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New Carbohydrate Specificity and HIV-1 Fusion Blocking Activity of the Cyanobacterial Protein MVL:

NMR, ITC and Sedimentation Equilibrium Studies

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Abstract

Carbohydrate-binding proteins that bind their carbohydrate ligands with high affinity are rare and therefore of interest because they expand our understanding of carbohydrate specificity and the structural requirements that lead to high-affinity interactions. Here, we use NMR and isothermal titration calorimetry techniques to determine carbohydrate specificity and affinities for a novel cyanobacterial protein, MVL, and show that MVL binds oligomannosides such as Man₆GlcNAc₂ with sub-micromolar affinities. The amino acid sequence of MVL contains two homologous repeats, each comprising 54 amino acid residues. Using multidimensional NMR techniques, we show that MVL contains two novel carbohydrate recognition domains composed of four non-contiguous regions comprising ~15 amino acid residues each, and that these residues make numerous intermolecular contacts with their carbohydrate ligands. NMR screening of a comprehensive panel of di-, tri-, and high-mannose oligosaccharides establish that high-affinity binding requires at least the presence of a discrete conformation presented by Manβ(1 → 4)GlcNAc in the context of larger oligomannosides. As shown by sedimentation equilibrium and gel-filtration experiments, MVL is a monodisperse dimer in solution, and NMR data establish that the three-dimensional structure must be symmetric. MVL inhibits HIV-1 Envelope-mediated cell fusion with an IC₅₀ value of ~30 nM.

Keywords

high-mannose oligosaccharides; gp120; HIV-1 envelope-mediated fusion; carbohydrate binding proteins; NMR chemical shift mapping

Introduction

The human immunodeficiency virus (HIV) enters a cell by way of an orchestrated series of recognition events between invading virus and target host cell.¹ These include binding of the surface Envelope (Env) glycoprotein gp120 to the target cell receptor CD4,² followed by

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subsequent interactions between gp120 and a chemokine receptor, CXCR4 or CCR5, depending on viral tropism.³ The conformational changes brought about by this multi-protein assembly facilitates gp41-mediated membrane fusion.

Active areas of current research in pharmaceutical companies and universities include engineering Env-derived or receptor-derived peptides or proteins that can block Env-mediated fusion as well as identifying completely novel proteins that inhibit diverse strains of HIV. Depending on their size and stability, such inhibitors have the potential to be used as antivirals *in vivo*, or as microbicides *ex vivo*. Examples of engineered proteins and peptides that inhibit viral entry include those that target the pre-fusogenic form of gp41 and those that target receptor-binding sites on gp120. Examples of nanomolar gp41 inhibitors include the peptides FuzeoneTM,⁴ C34⁵ and N36^[Mut],⁶ and the engineered proteins N_{CCG}-gp41,⁷ N35_{CCG}-N13⁸ and 5-helix.⁹ Other than antibodies, examples of natural proteins that potently inhibit HIV-1 Env-mediated fusion include cyanovirin-N (CVN),¹⁰ scytovirin,¹¹ and actinohivin.¹² Interestingly, all of these proteins were isolated from prokaryotic organisms, with CVN and scytovirin coming from the cyanobacteria *Nostoc ellipsosporum* and *Scytonema varium*, respectively, and actinohivin from an actinomycete strain. CVN, in particular, has been studied extensively at the structural and biochemical levels, and shown to recognize a stacked conformation of α -(1 $\rightarrow$ 2)-linked mannobiose^{13,14} or mannotriose structures.^{15,16} Consequently, CVN binds with high affinity GlcNAc₂Man₈ D1D3 and GlcNAc₂Man₉,^{13,17,18} the two mammalian high-mannose oligosaccharides that present these structures in an accessible manner; and it is through these interactions that CVN potently blocks HIV-1 Env-mediated fusion.^{13,18} While the mechanisms by which scytovirin and actinohivin inhibit HIV have not been elucidated fully, early studies suggest that these bacterially derived proteins may exert their antiviral activity through high-affinity, carbohydrate-mediated interactions with gp120.

Little is known about the prevalence or function of cyanobacterial carbohydrate-binding proteins. Recently, we have been engaged in both structural and functional studies of these proteins for a variety of fundamental reasons: they represent novel proteins in terms of amino acid sequence homology and three-dimensional structures, they can recognize precise carbohydrate structures and conformations with unusually high (nanomolar) affinity, and at least some of these proteins potently block HIV-1 Env-mediated fusion. Perhaps most importantly, however, complexes formed between these proteins and their carbohydrate ligands represent novel templates for protein—carbohydrate recognition, and increase our understanding of the interactions required to achieve high-affinity protein—carbohydrate binding. Here, we report on the HIV-1 fusion blocking activity of a novel carbohydrate-binding protein, MVL, which shares no detectable sequence homology with any known protein families, and demonstrate carbohydrate specificity and stoichiometry of binding through NMR chemical shift mapping experiments, and isothermal titration calorimetry (ITC) and sedimentation equilibrium experiments.

Results

Background

Microcystis viridis NIES-102 is a freshwater bloom-forming cyanobacterium that was observed to have transient hemagglutinating activity when grown in the laboratory under anaerobic conditions.¹⁹ This activity was traced to a 113 amino acid residue, 13 kDa protein, termed MVL. The amino acid sequence of MVL was determined by enzymatic digestion and sequencing, allowing for cloning of the *mvl* gene from template total genomic *M. viridis* DNA. The amino acid sequence of MVL was found to comprise two tandemly repeated homologous domains containing 54 amino acid residues with 50% identity with one another, but no similarity or homology to any reported protein sequence. The two domains are separated by a

stretch of six amino acid residues. MVL was originally classified as a mannan-binding protein on the basis of inhibition assays employing a number of different monosaccharides and polysaccharides, wherein yeast mannan inhibited MVL hemagglutinating activity at concentrations of $\sim 20 \mu\text{g ml}^{-1}$. However, owing to the heterogeneity and complexity of yeast mannan, this result did not illuminate details regarding carbohydrate specificity.

Determining carbohydrate specificity by NMR

To determine carbohydrate specificity for MVL in detail, we carried out a series of NMR titration studies by recording ^1H — ^{15}N correlation spectra on samples of uniformly ^{15}N -labeled MVL in the presence of increasing amounts of the various mannose-containing carbohydrates illustrated in Figure 1. These included α -linked mannobiose structures $\text{Man}\alpha(1 \rightarrow 2)\text{Man}\alpha(1)$, $\text{Man}\alpha(1 \rightarrow 3)\text{Man}\alpha(2)$, $\text{Man}\alpha(1 \rightarrow 4)\text{Man}\alpha(3)$, and $\text{Man}\alpha(1 \rightarrow 6)\text{Man}\alpha(4)$; their β -linked counterparts (structures not shown); the disaccharide $\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}(5)$; the tetrasaccharide $\text{Man}_2\text{A}(8)$, $\text{Man}\beta(1 \rightarrow 6)\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}-\beta(1 \rightarrow 4)\text{GlcNAc}$; and highmannose oligosaccharides $\text{Man}_3\text{GlcNAc}_2$, $\text{Man}_5\text{GlcNAc}_2$, $\text{Man}_6\text{GlcNAc}_2$ and $\text{Man}_9\text{GlcNAc}_2$ (Figure 1). Spectra of MVL in the presence of stoichiometric excesses of each of the abovementioned disaccharides were identical with that of free MVL (spectra not shown), indicating that MVL does not bind to any of these molecules. In contrast, addition of oligomannose structures such as $\text{Man}_3\text{GlcNAc}_2$ and $\text{Man}_6\text{GlcNAc}_2$ causes dramatic changes in the ^1H — ^{15}N correlation spectra. As seen in Figure 2(a) and (b), more than one-third of the backbone and side-chain amide resonances undergo large changes in chemical shift upon binding to these complex carbohydrates. Similarly, substantial changes in the ^1H — ^{15}N correlation spectra of MVL were observed upon addition of each of the high-mannose oligosaccharides listed in Table 1.

Each of the oligomannose structures shown to bind to MVL contains at least one of the conserved disaccharide or trisaccharide core structures $\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}(5)$, $\text{GlcNAc}\beta(1 \rightarrow 4)\text{GlcNAc}(6)$, GlcNAc_2 and $\text{Man}\alpha(1 \rightarrow 3)[\text{Man}\alpha(1 \rightarrow 6)]\text{Man}\beta$ (mannotriose, 7). To determine whether MVL recognizes these smaller substructures, we carried out titration studies with species 5, 6, and 7. NMR spectra for MVL in the presence of even a 20-fold excess of any of these carbohydrates were identical with that of free MVL where neither perturbation of chemical shifts nor changes in cross-peak intensity or line-width were detected. To simulate the larger oligomannose core structures, such as $\text{Man}_2\text{A}(8)$ and $\text{Man}_3\text{GlcNAc}_2$, we titrated in 1:1 mixtures of $\text{Man}\alpha(1 \rightarrow 6)\text{Man}\alpha:\text{GlcNAc}_2$, $\text{Man}\alpha(1 \rightarrow 3)\text{Man}\alpha(1 \rightarrow 3)\text{Man}\alpha:\text{GlcNAc}_2$, and $[\text{Man}\alpha(1 \rightarrow 6)]\text{Man}\beta:\text{GlcNAc}_2$. Again, NMR spectra were identical for free MVL *versus* MVL in the presence of excesses of each of these mixtures. These results indicate that in terms of natural oligosaccharides present in mammalian systems, the smallest structure MVL binds with high affinity is Man_2A . (It remains possible that MVL would bind the truncated trisaccharide $\text{Man}\alpha(1 \rightarrow 6)\text{Man}\beta(1 \rightarrow 6)\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}$ with similar affinity, but this carbohydrate was not available for binding studies.)

Direct comparisons of the ^1H — ^{15}N correlation spectra of MVL in the presence of excess amounts of the various oligomannosides revealed subtle differences in the spectra for binding to increasingly complex oligosaccharides. For example, superposition of the ^1H — ^{15}N correlation spectra for 1:4 complexes of $\text{MVL}:\text{Man}_3\text{GlcNAc}_2$ and $\text{MVL}:\text{Man}_6\text{GlcNAc}_2$ shows that roughly eight backbone and/or side-chain amide groups resonate at slightly different chemical shifts within the two complexes (Figure 2(c)). Comparison of $\Delta\delta$ values (Figure 3) shows that the same residues are involved in binding to both oligosaccharides, and that the $\Delta\delta$ values are slightly higher for amino acid residues 34–40 and 95–100 in the complex with $\text{Man}_6\text{GlcNAc}_2$ relative to the complex with $\text{Man}_3\text{GlcNAc}_2$. Spectra for complexes of 1:4 $\text{MVL}:\text{Man}_6\text{GlcNAc}_2$ and $\text{MVL}:\text{Man}_9\text{GlcNAc}_2$, on the other hand, are nearly identical (data not shown).

MVL binds oligomannosides through two binding sites in slow exchange

The concentrations of the NMR samples of MVL were determined spectrophotometrically from the extinction coefficient calculated for a denatured monomer having the sequence shown within Figure 3 (ϵ 26,600 M⁻¹ cm⁻¹). Spectral expansions of an upfield region of the ¹H—¹⁵N correlation spectra recorded at individual points during the titration of Man₃GlcNAc₂ to MVL are shown in Figure 2(d). As can be seen in the expansions, which contain cross-peaks for Gly10 and Gly69, addition of 0.5 equivalent of Man₃, based on the concentrations calculated for monomer, resulted in the appearance of two new cross-peaks, whose average volume integrates to approximately one-fourth that of resonances corresponding to free MVL (left-most panel, Figure 2(d)). Addition of a second 0.75 equivalent gives rise to cross-peaks whose relative intensity is approximately 1:2 for free *versus* bound resonances (middle panel, Figure 2(d)). Similar changes in cross-peak intensities were observed during the course of the titration until a twofold excess of carbohydrate had been added, at which time no further change in appearance or disappearance of cross-peaks was observed, nor was any change in volume or line-width observed (right-most panel, Figure 2(d)). In terms of stoichiometry, similar changes in the intensities of cross-peaks for the carbohydrate-bound state of MVL were observed in the ¹H—¹⁵N correlation spectra for individual points of the titrations of oligomannose-6 and oligomannose-9 to ¹⁵N-MVL (spectra not shown). Collectively, these results demonstrate that two carbohydrate-binding sites must be present in each MVL monomer, since addition of two equivalents of oligosaccharide was required before the protein was fully bound, and that MVL binds oligomannose structures with sufficiently high affinity to be in slow exchange on the NMR time-scale (see below).

Identification of carbohydrate-binding sites

In preparation for solving the three-dimensional solution structure of MVL in complex with a representative oligomannose structure, Man₃, we have completed backbone resonance assignments (including HN, N, H^a, C^a, H^b and C^b atoms) for both free MVL and a 1:2 MVL:Man₃ complex using multi-dimensional NMR techniques (our unpublished results). With these resonance assignments in hand, the difference in backbone HN and N chemical shifts for free and bound MVL were compared directly and plotted as a function of residue number (Figure 3). The presence of two homologous carbohydrate-binding sites is readily apparent from the plot, which shows similar patterns of Δv_{SUM} values present in the two tandemly repeated domains, where:

$$\Delta v_{\text{SUM}} = \sqrt{(\Delta v_{\text{HN}}^2 + \Delta v_{\text{N}}^2)}$$

Sequences displaying average Δv_{SUM} values greater than ~50 Hz are observed in four regions of the protein, with each region comprising approximately 15 amino acid residues. These include regions encompassing residues 10–25, 36–49, 67–83 and 95–108, all of which include one tryptophan residue. Comparison of the sequence alignments and the Δv_{SUM} values of the two tandem repeats thus suggest that the two carbohydrate-binding regions are composed of the conserved sequences GPLWSNXEAQXXGPX (corresponding to residues 10–24 and 69–83) and FTGQWXTXVEXXMSV (corresponding to residues 33–47 and 92–106). Backbone chemical shift assignments also revealed that Δv values range from less than 10 Hz to greater than 800 Hz for HN atoms. Thus, the lifetime of the MVL:Man₆ complex is $\gg 16$ ms ($(2\pi\Delta v)^{-1}$). The observation that only one set of resonances appeared during the course of each of the titrations indicated that the two carbohydrate-binding sites bind these various oligomannosides with comparable affinities under the conditions used for the NMR titrations (micromolar concentrations at pH 6.85).

Isothermal titration calorimetry

To determine the equilibrium association constants and thermodynamic parameters for MVL binding to representative oligomannosides, iso-thermal titration calorimetry (ITC) measurements were performed for several of the carbohydrates shown to bind to MVL by NMR. As seen in the representative equilibrium titration curves for MVL binding to Man₃GlcNAc₂ and Man₆GlcNAc₂ (Figure 4(a) and (b), respectively), each of these oligosaccharides bind MVL with large negative enthalpies (Table 2). While negative enthalpies do not unequivocally indicate purely electrostatic interactions, it is likely that electrostatic interactions contribute significantly to MVL—oligosaccharide binding, given the number of polar and charged amino acids present in the binding motifs. Calculations of the free energies of binding show that each of the three oligosaccharides binds MVL with favorable free energies ranging from -7.4 kcal mol⁻¹ to -9.0 kcal mol⁻¹ (Table 2). On the other hand, steady increases in entropic cost are observed for binding to MVL as the carbohydrate ligands become larger and more complex, with values ranging from -10.7 cal mol⁻¹ for Man₂A to -39.5 cal mol⁻¹ for Man₆. Curve fitting of the binding isotherms using a one independent site model for Man₂GlcNAc₂, Man₃GlcNAc₂ and Man₆GlcNAc₂ yielded a stoichiometry of binding equal to two carbohydrate molecules per monomer for each of these ligands (Table 2). Curve fitting was attempted using a two independent site model and a two sequential binding site model. However, fits to both models resulted in error values at least one order of magnitude greater than those generated by a one independent site model. When considered together with the results of the NMR titration experiments, wherein the positions of a large number of cross-peaks changed concurrently upon carbohydrate binding and a stoichiometry of 2:1 carbohydrate:MVL was observed, these measurements indicate that in terms of affinity, the two carbohydrate recognition domains present in MVL are very similar. These results are supported by the symmetry observed for the Δv values as a function of amino acid residue illustrated in Figure 3.

Oligomeric nature of MVL, which is a monodisperse dimer

As many carbohydrate-binding proteins exhibit oligomeric structures, we sought to examine the oligomeric state of MVL using biophysical techniques. Thus, a sample of MVL was analyzed by gel-filtration chromatography using a Superdex75 column calibrated as described.²⁰ Elution profiles clearly indicated that MVL was considerably larger than a monomer, and extrapolation from the linear plot of log molecular mass as a function of elution volume (Figure 5(a)) showed the protein to have a molecular mass of 25.4 kDa. Sedimentation equilibrium experiments were subsequently carried out on a sample of MVL at three different rotor speeds. In all cases, sedimentation equilibrium data were best modeled in terms of a single ideal solute (Figure 5(b)) yielding identical values of the buoyant molecular mass and indicating that the sample was monodisperse. A global analysis yields a value of 6170(±100) g mol⁻¹ for $M(1 - v\rho)$, which corresponds to a measured molecular mass of 23,340(±400) g mol⁻¹, and indicates that under these conditions MVL is dimeric ($M_{\text{calc}} = 12,237.4$ g mol⁻¹, $n = 1.9 \pm 0.03$). Together, these results firmly establish that MVL is a dimer in solution. Moreover, on the basis of the NMR data, the three-dimensional structure of MVL must be a symmetric dimer, because a single set of resonances (corresponding to the number expected from the amino acid sequence) are observed in the ¹H—¹⁵N correlation spectra of both free and fully bound MVL.

Additional sedimentation equilibrium measurements were made on three separate samples of 30 μ M MVL containing a fourfold molar excess of either oligomannose-3, oligomannose-6 or oligomannose-9. As MVL binds Man₃ and Man₆ with apparent equilibrium constants of 3.5 (±0.2) $\times 10^5$ M⁻¹ and 4.5(±0.3) $\times 10^6$ M⁻¹, respectively (Table 2), the MVL dimer is expected to be saturated with the oligosaccharide. In all cases, data were best fit in terms of a single ideal solute yielding experimental molecular masses corresponding with a monodisperse MVL dimer bound to at least four (average $n = 5.4 \pm 1.1$) oligomannose moieties (data not shown).

Although the resolution and error of the sedimentation method does not allow us to distinguish between four, five or six oligomannose species bound to an MVL dimer, the data appear to be consistent with the stoichiometry determined by NMR and ITC. Thus, at micromolar concentrations, these branched ligands do not appear to induce cross-linking of MVL. Moreover, these results are entirely consistent with specific recognition of the tetra- or pentasaccharides Man₂A and Man₃, structures located in the internal core (Figure 1) as opposed to the terminal branching arms of high-mannose structures. This mode of binding is illustrated in Figure 5(c), where multi-domain carbohydrate-binding proteins that recognize terminal structures can be cross-linked by the branching, providing the spacing separating binding sites is greater than that separating terminal saccharide units; and those recognizing internal core structures, or non-branching oligosaccharides are simply saturated with the ligand.

MVL inhibits HIV-1 Env-mediated fusion

To date, there are several reports of lectins exhibiting antiviral activity toward HIV.²¹ Complex and especially high-mannose type oligosaccharides account for nearly half of the molecular mass of gp120, and these antiviral lectins likely bind to oligosaccharides displayed over much of the envelope surface,²² thereby interfering with the fusion event. The magnitude of antiviral activity for various carbohydrate-binding proteins appears to correlate with the types of oligosaccharide present on the viral surface.^{23,24} Given the high affinities with which MVL can bind various high-mannose oligosaccharides, we evaluated its effect on HIV-1 Env-mediated fusion in a quantitative vaccinia virus reporter gene assay that faithfully reproduces the events that lead to membrane fusion.²⁵ MVL was tested for its ability to inhibit an HIV-1 T-cell tropic (T-tropic) strain LAV, and a macrophage tropic (M-tropic) strain SF162, with inhibition curves for both virus types shown in Figure 6(a). Titration data were best fit to a two-independent site model comprising two molecules of MVL per molecule of gp120, as fitting to a one independent site model gave systematic errors in the fit. Best fits of the inhibition curves for LAV and SF162 viruses yield respective IC₅₀ values of 30(±4) nM and 37(±6) nM (given by $(\sqrt{2} - 1)/K_A$).

Competition experiments with several oligosaccharides and MVL were carried out using 200 nM MVL in the presence of 1 μM carbohydrate. Pretreatment with oligomannose-3 and oligomannose-9 resulted in partially reduced inhibition of fusion by MVL with respective values of approximately 18% and 41%, while pre-treatment with one equivalent of gp120 (SF162) resulted in blocking MVL's inhibitory action by around 85%. Pretreatment of MVL with mannotriose had no effect on MVL's inhibitory activity, as expected from NMR and ITC results that demonstrated MVL does not bind to this trisaccharide. Although equilibrium association constants were not measured by ITC for Man₉, the nearly identical sets of spectra from the NMR titrations for Man₆ and Man₉ indicate that these two oligosaccharides may bind MVL with similar affinities. Given an IC₅₀ value of ~30 nM for MVL binding to gp120, and a K_a of $3.5 \times 10^5 \text{ M}^{-1}$ for Man₃ binding to MVL, the calculated %fusion for the above experimental conditions is 17%, within the error of the experimentally observed value (18%). In the case of Man₉, the same K_a measured for the binding of Man₆ to MVL ($4.5 \times 10^6 \text{ M}^{-1}$, Table 2) yields a calculated value of %fusion of 42%, which coincides with the experimentally observed value of 41%. (Thus, the affinity of Man₆ and Man₉ for MVL must be very similar.) These results indicate that Man₃ and Man₉ compete directly with gp120 for binding to MVL and support the notion that MVL inhibits fusion through carbohydrate-mediated interactions with high-mannose residues on gp120.

Discussion

Protein—carbohydrate interactions mediate a host of interactions that facilitate recognition and attachment among macromolecules, cells and foreign pathogens.^{26,27} For this reason,

molecules that block deleterious carbohydrate-mediated interactions, such as those governing viral entry or bacterial pathogenesis, have the potential to be useful therapeutics and/or mechanistic probes. In practice, however, one fundamental obstacle to realizing this concept is the intrinsically low affinities with which carbohydrate-binding proteins typically bind their saccharide ligands. One notable exception to this general trend was illustrated recently by the cyanobacterial protein cyanovirin-N (CVN), which, to our knowledge, is the only lectin that has been shown to bind a small carbohydrate ligand, namely the disaccharide $\text{Man}\alpha(1 \rightarrow 2)\text{Man}\alpha$, with nanomolar affinities.¹³ The structures of the two carbohydrate-binding sites present in CVN^{14,16} and the conformation that the disaccharide,¹⁴ as well as $\text{Man}\alpha(1 \rightarrow 2)\text{Man}\alpha$ -containing trisaccharides^{15,16} adopt when bound to CVN had not been observed, either free or bound. To begin to assess the prevalence of this phenomenon, we have since been engaged in studying multiple aspects of other cyanobacteria-derived carbohydrate-binding proteins to expand our knowledge in terms of protein—carbohydrate specificity and the structural requirements that lead to such rare but potentially useful high-affinity interactions.

Here, we have demonstrated that the novel cyanobacterial protein MVL contains two carbohydrate-binding sites per monomer, and exists as a stable monodisperse dimer in solution. The dimeric nature of MVL gives rise to a new tetravalent carbohydrate-binding protein where the carbohydrate-binding sites as detected by NMR titrations appear to be composed of the amino acid sequences GPLWSNXEAQXXGPX and FTGQWXTXVEXXMSV, both of which are duplicated in the first and second repeats of the protein sequence (Figure 3). These carbohydrate-binding sequences appear to be novel, since homology searches failed to retrieve protein or peptide sequences bearing any similarity to these carbohydrate recognition domains or to other regions of the amino acid sequence. In terms of tertiary structure, it cannot be assumed that the individual repeats of the protein sequence necessarily comprise structurally independent sub-domains. On the contrary, attempts to express recombinant versions of the N and C-terminal sequences (amino acid residues 1–60 and 55–113) demonstrated that the individual peptides cannot be reconstituted on their own (our unpublished results). With regard to the quaternary structure of the dimer, it is possible that MVL exists as a homo-dimer; or as observed for other carbohydrate-binding proteins such as CVN²⁰ and the anti-gp120 monoclonal antibody 2G12,²⁸ as a three-dimensional domain-swapped dimer. Ongoing structural studies should resolve these questions.

MVL recognizes with sub-micromolar affinity high-mannose oligosaccharides, and binds the smaller ligands Man_2A and Man_3 with low micro-molar affinities ($\sim 5 \mu\text{M}$, Tables 1 and 2). Subtle differences between the NMR spectra for 1:4 complexes of MVL with Man_3 , Man_6 and Man_9 indicate that relative to Man_3 , one or more of the non-reducing mannopyranose units of Man_6 (rings A—C of $\text{Man}_9\text{GlcNAc}_2$, Figure 1) contribute to binding to MVL, possibly accounting for the increased affinity to MVL for the larger oligosaccharides. MVL does not bind to α or β -linked dimannosides (1–4) or α -linked trimannosides that correspond to the D1, D2 and D3 arms of oligomannose-9; nor does it bind the disaccharides $\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}$ (5) and $\text{GlcNAc}\beta(1 \rightarrow 4)\text{GlcNAc}$ (6) or the trisaccharide mannotriose(7), each of which is present in the ubiquitous branching core of higher-mannose structures. Furthermore, although MVL binds oligomannose-2A and oligomannose-3 with equilibrium dissociation constants $< 5 \mu\text{M}$, it does not bind with measurable affinities mixtures of the individual carbohydrates $\text{Man}\alpha(1 \rightarrow 3)[\text{Man}\alpha(1 \rightarrow 6)]\text{Man}\beta$ or GlcNAc_2 that together form oligomannose-3, even when these carbohydrates are added simultaneously to MVL in both NMR and ITC experiments.

Oda *et al.* reported recently that the *Crocus sativus* lectin CSL recognizes $\text{Man}_3\text{GlcNAc}$ of N-glycan cores.²⁹ However, CSL was reported to bind the equivalent of Man_2A with a 65-fold decrease in affinity relative to Man_3 . Thus, MVL recognition must differ considerably from that of CSL given that MVL binds Man_2A and Man_3 with nearly equal K_A values (Table 2). These results suggest that MVL recognizes a specific conformation of Man_2A or Man_3 that is

presented by a $\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}$ structure embedded within the trisaccharide $\text{Man}\alpha(1 \rightarrow 6)\text{Man}\beta(1 \rightarrow 4)\text{GlcNAc}$ or Man_2A . This is in contrast to other so-called Man-specific or Glc-specific lectins that are known to preferentially bind mannotriose^{30,31} or GlcNAc_2 ,^{32,33} but not both.

It is known that lectins are able to overcome the weak affinities with which they bind a given carbohydrate ligand through multimerization. This strategy increases both specificity, brought about by spacing of individual carbohydrate recognition domains, and overall avidity, brought about by multivalency.^{34,35} Indeed, both plant and animal lectins generally occur as dimers, trimers or tetramers with two, three or four carbohydrate-binding sites, respectively. Examples of each include the homodimer galectin,³⁶ the homotrimer mannose-binding lectin³⁷ and the pH-dependent tetramer concanavalin A.³⁸ In this respect, cyanovirin-N again provided an exception, in that the three-dimensional fold of this relatively small 11 kDa protein displays C_2 pseudosymmetry brought about by the internal tandem repeats in its amino acid sequence.³⁹ This internal 2-fold symmetry manifests in the presence of two carbohydrate-binding sites within a single monomer,^{13,14} thereby making CVN a dimer in terms of ligand binding, and its three-dimensional domain-swapped dimeric form a tetramer.^{40,41} Although MVL shares no sequence similarity with CVN, it is interesting that it too contains tandem repeats within its amino acid sequence, which we have shown must accommodate two carbohydrate-binding sites per monomer, or four binding sites per dimer, the natural form of MVL. Cyanovirin is in preclinical development as a microbicide to prevent transmission of HIV and was reported recently to show 100% efficacy in a primate model.⁴² Those studies establish the potential of high-affinity carbohydrate-binding proteins as *ex vivo* therapeutics. While MVL potently blocks HIV-1 Env-mediated fusion, thereby offering similar potential, further high-resolution structural studies of MVL in complex with a high-affinity ligand promise to enhance our understanding of the nature of intermolecular interactions required to bring about fine specificity and high-affinity protein-carbohydrate interactions.

Materials and Methods

Cells

B-SC-1 cells (American Type Culture Collection) grown in DMEM 10% (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum) 2 mM L-glutamine and 50 mg ml⁻¹ of gentamycin (all from Gibco BRL, Bethesda, MD) were used for all assays.

Reagents

Recombinant vaccinia viruses used in this study were obtained from the AIDS Research and Reference program, Division of AIDS, NIAID, National Institutes of Health, and include the following recombinants (donor in parentheses): vCB-32 encoding HIV-1 Env from SF162 (C. Broder, P. Kennedy, E. Berger⁴³), vCB-41 encoding HIV-1 Env from LAV (C. Broder, P. Kennedy, E. Berger⁴³), vCBYF1-fusin encoding T-tropic receptor CXCR4 (C. Broder, P. Kennedy, E. Berger⁴⁴), vCB-CCR5 encoding M-tropic receptor CCR5 (a gift from Dr Christopher Broder⁴⁵), vCB21R-LacZ encoding β -galactosidase (C. Broder, P. Kennedy, E. Berger), and vP11T7gene1 encoding phage T7 polymerase. Chlorophenol-red- β -D-galactopyranoside (CPRG) was purchased from Roche (Nutley, NJ), and [¹⁵N]NH₄Cl was purchased from Cambridge Isotope Labs (Andover, MA). Mannobiose disaccharides were purchased from Sigma-Aldrich (St. Louis, MO), and GlcNAc_2 , mannotriose, and all other high-mannose complex carbohydrates were purchased from Glycotech (Rockville, MD).

Expression and purification of uniformly labeled MVL

Isolation and sequencing of the MVL gene from *M. viridis* NIES-102 strain has been described.¹⁹ An insert spanning the MVL gene was generated by polymerase chain reaction using MVL

pTV118N as template and purified primers 5'-cgggtgcgagcata tggcgagttacaa agtg and 5'-ggccacgctcgag ttagaaagtgtac ttg (Lofstrand Labs, Rockville, MD), which encode NdeI and XhoI restriction sites, respectively. The MVL expression vector was constructed following digestion with NdeI and XhoI, and cloning into pET11a expression vector (Novagen, Madison, WI) digested with the same endonucleases. The MVL insert was verified by DNA sequencing and expressed in *Escherichia coli* BL21(DE3), and the composition confirmed by mass spectrometry.

Uniformly ^{15}N -labeled protein was obtained by growing cells at 37 °C in M1 minimal medium containing $[^{15}\text{N}]\text{NH}_4\text{Cl}$ and $[^{12}\text{C}]\text{glucose}$ as nitrogen and carbon sources, respectively. Upon reaching an absorbance of ~ 1.0 , cells were induced with 1 mM isopropyl- β -D-thiogalactoside for three to four hours and harvested by centrifugation at 7500g for ten minutes. Following resuspension and homogenization in 50 mM Tris (pH 8), 50 mM NaCl, 1 mM benzamidine at 4 °C, cells were lysed in a microfluidizer, and a clear cell-free extract was obtained after centrifugation at 16,000g for one hour at 4 °C. The supernatant was applied directly to a HiLoadTM 26/10 Q-Sepharose anion-exchange column (Amersham Biosciences, Piscataway, NJ) equilibrated in 20 mM Tris (pH 8) at a flow-rate of 0.5 ml min⁻¹, and eluted with a linear gradient of 0–1 mM NaCl in 20 mM Tris (pH 8) over ten column volumes at a flow rate of 5 mL min⁻¹. Fractions containing MVL were combined and concentrated in a 5000 Da cutoff filter and applied to a Superdex75 26/60 column equilibrated with 20 mM sodium phosphate (pH 6.5) and eluted with the same buffer. Fractions containing pure MVL, as judged by SDS-PAGE on premade 20% Phast gels (Amersham Pharmacia Biotech), were combined and concentrated in a 5000 Da cutoff filter and stored at 4 °C. Concentrations of all samples were determined spectrophotometrically based on the extinction coefficient calculated for a denatured monomer having the sequence shown in Figure 1 (ϵ 26,600 M⁻¹ cm⁻¹ for monomer).⁴⁶ Note that no measurable difference in absorbances was observed for identical samples diluted into 6 M guanidine HCl or phosphate buffer.

NMR spectroscopy

All NMR experiments were recorded at 27 °C on Bruker DMX500 and DMX600 spectrometers equipped with *x*, *y*, *z*-shielded gradient triple-resonance probes or a *z*-shielded gradient triple-resonance cryoprobe. Spectra were processed and analyzed using the software package NMRPipe.⁴⁷ Titration experiments using 250 μl NMR samples of 0.15 mM $[^{15}\text{N}]\text{MVL}$ were performed by recording ^1H — ^{15}N heteronuclear single quantum coherence spectra of samples in the presence of varying stoichiometries of di-, tri- and oligosaccharides. Typically, 0.25 to 0.5 equivalent of ligand were added in 5 μl aliquots. All solutions were prepared in 20 mM sodium phosphate and the pH was adjusted to 6.85. ^1H , ^{13}C and ^{15}N backbone assignments were made for free MVL and 1:2 MVL:Man₃ using three-dimensional double and triple-resonance through-bond correlation and NOE experiments including CBCA(CO)NH, CBCANH, HNCA and ^{15}N separated NOE experiments.⁴⁸

Isothermal titration calorimetry measurements of oligomannose-MVL binding

ITC measurements and analysis were performed with a Microcal VP-ITC titration calorimeter and Origin software. In each experiment 1.484 ml of 10 μM MVL was present in the solution cell, and 20–30 5 μl aliquots of ligand were added *via* a 250 μl rotating stirrer-syringe every 180 seconds at 25 °C. The ligands included solutions of 800 μM Man₂A, Man₃ and Man₆, and 1.0 mM Mana(1 $\rightarrow$ 2)Mana(1 $\rightarrow$ 3)Man, Mana(1 $\rightarrow$ 2) Mana(1 $\rightarrow$ 6), mannatriose, GlcNAc₂, and an equimolar mixture of mannatriose:GlcNAc₂. All solutions were prepared in 10 mM Tris buffer (pH 6.5). Controls were performed for each experiment wherein the appropriate ligand solution was added to buffer only. In the case of all di- and trisaccharide ligands, the binding isotherms were indistinguishable from those of the controls and could not be integrated, indicating that binding to these carbohydrates does not occur.

Gel-filtration

Gel-filtration experiments were carried out on a Superdex75 10/30 column using an AKTA FPLC (both from Amersham Biosciences, Piscataway, NJ). Samples of dextran blue (120 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), Δ Q50-cyanovirin-N (22 kDa),⁴⁰ ribonuclease A (13.7 kDa), cyanovirin-N (11 kDa) and aprotinin (6.7 kDa) were used to calibrate the column eluting with a flow-rate of 0.8 ml min⁻¹ in PBS (pH 7.0).

Sedimentation equilibrium

Sedimentation equilibrium experiments were conducted at 25.0 °C and three different rotor speeds (12,000 rpm, 14,000 rpm and 16,000 rpm) on a Beckman Optima XL-A analytical ultracentrifuge. Protein samples were prepared in 20 mM sodium phosphate buffer (pH 6.85) and loaded into the ultracentrifuge cells at nominal loading concentrations of 0.8 A₂₈₀. Data were analyzed in terms of a single ideal solute to obtain the buoyant molecular mass, $M(1 - 2\nu\rho)$, using Sigma Plot 2002 (SPSS Inc.) The value for the experimental molecular mass M was determined using calculated values for the density ρ (determined at 25.0 °C using standard tables) and partial specific volume ν (calculated on the basis of amino acid composition).⁴⁹

Cell fusion assays

HIV-1 Env-mediated cell fusion assays on MVL were carried out as described.⁸ B-SC-1 cells were used for both target and effector cell populations. For experiments employing T-cell-tropic Env, target cells were co-infected with recombinant vaccinia viruses vCB21RLacZ and vCBYF1-fusin, and effector cells with vCB41 and vP11T7gene1, at a multiplicity of infection (MOI) of 2.5. In the experiments employing macrophage-tropic Env, target cells were co-infected with vCB-CCR5 and vCB21R-LacZ, and target cells with vCB21 and vP11T7gene1. Following infection, cells were incubated for 18 hours at 32 °C to allow for vaccinia virus-mediated expression of recombinant proteins. For inhibition studies, proteins or complexes were added to an appropriate volume of DMEM 2.5% and PBS to yield identical buffer compositions (100 μ l), followed by addition of 1 $\times$ 10⁵ effector cells (in 50 μ l of medium) per well and 1 $\times$ 10⁵ target cells (in 50 μ l of medium) per well. Soluble CD4 was added to the media of the target cells at a concentration of 800 nM to yield a final concentration of 200 nM soluble CD4 per well. Following 2.5 hours incubation at 37 °C, the β -galactosidase activity of cell lysates was determined from measurement of the absorbance at 570 nm (Molecular Devices 96-well spectrophotometer) upon addition of CPRG. The curves for % fusion *versus* MVL concentration were fit by non-linear, least-squares optimization using the program Kaleidagraph 3.5 (Synergy Software, Reading, PA).

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Abbreviations used

HIV-1, human immunodeficiency virus type 1; ITC, isothermal titration calorimetry; CPRG, chlorophenol-red- β -D-galactopyranoside.

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Figure 1.

Carbohydrates used for NMR titrations. Both alpha and beta-linked di-mannosides were used for titrations, but only alpha-linked structures are shown (1–4). Man α (1 $\rightarrow$ 4)GlcNAc (5) and GlcNAc β (1 $\rightarrow$ 4)GlcNAc (6, GlcNAc₂) are disaccharides corresponding to the core, and mannotriose (7) refers to the trisaccharide Man β (1 $\rightarrow$ 3) [Man α (1 $\rightarrow$ 6)]Man β , and Man₂A (8) to the tetrasaccharide Man α ((1 $\rightarrow$ 6)Man β (1 $\rightarrow$ 4)GlcNAc β (1 $\rightarrow$ 4)GlcNAc. Structures corresponding to Man₃GlcNAc₂ (Man₃), Man₆GlcNAc₂ (Man₆ and Man₉GlcNAc₂ (Man₉) are indicated with black, red and blue arrows, respectively, within the Man₉GlcNAc₂ structure, and standard designations for each pyranose are indicated in bold italics.

Figure 2.

Overlays and expansions of ^1H - ^{15}N correlation spectra for complexes of free and oligomannose-bound MVL. In (a), the spectra for free MVL and a complex of 1:4 MVL:Man₃GlcNAc₂ are colored black and blue, respectively; and in (b) the spectra for free MVL and a complex of 1:4 MVL:Man₆GlcNAc₂ are colored black and red, respectively. In (c), spectra of the complexes of Man₃ and Man₆ (blue and red spectra, respectively) are superimposed and regions with noticeable differences are indicated with an asterisk (*). (d) Expansion of the ^1H , ^{15}N correlation spectra for individual points in the Man₃ titration. Based on the concentration of monomeric MVL determined by UV absorbance ($\epsilon = 26,600 \text{ M}^{-1} \text{ cm}^{-1}$ for monomer), the integrated volume of cross-peaks corresponding to the bound state of

Gly10 and Gly69 and appearing during the course of the titration were approximately half that expected for full occupancy of one site. No further change in peak intensity or volume was observed upon addition of greater than 2.0 equivalents of Man₃.

Figure 3.

Amino acid residues involved in carbohydrate binding. Carbohydrate-binding regions were delineated by Δv_{SUM} values, where $\Delta v_{\text{SUM}} = \sqrt{(\Delta v_{\text{HN}}^2 + \Delta v_{\text{N}}^2)}$, and Δv_{HN} and Δv_{N} are the differences (in Hz) between pairs of free and bound HN and N resonances, respectively. Δv_{SUM} values for binding to Man₃ and Man₆ are shown in the top and bottom two panels, respectively, where the grey bar marks 0–60 Hz values. Note that Δv values for HN and/or N atoms could not be determined for Asn15 (Man₃ complex only), Gly35, Glu42, Asn74, and Gly94 due to overlap; these residues are indicated with triangles. Amino acid sequence and numbering appear along the x-axis where the tandem repeats are aligned by internal sequence

homology. Red and orange one-letter codes represent identical and conserved residues within the two tandem repeats, respectively. MVL comprises an N and a C-terminal region with 50% identity between the two 54 amino acid repeats that are connected by a sequence of six amino acids.

Figure 4.

ITC of binding of Man₃GlcNAc₂ (a) and Man₆GlcNAc₂ (b) to MVL. Raw data as a function of time for each oligomannoside are shown in the top panel, and plots of the total heat released as a function of the molar ratio of each ligand are shown in the bottom panel. The continuous lines represent the non-linear, least-squares best fits to the experimental data using a one-site model. Fitting to a two independent sites model yielded χ^2 values at least one order of magnitude greater than those observed for best fits to a one-site model. The values of the fitted parameters K_A and ΔH are provided in Table 2.

Figure 5.

Oligomeric nature and model of carbohydrate binding for MVL. The molecular mass of MVL was determined by (a) gel-filtration chromatography and (b) sedimentation equilibrium experiments. In (a), a plot of log molecular mass as a function of elution volume for the proteins indicated shows a linear relationship between the two giving an estimated molecular mass for MVL of 25.4 kDa. (b) Sedimentation equilibrium profile at 16,000 rpm and 25 °C shown as a distribution of ln(*A*₂₈₀) at equilibrium. The results are analyzed for the best single component $M(1 - v\rho)$ fit, corresponding to a measured molecular mass of 23,340(±400) g mol⁻¹. The distribution of the residuals to the best single-component fit is shown above the plot. (c) Model of multi-domain binding to oligosaccharides. The top panel illustrates specificity for core or

non-branching structures wherein each carbohydrate-binding site is saturated but not cross-linked, since the non-branching structures do not facilitate cross-linking; while the bottom panel illustrates specificity for saccharides located at the termini of the arms of branched oligosaccharides and are thus capable of inducing cross-linking.

Figure 6.

HIV-1 Env-mediated cell fusion by MVL and complexes. In (a) the effect of MVL on fusion is indicated with red filled circles for T-tropic virus (LAV) and with black open circles for M-tropic virus (SF162); error bars are shown in red and black, respectively. Continuous lines represent best fits to the data using the activity relationship of a two independent sites model:

$\% \text{fusion} = 100 / (1 + 2K_A [I] + K_A^2 [I]^2)$ where $[I]$ is the concentration of MVL and K_A is the equilibrium association constant for MVL binding to trimeric gp120.¹³ Best fits yield IC_{50} values, given by $(\sqrt{2} - 1) / K_A$, of $30(\pm 4)$ nM for LAV and $37(\pm 6)$ nM for SF162. (b) Effect on fusion-blocking activity of MVL (200 nM) upon pretreatment with 1 μ M mannitol, mannitol, Man3, Man9, and gp120.

Man₃GlcNAc₂ or Man₉GlcNAc₂, and 200 nM gp120 (SF162). The %fusion observed in the presence of MVL and competing sugars is consistent with the affinity of MVL for gp120 (IC₅₀ 30 nM) and the reported K_a values for oligomannose binding to MVL shown in Table 2. For Man₃, the calculated %fusion is 17%; for Man₉, the calculated value is 42% assuming that the K_a for Man₉ binding to MVL is the same as that for Man₆. Negative and positive controls were performed for all experiments and were used for normalization. Controls for each of the ligands in the absence of MVL were carried out and yielded results indistinguishable from those of the positive controls; that is, they had no effect on %fusion.

Table 1

Mannose-containing carbohydrates used for MVL binding studies

Carbohydrate	Binding to MVL
GlcNAc β (1 $\rightarrow$ 4)GlcNAc	—
Man α (1 $\rightarrow$ 2)Man	—
Man α (1 $\rightarrow$ 3)Man	—
Man α (1 $\rightarrow$ 4)Man	—
Man α (1 $\rightarrow$ 6)Man	—
Man β (1 $\rightarrow$ 2)Man	—
Man β (1 $\rightarrow$ 3)Man	—
Man β (1 $\rightarrow$ 4)Man	—
Man β (1 $\rightarrow$ 6)Man	—
Man β (1 $\rightarrow$ 4)GlcNAc	—
Man α (1 $\rightarrow$ 2)Man α (1 $\rightarrow$ 2)Man	—
Man α (1 $\rightarrow$ 2)Man α (1 $\rightarrow$ 3)Man	—
Man α (1 $\rightarrow$ 2)Man α (1 $\rightarrow$ 6)Man	—
Man α (1 $\rightarrow$ 3)[Man α (1 $\rightarrow$ 6)]Man	—
1:1 Man α (1 $\rightarrow$ 3)Man:GlcNAc ₂	—
1:1 Man α (1 $\rightarrow$ 6)Man:GlcNAc ₂	—
1:1 Man α (1 $\rightarrow$ 3)[Man α (1 $\rightarrow$ 6)]Man:GlcNAc ₂	—
Oligomannose-2A	✓
Oligomannose-3	✓
Oligomannose-5	✓
Oligomannose-6	✓
Oligomannose-9	✓

Table 2

Isothermal titration calorimetry data

Carbohydrate	Stoichiometry	K_a (M^{-1})	ΔH (kcal mol ⁻¹)	ΔG^a (kcal mol ⁻¹)	ΔS (cal mol ⁻¹)
Man ₂ A	2.04 ± 0.05	2.4(±0.2) × 10 ⁵	-10.5 ± 0.04	-7.4 ± 0.6	-10.7 ± 2.1
Man ₃	2.15 ± 0.02	3.5(±0.2) × 10 ⁵	-13.1 ± 0.1	-7.5 ± 0.4	-18.7 ± 5.1
Man ₆	2.06 ± 0.01	4.5(±0.3) × 10 ⁶	-20.8 ± 0.1	-9.0 ± 0.6	-39.5 ± 2.3

T = 298 K.

^a $\Delta G = -RT \ln K_A$