Published in final edited form as:

Chembiochem. 2017 April 18; 18(8): 764–771. doi:10.1002/cbic.201600665.

NMR insights into the conformational properties of Man-9 and its recognition by two HIV binding proteins

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Abstract

Man₉GlcNAc₂ (Man-9) present at the surface of HIV constitutes the binding sites of several HIV neutralizing agents and mammalian lectin DC-SIGN that is involved in cellular immunity and trans-infections. We describe the conformational properties of Man-9 in free state and when bound by an HIV entry inhibitor protein and define the minimum epitopes of two HIV binding proteins using NMR spectroscopy. In this regard we developed a robust expression system for production of ¹³C, ¹⁵N-labeled glycans in mammalian cells that facilitated the implementation of 3D ¹³C-edited spectra to deconvolute spectral overlap allowing for the solution structure determination of Man-9. The studies reveal that Man-9 interacts with HIV-binding proteins via distinct binding epitopes and adopts divergent conformations in the bound state. In combination with molecular dynamics we find that these receptor-bound conformations are sampled by Man-9 in the free state suggesting a mechanism for diverse recognition.

Sweet solutions

A robust method to produce uniform ¹³C, ¹⁵N-labeled *N*-linked glycans facilitates NMR spectroscopy of carbohydrates and 3-dimensional structural studies of glycans free and in complex with disease-relevant proteins.

Keywords

glycan conformation; molecular dynamics; isotope labeling; ¹³C-edited NMR; carbohydrate recognition

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Introduction

Glycans decorate the surfaces of pathogens such as bacteria and viruses and serve essential roles in the biology of these organisms. The HIV-1 glycan shield in which high mannose type glycans predominate illustrates the multiple roles sugars can play. Glycosylation is essential for proper folding of viral envelope proteins and subsequent incorporation into mature particles; moreover, HIV's glycan shield effectively masks the virus from the innate immune system, yet later in infection comprises a large portion of the epitopes recognized by broadly neutralizing antibodies (bNAb).^[1–2] Further, high mannose oligosaccharides (comprising Man_{5–9}GlcNAc₂ and referred to as Man-5 through Man-9) are the targets of mannose-recognizing lectins, including some of the most potent anti-HIV agents known,^[3–4] and recognition motifs for antigen presenting cells and receptors.^[5] Structural information defining these important glycan-mediated interactions is therefore desirable, but the flexibility and heterogeneity associated with glycans together with variable binding kinetics can pose barriers to structural characterization by methods such as x-ray crystallography.^[6–8]

NMR spectroscopy is a powerful method for studying carbohydrate structure and dynamics.^[9–10] However, making unambiguous assignments of chemical shifts needed for 3-dimensional structure determination can be challenging due to the abundance of overlapping ¹H, and to a lesser degree, ¹³C resonances. Overlapping signals can be resolved by using ¹³C-edited or ¹³C-coupled NMR experiments, but limitations in sensitivity generally require uniform isotopic labeling.^[11–13] In contrast to proteins, the lack of templated biosynthesis and the presence of heterogeneity in naturally occurring glycans has limited production of uniform isotopically labeled sugars to those present on the outer membrane of *Escherichia coli* or to oligomannosides produced in an engineered yeast strain.^[12, 14–16] Here, we sought to study the conformational properties of Man-9 (Figure 1a) because it constitutes the binding site of several HIV neutralizing agents, together with its recognition by two unrelated HIV binding proteins. These included the non-human lectin HIV entry inhibitor microvirin (MVN)^[17] and the mammalian lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) that facilitates HIV infection in trans.^[5] We show that Man-9 acts as a diverse biological receptor sometimes through interactions with core or alternatively with its termini. Further, in combination with molecular dynamics (MD) simulations and principle component analysis (PCA), we find that receptor-bound conformations of Man-9 exist during conformational simulations suggesting that bioactive conformations may preexist in situ.

Results and Discussion

To facilitate NMR studies we first developed a general method of producing uniform ¹³C,¹⁵N-labeled mammalian glycans in milligram quantities in standard cell lines. Previously Kato and co-workers engineered a yeast system to produce labeled oligomannose (primarily Man-8 and Man-9).^[15–16] To obtain Man-9 for structural studies, we chose to use an adenovirus-based expression system to produce uniformly ¹³C,¹⁵N-labeled HIV-1 gp120 for two reasons: first we showed previously that this is a high producing expression system that can yield as much as 40 mg gp120 L⁻¹ of media;^[18] and second, due to the ~24 N-

linked glycosylation sites half of gp120's mass is composed of high mannose-type glycans providing a good source of uniformly labeled sugars. We infected A549 lung carcinoma cells with an adenovirus construct encoding HIV-1 gp120.^[19] To increase the amount of Man-9, cells were treated with kifunensin (50 μ M), an α 1–2 mannosidase inhibitor that leads to increased production of Man-8 and Man-9, and gp120 was harvested by affinity and size exclusion chromatography.^[20] N-linked oligomannosides were enzymatically released from gp120 using peptide-N-glycosidase F and purified using size exclusion chromatography followed by HPLC. The final yield of purified glycan was approximately 10 mg L⁻¹ of media (gp120 expressed in 400 mL media yielded 4 mg of Man-9). HR-MS confirmed approximately 95% ¹³C and ¹⁵N incorporation into purified recombinant Man-9 (Figure S1, Supporting Information). We used the same method to produce unlabeled Man-9 by growing the A549 cells in standard cell culture media (DMEM with 10% fetal bovine serum) where yields were comparable. This approach represents a significant improvement in producing ¹³C-labeled glycans in relatively high yield, as well as production of Man-9 by inhibiting glycan processing.

To test the utility of ¹³C-incorporation in making chemical shift assignments, we compared standard approaches employing 1D and 2D NMR experiments on unlabeled Man-9 with C-C coupled 2D and 3D experiments used routinely in protein NMR on ¹³C-Man-9. A summary of NMR samples and experiments (Table S1), and a description of chemical shift assignments for unlabeled Man-9 are provided in the Supporting Information. Due to the high degree of spectral overlap in Man-9 and other oligosaccharides, only 60% of resonances could be unambiguously assigned on the unlabeled sample, even with the use of 1D Chemical Shift Selective Filter TOCSY (CSSF-TOCSY) and 1D CSSF-NOESY data (Fig S2, Supporting Information). However, the improvement both in sensitivity and extent of through-bond correlations using labeled Man-9 is evident in the comparison of a 2D ¹H-¹³C HSQC-TOCSY spectrum of unlabeled Man-9 with an HCCH-TOCSY spectrum of ¹³C Man-9 (Figures 1b–c). As seen in the anomeric region (4.6–5.4 ppm), TOCSY correlations within each mannose residue are observed only from H1 to H2 or H3, due to the small J_{H1-H2} and J_{H2-H3} that results from the axial position of the C-2 hydroxyl. In contrast, the spectrum from an HCCH-TOCSY experiment that utilizes the large ¹³C-¹³C couplings for magnetization transfer shows correlations for all protons and carbons in each pyranose ring. This facilitates more rapid and reliable assignments of ¹H and ¹³C resonances (Table S2, Supporting Information) for complete pyranose spin systems. Further, this allowed us to obtain 3-dimensional ¹³C-edited NOESY spectra in which overlapping ¹H resonances could be resolved through the added ¹³C dimension, that in turn provided distance restraints used for structure calculations (Table S3, Supporting Information).

We determined the solution structure of Man-9 using torsion angle dynamics/simulating annealing (Xplor-NIH^[21]). Inter-proton distances (Table S3, Supporting Information) were obtained from 2D NOESY spectra via 2D HSQC-NOESY and 3D ¹³C-edited NOESY experiments. Key inter-ring NOEs are shown in Figure 2a. Resonances in the terminal D1, D2 and D3 mannose rings showed the greatest degree of overlap; however the H1 and H2 atoms in all rings were clearly resolved. Thus, the three sequential NOEs between H1 of ManB, ManA and ManC with the H5 atoms of ManD1, ManD2 and ManD3, respectively, were assigned due to the absence of additional inter-arm NOEs that would be predicted.

Because 1–6 glycosidic bonds (Man4'-Man3 and ManB-Man4') have the greatest torsional freedom and are at branching points in the glycan, they are important in determining solution conformations. In NOESY spectra recorded with a short mixing time (τ_m 100 msec) equivalent NOE intensities are seen for H5–H6 and H5–H6' (Figure 1d–e), and small $^3J_{HH}$ values (3–4 Hz) between H5–H6 and H5–H6' are observed in a high resolution, phase sensitive HSQC spectrum (Figures 1f, S4–S5, Supporting Information). This combination indicated that the dominant rotamers possessed a gauche-gauche (gg) conformation with ω angles (O-C6'-C5'-O5') of approximately -60° for Man4' and Man3, and were observed in 89% and 75% of the structures, respectively.^[8, 22]

Superimpositions of the final ensemble of structures (Figure 2b) show the core of Man-9 to be well determined (Table S3, Supporting Information) with small variations in the glycosidic linkages. The Mana1–2Man termini of the D1 and D3 arms show the greatest spread. This can result from there being fewer observed NOEs, greater mobility in the termini, or a combination of both. The D2 arm however, shows a smaller degree of conformational flexibility. This arm adopts an orientation almost perpendicular to the axis of the core of Man-9, revealed by several weak NOEs between GlcNAc2 and H1 of Man4' and H6 of ManA. This observation is in agreement with earlier studies analyzing the conformational space of Man-9.^[16, 23–26] Recently Yamaguchi, *et al.* described an elegant approach using pseudo contact shifts (PCS) that could independently validate the presence and quantify the populations of this 'fold back' conformation.^[26] In addition the PCSs allowed for unambiguous assignments of otherwise overlapping resonances providing the first method to differentiate the terminal mannopyranose units.

To assess the quality of the calculated NMR ensemble, we explored the conformational space of Man-9 using unrestrained molecular dynamics (MD). The simulation of Man-9 was carried out for 1.6 μ s in explicit solvent without restraints using the CHARMM (c36) force field (see Supporting Information for experimental details).^[27] Overall we found that the conformers and their populations observed by MD shared global topologies^[23] with the lowest energy model from the NMR ensemble with an average root-mean-square distance (RMSD) of 2.86 ± 0.72 Å (with a minimum RMSD of 1.54 Å). We then computed the differences between interproton distances observed during the simulation versus their NOE-derived distances. The majority of violations correspond to NOEs observed between the D2 arm and the GlcNAc2 moiety (Figure 2c and Table S4, Supporting Information). Sampling of the Man4'-Man3 ω glycosidic torsion angle showed transient rotation of the D2 arm occurring at 400–500 ns during the simulation. The distribution of ω torsion angles revealed two distinct populations with ϕ and ω angles centered around $90^\circ/-75^\circ$ and $90^\circ/75^\circ$ respectively (Figure 2d). The first peak defining the gg rotamer was sampled more densely (96% of conformers) and was the most consistent with the observed NOEs, while the second peak defining the gauche-trans (gt) rotamer leads to the flipping of the D2 arm. Sampling of the ϕ/ψ torsion angles of the remaining glycosidic linkages had only a marginal effect on the NOE violations (see Figure S6, Supporting Information for remaining torsion angle distributions). The conformations of Mana1–6Man linkages have been investigated previously by NMR in oligosaccharides of various sizes using J couplings, NOEs and MD simulations. Flexibility in ω torsion angles depends on the level of branching. In the case of smaller oligosaccharides this linkage populates two different rotamers, gg and gt with equal

probability^[28–29] but in Man-9 the conformation is more restricted showing gg as a major rotamer,^[23–25] consistent with the experimental results reported here.

Having chemical shift assignments and a solution structure of Man-9, along with glycan and protein samples in labeled and unlabeled forms, we could determine solution conformations and/or binding epitopes of Man-9 in complex with two unrelated HIV-binding proteins, MVN and DC-SIGN. MVN inhibits HIV-1 infection through interactions with the termini of Man-8 or Man-9 on gp120.^[17, 30] Previously, we solved the solution structure of MVN and an MVN:Man α 1–2Man disaccharide complex and showed that MVN binds mannobiose monovalently, through a single dimannose binding site.^[30] This arrangement is expected to preclude intermolecular crosslinking and formation of large aggregates when binding to branched glycans such as Man-9, but it has not been shown experimentally. Having both ¹³C Man-9 and ¹⁵N MVN, we determined the stoichiometry and binding sites of both interacting molecules as well as determined the protein bound conformation of Man-9 (Figures S7 and S8, Supporting Information). We mapped the binding site of Man-9 onto MVN by performing NMR titrations where unlabeled Man-9 was added to ¹⁵N MVN and spectral changes were monitored by ¹H-¹⁵N HSQC experiments (Figures 3 and S8, Supporting Information). Several informative observations were made. First, addition of Man-9 led to chemical shift perturbations in the mannobiose-binding site only, and addition of one equivalent of Man-9 was sufficient to saturate that binding site. Addition of stoichiometric excesses of Man-9 did not lead to further changes in the NMR spectra. This confirmed that MVN binds even a branched high mannose glycan monovalently and without aggregation. Interestingly, in the ¹H-¹⁵N HSQC of MVN in complex with Man-9, every perturbed signal of MVN shifted to two distinct chemical shifts.

To explain this observation in more detail we performed complementary NMR titrations with three synthetic trisaccharides corresponding to the D1, D2 and D3 arms of Man-9 and monitored chemical shift perturbations in ¹H-¹⁵N HSQC spectra of ¹⁵N MVN (Figures 3 and S8, Supporting Information). MVN spectra in the presence of Man α 1–2Man α 1–3Man (D2 arm) and Man α 1–2Man α 1–6Man (D3 arm) were identical to those of MVN in the presence of mannobiose.^[30] In contrast when titrating Man α 1–2Man α 1–2Man comprising the D1 arm, two sets of chemical shifts present in an approximate 2:1 ratio were observed for the carbohydrate binding site residues. While the major set was superimposable with the spectra of MVN bound to α 1–2 mannobiose or trisaccharides corresponding to the D2 or D3 arms of Man-9, the second minor set of bound resonances showed larger chemical shift perturbations similar to those observed with binding to Man-9 (Figure 3). As with Man-9, no further changes were observed upon addition of more than one equivalent of Man α 1–2Man α 1–2Man (D1 arm). These results clearly show that interactions between MVN and the D1 arm of Man-9 are more extensive than interactions with the D2 or D3 arms.

Given these differences it is interesting to compare these results with the mode of binding of Man-9 to cyanovirin-N (CV-N) revealed by x-ray crystal structures.^[31] Like MVN, CV-N is a monomeric protein whose sequence contains two internal tandem repeats that give rise to *C*₂ pseudosymmetry.^[32] In the crystal form, CV-N is always observed as a three-dimensional domain swapped dimer (dsCV-N).^[31, 33] While monomeric CV-N contains two non-equivalent but symmetrically positioned mannobiose binding sites,^[34–35] the crystal

structure of dsCV-N reveals perturbations in the primary binding site located proximal to the hinge linker about which domain swapping takes place, and high mannose binding is observed only in the secondary binding site comprising residues Leu 1 to Thr 7, Glu 23 to Thr 25 and Asn 93 to Asp 95.^[31] Compared to the deep pocket that is the primary binding site of monomeric CV-N, this secondary site appears as a long groove.^[34] Interestingly in the crystal form only the D1 arm of Man-9 (and not the D2 or D3 arms) is bound to this site, and all three mannose residues comprising the D1 arm are involved in protein-Man-9 interactions. This is consistent with a separate study where NMR titrations of trisaccharides corresponding to the D1, D2 and D3 arms of Man-9 to monomeric CV-N showed that the D1 trisaccharide preferentially binds to the secondary binding site,^[36] which corresponds to the one occupied in Man-9-bound dsCV-N. In terms of structure it is interesting to note that the sole binding site in MVN most closely matches the secondary site of CV-N as best fit superimpositions preferentially align MVN and dsCVN with their carbohydrate binding sites superimposed.

To determine solution structures of Man-9 when bound to MVN, chemical shifts were assigned as described for free Man-9. MVN-bound conformation of Man-9 was determined from NOEs observed in ¹³C-separated NOESY spectra of a stoichiometric complex of ¹³C Man-9: MVN, and transferred NOE spectra of Man-9 in the presence of MVN. The conformational space of MVN-bound Man-9 was not significantly different from free Man-9. The only differences observed in the NOESY spectra between free and MVN-bound Man-9 were NOEs between H1-ManC/H3-Man3, and H1-ManA/H6-Man4' (Figure 4a). Consistent with these data superimpositions of the minimized average structures of free and MVN-bound Man-9 (Figures 4b and 4c) show the only difference to be in the orientation of the D1 arm at the ManC-Man4 linkage. These observations suggest that MVN may recognize a pre-existing solution conformation of Man α 1-2Man present at all three non-reducing termini of Man-9, and binding the D1 arm effects the conformation at the reducing linkage (ManC α 1-2Man4) possibly because this arm contains two minimum binding epitopes of MVN.

Last we investigated interactions between DC-SIGN and Man-9. DC-SIGN is a mammalian C-type lectin present on the surface of dendritic cells and phagocytes. Its interactions with oligomannosides have been implicated in phagocytosis, activation of CD4⁺ T-cells, and virus transmission. The crystal structure of the carbohydrate-recognition domain (CRD) of DC-SIGN bound to Man₃GlcNAc₂ showed that interactions centered around the mannotriose core,^[37] while alternate binding modes were observed with synthetic hexamannosides and other mannose fragments.^[38] To our knowledge the interaction between DC-SIGN and homogeneous Man-9 present at key sites of vulnerability on viral surfaces, has not been studied. Using ¹³C-Man-9 we monitored DC-SIGN binding by NMR. Upon addition of DC-SIGN resonances for the Man4 α 1-6Man3 β 1-4GlcNAc β 1-4GlcNAc core disappear (Figure 5). This indicates that DC-SIGN-Man-9 interactions predominantly involve core residues that are present in all oligomannosides, shows that the binding is on the time scale of the NMR chemical shift difference between the bound and unbound Man-9 (estimated around 100 Hz or 10 msec), and is consistent with the crystal structure of the DC-SIGN/Man₃GlcNAc₂ complex. Though these results reveal the glycan epitope recognized by DC-SIGN, the loss of ¹H signals located at the binding interface preclude structural studies of

the Man-9:DC-SIGN complex. To complement the NMR results, we measured the binding of DC-SIGN to two different samples of gp120, one that contains predominantly Man-9 (gp120_{Kif}) and the other that is enriched in Man-5 (gp120-GNTI^{-/-}, SI). Using surface plasmon resonance (SPR) we found that DC-SIGN binds gp120-GNTI^{-/-} with three-fold higher affinity compared to gp120_{Kif}, consistent with the notion that DC-SIGN may prefer processed mannosides where the core is more accessible (Figure S9, Supporting Information).

Given the divergent ways in which MVN and DC-SIGN engage Man-9, we were interested to probe whether protein-bound conformations of Man-9 are observed during MD simulations. Few crystal structures of high-mannose glycans are available; thus we were also interested in determining whether the conformation of Man-9 when bound to the HIV neutralizing antibody PGT128 exists during the simulations. PGT128 recognizes a region of variable loop 3 on HIV-1 gp120 that contains a conserved glycosylation site at Asn 332 that is occupied by Man-8 or Man-9. An X-ray structure has been solved for PGT128 bound to the outer domain of HIV-1 gp120 and good electron density was observed for most of Man-8 and showed the glycan to adopt a relatively compact conformation in the complex.^[39] As seen in Figures 4d and S10 (Supporting Information) we observe all three of these experimentally-determined, receptor-bound conformations of Man-9 during the simulations as shown by Principal Component Analysis (PCA) of all glycosidic torsion angles, where 25% of all the variance can be attributed to the first two components. This suggests that despite the dynamic nature of Man-9, the conformations recognized by receptors may pre-exist, offering one explanation for how such diverse recognition of this glycan can be accomplished in biological systems.

Conclusions

In total these studies show how unrelated proteins can engage Man-9 in different ways. Of key importance the terminal sugars of each branch or arm (D1, D2 or D3) of Man-9 may act as specific recognition elements, as can the core. In the former, binding to a single arm (MVN) at a time versus a pair (PGT128) leads to entirely different bound conformations of the branches. Finally we emphasize that this adenovirus infection system enabled production of multi-milligram quantities of ¹³C, ¹⁵N-labeled Man-9 from 200–250 mL cultures, and allowed us to employ ¹³C-edited 3D NMR experiments that are the main stay for determining 3-dimensional protein structures by NMR. This labeling scheme opens up the possibility to define precise binding epitopes for other glycans by monitoring spectral perturbations upon protein binding, removing a current limitation in biological NMR for carbohydrates.

Experimental Section

Production of uniformly labeled ¹³C, ¹⁵N Man-9 from HIV-1 gp120

A549 lung carcinoma cells used in this study were obtained from ATCC. High glucose containing Dulbecco's modified Eagle medium (DMEM) with HEPES and NaHCO₃, dialyzed fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin were obtained from Life Technologies (Carlsbad, CA). ¹⁵N/¹³C-CGM6750 (U-98%) containing

Hepes, NaHCO₃, ¹³C₆ (99%) D-glucose and ¹³C₉ (99%) sodium pyruvate were purchased from Cambridge Isotopes Laboratories (Andover, MA). Isotopically enriched HIV-1 core gp120 (HxBc2, residues 83–492) was expressed using an adenoviral coupled mammalian expression system.^[18, 40] Specifically, A549 lung carcinoma cells were seeded at 1.2–1.5 × 10⁷ cells/150 mm plate on day 1 in DMEM containing 10% heat inactivated, dialyzed FBS and 1% penicillin-streptomycin w/v. Cells were grown overnight at 37 °C in a 5% CO₂ atmosphere, and allowed to recover for 15–17 hours. The spent media was replaced with freshly prepared ¹⁵N/¹³C CGM6750 containing 10% heat inactivated FBS, 1% penicillin-streptomycin, and 20 µg/mL of the α-mannosidase inhibitor kifunensine to increase amount and homogeneity of Man-9. Cells were incubated for 3–4 hours at 37 °C and infected with the recombinant adenovirus containing the gene encoding core gp120 (rAdHxBc2 core). The culture supernatant was harvested 96–108 hr post infection, centrifuged for 30 min at 360 × g, and the supernatant filtered aseptically through a 0.2 µm filter. HIV-1 core gp120 (HxBc2) protein was purified by affinity chromatography using an anti-gp120 F105 monoclonal antibody-agarose resin (Zhou et al 2007). HxBc2 was eluted with IgG elution buffer containing 100 mM glycine-HCl, pH 2.5–3.0, 100 mM citric acid, pH 3.0. Fractions containing gp120 core were immediately neutralized, pooled, concentrated, and dialyzed against phosphate buffered saline (PBS), pH 7.4. Full length HMTGs were enzymatically released from gp120 using the endoglycosidase peptide-N-glycosidase F (PNGase). The glycans were separated from the polypeptide component of gp120 by size exclusion chromatography (Superdex 75) and purified by gel filtration and/or RP-HPLC. The purity of labeled Man-9 was confirmed by RP HPLC (C18 column, 5 micron pore size, detection at 210 nm), 1D ¹H NMR and mass spectrometry. HR-MS data confirmed approximately 95% ¹³C and ¹⁵N incorporation into Man-9 (Fig S1).

Production of unlabeled Man-9

Full-length HIV-1 gp120, strain ZM109 was expressed by transfecting 293 F cells with pVRC8400 expression vector^[19] in DMEM in the presence of 50 µg L⁻¹ kifunensine to produce unlabeled Man-9. The recombinant glycoprotein was purified by affinity chromatography using an anti-gp120 F105 monoclonal antibody-agarose resin. To release Man₉GlcNAc₂ from the glycoproteins, both isotopically labeled and unlabeled gp120 glycoproteins were treated with PNGase F at 37 °C for 16 hours. The enzymatically released Man-9 was purified chromatographically in two steps employing size exclusion chromatography. In the first step the hydrolyzed mixture was applied to a Superdex-75 gel filtration column (GE Healthcare) equilibrated in and eluted with PBS to give fractions containing free oligomannosides. These were combined, lyophilized and resuspended in water before applying to a peptide gel filtration column (GE Healthcare) equilibrated in and eluted with ultrapure H₂O. As for labeled Man-9, the purity of unlabeled Man-9 was confirmed by RP HPLC-MS (C18 column, 5 micron pore size, detection at 210 nm), 1D ¹H NMR and mass spectrometry. After purification the yield of glycan was approximately 10 mg L⁻¹ of media. (Gp120 expressed in 400 mL media yielded 4 mg of Man-9).

Recombinant protein production of MVN and DC-SIGN

Two versions of recombinant MVN, unlabeled and ¹⁵N labeled protein, were produced in the BL21 DE3 strain of *E. coli* and purified as described previously.^[30] The carbohydrate

recognition domain (CRD) and full-length DC-SIGN were obtained by transforming a DC-SIGN or DC-SIGN CRD encoding pET22b plasmid into the BLD21 strain of *E. coli*. Plasmids were a generous gift of the Sun and Wang laboratories. Protein expression was induced with 1 mM isopropyl β -thiogalactoside. Full length and the CRD of DC-SIGN^[41] were insoluble and present in inclusion bodies. After washing with Tris 7.4/0.5% NP-40, inclusion bodies were solubilized in 8 M urea and the proteins were refolded by dialysis against PBS.

NMR experiments and structure determination of Man-9

See Table S1, Supporting Information, for NMR samples and complexes used in these studies. NMR experiments were performed on Bruker Avance 600, Avance III 800, and Avance III 900 spectrometers equipped with z-gradient [^1H , ^{13}C , ^{15}N]-cryoprobes. All NMR samples were prepared in 20 mM NaPO_4 , 20 mM NaCl buffer, pH 6.8 (NMR Buffer). The pH of each sample was adjusted to 6.8 with 0.1 N NaOH or HCl . To prepare D_2O samples, we lyophilized NMR buffer, aliquots of Man-9 and protein solutions. For each, the lyophilized powder was dissolved in 99.99% D_2O . Samples were lyophilized a second time and dissolved in 99.99% D_2O . 2D HSQC-TOCY and HSQC-NOESY experiments on unlabeled samples were acquired using 70 and 500 ms mixing times, respectively. Key ^1H - ^1H couplings were verified using a 1D TOCSY with a chemical shift selective filter (CSSF); for all signals the ^1H - ^1H couplings observed in 2D HSQC experiments and 1D TOCSY experiments were in agreement. Selective saturation of signals in crowded regions of the spectra was achieved by using 110 CSSF increments. 2D-HCCH-TOCSY experiments were acquired using 1024, 1, and 512 data points for the F3 (^1H), F2 (^{13}C), and F1 (^{13}C) dimensions, respectively. In this experiment the optimal TOCSY mixing time was determined to be 50 ms after acquiring spectra with different spin lock times. Inter proton distance restraints were obtained from NOESY data summarized in Table S1. For the stoichiometric complexes, ^{13}C -edited NOESY spectra were acquired with a τ_m of 100 msec. For structure calculations five sets of NOE signals binned as very strong, strong, medium, weak and very weak and corresponding to lower limit inter proton distances of 2.5, 3.0, 3.5, 4.0 and 4.5 Å, respectively were employed. Structures were calculated using torsion angle dynamics/simulated annealing with the program XPLOR-NIH.^[21] Experimentally determined torsion angles were introduced as loose restraints ($\pm 30^\circ$) in the final stages of the structure calculations.

Molecular dynamics using ACEMD with the CHARMM force field

A mannose 9 molecule was built *de novo* using in-house software and then solvated in a water box of dimensions $50 \times 50 \times 50 \text{ \AA}^3$. The resulting system was parametrized using the TIP3P model^[42] for the water molecules and CHARMM (c36) for the mannose 9 molecule.^[43] The simulation was carried out using ACEMD.^[44] The cutoff for non-bonded interactions was set to 9 Å and long-range electrostatic forces were computed using the particle-mesh Ewald summation method with a grid size of smaller than 1 Å. We used a Langevin thermostat to control the temperature ($T=300 \text{ K}$). A hydrogen mass repartitioning scheme was used to achieve time-steps of 4 fs. The simulation involved 5000 steps of energy minimization followed by a 1.6 μs production phase.

Surface Plasmon Resonance (SPR)

A Biacore T100 instrument was used for SPR analysis to obtain K_d s for DC-SIGN binding to Man-9-enriched and Man-5-enriched gp120s. Each gp120 was immobilized through amine coupling in individual flow cells on a CM5 sensor chip to 300–320 RUs. To perform the kinetic analysis, DC-SIGN CRD was injected at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μ M in phase buffer onto the gp120-coated and reference surfaces at a flow rate of 60 μ L/min for 60 seconds (association phase) at which time equilibrium binding was observed. Proteins were dissociated by injecting buffer at the same flow rate for 5 minutes. Kinetic data were analyzed with the BIAevaluation program.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. L. Xu and G. Nabel for the adenovirus construct, J. Lloyd for HR-MS data, and W. DeGrado for helpful discussions. This work was supported by the NIH Intramural Research Program (NIDDK and NIAID), and the AIDS-Targeted Antiviral Program, Office of the Director, NIH (C.A.B.), and T.L. was supported by NIH Grant GM54616 (W.F.D.).

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Figure 1.

NMR data for $\text{Man}_9\text{GlcNAc}_2$ (Man-9). a) Symbol representation of Man-9 showing nomenclature for individual rings, glycosidic linkages and the D1, D2 and D3 arms; b) ^1H - ^{13}C HSQC-TOCSY spectrum of unlabeled Man-9 versus c) HCCH-TOCSY spectrum of ^{13}C -labeled Man-9 showing TOCSY correlations from H1 through H6/6' for each pyranose ring. (See Figure S3, Supporting Information, for an expansion.) d) Glycosidic torsion angle ω ($\text{O}5'-\text{C}5'-\text{C}6'-\text{O}$) of Man3 and Man4' rings (shown in blue) were determined from NOEs and scalar couplings: in e) overlay of HSQC-NOESY (blue) and HSQC (red) spectra of Man-9 showing NOEs of equal intensities from H5 of Man4' to H6 and H6' indicating an anti conformation for H5-O6, and respective dihedral angles of +60 and -60 for H5-H6 and H5-H6'. f) High-resolution (1.7 Hz/point, Figures S4-5, Supporting Information) HSQC spectrum showing two large H-H couplings for H4, which is anti to H3 and H5, compared to the large geminal and small gauche couplings for H6/6' of Man4 and Man4' consistent with dihedral angles determined by NOEs.

Figure 2.

NMR structure and MD simulations of Man-9. a) Key inter-ring NOEs used for structure calculations. Strong, medium and weak NOEs are designated by bold, regular and dashed lines, respectively. b) NMR ensemble of 30 structures calculated by Xplor-NIH using NOE derived distance restraints, and NOE- and J coupling-derived dihedral angle restraints. c) Temporal analysis comparing distances observed in 1.6 μ s simulations of Man-9 in explicit solvent versus NOE-derived distances. NOE-assigned distances appear along the x-axis and simulation time in nsec along the y-axis. $|\Delta_{NOE}|$ is defined as the absolute difference between the distance measured during the free simulation and the NOE-computed distance (used as restraints in solving the free Man-9 NMR structure). d) Distributions of the glycosidic ω torsion angles for the unrestrained MD simulation. ω , ϕ , ψ torsion angles were defined as O-C6'-C5'-O5', H1-C1-O-C6' and C1-O-C6'-C5', respectively.

Figure 3.

Binding site, stoichiometry and modes of binding of Man-9 and terminal trisaccharides to MVN. a) Overlay of ^1H - ^{15}N HSQC spectra of free MVN (black) and MVN in the presence of stoichiometric amounts of Man-9 (red). b–e) Expansion of Asn55 resonance showing different effects on MVN in the presence of (d) D2 and (e) D3 arm trisaccharides compared to (b) Man-9 and (c) the D1 arm trisaccharide. f) Surface representation of MVN showing the Man-9 D1 arm binding site (blue) is more extensive than Man α 1-2Man (pink surface). Full description is shown in Figure S8, Supporting Information.

Figure 4. NMR-determined conformation of MVN-bound Man-9 and principal component analysis of receptor-bound Man-9 structures. a) NOESY spectra of free (left) and MVN-bound (right) Man-9 showing minor differences in NOE patterns. b) NMR ensemble of Man-9 in the presence of MVN. c) Superposition of restrained minimized, mean NMR structures of Man-9 (blue) and MVN-bound Man-9 (green). d) PCA of MD-observed and NMR-derived glycosidic torsion angles.

Figure 5.

Man-9 binding to DC-SIGN. a) Anomeric region of HSQC spectrum of ^{13}C -Man-9 free (blue) and in a 1:1 complex with DC-SIGN (red). Resonances that disappear upon DC-SIGN binding are labeled. b) Region of Man-9 in contact with DC-SIGN in solution.