Fig. 5 The interaction of LacDAN with galectin-1. The upper spectrum shows the regular 500 MHz ^{1}H NMR spectrum of LacDAN at 300 K in the presence of galectin-1 (40 : 1). The lower spectrum shows the STD spectrum (saturation time 2 s, on resonance irradiation at $\delta = -1$ ppm), showing major enhancement of the Gal protons, followed by those of the Glc moiety with smaller transfer to the linker and no transfer to the DAN fragment. The binding epitope is the lactose moiety.

STD effects for protons of the Glc residue. Small enhancements were even evident at the linker, while the DAN protons showed only tiny enhancements at long saturation times, indicating a minor interaction of this section of the derivative with galectin-1. Analogous experiments were performed for LacDAN and the CRD of galectin-3, which showed even smaller transfer to the Glc moiety and to the linker fragments (Fig. 6).

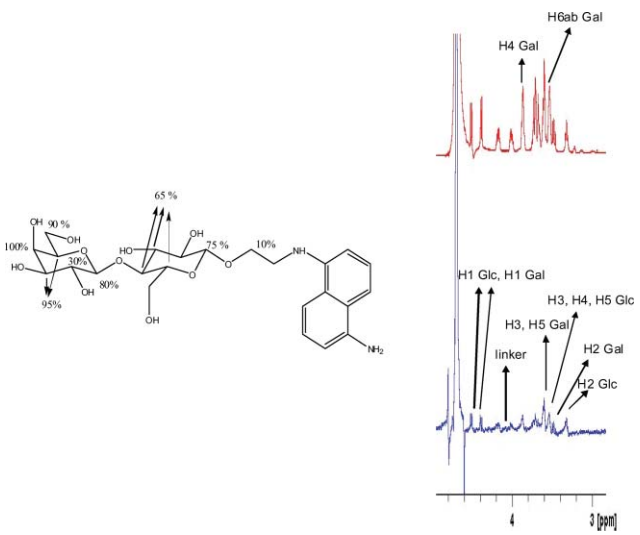

Fig. 6 The interaction of LacDAN with galectin-3. The upper spectrum shows the regular 500 MHz ^{1}H NMR spectrum of LacDAP at 300 K in the presence of galectin-3 (30 : 1). The lower spectrum shows the STD spectrum (saturation time 2 s, on resonance irradiation at $\delta = -1$ ppm), showing major enhancement of the Gal protons, followed by those of the Glc moiety with smaller transfer to the linker and no transfer to the DAN fragment. The binding epitope is the lactose moiety.

Minor interactions of the side chains with both galectins were evident, with major transfers to the Gal moiety, quantitatively followed by the Glc residue. As deduced from the STD experiments, subtle differences are seen, with more contacts for the Glc unit with galectin-1 than galectin-3.

Since the efficiency of STD transfer depends on the effective correlation time of the complex, which in turn depends on the molecular size of the system under investigation, the lower molecular weight of the proteolytically truncated galectin-3 relative to homodimeric galectin-1 could be a factor in this result. Having mapped contact sites, we next assessed the conformation bound by the lectins.

trNOE experiments were performed for the complex formed by LacDAN with galectin-1. In the bound state, strong and negative NOE cross-peaks were observed for the lactose derivative in the presence of this galectin at a 20 : 1 ligand : receptor molar ratio. This observation contrasts with findings noted for the free state, for which NOE cross-peaks were exclusively positive (Fig. 7). The analysis of the observed cross peaks indicated that basically the free-state conformation of LacDAN is accommodated into the binding site of galectin-1, since the cross-peak pattern was indeed very similar to that described for the free state previously[4] for the free saccharide. Interestingly, no H1Glc-CH_2–linker NOEs were observed with negative sign, indicating that protons at this region of the molecule display different effective correlation times relative to those at the lactose moiety, fully in accord with STD data revealing no tight contacts between the linker and protein. The application of these two methods converges in detecting the global low-energy conformation in the bound state with no major interaction with the non-glycosidic part.

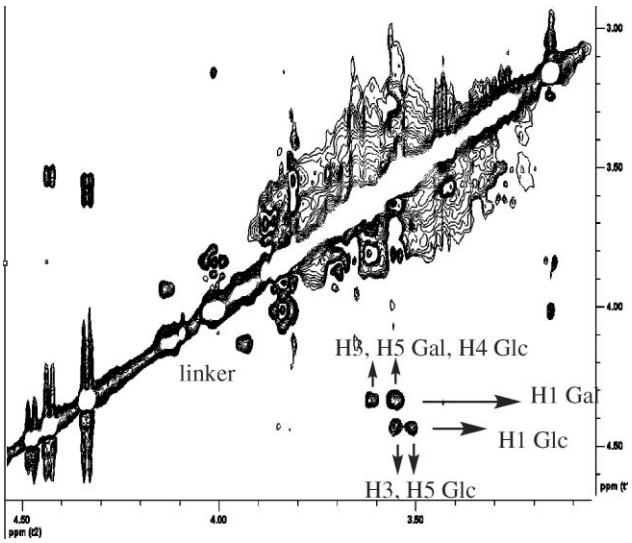

Fig. 7 Section of the trNOESY (mixing time, 200 ms) for the LacDAN/galectin-1 complex (20 : 1 molar ratio) at 500 MHz and 300 K. Negative NOE cross peaks are observed and those for H1Glc and H1Gal are clearly observed for both anomeric protons. The observed NOEs are basically identical to those observed in the free state (*i.e.*, H1Gal–H4Glc (overlapped), H1Glc–H3,H5Glc).

Docking calculations

As the final part of this study, the major conformer of lactose, as deduced by NMR, was docked into the binding site using the programme AUTODOCK.[26] Being homologous proteins, the architecture is well conserved, encompassing a central Trp residue

to provide stacking interactions with the Gal residue. Asn and His residues provide hydrogen bonding interactions with the Gal moiety, while Arg and Gln lateral chains provide simultaneous contacts to both the Gal and Glc units. For galectin-3, one additional Arg (Arg 186) binds to the Glc residue, while for galectin-1, His52 closes the binding site in the surroundings of the Glc unit. As seen respectively in Fig. 8 and Fig. 9 rigid-body docking could be performed with remarkable fit.

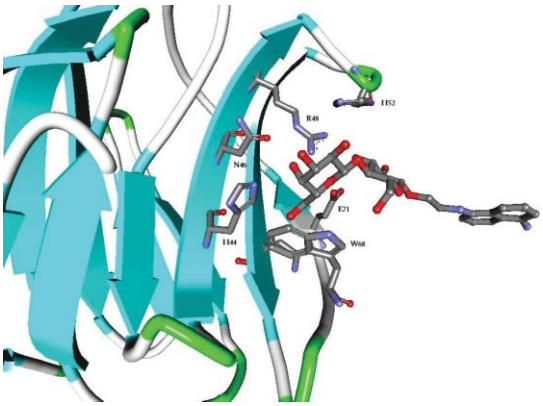

Fig. 8 A 3D model of the complex of the CRD domain of galectin-3 with LacDAN (coordinates taken from the Protein Data Bank, code 1A3K) as deduced by docking experiments with AUTODOCK. The lactose moiety is recognized by the lectin, but the major contacts concern the Gal residue. The linker and the aminopyridine residue are in contact with the water solvent. Different perspectives are shown.

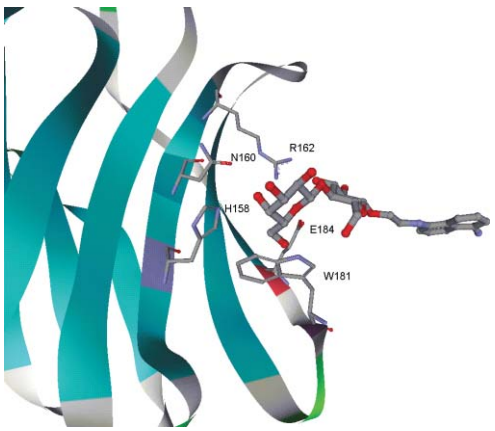

Fig. 9 A 3D model of the complex of galectin-1 (coordinates taken from the Protein Data Bank, code 1GZW) with LacDAN. The AUTODOCK docking experiment was performed as described in the experimental part. The figure shows the best result. No major contacts between the aromatic part of the linker or the DAN moiety and the protein are observed.

No major contacts between the linker and fluorescent tag with the lectins could be deduced for the best-fit models in both cases, further supporting the experimental NMR observations.

Conclusions

A fluorescent glycan (LacDAN), which can be easily immobilized on a surface, has been prepared. By using SPR, STD/trNOESY and docking measurements, it was possible to analyze galectin–LacDAN interactions in a sensitive, systematic, reliable and quantitative manner. For these studies we have chosen three members of the family of adhesion/growth-regulatory galectins (galectin-1, -3 and CG-1A). In all cases, the bioactivity of the disaccharide was maintained. The attachment of this new fluorescent glycoconjugate to the surface results in a well-defined interface, enabling desired orientational flexibility and enhanced access of binding partners. The results indicate that this new glycoconjugate exhibits binding affinity to galectin-1, -3 and CG-16. No major contacts between the linker and fluorescent tag with the lectins could be deduced for the best-fit models in both cases, further supporting the experimental NMR observations. No major conformational rearrangement of lactose as ligand is required. Fluorescent tagging thus is feasible to maintain bioreactivity. The specificity to the sugar part is not compromised by the chemical tagging.

This approach leads to new fluorescent glycoconjugates that could be broadly applied to a wide variety of biologically relevant low or high-affinity systems beyond the carbohydrate–galectin interaction studies shown here.

Experimental

Materials and methods

NMR spectra were recorded on a Varian 500 MHz spectrometer. Samples were dissolved in the appropriate deuterated solvent (D_2O), and chemical shifts (δ) are expressed in parts per million (ppm). Determination of protein concentration was carried out by the dye-binding method.[27] Surface Plasmon Resonance (SPR) experiments were performed in a BIACORE-3000, the SPR sensor chips and solutions used for the SPR assays were purchased from BIAcore. The sensorgrams and fittings were processed by the software BIAEvaluation (version 4.1, 2003, Biacore). Compound LacDAN was synthesized as previously described.[4]

Lectin purification and quality controls

The starting material for purification of galectins from bovine heart (galectin-1), chicken liver (C-16, CG-16, now called CG-1A) and murine galectin-3 was obtained either from extracts of fresh bovine heart or from recombinant production.[28-30] Following affinity chromatography on lactose-bearing Sepharose 4B, obtained by resin activation with divinyl sulfone, the proteins were checked for purity by one- and two-dimensional gel electrophoresis and nanoESI mass spectrometry, for quaternary structure by gel filtration and for activity by haemagglutination and cell-binding assays.[31-34] Proteolytic truncation of murine galectin-3 by digestion with commercial collagenase D was carried out as described followed by rechromatography and product analysis by mass spectrometry.[29,35] The obtained C-terminal section constitutes the carbohydrate recognition domain (CRD) of galectin-3.

Immobilization of synthetic glycan derivatives on a carboxylated dextran matrix CM5 (Biacore)

The first flow cell (Fc2) from a standard CM5 chip (Biacore) was used for the immobilization of the lactose derivative LacDAN. For that purpose, the surface was activated by injection of the reagents of the amino coupling kit (Biacore): EDC/NHS, 35 µL,

at a flow rate of 5 μL/min, at 28 °C, and HBS-P (0.01 M HEPES, 0.15 M NaCl, pH 7.4) as running buffer. At the same conditions of flow and temperature, a solution of LacDAN (1 mM, 180 μL) in sodium acetate buffer (10 mM, pH 4.5) was injected three consecutive times for maximum extent of immobilization, followed by the injection of ethanolamine (1 M, 35 μL) to block remaining active sites on the dextran matrix. The final response reached was 660 RU. As control the flow cell Fc1 was processed by activation and blocking to serve as negative control.

Interaction assay with human galectin-1 by SPR

Solutions of different concentrations of galectin-1 (0.9, 1.8, 3.6, 7.2, 14.3, 28.7, 57.3 and 114.7 μM) in HBS-P buffer were injected (15 μL) to flow over the sensor chip surface, at a flow rate of 5 μL/min at 25 °C. After a suitable period for dissociation, HBS-P (15 μL) was injected twice with the same flow and temperature conditions to wash the surface. Sensorgrams obtained in each case and for each protein were analyzed and kinetic parameters of the interaction were determined with BIAEvaluation software.

Interaction assay with avian CG-1A by SPR

Solutions of different concentrations (1.9, 3.9, 9.4, 15.6, 31.2 and 62.5 μM) of the avian galectin-CG-1A in HBS-P buffer were injected (15 μL) over the sensor chip surface, at a flow rate of 5 μL/min at 25 °C. After a suitable period for dissociation, a solution containing NaCl 1 M and NaOH 50 mM (5 μL) was injected with the same flow and temperature conditions, followed by an injection of HBS-P (15 μL) to regenerate the surface for the next protein sample.

Interaction assay with murine Galectin-3 (Carbohydrate Recognition Domain) by SPR

For the interaction assays, solutions of different concentrations (0.5, 1.7, 3.4, 6.8, 8.5, 10.2, 11.8 μM) of galectin-3 in HBS-P buffer were injected (15 μL) over the sensor chip surface, at a flow rate of 5 μL/min at 25 °C. After a suitable dissociation phase, HBS-P (15 μL) was injected twice with the same flow and temperature conditions to wash the surface before the next sample injection.

Molecular recognition studies by NMR

STD experiments[36] were performed at molar ratios between 20 : 1 and 50 : 1 of the corresponding compound/lectin. A series of Gaussian-shaped pulses of 50 ms was run, with a total saturation time of the protein of 2 s and a maximum B1 field strength of 50 Hz. An off-resonance frequency of $\delta = 40$ ppm and on-resonance frequency of $\delta = -1.0$ ppm (protein aliphatic signals region) were applied. In all cases, before performing the experiment, line-broadening of ligand protons was monitored. Water suppression was done by using a Watergate module at the end of the pulse sequence. Routinely, STD experiments were repeated twice with comparable results. In each case, the intensities were normalized with respect to the highest response.

For the bound ligands, trNOE experiments were performed as described previously.[37] Measurements were done with a freshly prepared ligand/lectin mixture, with mixing times of 100 and 200 ms, at an approximately 30 : 1 molar ratio of ligand/galectin. A concentration between 2–3 mM of the ligand was employed in all cases. No purging spin-lock period was employed to remove the NMR signals of the macromolecule background. First, line-broadening of the ligand protons was monitored after addition of the lectin. Strong negative NOE cross-peaks were observed, in contrast to respective free state signals, indicating binding of the sugar derivatives to the proteins.[38]

Molecular mechanics and dynamics calculation

The conformational analysis of the lactose derivative (LacDAN) has been already described,[3,4] using NMR and the MM3* force field,[39] with the generalized Born GB/SA continuum solvent model.[40] The torsion angles Φ, Ψ are defined as Φ: H1Gal–C1Gal–O–C4Glc and Ψ: H4Glc–C4Glc–O–C1Gal. For the pendant chain, Φchain is H1Glc–C1Glc–O–CH2 and Ψchain is C1Glc–O–CH2–CH2. The torsion angle around the C5–C6 linkage (ω) is defined as O5–C5–C6–O6. Two different conformers were considered gt ($\omega = +60°$) and gg ($\omega = -60°$) for the Gal and Glc moieties, respectively. The major low-energy conformer in solution is selected for binding to allow its use in docking analysis.

Docking calculations

The prevalent conformer of LacDAN (as determined by NMR) was manually docked into the carbohydrate-binding sites of the mammalian galectins by superimposing the terminal Gal residue with that of the crystallographic co-ordinates (PDB codes 1GZW for galectin-1 and 1A3K for the CRD of galectin-3). Then, different possibilities of arranging the side chain of disaccharide derivative were used as input geometries for AUTODOCK 3.0 simulations[41] with the multiple Lamarckian Genetic Algorithm. Local searches were also performed centred in the experimental galactoside-specific X-ray sites. The AUTOGRID program present in AUTODOCK 3.0 generated grids of probe atom interaction energies and electrostatic potential. Grid spacing of 0.6 and 0.375 Å were used for the global and local searches, respectively. For each calculation, 100 docking runs were performed using a population of 200 individuals and an energy evaluation number of 3×106.

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