Solution NMR Analyses of the C-type Carbohydrate Recognition Domain of DC-SIGNR Reveal Different Binding Modes for HIV-derived Oligosaccharides and Smaller Glycan Fragments*

Fay Probert 1, Sara B-M. Whittaker 2, Max Crispin 3, Daniel A. Mitchell 4, and Ann M. Dixon 5

1 MOAC Doctoral Training Centre, 4 Warwick Medical School, and 5 Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.

2 HWB-NMR, Birmingham Cancer Research UK Centre, School of Cancer Sciences, University of Birmingham, Vincent Drive, Edgbaston, Birmingham, B15 2TT, UK.

3 Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

*Running title: NMR analyses of free and ligand-bound DC-SIGNR

SUMMARY

The C-type lectin DC-SIGNR (Dendritic Cell-Specific ICAM-3-Grabbing Non-integrin-Related; L-SIGN; CD299) is a promising drug target due to its ability to promote infection and/or within-host survival of several dangerous pathogens (e.g. HIV, SARS) via interactions with their surface glycans. Crystallography has provided excellent insight into the mechanism by which DC-SIGNR interacts with small glycans such as (GlcNAc)2Man3, however direct observation of complexes with larger, physiological oligosaccharides such as Man9GlcNAc2 remains elusive. We have utilised solution-state nuclear magnetic resonance spectroscopy to investigate DC-SIGNR binding and herein report the first backbone assignment of its active, calcium-bound carbohydrate recognition domain (CRD). Direct interactions with the small sugar fragments Man3, Man5 and (GlcNAc)2Man3 were investigated alongside Man9GlcNAc derived from recombinant gp120 (present on the HIV viral envelope), providing the first structural data for DC-SIGNR in complex with a virus-associated ligand, and unique binding modes were observed for each glycan. In particular, our data show that DC-SIGNR has a different binding mode for glycans on the HIV viral envelope compared with the smaller glycans previously observed in the crystalline state. This suggests that using the binding mode of Man9GlcNAc, instead of those of small glycans, may provide a platform for the design of DC-SIGNR inhibitors selective for high-mannose glycans (like those on HIV). 15N relaxation measurements provided the first information on the dynamics of the CRD, demonstrating that it is a highly flexible domain that undergoes ligand-induced conformational and dynamics changes which may explain the ability of DC-SIGNR to accommodate a range of glycans on viral surfaces.

To whom correspondence should be addressed: Ann M. Dixon, Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK. Tel.: +44 2476 150037; FAX: +44 2476 524112; E-mail: ann.dixon@warwick.ac.uk

Keywords: C-type lectin; glycan binding affinities; gp120 Man5GlcNAc2; oligosaccharide interactions; solution NMR dynamics

Background: DC-SIGNR, a C-type lectin which promotes infection of pathogens such as HIV, is a promising drug target.

Results: Carbohydrate recognition domain of DC-SIGNR is highly dynamic, displaying unique binding modes for individual glycans.

Conclusion: More complex, disease-associated glycans have different binding modes than smaller glycans previously studied.

Significance: Understanding ligand-binding properties and solution dynamics of DC-SIGNR will facilitate therapeutic design.
high selectivity via calcium-dependent carbohydrate recognition domains (CRDs) (3). The C-type lectin DC-SIGNR (dendritic-cell specific ICAM3 grabbing non-Integrin related, known also as L-SIGN and CD299) is a type II transmembrane protein that recognizes high-mannose N-linked oligosaccharides on viral envelopes and host glycoproteins (4). Although DC-SIGNR is known to bind high-mannose ligands, little is known about this molecule's biological function, owing partly to difficulties studying its specialized and often inaccessible cell types in which it is natively expressed. Furthermore, functional orthologues of DC-SIGNR in model species such as mice are not clear cut, thus restricting the value and meaning of gene-targeting studies such as knockout animals. In humans, it is expressed on specialized endothelia found in liver sinusoids, lymph nodes and placental capillaries, suggesting important roles in leukocyte adhesion and migration (5). Expression has also been found on precursor lung epithelial cells (6), a site where there is potential exposure to airborne viruses. Despite the lack of understanding of its biological function, important disease associations have been reported for DC-SIGNR such as vertical transmission of human immunodeficiency virus (HIV) (7) and severe acute respiratory syndrome coronavirus (SARS) infection (8). Recent work also indicates involvement of DC-SIGNR in respiratory syncytial virus (RSV) infection (9), influenza (10) and within-host dynamics of Dengue infection (11). The affinity of DC-SIGNR for glycoproteins on the surface of viruses, as well as its localization at the primary sites of virus replication, promotes in trans infection by viruses such as HIV. Specifically, DC-SIGNR is believed to promote HIV infection by transferring the virus to adjacent CD4+ T-cells, where the HIV glycoprotein gp120 binds to the CD4 receptor on these cells promoting entry of the virus into host T-cells. This reinforces the important role of this protein in immunity as well as its considerable potential as a drug target.

Therapeutic strategies designed to inhibit or stimulate the function of C-type lectins such as DC-SIGNR are scarce considering the scale of the diseases involved in their biology. Of particular interest is the interaction of the DC-SIGNR CRD (residues 262-400) with Man$_9$GlcNAc$_2$, one of the dominant oligosaccharide present on the HIV envelope glycoprotein gp120. It has been speculated that direct blockade of DC-SIGNR could provide a topical barrier against primary HIV infection. Therefore, a detailed understanding of the interaction between the DC-SIGNR CRD and the oligosaccharides present on viral glycoproteins is valuable for design of compounds that could act as antiviral drugs.

Thus far, X-ray crystallography has been the primary method employed for atomic-level study of the DC-SIGNR CRD structure. To date four crystal structures have been deposited for the DC-SIGNR CRD: 1) in complex with the branched pentasaccharide (GlcNAc)$_2$Man$_3$ (PDB ID: 1K9J); 2) in the absence of ligand (but with one Ca$^{2+}$) and containing a portion of the N-terminal α-helical neck region (1XPH); 3) with two repeats of the α-helical neck region and one sodium ion bound (1XAR); and 4) in complex with Lewis-x trisaccharide and containing a portion of the neck (1SL6). The DC-SIGNR CRD adopts a typical ‘lectin fold’ consisting of alpha helices and anti-parallel beta sheets connected by irregular loops which are stabilised by disulphide bonds and calcium ions (2). The structure of the DC-SIGNR CRD in complex with (GlcNAc)$_2$Man$_3$ provides insight into the CRD structure and potential ligand binding mechanism, notably that an extended binding site exists which is composed of α-helix 2 and a solvent exposed F325 residue. The C-terminal end of α-helix 2 packs against the loop joining β-sheets 6 and 7 forming a continuous binding surface (see Figure 1A). The ‘shelf’ formed by α-helix two and F325 creates a complementary shape to the Man$_9$-6Man moiety which forms van der Waals contacts with F325 and hydrogen bonds with S372. The F325 residue is also thought to be responsible for the selective binding of DC-SIGNR to the outer branched tri-mannose moiety of high-mannose structures (such as Man$_9$GlcNAc$_2$) as it sterically hinders binding to the inner branched mannose (4).

In addition, co-ordination bonds via the primary Ca$^{2+}$ binding loop (residues 356-364) and contacts with residues in β-sheets 6 and 7 are described (4). Regions of interest that form contacts with (GlcNAc)$_2$Man$_3$ are shown in Figure 1B and listed in Table S1. However, crystal structures of the DC-SIGNR CRD bound to larger, physiologically relevant oligosaccharides such as Man$_9$GlcNAc$_2$ have proved to be unattainable thus far. This may be due to as yet uncharacterized conformational/dynamic factors that prohibit crystal growth and diffraction.
High-field nuclear magnetic resonance (NMR) studies of the DC-SIGNR CRD have not been reported previously, although NMR studies of ligand interactions with the homologous protein DC-SIGN have started to emerge (12-18). These largely ligand-based studies have been similarly restricted to the use of small glycans and sugar mimetics and have not approached conformational or dynamic properties of DC-SIGN in solution or included larger physiological glycans such as Man$_9$GlcNAc$_2$ or similar. As a result, binding of disease-associated ligands such as Man$_9$GlcNAc$_2$ to molecules such as DC-SIGNR and DC-SIGN has been assumed to be consistent with the binding modes observed for smaller glycan fragments co-complexed in the crystalline state (19, 20).

We aimed to increase current understanding of DC-SIGNR-glycan interactions by investigating the binding of the DC-SIGNR CRD to a number of oligosaccharides in solution using heteronuclear solution-state NMR techniques that can better deal with issues of dynamics that we surmise to be restricting the rate of progress in DC-SIGNR crystallography. Here we present the backbone assignment of the DC-SIGNR CRD as well as the first structural data for binding of a disease-associated ligand, namely Man$_9$GlcNAc. These results are extended using dynamics measurements ($^{13}$N T$_1$ and T$_2$), which suggest that the same regions of the DC-SIGNR CRD are highly dynamic in both holo and ligand-bound forms and interconvert between a number of conformations at similar rates. Our results support the location of the extended binding site observed in the crystal structure of DC-SIGNR CRD-(GlcNAc)$_2$Man$_3$, complex (1K9J). However, our data also demonstrate that DC-SIGNR employs a different binding mode for Man$_9$GlcNAc, suggesting that DC-SIGNR may interact with the HIV glycoprotein gp120 in a different way to that previously observed for smaller glycans. Dynamics data provide new information on the flexible nature of DC-SIGNR and highlight new regions of the protein that are potentially important for ligand recognition.

**EXPERIMENTAL PROCEDURES**

Expression and purification of $^{13}$C/$^{15}$N isotopically labelled DC-SIGNR CRD - The pT5T overexpression plasmids containing modified cDNA inserts encoding the human DC-SIGNR CRD sequence (Q9H2X3 (CLC4M_HUMAN; residues 262-400); cDNA provided by Elizabeth Soilleux, University of Oxford, UK) were prepared as described previously (21) and subjected to DNA sequencing in order to confirm sequence integrity. The plasmids were then used to transform *Escherichia coli* strain BL21(DE3) and frozen bacterial stocks were prepared in 15% glycerol and stored at -80°C. Protein expression was carried out in M9 minimal media (22) (pH 7.3) containing 50 μg/ml ampicillin, 1 g of $^{15}$N ammonium chloride, and 2 g $^{13}$C glucose per litre of culture. Two 100 ml starter cultures were inoculated using colonies from an M9/ampicillin plate. Starter cultures were grown at 37°C with shaking at 200 rpm for 24 hours before dilution into 1 L of M9 minimal media to a starting OD$_{600nm}$ of ~0.2. Once an OD$_{600nm}$ = 0.7 was reached, the 1 L culture was induced with isopropyl-$eta$-D-thiogalactoside (IPTG) to a final concentration of 350 μM. Refolding of the CRD from inclusion bodies was performed as described (21). The protein CRD fragment was purified using a 2 ml mannose-Sepharose column (kindly provided by Dr. Russell Wallis, University of Leicester, UK) equilibrated with 25 mM HEPES, 5 mM CaCl$_2$, 150 mM NaCl pH 7.8 (loading buffer) as described (21). Protein purity and oligomeric state were assessed using mass spectrometry, SDS-PAGE and circular dichroism (CD) spectroscopy. The protein predominantly migrated near its monomeric molecular weight of 17.1 kDa (for the isotopically-labeled protein) and yielded a CD spectrum which is in good agreement with previous reports (21).

Carbohydrates - All carbohydrate fragments (GlcNAc)$_2$Man$_3$, (M592), Man$_9$ (M336), Man$_5$ (M536)) were purchased from Dextra Labs [Dextra, Reading, UK.]. The Man$_9$GlcNAc was prepared by digestion of recombinant gp120 with Endoglycosidase H using gp120 glycoprotein harvested from 10 μM kifunensine-treated HEK 293T cultures as described (23). The gp120 glycoform was characterized by mass spectrometry as shown in Figure S1.

Nuclear magnetic resonance spectroscopy (NMR) - Purified protein samples were subjected to extensive dialysis into water (1 week with 12 buffer changes) before lyophilization. The protein was then dissolved in 180 μl of 20 mM d-HEPES, 20 mM NaCl pH 6.8 in 10% D$_2$O / 90% H$_2$O to a final concentration of 0.7 mM DC-SIGNR CRD.
NMR experiments were carried out at 37°C on either a 700 MHz Bruker Avance spectrometer fitted with cryoprobe (University of Warwick) or a 5 mm triple resonance cold probe-equipped Varian Unity Inova 800 MHz spectrometer (HWB-NMR, University of Birmingham). Proton chemical shifts were referenced against external DSS while nitrogen chemical shifts were referenced indirectly to DSS using the absolute frequency ratio (24). One-dimensional (1D) proton spectra were acquired using the pulse sequence described by Liu et al (25). 2D $^1$H-15N HSQC spectra (26) were recorded with 128 increments in the $t_1$ domain and 1k data points in the $t_2$ dimension. The sweep width was 18.0 ppm in the $^1$H dimension and 31.8 ppm in the 15N dimension. The triple resonance ($^1$H-13C-15N) experiments (CBCA(CO)NH (27), CBCANH (28), HNCA (29), HN(CO)CA (30), HNCO (29), HN(CA)CO (31), were recorded with 128 increments in the $t_1$ domain, 40 increments in the $t_2$ domain and 2k increments in the $t_3$ domain. Spectra were processed using Toppin 2.0 (unless otherwise stated) and analysed using CCPN Analysis software version 2.1.5 (32, 33) and SPARKY 3 (34). Secondary structure predictions based on the chemical shift index were carried out using TALOS+ (35).

Un-labelling of specific residues, by addition of 100 mg unlabelled amino acid to double labelled M9 media, was used to assign difficult residues (such as the highly mobile residues N102 and N103 in the primary calcium binding loop).

Carbohydrate titrations - Titration experiments were carried out by adding increasing amounts of carbohydrate (0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 mM Man$_9$, 1.0, 2.0, 5.0, 10.0 mM Man$_3$ and (GlcNAc)$_2$Man$_3$ and 0.1, 0.3, 0.7, 1.0, 1.5, 2.0, 5.0 and 10.0 mM Man$_9$GlcNAc) to 0.7 mM [U-15N,13C] - DC-SIGNR CRD at pH 6.8 and acquiring a series of 2D $^1$H-15N HSQC spectra at 37°C. The pH and temperature were held constant throughout the experiments. The three sugar fragments reached saturation by 10 mM while relaxation properties prevented determination of Man$_9$GlcNAc saturation.

The total chemical shift perturbation per residue ($\Delta\delta_{\text{total}}$) was calculated using Equation 1,

$$\Delta\delta_{\text{total}} = \sqrt{(\Delta\delta_{\text{NH}})^2 + (0.1\Delta\delta_{N})^2} \quad \text{(1)}$$

where $\Delta\delta_{\text{NH}}$ and $\Delta\delta_{N}$ are the chemical shift differences in the $^1$H and 15N dimensions respectively. The weighting factor of 0.1 applied to the nitrogen chemical shift corresponds to the difference in magnetogyric ratios of 15N with $^1$H nuclei. While a weighting factor of 0.15 can also be used, Schumann et al. found little difference between the two weighting factors and concluded that either is sufficient (36). Residues significantly perturbed by ligand addition were determined by calculating the standard deviation of the chemical shift perturbations across all residues for each carbohydrate and using this as a cut-off (36).

To determine the dissociation constant ($K_p$), titration curves were fit to Equation 2 valid for 1:1 complex formation in fast exchange on the NMR chemical shift time scale,

$$\Delta\delta_{\text{total,}x} = \frac{\Delta\delta_{\text{max}}}{\frac{1}{K_p} + \frac{1}{[P]} - \frac{1}{[P]}[x]} \quad \text{(2)}$$

where $x$ and $[P]$ are the ligand and protein concentration respectively, $\Delta\delta_{\text{total,}x}$ is the total chemical shift perturbation at ligand concentration $x$ and $\Delta\delta_{\text{max}}$ the total chemical shift perturbation at saturation of ligand (37, 38). The fit was carried out and analysed in Origin 8.5 using the non-linear least squares method.

Backbone dynamics - 13N longitudinal ($T_1$) and transverse ($T_2$) relaxation times were measured using the procedures of Kay et al (39) and Farrow et al (40). For $T_1$ measurements, 11 spectra were recorded with relaxation delays between 0.01-0.750 seconds. Matrices of 1024 x 128 complex data points were acquired, using 32 scans per $t_1$ increment and a recycle delay of 3 seconds. For $T_2$ measurements, 10 spectra were recorded with relaxation delays of 0.0077-0.0850 seconds. Matrices of 1024 x 128 complex data points were recorded, using 32 (holo protein) or 64 (Man$_9$-bound protein) scans per $t_1$ increment and a recycle delay of 3 seconds. Sample heating owing to cold probe sensitivity was compensated for by application of continuous wave irradiation to 15N nuclei for a variable time period during the recycle delay (41). Spectral widths of 13008.1 Hz ($^1$H) and 2500 Hz (15N) were measured. Relaxation data were acquired in an interleaved manner to minimise the effects of sample heating, and three repeat measurements for each of the $T_1$ and $T_2$ datasets were made for determination of peak
height uncertainties. Relaxation spectra were processed in NMRpipe (42) and peak heights calculated and fit to a mono-exponential decay in SPARKY 3 (34).

RESULTS

Assignment of Ca^{2+}-bound DC-SIGNR CRD - To investigate the structure, dynamics, and ligand-binding modes of the human DC-SIGNR CRD domain in solution, solution-state NMR was used. A 0.7 mM sample of the recombinant 138-residue fragment (containing CRD residues 262-399) was prepared with uniform $^{15}$C/$^{15}$N-labelling in the presence of 4 mM Ca^{2+} (pH 6.8). The purity, secondary structure, and oligomeric state were probed using SDS-PAGE, mass spectrometry and circular dichroism, and in all cases we observed pure, monomeric protein. Purification of the CRD using a mannose-Sepharose column served as an effective screen for correct and functional folding of the CRD, as incorrectly folded protein fails to bind to immobilized mannose on the column and elutes much earlier.

The $^1$H-$^1$N HSQC spectrum of the calcium-bound DC-SIGNR CRD is shown in Figure 2, and the sequential assignment of the N, C, and H nuclei along the protein backbone was achieved using a full suite of triple resonance experiments (see Materials and Methods). The assignments are given in Figure 2 and Table S2. Of the 138 amino acids in the CRD, 5 proline residues do not appear in the HSQC spectrum due to their lack of a backbone amide group and 17 residues (8 at the N terminus and 9 at the C terminus) were also not present. Of the observable residues present in the HSQC, 96% have been unambiguously assigned (80% of the total CRD).

The C, H, and N chemical shift assignments (deposited at BMRB, ID 19297) facilitated determination of secondary structure using the TALOS+ program (35) to designate regions containing α-helical/β-sheet structure based on dihedral angle predictions. The secondary structure prediction is in good agreement with crystal data (1K9J) (Figure S2). Therefore we have used published crystal structures as a foundation for comparison of solution data in this work.

Binding modes for three glycan fragments in complex with DC-SIGNR CRD - The HSQC spectrum shown in Figure 2 provides a ‘map’ of the CRD in the calcium bound state. To investigate a range of glycan-CRD interactions in solution, with an eye toward difficult-to-crystallize glycans, a series of HSQC spectra were acquired upon titration of the oligosaccharide fragments Man$_3$, Man$_5$, or (GlcNAc)$_2$Man$_3$ (see Figure 3) into the DC-SIGNR CRD. Analysis of (GlcNAc)$_2$Man$_3$, the sugar present in the 1K9J structure, allowed the direct comparison of results in solution to published crystal data. As shown in Figures 4 and S3-S5, binding of all three sugars resulted in significant chemical shift perturbations along the length of the CRD. The standard deviation of the chemical shift perturbation across all residues (dashed line in Figure 4) was used (36) to determine residues most affected by binding. Perturbations above this threshold were considered significant and are mapped onto the (GlcNAc)$_2$Man$_3$-CRD structure (1K9J) as shown in Figure 5.

Binding of all three sugar fragments was consistent with a principal glycan binding site composed of the primary Ca$^{2+}$-binding loop (residues 356-364), previously proposed to directly interact with the glycan (3,4,19, 20), and residues in β-sheets 6 and 7 at the core of the protein fold (residues 368-379, Figure 1B).

However, binding of the glycan fragments was not universally consistent with the proposed ‘shelf’ formed by α-helix 2 and F325. The three glycan fragments show marked differences in the degree of perturbation of residues in α-helix 2 (residues 308-323), as shown by the circle in Figure 5A. Only binding of (GlcNAc)$_2$Man$_3$, the glycan bound in the 1K9J structure, yielded appreciable chemical shift perturbations in α-helix 2 (specifically residues 314-324). Even then, the F325 residue in α-helix 2 shown to form a direct contact with (GlcNAc)$_2$Man$_3$ in the crystal was not perturbed significantly in solution. Even fewer perturbations in α-helix 2 were observed upon binding of Man$_3$ and Man$_5$, and neither sugar induced any perturbation of the F325 peak. However, Man$_3$ and Man$_5$ binding significantly affect S326, adjacent to F325, while (GlcNAc)$_2$Man$_3$ binding does not.

The chemical shift perturbations observed here in solution also highlighted changes in additional regions of the CRD, distal to the proposed glycan binding site. Man$_3$ and Man$_5$-binding induced perturbation of residues 270 & 271 at the N-terminus of the CRD (solid box, Figures 5B-C), whilst (GlcNAc)$_2$Man$_3$-binding exhibited unique perturbations in the loop region consisting of residues 382-385.
(Figure 5A, dashed box). This suggests that perturbations in this region may be due to the GlcNAc moieties, possibly in conjunction with their β-(1,4) linkages to the trimannose core. These residues are not highlighted in previous glycan-binding studies, suggesting that a conformational or dynamics change is taking place in this region.

More broadly, when our results are compared to the existing structural data, the number of chemical shifts affected by ligand binding in all cases is greater than was expected based on the size of the canonical binding site in the crystal (see summary in Table S1). This, along with the fact that there are chemical shift perturbations distal to the canonical glycan binding surface, suggests that significant changes in conformation and/or dynamics occur upon ligand binding in solution. This is in contrast to results obtained from crystallography, which yield virtually identical average structures for the free and ligand-bound states (4,43) (see Figure 1A for an overlay of two representative structures for these opposing states).

**Binding of Man₉GlcNAc derived from HIV gp120 to the DC-SIGNR CRD** - To extend current knowledge of the DC-SIGNR CRD to binding of more complex, physiologically important and/or disease-associated ligands, and provide a better understanding of DC-SIGNR-HIV interactions, similar titration experiments were carried out using Man₉GlcNAc derived from the gp120 protein of HIV. Man₉GlcNAc is very closely related to Man₉GlcNAc₂ on HIV gp120, differing by a single GlcNAc unit at the reducing terminus which would be anchored to the polypeptide backbone and hence less likely to play a crucial role in DC-SIGNR binding (Figure 3).

Similar to the glycan fragments, chemical shift perturbation data upon Man₉GlcNAc-binding (Figures 4D, 5D, and S6) is consistent with a principal binding site containing residues in β-sheets 6 and 7 and the primary calcium binding loop. The same effects on distal regions of the CRD (e.g. N-terminal residues 270-271, solid box in Figure 5D) are also observed. Man₉GlcNAc binding results in no chemical shift changes for residues in the region thought to compose a ‘shelf’ formed by α-helix 2 and F325. This is interesting as crystal structures (on small fragments of DC-SIGNR and the homologous protein DC-SIGN) highlight this shelf as forming part of the extended binding site. The NMR data presented here show that, in solution, α-helix 2 is involved in binding to (GlcNAc)₉Man₅ (and possibly to the smaller glycans) which is in good agreement with 1K9J structure. However, α-helix 2 is not involved in binding to Man₉GlcNAc suggesting that it has a different mode of binding to the CRD than does (GlcNAc)₂Man₃, and that DC-SIGNR may interact with the HIV glycoprotein gp120 in a different way than observed in the (GlcNAc)₂Man₃-CRD complex. Ongoing work to determine a high-resolution solution structure of DC-SIGNR CRD bound to this ligand will shed further light upon this.

**Ligand binding affinities in solution** - NMR titration data was also used to provide detailed affinity information. Figures S3-S5 show the HSQC spectra of the CRD acquired at increasing concentrations of (GlcNAc)₂Man₃, Man₉ or Man₅, respectively. The three sugar fragments behaved similarly upon titration, displaying linear chemical shift perturbations and no line broadening as ligand concentration was increased. This behaviour is characteristic of fast exchange between free and bound protein on the NMR chemical shift timescale, and suggests that the interaction between the CRD and sugar fragments is weak. This weak binding of our small sugar fragments is unsurprising in light of several reports that DC-SIGNR binds preferentially to larger, highly-branched oligosaccharides (4,19,43). Fitting the chemical shift perturbations to a 1:1 binding model (see Materials and Methods), which produced the best fits to the data, provides estimates of the dissociation constants for each sugar (Figure 6). Table 1 shows that (GlcNAc)₂Man₃, Man₉ and Man₅ all bind with similar, weak affinities. $K_D$ values ranged from 1.57-2.2 mM. This value is considerably weaker than binding of similar simple sugars to other lectin CRD domains such as galectin-1 (lactose bound with a $K_D = 40 \mu M$ (44) – 520 μM (45)), galectin-3 (lactose bound with a $K_D = 231 \mu M$ (46)), BclA (methyl-α-D-mannoside bound with a $K_D = 2.75 \mu M$ (47)), and the asialoglycoprotein receptor (binding constants of 66-539 μM were reported for a variety of simple sugars (48)). However, these $K_D$ values are in line with the IC₅₀ values of the analogous protein DC-SIGN for fucose (1.2 mM) and mannose (1.8 mM) (16).

Unlike the glycan fragments, binding of Man₉GlcNAc caused substantial broadening and disappearance of a number of CRD peaks.
studies reporting a complex is increased, and supports previous fragments, since we move from fast exchange to intermediate exchange as the lifetime of a complex is increased, and supports previous studies reporting a $K_i$ value of 200 $\mu$M (21) for the DC-SIGNR-Man$_5$GlcNAc$_2$ complex. Studies of other lectins (e.g. galectin-1) also report increased affinity for larger, more complex glycans (45). However, due to the severe line broadening at the highest Man$_5$GlcNAc concentrations (5 mM), an accurate $K_d$ could not be estimated using NMR under these conditions.

**NMR dynamics** – The high degree of dynamics in the DC-SIGNR CRD was first suspected after measurement of the HSQC spectrum of the Ca$^{2+}$-free (apo) form (Figure S7), in which variable signal intensities prevented further study using three-dimensional NMR methods. Weak or missing signals suggest that, in its Ca$^{2+}$-free form, the protein can exchange between an ensemble of conformational states with a rate corresponding to the intermediate exchange regime on the NMR chemical shift timescale. Addition of Ca$^{2+}$ to the intermediate exchange regime on the conformational states with a rate corresponding on the NMR chemical shift timescale (Figure S6) consistent with intermediate exchange on the NMR chemical shift time scale (49,50). This suggests higher-affinity binding of Man$_5$GlcNAc compared with the sugar fragments, since we move from fast exchange to intermediate exchange as the lifetime of a complex is increased, and supports previous studies reporting a $K_i$ value of 200 $\mu$M (21) for the DC-SIGNR-Man$_5$GlcNAc$_2$ complex. Studies of other lectins (e.g. galectin-1) also report increased affinity for larger, more complex glycans (45). However, due to the severe line broadening at the highest Man$_5$GlcNAc concentrations (5 mM), an accurate $K_d$ could not be estimated using NMR under these conditions.

Per residue $^{15}$N $T_1$ and $T_2$ relaxation times were measured for the CRD in the absence (holo) and presence of 10 mM Man$_5$ (Figure 7, Table II) to map regions of the CRD where dynamics is altered upon ligand binding. Man$_5$ was selected for this study because binding of Man$_5$GlcNAc produced severely exchange-broadened spectra, preventing accurate measurement of relaxation parameters. The $T_1$ data for the holo and Man$_5$-bound states, showing a similar trend across the protein, and average values of 788.26 $\pm$ 22.4 ms (holo) and 806.22 $\pm$ 40.35 ms (Man$_5$-bound). Larger differences were observed in the transverse relaxation time constants ($T_2$). For the holo CRD, though most of the $T_2$ values fall near the average (76.27 $\pm$ 2.27 ms), residues in both Ca$^{2+}$ binding loops, β-sheets 6 and 7, and at the N-terminus (see shaded regions in Figure 7) display significantly shorter $T_2$ relaxation times suggesting that these regions are undergoing motions on the $\mu$-ms timescale due to conformational exchange processes (51). A similar trend is seen in the $T_2$ data for the Man$_5$-bound CRD, but with a slightly lower average $T_2$ (66.54 $\pm$ 8.23 ms) and more pronounced reduction of $T_2$ values for the shaded regions in Figure 7. These data suggest that $\mu$-ms motions present in the holo- form of the CRD still persist upon glycan binding, albeit at a slightly increased rate.

Asterisks in Figure 7 indicate residues in the Ca$^{2+}$ binding loops whose signals are so severely broadened as to be unobservable in these experiments, supporting the rapid relaxation of these regions. Specifically, these residues included E359, N361 and N362 in the primary Ca$^{2+}$ binding loop, which make up the EPN motif conserved among all mannose-binding C-type lectins (52). This binding at the primary Ca$^{2+}$ site is well characterised (it is a distinguishing feature of C-type lectin binding) and it has been confirmed that the EPN sequence is responsible for mannose specificity (52). In addition to the EPN motif, residues across the entire primary Ca$^{2+}$ binding loop and β-sheets 6 and 7 display enhanced transverse relaxation in both the holo and ligand bound forms. For the holo form, the increased exchange contribution is possibly driven by the kinetics of Ca$^{2+}$ binding, confirming that these regions are near the calcium binding sites. A further reduction in $T_2$ values of residues in the primary calcium binding loop are observed upon addition of Man$_5$. As there is little change in the average $T_2$ value between holo and Man$_5$-bound forms, this enhanced relaxation is likely due to an increased conformational exchange contribution as a result of Man$_5$ binding kinetics or hindered motions as a result of Man$_5$ interacting with the principal binding site. This supports previous reports that this is the site of key CRD-mannose interactions.

Interestingly, no dynamics changes were observed for residues in α-helix 2 (thought to form the extended glycan-binding ‘shelf’) upon addition of Man$_5$.

The relaxation data presented here also highlighted new regions in the CRD that have not yet been implicated in binding to sugars, namely the secondary calcium binding loop and α-helix 1. Residues all along the length of α-helix 1 show a subtle but significant reduction in $T_2$ (as compared to the average) in the holo CRD, and a further reduction upon binding of Man$_5$. α-helix 1 is positioned towards the N-terminus of the CRD, where we also see
increased transverse relaxation rates for residues 269-272. The secondary calcium binding site also responds to glycan binding, despite the fact that this region has not (to our knowledge) been implicated in glycan binding previously. In the holo protein, the reduced T2 values in this region (T337 was broadened beyond detection) were attributed to slow internal motions of the loop (on the ms-μs timescale) upon binding of Ca2+. The further enhancement in transverse relaxation rates upon ligand binding is less obvious, since this region of the CRD has not been shown previously to form part of the extended glycan binding site.

The average T2 value decreased significantly from 76.27 to 66.54 ms upon ligand binding. We have attributed this reduction in T2 to slower tumbling of the ligand-bound protein compared to the holo protein in solution. This was confirmed by using the T1/T2 ratio (Figure 7) to estimate the overall rotational correlation time (τc) by first excluding residues that contained values more than one standard deviation from the average (and thus experience a significant contribution from either chemical exchange or internal motion (53)) and then calculating as described (54). A value for the relaxation-derived τc of 10.4 ± 0.4 ns was obtained for the holo CRD, which is only slightly longer than the expected value of 8.55 ns obtained using the general rule of 0.5 ns τc per 1 kDa molecular weight (53-55). This deviation from the ideal value is not large enough to infer oligomerization of the CRD, but may reflect a non-spherical shape of the monomeric protein. There is a ~17% increase in the rotational correlation time from 10.4 - 12.5 ns upon binding of Man5 to the CRD (Table II). While we acknowledge that a small (~2% as estimated using a published model (56)) increase in solution viscosity upon addition of 10 mM Man5 may contribute to this change, and the additional size imparted by the bound sugar may also yield a very small increase, these two factors are unlikely to fully account for the increase in rotational correlation time. Likewise, binding induced aggregation would result in a much larger increase in τc, suggesting that the CRD adopts a more ‘open’ conformation as a result of Man5 binding.

In broad terms, comparison of the relaxation and chemical shift perturbation data demonstrate that, while several regions in the CRD display μs-ms timescale dynamics that persist upon glycan binding, many more residues display chemical shift perturbations. This suggests that there is a ligand-induced conformational change in the CRD. The extent of this conformational change warrants further investigation, as thus far no conformational changes have been observed in any published crystal structures for DC-SIGNR CRD upon ligand binding.

DISCUSSION

Complex carbohydrate binding events that occur within the human immune system are vital to healthy immune function and proper host responses to a wide variety of pathogens. Greater understanding of this essential glycoimmunology promises to provide important insights into major world health risks such as HIV, tuberculosis and Gram-negative multiresistance-diseases. C-type lectins represent some of the most important receptors for complex carbohydrates and their roles in contributing to sophisticated pathogen recognition and cellular response mechanisms are only just beginning to emerge. Structural studies have provided insights into the mechanisms via which C-type lectins assemble and bind to their targets with specificity, displaying a range of strategies including oligomerization, monosaccharide selectivity and, in some cases, extended binding sites incorporating multiple protein-glycan contacts. However, characterisation of the interaction of larger, disease-associated glycans with C-type lectins (especially HIV-derived Man9GlcNAc with the human C-type lectin DC-SIGN) has not been reported thus far.

Here we have described the first solution state NMR backbone assignment of the carbohydrate recognition domain of human DC-SIGN, and have used this spectrum as a platform upon which to characterize the solution-state binding properties of a variety of glycan ligands, including Man9GlcNAc. We have also used solution NMR methods to begin to characterise the molecular dynamics of the CRD in its free and ligand-bound states for the first time. These data have revealed several interesting properties of the DC-SIGNR CRD summarized below.

Different binding modes and affinities for small glycans vs.Man9GlcNAc - The C-type lectin family of proteins is striking in that substantial portions of the C-type lectin domain (CTLD) do not adopt regular secondary structure
and typically the ligand binding properties of the CTLDs are located within these nonregular regions. Furthermore, it has been shown that a number of transmembrane human C-type lectins are capable of binding multiple ligands via discrete binding sites and can transduce different intracellular signals through the same receptor molecule depending upon the type of ligand engaged at the extracellular face (57,58).

Another key feature of the C-type lectin family is its enormous potential for ligand binding diversity, brought about largely through the ability of the CTLD scaffold to accommodate a substantial variety of nonregular polypeptide loops at several distinct regions within the domain fold (59). It is very likely that, for these regions, the C-type lectin family has evolved into a range of homologous proteins with a very broad spectrum of ligand specificities, including targets of both exogenous and endogenous origin.

The different binding modes for the four glycans studied here, as indicated by four unique patterns of chemical shift perturbations, support the structural plasticity proposed for C-type lectins, which allows them to accommodate a wide range of diverse ligands and heterogeneously-glycosylated surfaces (60). All four glycans caused perturbation of protein regions near the principal glycan binding site, namely the primary Ca\(^{2+}\)-binding loop and β-sheets 6 and 7. However, each glycan had a unique set of additional perturbations in α-helices 1 and 2, the N-terminus of the CRD, the loop region consisting of residues 382-385, and the secondary Ca\(^{2+}\)-binding loop. For example, the majority of residues in α-helix 2 were preferentially engaged during binding of (GlcNAc)\(_2\)Man\(_3\) (circled Figure 5A), which supported its role as a critical region (along with F325) in forming a ‘shelf’ complimentary to (GlcNAc)\(_2\)Man\(_3\) in the binding site. However, this region does not appear to interact with Man\(_5\)GlcNAc. Overall, the data suggest that the use of small glycans as models for binding of larger, branched physiological ligands should be treated with caution, and demonstrate that solution state NMR is highly accommodating, informative and essential for the design of drug molecules that could inhibit binding of large, disease-associated carbohydrates.

The NMR data presented here also allowed us to report the first dissociation constants for direct binding of the three glycan fragments to the DC-SIGNR CRD. The three glycan fragments displayed 1:1 binding to a single binding site with similar, weak affinities. Man\(_5\)GlcNAc has a higher affinity for the CRD, as indicated by severe broadening of selected NMR signals unique to this ligand, characteristic of intermediate exchange on the NMR timescale and longer lifetimes for the complex. This higher affinity may explain the fact that Man\(_5\)GlcNAc (the largest of the ligands tested) yielded the smallest number of chemical shift perturbations, i.e. the binding site in the DC-SIGNR CRD may have evolved around this ligand and does not need to rearrange significantly in order to accommodate it.

**NMR dynamics reveal a high degree of flexibility for the DC-SIGNR-CRD and suggest new binding regions** - Whilst several structural analyses of mammalian C-type lectins have revealed substantial spatial information on glycan ligand binding, the level of dynamics data relating to these carbohydrate-binding proteins is surprisingly limited. Given the considerable structural diversity of C-type lectins in nature, especially within regions of nonregular secondary structure, it follows that diversity in the dynamic characteristics of these proteins may play an important role in defining ligand interactions and specificity. Previous studies on the tunicate C-type lectin TC14 have shown that the nonregular sequences in the CTLD are rigid (61). However, just as primary sequences and ligand specificity for the C-type lectin family are many and varied, so too could be the dynamic properties of the assorted domain family members.

Our data indicate that, unlike TC14, DC-SIGNR shows considerable flexibility within its nonregular sequences and this may contribute to its ability to interact with large, flexible glycans and transduce intracellular signals. The apo CRD appeared to be very dynamic, probably exchanging between a broad ensemble of conformations.

Binding of Ca\(^{2+}\) and Man\(_5\) lead to enhanced transverse relaxation rates in the primary Ca\(^{2+}\)-binding loop and β-sheets 6 and 7, known sites of key CRD-mannose interactions. This rapid relaxation could suggest direct binding and thus more hindered (yet persistent) μs-ms motions in these regions or an enhanced conformational / chemical exchange contribution. This exchange could be compatible with the association / dissociation kinetics of Ca\(^{2+}\) ions or Man\(_5\), although there is no existing data in this area. *Cis-trans* isomerisation about...
the peptide bond of the conserved proline (P360) in the EPN motif (61-65) has been reported for several other C-type lectins in their apo-form, and this could also result in the conformational exchange observed in apo DC-SIGNR-CRD.

Previous studies have suggested that Ca\(^{2+}\)- and Man\(_9\)-induced changes in the CRD distal to the proposed glycan binding site, namely in the secondary Ca\(^{2+}\)-binding loop, α-helix 1 and the N-terminus (most pronounced for D271). Previous studies have suggested that Ca\(^{2+}\)-binding in the secondary loop is enhanced by glycan-binding (43). This type of behaviour could explain the reduction in T\(_2\) as we go from holo- to Man\(_9\)-bound forms, with enhanced Ca\(^{2+}\)-binding in the secondary loop (in the presence of Man\(_3\)) leading to a more stabilized loop structure. The secondary Ca\(^{2+}\)-binding loop lies in close proximity to the proposed binding site, and given the structural plasticity proposed for C-type lectins which allows them to accommodate a wide range of diverse ligands, it is also possible that Man\(_3\) has a different mode of binding to the DC-SIGNR CRD than was shown for (GlcNAc)\(_2\)Man\(_3\) which includes the secondary Ca\(^{2+}\)-binding loop.

The N-terminus of the CRD connects to the α-helical neck in the full-length protein, and others have proposed that this region forms a flexible ‘hinge’ (43,66,67) allowing the CRD to sample multiple orientations with respect to the neck. Taking this into account, one tentative explanation for the enhanced relaxation in α-helix 1 and the N-terminus is that binding of Ca\(^{2+}\) and Man\(_9\) increasingly reduces the rate of conformational interconversion of this region. Such dynamic behaviour may influence ligand-induced conformational changes throughout the entire DC-SIGNR molecule, including the neck and cytoplasmic region. Alternatively, ligand binding could alter the orientation of the CRD with respect to the neck promoting multi-valent binding by adjacent CRDs. Structural analyses of the human C-type lectin CLEC5A allude to similar possibilities that dynamic changes in the CRD, attributable to distal glycan binding, could contribute to the transmission of conformational information and signalling beyond the target binding site to the intracellular regions of the native polypeptide (68). In the case of DC-SIGNR, a receptor previously believed to be involved primarily in adhesion, evidence of signalling activity has been demonstrated in the context of respiratory syncytial virus glycoprotein binding (9).

Although dynamics data could not be acquired in the case of Man\(_9\)GlcNAc, binding of this glycan was unique in that NMR spectra displayed severe line-broadening, which could be due to extensive μs-ms dynamics in the CRD (compared with Man\(_3\)-associated CRD). It follows that the larger, higher-affinity Man\(_9\)GlcNAc could restrict the motions of the CRD more than the smaller, low affinity Man\(_3\), however more work is needed to improve the solution behaviour of this complex.

Together the dynamics and chemical shift perturbation data suggest that more residues are affected by ligand binding than can be explained by direct interaction of the protein with the oligosaccharides. Our interpretation of these data leads us to portray the DC-SIGNR CRD as a highly flexible, dynamic domain that can interconvert between a number of conformations over a range of timescales. However, the crystal structures for free and ligand-bound DC-SIGNR CRD (Figure 1A) do not suggest conformational rearrangement upon ligand binding. Future work will involve solving the solution structures of holo- and ligand-bound states in an effort to characterize these conformational changes. An alternative interpretation is that glycans bind to multiple binding sites or experience multivalent binding. For the homologous protein DC-SIGN, crystallography has revealed multiple binding modes for the smaller glycans Man\(_3\) and Man\(_6\). However, only a single binding mode was observed in crystals of (GlcNAc)\(_2\)Man\(_3\) with DC-SIGNR, and no non-linear chemical shift perturbations or broadening (which would result from multiple binding sites and / or modes) was observed after addition of the three sugar fragments. The linear chemical shift perturbations also fit very well to a one-site binding model. Man\(_9\)GlcNAc binding did result in broadening of signals, therefore we cannot rule out multiple binding modes for this ligand, but taken together with the rest of the data we conclude that significant changes (both dynamic and structural) are taking place in the CRD as a result of ligand binding in solution that cannot be sampled in the crystal structures. This inherent flexibility may enhance the ability of DC-SIGNR to accommodate a variety of ligands including those of the HIV envelope.
REFERENCES

1. Weis, W. I., Taylor, M. E., and Drickamer, K. (1998) The C-type lectin superfamily in the immune system. *Immunol Rev* 163, 19-34
2. Drickamer, K. (1997) Making a fitting choice: Common aspects of sugar-binding sites in plant and animal lectins. *Structure* 5, 465-468
3. Drickamer, K. (1999) C-type lectin-like domains. *Curr Opin Struct Biol* 9, 585-590
4. Feinberg, H., Mitchell, D. A., Drickamer, K., and Weis, W. I. (2001) Structural basis for selective recognition of oligosaccharides by DC-SIGN and DC-SIGNR. *Science* 294, 2163-2166
5. Soilleux, E. J., Barten, R., and Trowsdale, J. (2000) DC-SIGN; a related gene, DC-SIGNR; and CD23 form a cluster on 19p13. *J Immunol* 165, 2937-2942
6. Chen, Y., Chan, V. S., Zheng, B., Chan, K. Y., Xu, X., To, L. Y., Huang, F. P., Khoo, U. S., and Lin, C. L. (2007) A novel subset of putative stem/progenitor CD34+Oct-4+ cells is the major target for SARS coronavirus in human lung. *J Exp Med* 204, 2529-2536
7. Boily-Larouche, G., Iscache, A. L., Zijenah, L. S., Humphrey, J. H., Mouland, A. J., Ward, B. J., and Roger, M. (2009) Functional genetic variants in DC-SIGNR are associated with mother-to-child transmission of HIV-1. *PLoS One* 4, e7211
8. Chan, V. S., Chan, K. Y., Chen, Y., Poon, L. L., Cheung, A. N., Zheng, B., Chan, K. H., Mak, W., Ngan, H. Y., Xu, X., Screaton, G., Tam, P. K., Austyn, J. M., Chan, L. C., Yip, S. P., Peiris, M., Khoo, U. S., and Lin, C. L. (2006) Homozygous L-SIGN (CLEC4M) plays a protective role in SARS coronavirus infection. *Nat Genet* 38, 38-46
9. Johnson, T. R., McLellan, J. S., and Graham, B. S. (2012) Respiratory syncytial virus glycoprotein G interacts with DC-SIGN and L-SIGN to activate ERK1 and ERK2. *J Virol* 86, 1339-1347
10. Londrigan, S. L., Turville, S. G., Tate, M. D., Deng, Y. M., Brooks, A. G., and Reading, P. C. (2011) N-linked glycosylation facilitates sialic acid-independent attachment and entry of influenza A viruses into cells expressing DC-SIGN or L-SIGN. *J Virol* 85, 2990-3000
11. Dejnirattisai, W., Webb, A. I., Chan, V., Jumnainsong, A., Davidson, A., Mongkolsapaya, J., and Screaton, G. (2011) Lectin switching during dengue virus infection. *J Infect Dis* 203, 1775-1783
12. Mari, S., Serrano-Gomez, D., Canada, F. J., Corbi, A. L., and Jimenez-Barbera, J. (2005) 1D saturation transfer difference NMR experiments on living cells: The DC-SIGN/oligomannose interaction. *Angew Chem Int Edit* 44, 296-298
13. Reina, J. J., Sattin, S., Invernizzi, D., Mari, S., Martinez-Prats, L., Tabarani, G., Fieschi, F., Delgado, R., Nieto, P. M., Rojo, J., and Bernardi, A. (2007) 1,2-mannobioside mimic: Synthesis, DC-SIGN interaction by NMR and docking, and antiviral activity. *Chemmedchem* 2, 1030-1036
14. Angulo, J., Diaz, I., Reina, J. J., Tabarani, G., Fieschi, F., Rojo, J., and Nieto, P. M. (2008) Saturation Transfer Difference (STD) NMR Spectroscopy Characterization of Dual Binding Mode of a Mannose Disaccharide to DC-SIGN. *ChemBioChem* 9, 2225-2227

15. Reina, J. J., Diaz, I., Nieto, P. M., Campillo, N. E., Paez, J. A., Tabarani, G., Fieschi, F., and Rojo, J. (2008) Docking, synthesis, and NMR studies of mannosyl trisaccharide ligands for DC-SIGN lectin. *Org Biomol Chem* 6, 2743-2754

16. Timpano, G., Tabarani, G., Anderluh, M., Invernizzi, D., Vasile, F., Potenza, D., Nieto, P. M., Rojo, J., Fieschi, F., and Bernardi, A. (2008) Synthesis of novel DC-SIGN ligands with an alpha-fucosylamide anchor. *ChemBioChem* 9, 1921-1930

17. Guzzi, C., Angulo, J., Doro, F., Reina, J. J., Thepaut, M., Fieschi, F., Bernardi, A., Rojo, J., and Nieto, P. M. (2011) Insights into molecular recognition of Lewis(X) mimics by DC-SIGN using NMR and molecular modelling. *Org Biomol Chem* 9, 7705-7712

18. Prost, L. R., Grim, J. C., Tonelli, M., and Kiessling, L. L. (2012) Noncarbohydrate Glycomimetics and Glycoprotein Surrogates as DC-SIGN Antagonists and Agonists. *ACS Chem Biol* 7, 1603-1608

19. Guo, Y., Feinberg, H., Conroy, E., Mitchell, D. A., Alvarez, R., Blixt, O., Taylor, M. E., Weis, W. I., and Drickamer, K. (2004) Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. *Nat Struct Mol Biol* 11, 591-598

20. Feinberg, H., Castelli, R., Drickamer, K., Seeberger, P. H., and Weis, W. I. (2007) Multiple modes of binding enhance the affinity of DC-SIGN for high mannose N-linked glycans found on viral glycoproteins. *J Biol Chem* 282, 4202-4209

21. Mitchell, D. A., Fadden, A. J., and Drickamer, K. (2001) A novel mechanism of carbohydrate recognition by the C-type lectins DC-SIGN and DC-SIGNR - Subunit organization and binding to multivalent ligands. *J Biol Chem* 276, 28939-28945

22. Sambrook, J., and Russell, D. W. (2001) Molecular cloning : A laboratory manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

23. Dunlop, D.C., Bonomelli, C., Mansab, F., Vasiljevic, S., Doores, K.J., Wormald, M.R., Palma, A.S., Feizi, T., Harvey, D.J., Dwek, R.A., Crispin, M., Scanlan, C.N. (2010) Polysaccharide mimicry of the epitope of the broadly neutralizing anti-HIV antibody, 2G12, induces enhanced antibody responses to self oligomannose glycans. *Glycobiology* 20, 812-823

24. Wishart, D. S., Bigam, C. G., Yao, J., Abildgaard, F., Dyson, H. J., Oldfield, E., Markley, J. L., and Sykes, B. D. (1995) H-1, C-13 and N-15 Chemical-Shift Referencing in Biomolecular NMR. *J Biomol NMR* 6, 135-140
25. Liu, M. L., Mao, X. A., Ye, C. H., Huang, H., Nicholson, J. K., and Lindon, J. C. (1998) Improved WATERGATE pulse sequences for solvent suppression in NMR spectroscopy. *J Magn Reson* **132**, 125-129

26. Davis, A. L., Keeler, J., Laue, E. D., and Moskau, D. (1992) Experiments for Recording Pure-Absorption Heteronuclear Correlation Spectra Using Pulsed Field Gradients. *J Magn Reson* **98**, 207-216

27. Grzesiek, S., and Bax, A. (1992) An Efficient Experiment for Sequential Backbone Assignment of Medium-Sized Isotopically Enriched Proteins. *J Magn Reson* **99**, 201-207

28. Wittekind, M., and Mueller, L. (1993) HNCACB, a High-Sensitivity 3D NMR Experiment to Correlate Amide-Proton and Nitrogen Resonances with the Alpha-Carbon and Beta-Carbon Resonances in Proteins. *J Magn Reson Ser B* **101**, 201-205

29. Kay, L. E., Ikura, M., Tschudin, R., and Bax, A. (1990) 3-Dimensional Triple-Resonance NMR-Spectroscopy of Isotopically Enriched Proteins. *J Magn Reson* **89**, 496-514

30. Bax, A., and Ikura, M. (1991) An efficient 3D NMR technique for correlating the proton and $^{15}$N backbone amide resonances with the alpha-carbon of the preceding residue in uniformly $^{15}$N/$^{13}$C enriched proteins. *J Biomol NMR* **1**, 99-104

31. Clubb, R. T., Thanabal, V., and Wagner, G. (1992) A Constant-Time 3-Dimensional Triple-Resonance Pulse Scheme to Correlate Intraresidue H-1(N), N-15, and C-13(1) Chemical-Shifts in N-15-C-13-Labeled Proteins. *J Magn Reson* **97**, 213-217

32. Fogh, R., Ionides, J., Ulrich, E., Boucher, W., Vranken, W., Linge, J. P., Habeck, M., Rieping, W., Bhat, T. N., Westbrook, J., Henrick, K., Gilliland, G., Berman, H., Thornton, J., Nilges, M., Markley, J., and Laue, E. (2002) The CCPN project: an interim report on a data model for the NMR community. *Nat Struct Biol* **9**, 416-418

33. Vranken, W. F., Boucher, W., Stevens, T. J., Fogh, R. H., Pajon, A., Llinas, P., Ulrich, E. L., Markley, J. L., Ionides, J., and Laue, E. D. (2005) The CCPN data model for NMR spectroscopy: Development of a software pipeline. *Proteins* **59**, 687-696

34. Kneller, D. G., and Goddard, T. D. (1993) UCSF Sparky - an NMR Display, Annotation and Assignment Tool. University of California, San Francisco

35. Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) TALOS plus : A hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J Biomol NMR* **44**, 213-223

36. Schumann, F. H., Riepl, H., Maurer, T., Gronwald, W., Neidig, K. P., and Kalbitzer, H. R. (2007) Combined chemical shift changes and amino acid specific chemical shift mapping of protein-protein interactions. *J Biomol NMR* **39**, 275-289

37. Sudmeier, J. L., Evelhoch, J. L., and Jonsson, N. B. H. (1980) Dependence of NMR Lineshape Analysis Upon Chemical Rates and Mechanisms - Implications for Enzyme Histidine Titrations. *J Magn Reson* **40**, 377-390
NMR analyses of free and ligand-bound DC-SIGNR

38. Fielding, L. (2007) NMR methods for the determination of protein-ligand dissociation constants. *Prog Nucl Mag Res Sp* **51**, 219-242

39. Kay, L. E., Nicholson, L. K., Delaglio, F., Bax, A., and Torchia, D. A. (1992) Pulse Sequences for Removal of the Effects of Cross-Correlation between Dipolar and Chemical-Shift Anisotropy Relaxation Mechanism on the Measurement of Heteronuclear T1 and T2 Values in Proteins. *J Magn Reson* **97**, 359-375

40. Farrow, N. A., Muhandiram, R., Singer, A. U., Pascal, S. M., Kay, C. M., Gish, G., Shoelson, S. E., Pawson, T., Formankay, J. D., and Kay, L. E. (1994) Backbone Dynamics of a Free and a Phosphopeptide-Complexed Src Homology-2 Domain Studied by N-15 NMR Relaxation. *Biochemistry* **33**, 5984-6003

41. Demers, J. P., and Mittermaier, A. (2009) Binding mechanism of an SH3 domain studied by NMR and ITC. *J Am Chem Soc* **131**, 4355-4367

42. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NmrPipe - a Multidimensional Spectral Processing System Based on Unix Pipes. *J Biomol NMR* **6**, 277-293

43. Snyder, G. A., Colonna, M., and Sun, P. D. (2005) The structure of DC-SIGNR with a portion of its repeat domain lends insights to modeling of the receptor tetramer. *J Mol Biol* **347**, 979-989

44. Nesmelova, I. V., Ermakova, E., Daragan, V. A., Pang, M., Menendez, M., Lagartera, L., Solis, D., Baum, L. G., and Mayo, K. H. (2010) Lactose Binding to Galectin-1 Modulates Structural Dynamics, Increases Conformational Entropy, and Occurs with Apparent Negative Cooperativity. *J Mol Biol* **397**, 1209-1230

45. Miller, M. C., Nesmelova, I. V., Platt, D., Klyosov, A., and Mayo, K. H. (2009) The carbohydrate-binding domain on galectin-1 is more extensive for a complex glycan than for simple saccharides: implications for galectin-glycan interactions at the cell surface. *Biochem J* **421**, 211-221

46. Diehl, C., Engstrom, O., Delaine, T., Hakansson, M., Genheden, S., Modig, K., Leffler, H., Ryde, U., Nilsson, U. J., and Akke, M. (2010) Protein flexibility and conformational entropy in ligand design targeting the carbohydrate recognition domain of galectin-3. *J Am Chem Soc* **132**, 14577-14589

47. Lameignere, E., Malinovska, L., Slavikova, M., Duchaud, E., Mitchell, E. P., Varrot, A., Sedo, O., Imberty, A., and Wimmerova, M. (2008) Structural basis for mannose recognition by a lectin from opportunistic bacteria Burkholderia cenocepacia. *Biochem J* **411**, 307-318

48. Onizuka, T., Shimizu, H., Moriwaki, Y., Nakano, T., Kanai, S., Shimada, I., and Takahashi, H. (2012) NMR study of ligand release from asialoglycoprotein receptor under solution conditions in early endosomes. *FEBS J* **279**, 2645-2656

49. Keeler, J. H. (2005) Understanding NMR spectroscopy, John Wiley, Chichester
NMR analyses of free and ligand-bound DC-SIGNR

50. Lian, L.-Y., and Roberts, G. C. K. (2011) Protein NMR spectroscopy: Practical techniques and applications, Wiley, Hoboken, NJ

51. Csizmok, V., Felli, I. C., Tompa, P., Banci, L., and Bertini, I. (2008) Structural and dynamic characterization of intrinsically disordered human securin by NMR spectroscopy. J Am Chem Soc 130, 16873-16879

52. Drickamer, K. (1992) Engineering Galactose-Binding Activity into a C-Type Mannose-Binding Protein. Nature 360, 183-186

53. Clore, G. M., Driscoll, P. C., Wingfield, P. T., and Gronenborn, A. M. (1990) Analysis of the backbone dynamics of interleukin-1 beta using two-dimensional inverse detected heteronuclear $^{15}$N-$^1$H NMR spectroscopy. Biochemistry 29, 7387-7401

54. Kay, L. E., Torchia, D. A., and Bax, A. (1989) Backbone Dynamics of Proteins as Studied by N-15 Inverse Detected Heteronuclear NMR-Spectroscopy - Application to Staphylococcal Nuclease. Biochemistry 28, 8972-8979

55. Copie, V., Battles, J. A., Schwab, J. M., and Torchia, D. A. (1996) Secondary structure of beta-hydroxydecanoyl thiol ester dehydrase, a 39-kDa protein, derived from H alpha, C alpha, C beta and CO signal assignments and the Chemical Shift Index: comparison with the crystal structure. J Biomol NMR 7, 335-340

56. Chirife, J., and Buera, M. P. (1997) A simple model for predicting the viscosity of sugar and oligosaccharide solutions. J Food Eng 33, 221-226

57. Hibbert, R. G., Teriete, P., Grundy, G. J., Beavil, R. L., Reljic, R., Holers, V. M., Hannan, J. P., Sutton, B. J., Gould, H. J., and McDonnell, J. M. (2005) The structure of human CD23 and its interactions with IgE and CD21. J Exp Med 202, 751-760

58. Gringhuis, S. I., den Dunnen, J., Litjens, M., van der Vlist, M., and Geijtenbeek, T. B. (2009) Carbohydrate-specific signalling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. Nat Immunol 10, 1081-1088

59. Drickamer, K., and Taylor, M. E. (2005) Targeting diversity. Nat Struct Mol Biol 12, 830-831

60. Bonomelli, C., Dooris, K.J., Dunlop, D.C., Thaney, V., Dwek, R.A., Burton, D.R., Crispin, M., and Scanlan, C.N. (2011) The Glycan Shield of HIV Is Predominantly Oligomannose Independently of Production System or Viral Clade. PLoS ONE 6, e23521

61. Poget, S. F., Freund, S. M. V., Howard, M. J., and Bycroft, M. (2001) The ligand-binding loops in the tunicate C-type lectin TC14 are rigid. Biochemistry 40, 10966-10972

62. Ng, K. K. S., Park-Snyder, S., and Weis, W. I. (1998) Ca$^{2+}$-dependent structural changes in C-type mannose-binding proteins. Biochemistry 37, 17965-17976

63. Pavlicek, J., Sopko, B., Ettrich, R., Kopecky, V., Baumruk, V., Man, P., Havlicek, V., Vrbacky, M., Martinkova, L., Kren, V., Pospisil, M., and Bezouska, K. (2003) Molecular characterization of binding of calcium and carbohydrates by an early activation antigen of lymphocytes CD69. Biochemistry 42, 9295-9306
64. Nielbo, S., Thomsen, J. K., Graversen, J. H., Jensen, P. H., Etzerodt, M., Poulsen, F. M., and Thogersen, H. C. (2004) Structure of the plasminogen kringle 4 binding calcium-free form of the C-type lectin-like domain of tetranectin. *Biochemistry* **43**, 8636-8643

65. Ho, M. R., Lou, Y. C., Wei, S. Y., Luo, S. C., Lin, W. C., Lyu, P. C., and Chen, C. P. (2010) Human RegIV Protein Adopts a Typical C-Type Lectin Fold but Binds Mannan with Two Calcium-Independent Sites. *J Mol Biol* **402**, 682-695

66. Yu, Q. D., Oldring, A. P., Powlesland, A. S., Tso, C. K. W., Yang, C., Drickamer, K., and Taylor, M. E. (2009) Autonomous Tetramerization Domains in the Glycan-binding Receptors DC-SIGN and DC-SIGNR. *J Mol Biol* **387**, 1075-1080

67. Leckband, D. E., Menon, S., Rosenberg, K., Graham, S. A., Taylor, M. E., and Drickamer, K. (2011) Geometry and Adhesion of Extracellular Domains of DC-SIGNR Neck Length Variants Analyzed by Force-Distance Measurements. *Biochemistry* **50**, 6125-6132

68. Watson, A. A., Lebedev, A. A., Hall, B. A., Fenton-May, A. E., Vagin, A. A., Dejnirattisai, W., Felce, J., Mongkolsapaya, J., Palma, A. S., Liu, Y., Feizi, T., Screaton, G. R., Murshudov, G. N., and O'Callaghan, C. A. (2011) Structural flexibility of the macrophage dengue virus receptor CLEC5A: implications for ligand binding and signaling. *J Biol Chem* **286**, 24208-24218
Acknowledgements - The authors wish to acknowledge Prof. M. Overduin (HWB-NMR, University of Birmingham) and Dr. I. Prokes (Warwick Chemistry) for NMR assistance, Dr. R. Wallis for mannose-Sepharose columns, Snezana Vasiljevic and Camille Bonomelli (University of Oxford) for technical assistance and Dr C. Scanlan for guidance on glycan preparation. MC acknowledges funding from IAVI and CHAVI-ID. FP gratefully acknowledges funding for a studentship from the Engineering and Physical Sciences Research Council through the MOAC Doctoral Training Centre.

FOOTNOTES

1 MOAC Doctoral Training Centre, 2 Warwick Medical School, and 4 Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.
2 HWB-NMR, Birmingham Cancer Research UK Centre, School of Cancer Sciences, University of Birmingham, Vincent Drive, Edgbaston, Birmingham, B15 2TT, UK.
3 The Abbreviations used are: DC-SIGNR, Dendritic Cell-Specific ICAM-3-Grabbing Non-integrin-Related; carbohydrate recognition domain, CRD; severe acute respiratory syndrome coronavirus, SARS; respiratory syncitial virus, RSV; isopropyl-β-D-thiogalactoside, IPTG; $K_D$, dissociation constant.
FIGURE LEGENDS

FIGURE 1. Insight from current crystal structures. (A) Comparison of holo (1XPH, black) and (GlcNAc)$_2$Man$_3$-bound (1K9J, white) crystal structures. Ca$^{2+}$ ions are represented by spheres. All published crystal structures adopt nearly identical conformations suggesting that the DC-SIGNR CRD adopts the same conformation during crystal formation with or without glycan. (B) Residues that form direct contacts/bonds with the glycan in the structure of the (GlcNAc)$_2$Man$_3$-CDR complex (1K9J; ligand not shown) are highlighted in red, disulphide bonds are shown in blue, and bound calcium ions are shown in green.

FIGURE 2. Assignment of DC-SIGNR CRD. HSQC spectrum and annotated backbone assignment of holo DC-SIGNR CRD (20 mM d-HEPES, 20 mM NaCl, 4 mM CaCl$_2$, pH 6.8, 37°C), showing 96% of the observed $^1$H, $^{15}$N, and $^{13}$C resonances assigned.

FIGURE 3. Schematic representation of glycans used in this study. Binding of all four glycans to the DC-SIGNR CRD was measured using chemical shift perturbation analyses.

FIGURE 4. Chemical shift perturbation upon ligand-binding. Total chemical shift perturbation per residue upon addition of (A) (GlcNAc)$_2$Man$_3$, (B) Man$_3$, (C) Man$_5$, and (D) Man$_9$GlcNAc was calculated according to Equation 1. Dashed horizontal lines represent the 1 $\times$ standard deviation cut-off used in each data set, above which a change was considered significant.

FIGURE 5. Regions affected by glycan binding. Chemical shifts with perturbations greater than 1 $\times$ standard deviation on the addition of 5 mM (A) (GlcNAc)$_2$Man$_3$, (B) Man$_3$, (C) Man$_5$, and (D) Man$_9$GlcNAc are shown in red mapped onto the structure of the (GlcNAc)$_2$Man$_3$-CDR complex (1K9J). A schematic of the bound glycan is given in the lower right corner of each panel. These maps highlight the conserved binding regions as well as regions unique to each glycan.

FIGURE 6. Affinity of glycans for CRD. Fit of chemical shift perturbations vs. glycan concentration to a single site binding model for (A) (GlcNAc)$_2$Man$_3$, (B) Man$_3$, and (C) Man$_5$. Only data derived from residues near $\alpha$-helix 2 are shown.

FIGURE 7. Dynamics of the CRD. Plot of per residue values for (A) $^{15}$N $T_1$, (B) $^{15}$N $T_2$, and (C) $^{15}$N $T_1/T_2$ for holo (solid squares) and Man$_3$-bound (open circles) DC-SIGNR CRD. Asterisks along the bottom of each panel denote residues which are not observed due to fast relaxation or exchange.
**Table I.** *Dissociation constants calculated from chemical shift perturbations.*

| Glycan          | $K_D$ (mM)   |
|-----------------|-------------|
| (GlcNAc)$_2$Man$_3$ | 1.57 ± 0.46 |
| Man$_3$         | 2.04 ± 0.54 |
| Man$_5$         | 2.20 ± 0.43 |

**Table II.** *Average relaxation parameters of holo and Man$_5$ bound CRD.*

|                    | Holo CRD        | Man$_5$ bound CRD |
|--------------------|-----------------|-------------------|
| Average $T_1$ (ms) | 788.26 ± 22.4   | 806.22 ± 40.35    |
| Average $T_2$ (ms) | 76.27 ± 2.27    | 66.54 ± 8.23      |
| Average $T_1/T_2$  | 10.67 ± 0.43    | 13.19 ± 1.75      |
| Rotational correlation time ($\tau_c$) (ns) | 10.4 ± 0.4 | 12.5 ± 1.36 |
NMR analyses of free and ligand-bound DC-SIGNR

Figure 1
Figure 2

NMR analyses of free and ligand-bound DC-SIGNR
Figure 3

NMR analyses of free and ligand-bound DC-SIGNR
Figure 4

(A) 

(B) 

(C) 

(D)
Figure 5
Figure 6

(A) Residue
- 319
- 320
- 321
- 328
- 356
- 364

Total chemical shift perturbation (ppm)

GlcNAc-Man$_3$ concentration (M)

(B) Residue
- 321
- 322
- 326
- 328

Total chemical shift perturbation (ppm)

Man$_3$ concentration (M)

(C) Residue
- 321
- 322
- 323
- 324
- 326
- 327
- 328

Total chemical shift perturbation (ppm)

Man$_5$ concentration (M)
Figure 7
Solution NMR analyses of the C-type carbohydrate recognition domain of DC-SIGNR reveal different binding modes for HIV-derived oligosaccharides and smaller glycan fragments

Fay Probert, Sara B-M. Whittaker, Max Crispin, Daniel A. Mitchell and Ann M. Dixon

*J. Biol. Chem.* published online June 20, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.458299

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2013/06/20/M113.458299.DC1