Molecular insights into DC-SIGN binding to self-antigens: the interaction with the blood group A/B antigens

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KEYWORDS. DC-SIGN, molecular recognition, A and B histo blood group antigens, NMR, αGal epitope.

ABSTRACT: The dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is an important receptor of the immune system. Besides its role as pathogen recognition receptor (PRR), it also interacts with endogenous glycoproteins through the specific recognition of self-glycan epitopes, like Le^x. This lectin however, represents a paradigmatic case of glycan binding promiscuity, and has been shown to also recognize antigens with α1-2 linked fucose, such as the histo blood group antigens, with similar affinities to Le^X. Herein, we have studied the interaction in solution between DC-SIGN and the blood group antigens A and B, to get insights into the atomic details of such interaction. With a combination of different NMR experiments, we demonstrate that the Fuc coordinates the primary Ca^{+2} ion with a single binding mode through 3-OH and 4-OH. The terminal αGal/αGalNAc affords marginal direct polar contacts with the protein, but provides a hydrophobic hook in which V351 of the lectin perfectly fits. Moreover, we have found that αGal, but not αGalNAc, is a weak binder itself for DC-SIGN, which could endow an additional binding mode for the blood group B antigen, but not for the blood group A.

DC-SIGN is one of the most prominent and studied members of the C-type lectin receptor (CLR) family. These carbohydrate binding proteins are profusely distributed on the surface of antigen-presenting cells, where they act as pathogen recognition receptor (PRRs) through the specific recognition of glycans on the pathogen surface.[1,2] Infections by *M. tuberculosis*,[3,4] *C. Albicans*,[5] *Leishmania,*[6] dengue,[7] Ebola[8] or HIV[3] for instance, have been reported to involve the participation of DC-SIGN. Interestingly, at the same time, this lectin plays important roles in the immune modulation and homeostasis through its interaction with endogenous glycoproteins where it recognizes self-glycans.[9]

The glycan binding specificity of this lectin is remarkably broad. Besides, interestingly, different glycan array data have reached contradictory results.[10,11,12,13] They mostly agree in the facts that ligands include certain fucose-containing antigens, as well as high mannose and complex type N-glycans, although very subtle structural differences may influence the binding event.[13] The presentation of glycan epitopes is extremely important for their interactions with receptors[14] and this seems to be also a key element in DC-SIGN recognition events. For instance, the histo blood group antigens A/B (BGA, BGB) have been described to be recognized by DC-SIGN when presented on a long linker. However, only BGB was recognized when presented with a short one, while BGA was not.[10]

The interaction of DC-SIGN with endogenous partners remains highly unexplored. The Lewis^X (Le^X) antigen has been shown to be recognized on cell surfaces,[11,] and the epitope recognized on the ICAM-3 glycoprotein,[15] one of the known endogenous ligands of DC-SIGN. Obviously, the broad glycan specificity of the lectin raises the question of the biological significance of the interaction with other fucosylated self-epitopes. Fucose is commonly found in mammalian glycans as a terminal modification as part a of the blood group antigens: Lewis (α1-3 and α1-4 linked) and ABH types (α1-2 linked). Recently, the detection of terminal αGal residues and higher fucosylation patterns in oral cancer cells has suggested a role of the blood group B antigen (BGB), which could be related to its enhanced recognition by DC-SIGN and the escape of the cancer cells to the immune systems mediated by DC-SIGN.[16]

Herein, we have studied the structural details of the molecular recognition in solution of the A and B histo blood group antigens by the carbohydrate recognition domain of DC-SIGN (CRD DC-SIGN) by using nuclear magnetic resonance (NMR) and molecular modeling methods.[17] The interaction between Le^X and DC-SIGN, characterized by X-ray crystallography and NMR,[10, 18] takes place through the Fuc residue, which binds at the primary calcium binding site by coordinating the Ca^{+2} ion with hydroxyls 3-OH and 4-OH. Interestingly, the homologous C-type lectin langerin,[19] with a similar glycan recognition profile with respect to fucosylated oligosaccharides,[12] has been shown to also bind to the blood group B antigen.[20] In this case however, the Fuc residue is attached to the Ca^{+2} ion through 2-OH and 3-OH.

In this scenario, and given the promiscuity and plasticity of this particular system, we aimed at shedding light into the molecular details of the recognition of the histo blood group antigens A and B by CRD DC-SIGN in solution. Our data show that both blood group A and B type VI tetrasaccharide antigens (compounds **1** and **2**, respectively) are recognized with a similar presentation and with comparable affinities, and disclose the relevant structural requirements for the interaction. In addition, our data suggest that for **2**, and not for **1**, a second binding mode may also take place, in which the terminal αGal residue is bound at the primary Ca^{+2} binding site. Indeed, a simple αGal epitope is also a ligand for DC-SIGN through a weak, but specific interaction.

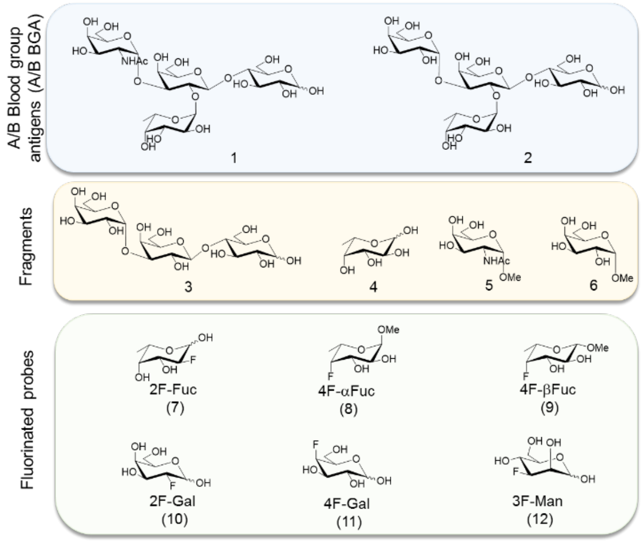


CHART 1. Glycan structures used in this study: histo blood group antigens A and B type VI tetrasaccharides (**1** and **2**, respectively); the fragments: galili-type VI trisaccharide (**3**), and the monosaccharides Fuc (**4**), GalNAc-α-OMe (**5**) and Gal-α-OMe (**6**); and monofluorinated probes of Fuc (**7**, **8**, **9**), Gal (**10**, **11**) and Man (**12**).

RESULTS

Lectin chemical shift perturbation analysis. ^{1}H-^{15}N HSQC titration experiments on ^{15}N-labeled CRD DC-SIGN were carried out in order to deduce the protein region involved in ligand binding and to estimate binding affinities. Data were acquired for the blood group tetrasaccharides (**1** and **2**), as well as for the monosaccharide Fuc (**4**). The addition of the tetrasaccharides produced very similar chemical shift perturbation profiles on the protein ^{1}H-^{15}N backbone resonances for the same number of equivalents (fig. 1a-c). As expected, the residues around the primary Ca^{+2} binding site, namely, the residues in the long loop and β-strands 3 and 4, were the ones affected the most. Additionally, residues F313, T314 and L371, in the nearby loops were also perturbed (fig. 1a). In general, the observed chemical shift perturbations were more pronounced for the B tetrasaccharide **2**, with the only exception of K368, which was more affected in the presence of **1**, the A antigen. The affected protein region is large enough to think about the existence of an extended interaction surface on the protein, where besides the monosaccharide attached to the primary Ca^{+2} binding site, additional glycan residue/s would establish further contacts with the protein, as reported for Le^X. However, the chemical shift perturbation produced by the simple monosaccharide Fuc (fig. 1d) is strikingly similar to that for the tetrasaccharides (fig. 1c). In particular, no further residues are affected by the tetrasaccharides compared to Fuc. This result suggests that the molecular recognition of the tetrasaccharides primarily involves the interaction with the Fuc moiety.

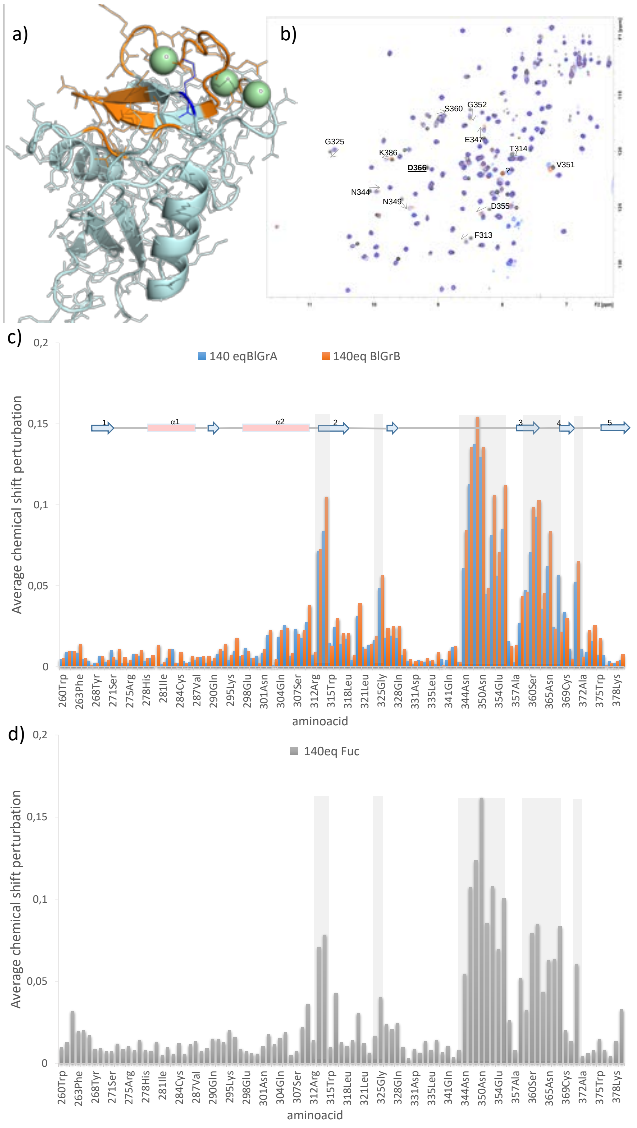


FIGURE 1. Chemical shift perturbations for the interaction of CRD DC-SIGN with tetrasaccharides **1**, **2** and with Fuc (**4**). a) In orange aminoacids with chemical shifts most perturbed upon the addition of BGB and BGA. In blue K368, affected more with

BGA than with BGB. b) Superimposition of ^{1}H-^{15}N HSQC spectra: in black, apo DC-SIGN; in orange, in the presence of 140 equivalents of BGB; in blue, in the presence of 140 equivalents of BGA. Some affected crosspeaks are annotated. Residue D366 that disappear in the middle points of the titration is underlined. c) and d) Average chemical shift perturbation upon the addition of BGA, BGB and Fuc. D366 is not included in the plot. Average chemical shift perturbations were calculated using the formula $\sqrt{\frac{1}{2}\left[\delta_H{}^2 + 0.2\delta_N{}^2\right]}$ where δ_H and δ_N are the chemical shift change in ^{1}H and ^{15}N respectively in ppm between the *apo* and bound forms.

The titration curves allowed to estimate the binding affinities. The best binder is **2** (K_D = 2.3 ± 0.6 mM) followed by Fuc (K_D = 4.1 ± 0.7 mM) and **1**, which displays the weakest affinity (K_D = 7.6 ± 1.4 mM). A detailed inspection of the titration curves, highlighted some remarkable issues. In the case of Fuc, a non-linear chemical shift perturbation was clear for certain residues (Fig. S1). For **2**, although the non-linear trajectories were not that obvious, however, the curve fitting (average chemical shift perturbation *vs* protein:ligand ratio) showed a step in the experimental data at ca. 1:50 lectin:ligand equivalents, an effect not observed for **1** (Fig. S2). At the same time, for both tetrasaccharides, some cross peaks diminish their intensity or disappear in the intermediate points of the titration, while others increase their intensity in the presence of the ligands (Fig. S1). Thus, although the recognition site on the lectin is basically the same for the three ligands, different dynamic processes take place, depending on the particular sugars.

Saturation-Transfer Difference (STD-NMR): the ligand point of view. ^{1}H-NMR STD experiments[21] were carried out in order to get information on the ligand epitopes for the different analogues. Higher STD intensities were obtained for protein irradiation in the aliphatic region, while temperature did not significantly affect the STD response. Because of the mutarrotation at the reducing-end residue (Glc), two separate resonances (for α and β anomers) are observed for some protons. The strongest STD was observed for Fuc H2, for both ligands **1** and **2**. Weaker STD intensities were observed for Fuc H1, αGalNAc H4, and αGalNAc H3, for **1**, and for Fuc H1, αGal H4, and αGal H3/H2 (overlapped) for **2**. No STDs were observed neither for the Fuc methyl group, nor for any of the protons of the Glc reducing-end residue. These data indicate that both tetrasaccharides are presented in a similar way to interact with DC-SIGN, displaying Fuc H2 close to the protein aliphatic sidechain(s).[22]

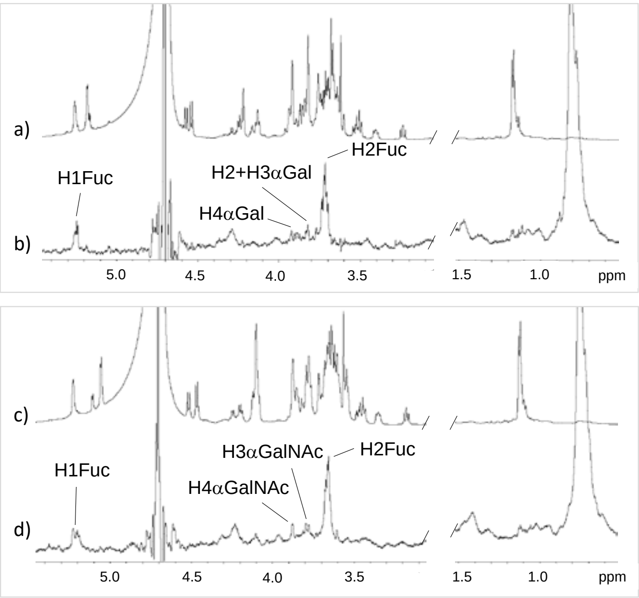


FIGURE 2. ^{1}H STD-NMR experiments for the interaction of CRD DC-SIGN with **2** (a) and (b) and **1** (c) and (d). (a) and (c) reference (off-resonance) spectra. (b) and (d) STD spectra with on-resonance irradiation in the aliphatic region, using a 50ms Gaussian pulse.

The bound ligand conformation: Transferred NOESY. trNOESY experiments[23] were acquired for 1/5 ratio samples of CRD DC-SIGN/tetrasaccharide and compared to the NOESY spectra of the free ligands. At 298K and 600MHz, the free ligands showed NOE effects close to zero, with both positive and negative NOEs and crosspeaks dominated by a double-quantum contribution, even at long mixing times. At 800 MHz, both tetrasaccharides exhibited weak negative NOE effect. On the contrary, in the presence of the protein, all NOEs became strong and negative at either field, indicating that they are trNOEs.

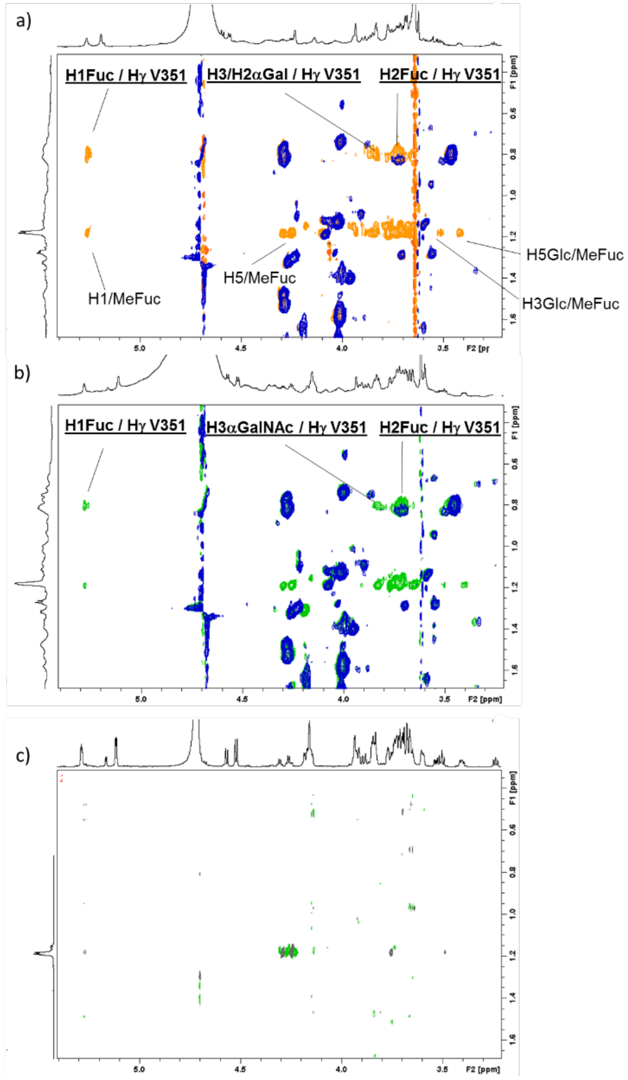


FIGURE 3. The quest of protein/ligand intermolecular NOEs. a) Superimposition of: the NOESY of the CRD of DC-SIGN (blue), and the trNOESY of the complex of the CRD DC-SIGN with **2** (lectin/ligand molar ratio is 1/5, orange). The new crosspeaks in the orange spectrum correspond to intramolecular NOE of the ligand (all those from MeFuc, some of which are annotated) and intermolecular NOE (underlined) b) Superimposition of: the NOESY of the CRD of DC-SIGN (blue), and the trNOESY of the complex of the CRD DC-SIGN with **1** (lectin/ligand molar ratio is 1/5, green). C) NOESY spectrum of tetrasaccharide **1**. All spectra were acquired at 298 K at 800 MHz and with 400 ms of mixing time.

The comparison of the interglycosidic ROEs/NOEs between the free and bound ligands indicated that the bound conformations remains the same as that existing in solution for both **1** and **2** tetrasaccharides. These branched glycans are known to be fairly rigid and they display a single very major conformation in solution, in which Fuc H1 and H2 are packed against the α-face of the αGal/αGalNAc residue. This is also the bound conformation in the complex with DC-SIGN, as confirmed by the key trNOEs between αGal/αGalNAc H1 with βGal H4 and H3, Fuc H1 with βGal H2 and H3 and αGal/αGalNAc H3 (Fig S3). The analysis of the trNOESY spectra however revealed a foremost issue: intermolecular NOE crosspeaks between protein and ligand protons. As shown in figure 3 (panels a and b), H1 Fuc (δ 5.26 ppm) has a clear NOE with protein signals at δ 0.77 ppm. Indeed, no ligand protons are present at that chemical shift and this cross peak is not present in the NOESY spectrum of the free protein (spectrum superimposed in blue in Figure 3a and 3b). Moreover, additional NOE crosspeaks between Gal H3α and Fuc H2 and the protein signals at δ 0.77ppm were also observed, which again are not present in the NOESY spectrum of the free protein. This high field region (<1 ppm) is exclusive of the methyl protons of Val, Leu, and Ile residues. The herein employed CRD construct of DC-SIGN contains 3 Ile, 11 Leu, and 6 Val residues. Among those, only V351 is located at the primary Ca^{+2} binding site and expected to be close to the ligand. Thus, these protein signals should correspond to the Hγ of V351, which display intermolecular NOEs with Fuc H1 and H2 and with αGal H3. This assignment was confirmed by ^{1}H-^{15}N HSQC-TOCSY (Fig S4). Interestingly, V351 has been reported to be essential for binding to one of the known endogenous glycoprotein receptors, ICAM-3. Fittingly, the V351G DC-SIGN mutant was unable to bind ICAM-3.[24] The X-ray structure of the complex with Le^{X} also highlights the key van der Waals contact.[20]

Monofluorinated probes. The substitution of a pyranose hydroxyl group by a fluorine atom has long been used to explore its participation in direct contacts with receptors.[25, 26] Since ^{19}F NMR experiments are extremely useful to monitor glycan-lectin interactions,[27] we have herein used a small library of simple monofluorinated monosaccharides and ^{19}F CPMG experiments[28] to define the hydroxyl pair coordination to the Ca^{+2} ion at the primary DC-SIGN binding site. In particular, we have employed 2-F-Fuc (**7**) and the α and β methyl glycosides of 4-F-Fuc (**8** and **9**), hypothesizing that the presence of fluorine at key positions of these molecules would abrogate the binding event through the 2-OH/3-OH and 3-OH/4-OH pairs, respectively. Additionally, we have tested the corresponding Gal probes, 2-F-Gal and 4-F-Gal (**10** and **11**, respectively), and 3-F-Man (**12**) as negative control, since this molecule is completely unable to coordinate calcium in either way.[29] As described, the variations in ^{19}F transverse relaxation rates (R2), in the absence and presence of the lectin, can be used as a probe for detecting binding through simple ^{19}F CPMG experiment. Figure 5a shows three NMR spectra (at the same scale) of the corresponding ^{19}F CMPG experiment carried out for a mixture of the monofluorinated compounds in the presence of CRD DC-SIGN. In the spectrum below, acquired with a total echo time of 8ms, all the ^{19}F NMR signals corresponding to every monosaccharide in the library are observed. In the spectrum in the middle (total echo time of 2000 ms), the ^{19}F NMR signals for β2-F-Gal, α2-F-Gal, α2-F-Fuc and β2-F-Fuc (highlighted with an asterisk) have disappeared, while those for 3F-Man, 4F-Gal and 4F-Fuc remain. All the signals were recovered after the addition of EDTA (see also Fig S6). Panel b gathers the complete ^{19}F T2 decay for each species, in the absence and in the presence of the lectin.

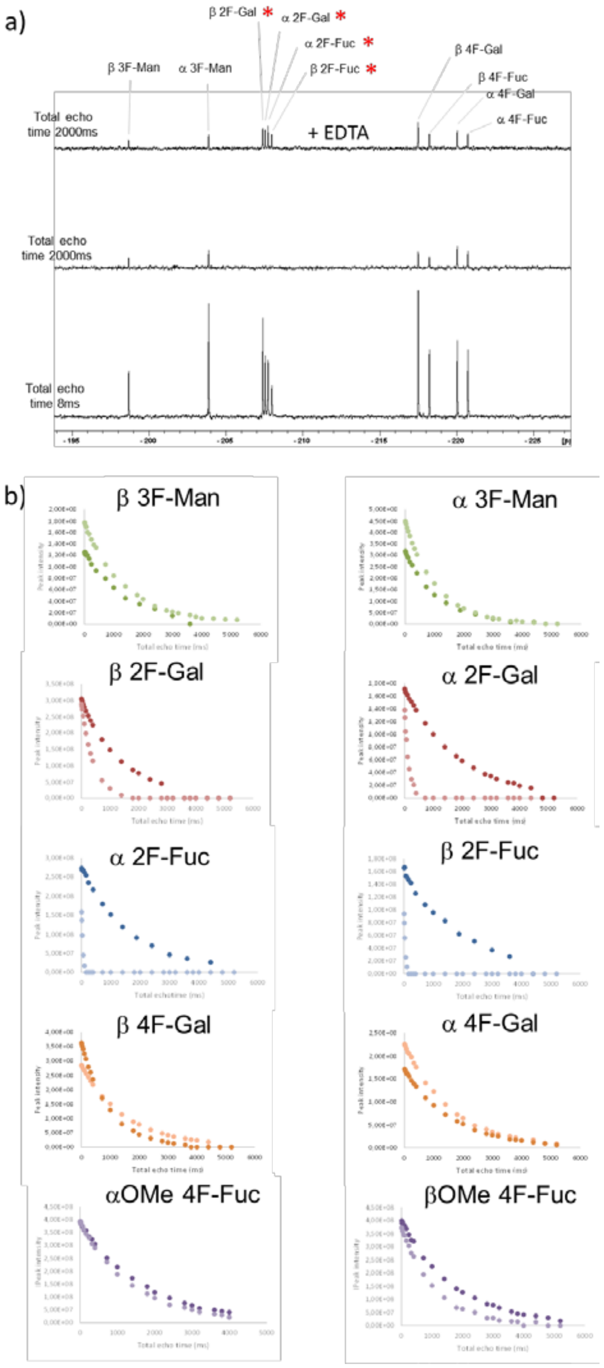


FIGURE 4. The ^{19}F CPMG experiments for the fluorinated monosaccharide small library: a) the employed concentrations were 40uM CRD DC-SIGN and 0.8mM of each monosaccharide. Bottom: the first spectrum was acquired with 8ms total echo time. Middle: the spectrum was acquired with 2000ms echo time. Top: the spectrum with 2000ms echo time after the addition of 20mM EDTA-d12. The red asterisks highlights those ^{19}F signals whose T2 values show a faster decay. b) Representation of the ^{19}F T2 decay curves obtained for every species in the mixture, in the absence (dark color) and in the presence of DC-SIGN (light color).

From these data it becomes clear that 2-F-Fuc is a DC-SIGN binder (both α and β anomers), due to the pronounced change in the T2 curves (free and bound). Remarkably, neither α- nor β-OMe-4-F-Fuc bind DC-SIGN. This result unambiguously indicates that Fuc binds DC-SIGN exclusively through 3-OH and 4-OH. Strikingly, the ^{19}F T2 of 2-F-Gal, especially for the α-anomer, was importantly affected by the presence of the protein, indicating that αGal is also recognized by DC-SIGN. Thus, in this case, Ca^{+2} coordination also takes place through 3-OH and 4-OH hydroxyls.

The interaction with the antigen fragments. In order to further corroborate whether α-Gal is a ligand for DC-SIGN, we carried out STD experiments with Galα-OMe (**6**), the Galα1-3Galβ1-4Glc trisaccharide (**3**) present in the B antigen, with GalNAcα-OMe (**5**) (present only in the A antigen), and L-Fuc (**4**) (figure 5). In addition to the expected STD for Fuc, both GalαOMe and Galα1-3Galb1-4Glc showed STD effect, that disappeared upon addition of EDTA to the sample (Fig. S7). On the contrary, GalNAcαOMe showed no STD signals. For both αGal containing moieties, exclusive STDs were observed for Gal H2. Although the Galβ anomer, also showed some perturbation in the T2 ^{19}F relaxation, STD experiments with lactose (Galβ1-4Gc) (data not shown) showed no STD signal, in agreement with previous reports.

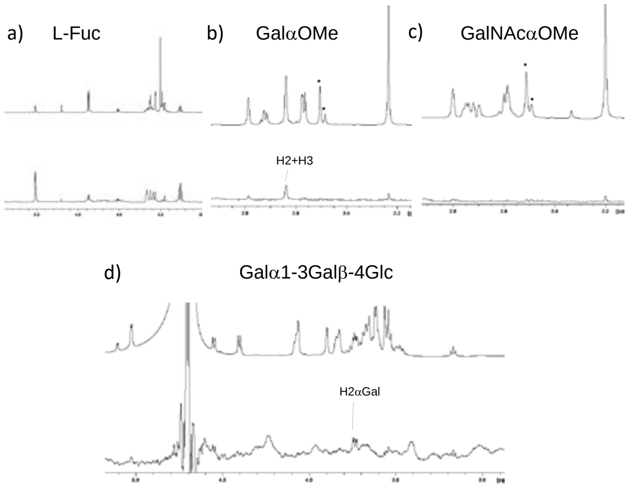


FIGURE 5. ^{1}H NMR STD experiments with fragments of the blood group antigens: STD (below) and reference NMR spectra (top) recorded for the interaction of the CRD of DC-SIGN with a) L-Fuc (**4**), b) Galα-OMe (**6**), c) GalNAcα-OMe (**5)** and d) the Galα1-3Galβ1-4Glc trisaccharide (**3**).

One additional proof of the interaction of trisaccharide **3** with DC-SIGN was obtained by monitoring the chemical shift perturbations of the lectin signals in the ^{1}H-^{15}N HSQC spectrum of the CRD of DC-SIGN upon addition of an excess of the trisaccharide. Figure 6 shows the superimposition of the spectra in the absence and in the presence of 40 equivalents of **3**. Even though the chemical shift perturbations are smaller in magnitude with respect to those observed for the interaction with simple Fuc and with the Fuc-containing glycans **1** and **2**, specific crosspeaks were affected by the presence of the trisaccharide. Without being exhaustive, the affected region is essentially the same as for the interaction with ligands **1, 2** and **4,** with a slightly different profile (Fig. S5). In particular, N349, N350, V351, D366 and K368 were the most affected residues (also affected with ligands **1**, **2** and **4**). Thus, in agreement with the ^{19}F-T2 relaxation and the ^{1}H-STD experiments, the Galα moiety is indeed a ligand for DC-SIGN, although the interaction is weaker than that for the blood group A and B antigens, and also for Le^x.

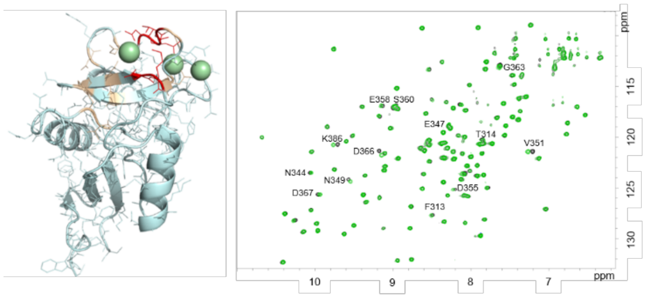


FIGURE 6. ^{1}H-^{15}N HSQC spectra recorded for the CRD of DC-SIGN without ligand (black) and upon the addition of 40 equivalents of compound **3** (green). On the left hand side, the 3D structure of the lectin where residues affected by the addition of **3** have been highlighted in wheat, and in red (the most affected ones).

Modeling. The analysis of the different experimental NMR data permitted to gather key information for building structural models for the complexes of **1** and **2** with DC-SIGN. The trNOESY data strongly suggest that Fuc H2 is oriented towards Val351. The observed STD fully support this scenario, in which Fuc H2 shows, by far, the strongest STD response when the on-resonance irradiation frequency is set on the protein aliphatic region. At the same time, the ^{19}F T2 experiments indicate that the Ca^{+2} coordination occurs through 3-OH and 4-OH of Fuc, and not using the 2-OH/3-OH pair. The Fuc binding mode found in the X-ray crystallographic structure of the complex DC-SIGN/Le^x fully comply with these observations. In addition, the chemical shift perturbations on the protein backbone resonances are essentially the same for the simple monosaccharide Fuc and for the tetrasaccharides. Thus, a manual docking protocol was employed for fitting the Fucose residues of **1** and **2** in the binding site of DC-SIGN. The obtained starting geometries were minimized and subjected to MD simulations. Both complexes were stable along the whole 100 ns MD run. The analysis of the structural parameters for both complexes was essentially the same.

For the weak interaction with the αGal moiety, the NMR data indicate that Gal H2 is positioned close to V351, while the Ca+2 coordination occurs through the 3-OH/4-OH pair. Thus, the starting geometry of the Galα1-3Galβ1-4Glc trisaccharide bound to DC-SIGN binding site was generated compiling with these structural prerequisites. The complex was stable along 200 ns MD simulation. The same geometry was then used for docking **2** into DC-SIGN, but with the terminal αGal in the primary binding site. Again, the complex was stable all throughout the complete MD simulation (Fig. S8).

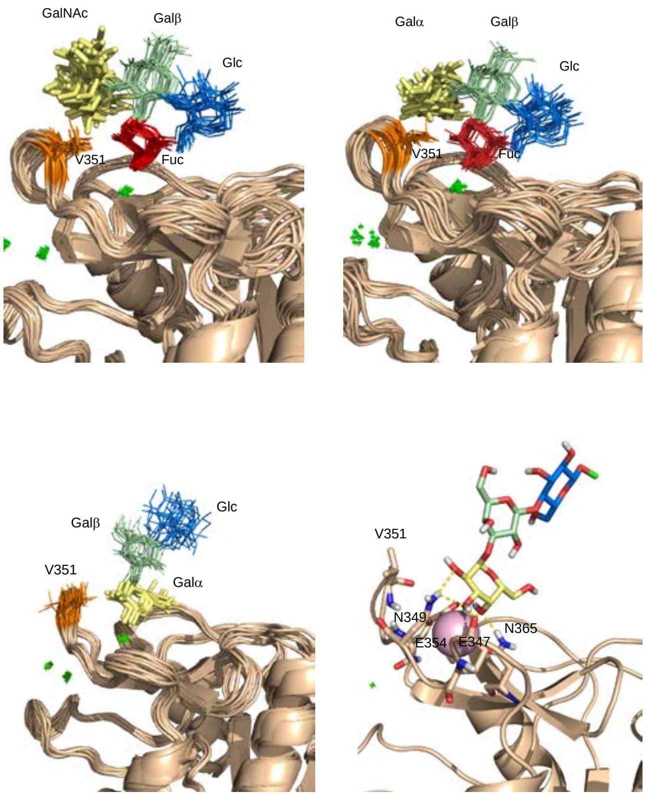


FIGURE 7. Structural models for the complexes of CRD DC-SIGN with tetrasaccharides **1** and **2**, and trisaccharide **3** obtained from MD simulations: A) and B) superimposition of 20 frames extracted from the 100 ns MD simulations performed for the complexes with **1** and **2**, respectively, where the binding to the calcium ion takes place through the Fuc residue. C) and D) The complex for the Galα1-3Galβ1-4Glc trisaccharide **3,** as deduced from the corresponding MD simulations.

DISCUSSION

DC-SIGN can be considered as a paradigmatic case of glycan binding promiscuity. This behavior has been related to its different biological roles that include both pathogen recognition and interaction with self-glycoproteins. In both cases, DC-SIGN is involved in the recognition of either pathogenic or self-glycans. How these different recognition processes are regulated at a molecular level is not fully understood. The understanding of the structural differences at the molecular level is an important piece of information. DC-SIGN is known to recognize certain fucosylated as well as high-mannose glycans, which implies a large number of structures present both in host and pathogenic cells. The atomic level structural information provided by X-ray crystallography has shown that Man or Fuc residues bind at the primary Ca^{+2} recognition site. Interestingly, Man has been shown to be recognized in two alternative binding modes,[30] which would be in dynamic equilibrium in solution,[31] and would contribute to enhance the affinity for high mannose N-glycans. For Fuc, a single binding mode has been reported, involving Ca^{+2} coordination through 3-OH and 4-OH, although Langerin, a homologous lectin, recognizes Fuc through 2-OH and 3-OH. Avidity and multivalence have been reported to be important elements in the recognition events by DC-SIGN, where these phenomena highly increase the low affinities observed for the monosaccharides. These effects are presumably amplified for the full length form of DC-SIGN, which occurs when the neck domain is present, promoting DC-SIGN arrangement into tetramers.[32]

Herein, we have used the CRD of DC-SIGN for studying the molecular recognition in solution of the blood group antigens A and B. We show that BGB is recognized with an affinity similar to that for the Le^X antigen, while the BGA binds slightly weakly. In both cases, the interaction is based on the attachment of Fuc, which coordinates the primary calcium exclusively with 3-OH and 4-OH. MD simulations show a stable hydrogen bonding network in which 4-OH act as an H-bond donor to E347 and acceptor from N350, 3-OH is donor to E376 and acceptor to N365, and 2-OH is donor to E354, in agreement with previous structures. Additionally, occasional hydrogen bonds involve the terminal Galα residue, in particular 6-OH with NH of V351, and 6-OH or 2-OH with K368. Importantly, our experimental data highlights the significant role of V351, whose side chain perfectly packs against the hydrophobic cleft formed by the α-face of αGal and H1/H2 Fuc, confirming this interaction as key contact for branched fucosylated oligosaccharides.

We demonstrate that, opposite to mannose, fucose binds DC-SIGN through a single binding mode.[33] However, we found that the αGal epitope, present in the BGB is also able to weakly bind DC-SIGN. Indeed, ^{1}H-STD and ^{19}F-R2 experiments indicate that αGal and the trisaccharide Galα1-3Galβ1-4Glc interact with the lectin. On the contrary, αGalNAc, present in the BGA, does not bind DC-SIGN, probably due to the steric clash of the NHAc group on C2 precisely with the V351 side chain. Even though the affinity for this interaction is very low, it could be relevant in the biological context, where glycan density on cell surface can be very high. Surface glycan density has been in fact proposed to be one of the regulatory mechanisms of immunological lectins[34] that could act as another level of regulation besides the loose preferences of DC-SIGN for individual glycan epitopes. In fact, this additional weak interaction for αGal may be at the origin of the differences observed in glycan array data, where the BGB was shown to bind DC-SIGN regardless the chemical nature and length of the linker, while the BGA was shown to be binder only when presented with a long linker and not with a shorter one. In this way, longer linkers would make the main epitope, Fuc, fully accessible, and thus both BGB and BGA would bind. However, with shorter linkers, this key epitope could be hidden, and thus BGB could still bind through the αGal epitope, while BGA could not.

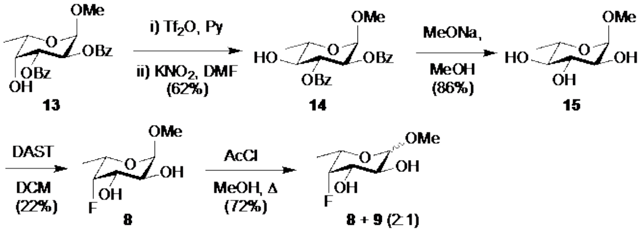
This work discloses fine atomic structural details for the interaction between the fucosylated A/B blood group self-antigens with DC-SIGN. Moreover, our findings highlights once more the promiscuity of this lectin at the molecular level, revealing a weak interaction also with the αGal, but not the αGalNAc epitope, which may have key implications in different immunological events from cancer[16] to allergy.[35]

MATERIALS AND METHODS

Glycans. Compounds **1**-**4** were purchased from Elycityl, compound **5** from Toronto Research Chemicals and compound **6** from Sigma. Fluorinated monosaccharides were purchased from Carbosynth, except for compounds **8** and **9** that were synthesized as described below.

Synthesis of the methyl 4-deoxy-4-fluorofucoside anomers ***8*** *and* ***9****.*

The synthesis of **8** and **9** (Scheme 1) started from the partially protected methyl fucoside[36] **13** by Lattrell-Dax inversion of the 4-OH group. Surprisingly, fluorination of **14** was unsuccessful, but after benzoate removal to methyl-α-L-quinovoside **15**, selective fluorination proceeded in low yield on gram scale to give the desired methyl 4-deoxy-4-fluoro-α-L-fucoside **8** next to unreacted **15** (37%). The β-anomer was subsequently obtained as a mixture of anomers (α:β 2:1) by acid-catalyzed equilibration.



SCHEME 1. Synthesis of **8** and **9**.

Protein expression and purification. The DNA fragment coding for the CRD DC-SIGN fragment (H254-A404 with an additional His-Tag at the N-terminus) was inserted into a pET15b plasmid (Thermo Fischer Scientific), amplified in *E. Coli* DH5α cells, and subsequently transformed into *E. Coli* BL21/DE3 cells. The culture was grown overnight at 37ºC in LB medium in the presence of ampicillin. When optical density reached 0.6 (600 nm), protein expression was induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG) and the culture was allowed to grow for 3 hours. For ^{13}C,^{15}N-labeled and ^{15}N-labeled samples, a little pre-culture in LB grown overnight at 37ºC was transferred to M9 minimal medium containing 1 g/L $^{15}NH_4Cl$ and 20% w/v U-^{13}C-glucose, or 1 g/L $^{15}NH_4Cl$ respectively. The culture was harvested by centrifugation at 4500 rpm, and the final pellet was re-suspended in lysis buffer (10 mM Tris-HCl pH 8) and sonicated at 4^0 C. The inclusion bodies were recovered by ultracentrifugation at 30000 rpm for 1 hour. The pellet was then dissolved in Tris-HCl 100 mM (pH 8) with 6 M urea and 0.01% v/v β-mercaptoethanol. The residual insoluble fraction was sedimented by ultracentrifugation at 40000 rpm × 2.5 hours, and the supernatant was decanted. The protein was then refolded through subsequent dialysis against 4 M, 2M and no urea (in 100 mM Tris-HCl pH 8, 100 mM NaCl, 10 mM $CaCl_2$, 0.01% v/v β-mercaptoethanol). Residual unfolded proteins were removed by ultracentrifugation, and the soluble folded protein was then purified through mannose-sepharose affinity chromatography and further purified by size exclusion chromatography in a HiLoad 16/60 Superdex 75 column eluting with 25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8. Finally, the fractions containing the pure monomeric CRD were washed with 25 mM Tris-HCl, 150 mM NaCl, 4 mM $CaCl_2$, 2 mM DTT, pH 8 to remove the EDTA, and concentrated using 10 MWCO membrane filters. For NMR experiments the buffer was eventually changed to 25 mM Tris-d_{11}, 150 mM NaCl, 4 mM $CaCl_2$, 2 mM DTT-d_{10}, in H_2O/D_2O 9:1 or D_2O. The presence of the lectin was monitored by 4-12% SDS-PAGE (band at 18-19 kDa) and the protein concentrations were quantified by the absorbance at 280 nm ($\varepsilon_\lambda = 52855\ M^{-1}\ cm^{-1}$, estimated from ProtParam).

NMR experiments. The backbone resonance assignment of the CRD DC-SIGN was carried out at 37 ºC on a 800 MHz Bruker spectrometer equipped with a cryoprobe, on a sample containing 200uM of double-labeled protein in the presence of 30 mM D-mannose (133 eq). 3D HNCO, HN(CA)CO, HN(CO)CA and HNCA, and HN(CO)CACB and HNCACB experiments were acquired and assigned. The entire analysis provided the unambiguous identification of an 86% of the expected NH signals for the CRD. The spectra were processed with Bruker TopSpin 3.5 and analyzed via CARANMR 1.9.1. Most of the assignments agreed with those[18] deposited in the *Biological Magnetic Resonance Data Bank* (BMRB 19931) with minor discrepancies. The assignment have been deposited in the BMRB d with the accession code 27854.

^1H-^{15}N-HSQC-based were performed on 120 μM ^{15}N-labeled CRD samples, at 37 ºC. Six or ten points were recorded for each ligand. Averaged chemical shift perturbation (CSP) and dissociation constants (K_D) were calculated using the CcpNmr Analysis 2.4.2 software.[37]

STD experiments were carried out as described[38] at 600 MHz using standard Bruker pulse sequences without water suppression nor protein filter. Protein saturation was achieved with a Gaussian-shaped pulse of 50 ms at both the aliphatic and aromatic regions. Saturation build-up curves were obtained with saturation times from 0.5 s to 3.5 s in steps of 0.5 seconds. Samples contained 60 μM of protein and 4.2 mM glycan (1:70 lectin/ligand) in buffered D_2O (pD 8).

Transferred NOESY spectra were acquired at 600 and 800 MHz with 1/5 protein/tetrasaccharide ratio samples (183 μM of protein), with mixing times of 200 ms, 400 ms, 600 ms and 800 ms, at 298 K.

^{19}F CPMG spectra were acquired in a 600 MHz spectrometer equipped with a Bruker Selective ^{19}F-^1H Decoupling (SEF) probe at 298 K, on samples containing 40 uM of protein and 0.8 mM of the fluorinated probes. The standard CPMG Bruker pulse sequence was modified as described.[39] 24 points were acquired with total echo times from 8-5200ms, with τ=2ms. Data were analyzed with the T1T2 relaxation module of Topspin3.5.

Modeling. MD simulations were run using Amber 12.[40] The initial pdb coordinates for CRD DC-SIGN were derived from the crystal structure PDB 1SL5. The magnesium ion was replaced by calcium, and the desired glycan manually docked into the binding site. The glycan structures were built in the Glycam web. The complexes were prepared in explicit water (TIP3P model) and minimized in two steps before starting the simulations. ff99SB and GLYCAM_06h force fields were used for treating the protein and the ligands, respectively. MD simulations of 100 or 200ns were analyzed with cpptraj.

ASSOCIATED CONTENT

Supporting Information. Figures S1-S8, detailed synthetic procedures and characterization data. "This material is available free of charge via the Internet at http://pubs.acs.org."

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The manuscript was written through contributions of all authors.

Funding Sources

Agencia Estatal de Investigacion of Spain: Grants CTQ2015-64597-C2-1P (to JJB), CTQ2015-64597-C2-2P (to FJC), SEV-2016-0644 Severo Ochoa Excellence Accreditation (to CIC bioGUNE), Ramón y Cajal fellowship RYC-2014-15969 (to AA), and FPU14/10147 (to PV). European Research Council (RECGLYCANMR, Advanced Grant no. 788143 to JJB). Industrial Biotechnology Catalyst (Innovate UK, BBSRC, EPSRC, BB/M028941/1); EPSRC (EP/K039466/1).

ACKNOWLEDGMENT

We thank Agencia Estatal de Investigacion of Spain and the European Research Council for financial support. BL, J-BV and JM thank the Industrial Biotechnology Catalyst (Innovate UK, BBSRC, EPSRC, BB/M028941/1) and the EPSRC (core capability EP/K039466/1) for funding. We also thank Dr. J.F. Espinosa (Lilly Research Laboratories, Alcobendas, Spain, for providing the modified NMR pulse sequence for the CPMG experiments).

ABBREVIATIONS

BGA/BGB: Blood group A type 6/Blood group B type 6. CLR: C-type lectin receptor. CPMG: Carr-Purcell-Meiboom-Gill sequence experiment. CRD: Carbohydrate recognition domain. DC: Dendritic cell, DC-SIGN: Dendritic Cell-Specific ICAM-3 grabbing non-integrin. Fuc: L-fucopyranose. Gal: D-galactopyranose, Glc: D-glucopyranose. GlcNAc: N-Acetyl-2-D-glucosaminopyranose. HIV: Human immunodeficiency virus. HSQC: Heteronuclear single quantum spectroscopy. ICAM-3: Intracellular adhesion molecule 3. Le^X: Lewis X trisaccharide. MD: Molecular dynamics. NOE: Nuclear overhauser effect. NOESY: Nuclear overhauser effect spectroscopy. PRR: Pathogen recognition receptors. ROE: Rotating-frame overhauser enhancement. STD: Saturation transfer difference.

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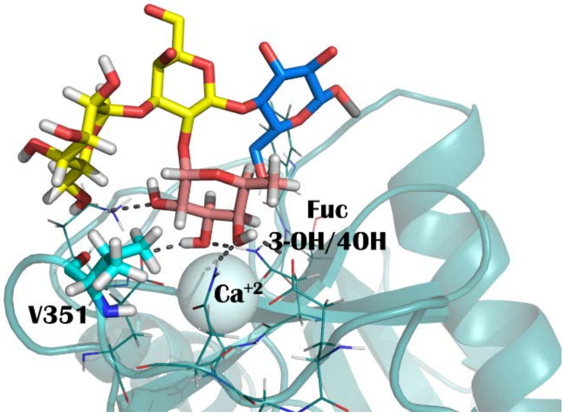

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