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Binding Site Geometry and Subdomain Valency Control Effects of Neutralizing Lectins on HIV-1 Viral Particles

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Abstract

Carbohydrate binding proteins such as griffithsin, cyanovirin-N, and BanLec are potent HIV entry inhibitors and promising microbicides. Each binds to high-mannose glycans on the surface envelope glycoprotein gp120, yet the mechanisms by which they engage viral spikes and exhibit inhibition constants ranging from nanomolar to picomolar are not understood. To determine the structural and mechanistic basis for recognition and potency, we selected a panel of lectins possessing different valencies per subunit, oligomeric states, and relative orientations of carbohydrate binding sites to systematically probe their contributions to inhibiting viral entry. Cryo-electron micrographs and immuno gold staining of lectin-treated viral particles revealed two distinct effects—namely, viral aggregation or clustering of the HIV-1 envelope on the viral membrane—that were dictated by carbohydrate binding site geometry and valency. “Sandwich” surface plasmon resonance experiments revealed that a second binding event occurs only for those lectins that could aggregate viral particles. Furthermore, picomolar K_d values were observed for the second binding event, providing a mechanism by which picomolar IC_{50} values are achieved. We suggest that these binding and aggregation phenomena translate to neutralization potency.

Graphical Abstract

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Author Contributions

S.L., K.L., J.M.-L., and R.G. performed experiments; S.L. and C.A.B. conceived of and designed the research with input from J.M.-L., J.H., and R.G. on cryo-EM and biophysical experiments; S.L. and C.A.B. wrote the paper with input from all authors.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.6b00139.

Additional materials including glycan array profiling, neutralization curves, DLS, cryo-EM images for BanLec and GNA, TEM images showing Au immunostaining, SPR for GRFT mutants, and analytical ultracentrifugation for all proteins used in this study (PDF)

The authors declare no competing financial interest.

Keywords

cryo-electron microscopy (cryo-EM); envelope glycoprotein; HIV gp120 (gp120); surface plasmon resonance (SPR); electron microscopy (EM); multivalency

The glycoprotein spikes displayed on the surfaces of viral membranes facilitate infection by binding to target cell membranes, specific host cell receptors, or a combination of both. Examples of enveloped viruses that exploit these interactions to gain entry include HIV, HCV, and influenza. An understanding of the structural and molecular basis of these early events is important for the discovery or design of small-molecule entry inhibitors^{1,2} and the development of immunogens useful in vaccine design.^{3,4}

Among viral envelope glycoproteins HIV gp120 is one of the most heavily glycosylated proteins known, containing on average 24 N-linked and many fewer O-linked glycans. Because the human glycosylation machinery is used to install and subsequently process these glycans, dense patches of high-mannose sugars are displayed on the HIV envelope (Env) (Figure 1) and comprise half of the molecular weight of gp120.⁶ This dense array of carbohydrates forms a so-called glycan shield that contributes to viral fitness by facilitating protein folding and virus maturation, masking the virus from immune surveillance and providing a physical barrier to inhibition. Paradoxically, the immune system has subverted this glycan shield by evolving broadly neutralizing antibodies (bNAbs) that directly target unique clusters of glycans not present on other human proteins (for example, 2G12⁷ and PGT128⁸) or accommodate glycosylation within or surrounding their combining sites (such as PG9,⁹ PG16,¹⁰ PGT135,¹¹ and VRC01⁵).

Another class of entry inhibitors includes carbohydrate-binding agents (CBAs) that exert their antiviral effects by binding to surface displayed glycans on viral envelope proteins. CBAs are diverse in size and structure and include synthetic compounds, small-molecule natural products, and lectins - non immune proteins that bind carbohydrates.^{12,13} Several man-nose-binding lectins in particular are extremely potent and possess traits that are attractive for therapeutic development.^{4,14-17} The widely studied lectin cyanovirin (CV-N), for example, shows broad-spectrum antiviral activity inhibiting HIV, influenza, Ebola,

hepatitis C, and herpes viruses.^{18–22} In addition, lectins can present a high barrier to resistance where mutation or removal of a single glycosylation sequon is insufficient to confer resistance.¹⁴ Rather, some viruses must undergo extensive mutation, deleting as many as four to five glycosylation sites to escape the inhibitory effects of CBAs, which in turn may decrease viral fitness. This is unique to lectins as antiviral agents, making them attractive microbicide candidates.²³

Although key studies have shown that mannose-binding lectins can inhibit viral entry through glycan-mediated interactions with gp120, the mechanisms that lead to modest (μM) versus supreme (pM) potency are poorly understood. Potency is typically attributed to multivalency; however, the majority of antiviral lectins are oligomeric, so multivalency alone cannot explain observed differences in efficacy. Here we sought to investigate the structural and biophysical properties required for potent inhibition of HIV by studying interactions with viral particles and fusion-competent HIV envelope glycoproteins. To rationally address these questions, we selected five lectins that differ in carbohydrate specificity and valency, oligomeric state, and binding-site geometry. After comparing their binding affinities to gp120 and potency in HIV infectivity assays, we used cryo-electron microscopy (cryo-EM) and dynamic light scattering (DLS) to observe their effects on viral particles. For the first time our data show that lectins have very different effects on viral particles, and these can be explained by the geometry and valencies of individual carbohydrate-binding sites. Our results suggest that specificity for the HIV glycan shield is achieved through multiple layers of multivalency that are so far unique to non-mammalian lectins.

RESULTS AND DISCUSSION

Structures and Carbohydrate-Binding Site Geometries of Selected Lectins.

The chemical and structural bases of lectin—glycan interactions that lead to differences in affinity and neutralization potency are not well understood. We hypothesized that determinants such as binding-site valency, protein oligomeric state, and relative binding-site geometry would govern mechanisms of binding that may translate to potency. To rationally investigate these traits, we selected five unrelated mannose-binding lectins, including GRFT, BanLec, CV-N, and GNA, and a monomeric form of GRFT (mGRFT), which served as a negative control. The carbohydrate-binding specificities of each lectin have been determined by NMR titration studies, glycan array profiling, and isothermal titration calorimetry. (Glycan array binding profiles for each lectin are shown in Figure S1 of the Supporting Information.) The three-dimensional structures, carbohydrate recognition epitopes, and multivalent features of each are compared in Figure 2. GRFT, the most potent anti-HIV protein known to date, is a homodimer. Each subunit contains three cofacial mannoside-binding sites that are arranged in an equilateral triangle, and the carbohydrate-binding faces of each monomer are approximately 180° opposed.²⁴ CV-N is a C_2 pseudo symmetric monomer containing two carbohydrate-binding sites specific for a minimal disaccharide epitope, Man α 1–2Man. These structures terminate the arms of high-mannose-type glycans (Man $_8$ GlcNAc $_2$ and Man $_9$ GlcNAc $_2$). The binding sites are located on the same face of the protein and separated by 36 Å.²⁵ BanLec is an oligomeric protein possessing the plant lectin beta prism I2 fold.²⁶ Each subdomain contains two predominant mannose-binding sites, and

BanLec's carbohydrate-binding faces are nearly opposed. GNA, on the other hand, is a tetrameric protein with mannose binding sites distributed throughout the protein surface, displayed in all directions.²⁷

Lectins Neutralize HIV-1 with Large Differences in Potency.

We assessed the neutralization potency of GRFT, CV-N, BanLec, GNA, and mGRFT in single-round HIV entry assays using two subtype B Env pseudoviruses, SF162 and YU-2, and TZM-bl target cells as previously described.²⁸ All lectins inhibited HIV strains in a dose-dependent manner (Figure S2), and their 50% inhibitory concentrations (IC₅₀) are summarized in Table 1. GRFT, BanLec, and CV-N showed sub-nanomolar IC₅₀ values and were uniformly less potent toward the primary virus strain YU-2, with BanLec and CV-N displaying low nanomolar IC₅₀ values and GRFT, picomolar IC₅₀ values. These values are consistent with published data and our own unpublished results where we reproducibly find GRFT to be the most potent HIV inhibitor among many other lectins. GNA and mGRFT were the least potent.

Lectins Bind Immobilized HIV-1 Env Trimer gp140 with Comparable Affinities.

Each of these lectins blocks HIV infection through interactions with the HIV Env glycoprotein gp120. To compare lectin—Env interactions, we used surface plasmon resonance (SPR) to measure affinity and kinetics of binding of the lectins to trimeric HIV-1 Env protein gp140. Recombinant glycosylated HIV-1 gp140 was immobilized at low density to prevent mass transfer and on-chip cross-linking that may occur through gp140—lectin interactions (see Material and Methods). Binding experiments were carried out using serial dilutions of lectins. Sensorgrams and best-fit curves are shown in Figure 3. The K_d values for lectin binding to gp140 were quite similar, ranging from 2 to 20 nM (Table 1). Thus, in contrast to disparate potencies for inhibition of viral infection (Table 1), there is little variability in affinities among these lectins when binding their target protein gp140. Comparisons between K_d and IC₅₀ values for individual lectins also were revealing; whereas these values differ by only 2–3-fold for BanLec and CV-N, the fold difference between affinity and inhibition for GRFT ranges from 100- to >700-fold. These data are consistent with the notion that differential modes of multivalent binding and inhibition may be occurring among these structurally diverse proteins.

GRFT and BanLec Facilitate Aggregation of Viral Particles.

Some classes of lectins are known to agglutinate cells and pathogens by binding to glycans on the cell surface. We thus sought to determine whether any of these mannose-binding lectins were capable of aggregating viral particles, which could shed light on the effects that valency and binding-site location may play. We used DLS to measure the particle size of HIV-1 virions in the presence and absence of lectins. As summarized in Table 2, only GRFT and BanLec were able to aggregate HIV-1 virions to a measurable extent compared to untreated virions. Viral particles in the absence of lectin had a mean diameter of 180 nm (Table 2), whereas in the presence of GRFT and BanLec the mean diameters were 240 and 260 nm, respectively. In the presence of CV-N, mGRFT, or GNA, no change in the mean virion diameter was observed.

Geometric Constraints Lead to Distinct Effects on Viral Particles.

DLS data indicated that BanLec and GRFT were able to aggregate HIV particles. We hypothesized that this was due to interviral interactions mediated by GRFT or BanLec and high-mannose-type glycans on gp120s. However, it remained possible that lectin—gp120 interactions could manifest in other ways including interactions between Env spikes within virions or within Env trimers. To visualize the effects of each lectin, viral particles were combined with individual lectins and visualized using cryo-EM. Control experiments in the absence of lectin showed spherical virions with an approximate diameter of 100 nm. In the absence of lectin, virions appeared as individual particles and were uniformly distributed in the imaging field (Figure 4a–c and Table 3). The inner and outer leaflets of the viral membrane were ordered, and individual Env spikes could be identified, consistent with previous studies.^{29,30} Cryo-EM showed that addition of lectins to virions had no effect on virion size or integrity. However, treatment with GRFT led to the formation of viral aggregates, and density corresponding to protein was present at the interfaces between neighboring virions (Figure 4d–f). The effects of BanLec on viral particles were similar to those of GRFT except that layers of particles could be viewed in some fields (Figure S4, Supporting Information). In contrast, CV-N did not induce virion aggregation; instead, remarkable differences were observed in the size and localization of Env spikes. After treatment with CV-N, extensive patches of electron density measuring up to 50 nm in width were observed on the viral membrane (Figure 4g–i). This indicated that CV-N is capable of clustering Env trimers on the surface of viral particles. Micrographs of GNA-treated virions showed mostly monodisperse particles (Figure S4, Supporting Information). Although some particles appeared to have spikes with slightly higher density and irregular shape compared to the control, the changes were modest compared to the effects of CV-N.

To support the cryo-EM studies, we used TEM and immunostaining to localize CV-N and GRFT on lectin-treated virions. This was accomplished by probing CV-N or GRFT-treated virions with primary antibodies against their corresponding lectins and secondary antibodies coupled to gold nanoparticles. The results supported the cryo-EM and light scattering data. For CV-N gold nanoparticles were localized to large patches of electron density at the virion surface, and for GRFT nanoparticles were localized between individual particles that were part of larger aggregates (Figure S5, Supporting Information). Together these data indicate that the relative location of carbohydrate-binding sites on lectins dictates their effect on viral particles. GRFT and Ban-Lec, lectins in which multivalent carbohydrate-binding domains are opposed, are able to bridge viral particles, leading to aggregation, whereas CV-N, the carbohydrate-binding sites of which are located on the same face of the protein, effectively clusters Env spikes on the surface of single virions.

Lectin-Mediated Cross-Linking of gp140 Correlates with Aggregation of Viral Particles.

SPR measurements showed that all lectins bind trimeric HIV-1 gp140, whereas cryo-EM and DLS experiments showed virus aggregation to be a function of carbohydrate-binding site geometries. To independently establish whether virus aggregation is mediated by the ability of lectins to cross-link trimeric Env or gp140, we designed a two-step SPR binding and capture experiment shown schematically in Figure 5. As in the first direct binding experiment (Figure 3), gp140 was immobilized on a CM5 chip and solutions of each lectin

were injected, allowing capture by gp140 and formation of a gp140:lectin complex. Immediately following lectin capture, various concentrations of gp140 were subsequently injected into each flow cell, allowing for binding to the gp140:lectin complex. Lectins capable of cross-linking gp140 are predicted to show a second binding event, and those incapable of cross-linking would not. The sensorgrams are shown in Figure 5. As predicted by the DLS and cryo-EM results, we found that GNA and mGRFT were unable to bind a second gp140 molecule. In contrast, GRFT and BanLec showed appreciable binding with the second injection of gp140, indicating the occurrence of intermolecular cross-linking, consistent with their effects on viral particles. CV-N was able to capture a second gp140 molecule albeit to a small degree compared to GRFT or BanLec. The dissociation rates for the ternary complexes comprising gp140:lectin:gp140 were extremely slow, preventing measurement of the off-rates and corresponding affinity constants for the second binding event. However, qualitative estimates could be made by fitting the data for the gp140 capture to a 1:1 binding model to give the values listed in Figure 5. It is interesting to note that for GRFT, in particular, binding of the second equivalent of gp140 (that corresponds to cross-linking) occurs with a much slower off-rate compared to the first binding event, and a picomolar K_d is estimated. A similar result is seen for BanLec and CV-N with estimated K_d values for a second binding event being lower than a single one. Others have made similar observations of significant decreases in dissociation rates driving increases in avidity for bi- or multivalent interactions.^{31–33}

Effects of Subdomain Valency on gp140 Cross-Linking and Virus Aggregation by GRFT.

Results from the SPR capture and cryo-EM experiments indicate that virus aggregation occurs when carbohydrate-binding faces have opposing orientations but do not address the effects that subunit valency have on cross-linking or aggregation. Li-Wang and co-workers previously showed that disruption of any one mannose-binding site on GRFT reduced potency by 1–2 orders of magnitude.³⁴ To determine the effect of carbohydrate-binding site valency on gp140 cross-linking and aggregation of viral particles, we evaluated single-site GRFT mutants using the SPR capture assay and DLS, respectively. Shown in Figure S6 (Supporting Information), none of the mannose-binding site mutants were able to capture a second gp140 trimer, nor did they aggregate virions (data not shown). Thus, in addition to possessing opposing glycan-binding faces, trivalency is necessary for cross-linking of Env and virion aggregation.

DISCUSSION

Compared to the enormous number of lectins found in nature, a very small subset shows great promise as antivirals. To realize their therapeutic potential, it is important to define the specific mechanisms by which they block viral infection, attain potency, and achieve selectivity for enveloped viruses over normal cells. When considering carbohydrate–protein interactions in biology, it is well appreciated that multivalency is employed. However, the chemical and structural details that lead to carbohydrate-receptor recognition have largely been overlooked. Here, using complementary biophysical methods and cryo-EM, we have shown that a select group of mannose-binding lectins is uniquely capable of cross-linking HIV Env glycoproteins, and their subsequent effects on actual virions is critically dependent

on a combination of geometric constraints enforced by both neutralizing lectin and carbohydrate, as well as valency. For GRFT and BanLec, an architecture wherein carbohydrate recognition faces are oriented in opposing directions permits bridging of separate virions that facilitates virus aggregation. However, it is not so simple because an additional level of multivalent interactions is required. In the case of GRFT, studies with rationally designed glycopeptides that recapitulate Man-9 binding and function,³⁵ as well as crystal structures with synthetic mannosides,²⁴ established that stoichiometric binding between the three arms of Man-9 and one GRFT subunit was impossible. Instead, those studies indicated that two arms of Man-9 could engage any two of the three mannose-binding sites while the third site binds to a second Man-9. This unique arrangement is depicted in Figure 6.

BanLec and GRFT had similar effects on Env glycoproteins and virions. Although each subunit of BanLec contains two rather than three closely spaced high-affinity mannose-binding sites (Figure 2), a recent study concluded that BanLec engages separate glycans in each of its two binding sites. Thus, BanLec should be engendered with the ability of participating in layers of multivalent binding, facilitating intermolecular cross-linking interactions comparable to GRFT and consistent with our results.

The finding that CV-N preferentially clusters viral spikes present on the surface of virions, rather than aggregating viral particles, can be rationalized by its carbohydrate specificity and binding-site architecture. CV-N contains two binding sites that strictly require the minimal disaccharide epitope $\text{Man}\alpha(1-2)\text{Man}$ for binding. These structures comprise the terminal branches of Man-8 and Man-9. Because the two binding sites are separated by a distance that exceeds the distance between any two arms of Man-8 or Man-9, stoichiometric binding is precluded and intermolecular CV-N:Man-9 cross-linking occurs.³⁶ Distinct from GRFT and BanLec, however, the two carbohydrate-binding sites on CV-N are located on the same face of the protein. This combination of binding site locations and the inability of CV-N to bind single terminal mannose units must place greater restrictions on the orientation and display of the high-mannose glycans that CV-N can simultaneously recognize. The cryo-EM images indicate that for virions this stringency manifests in CV-N tethering neighboring viral spikes as opposed to another virion (Figure 6). It is interesting to note that Moulai et al. recently described GRFT tandemers wherein monomeric domains were tethered by linkers.²⁹ In those studies cryo-EM showed similar clustering of glycoprotein spikes but only when treated with a construct comprising two GRFT monomers separated by a flexible linker, an arrangement that is more similar to CV-N.

In closing, cumulative data now support a view wherein multiple layers or dimensions of multivalency are required for potent HIV neutralization. These can be dissected through a combination of chemical and structural studies, paired with functional assays and biophysics. Moving forward, GRFT has shown the greatest promise for therapeutic development, as independent laboratories have demonstrated its lack of toxicity and immunogenicity.^{15,37,38} Although in vivo evaluations of CV-N support its safety as a microbicide,³⁹ others have noted toxicity for CV-N and BanLec in in vitro experiments.^{26,40} However, a recent study showed that for BanLec cytotoxicity can be decoupled from mitogenic activity through site-specific mutations in and around the carbohydrate-binding

sites.²⁶ It is through these detailed studies that the outstanding antiviral potential of non-human lectins may be realized.

MATERIALS AND METHODS

Protein Production.

Synthetic genes encoding 6H-2G-GRFT, 6H-BanLec, 6H-G2-mGRFT, or CV-N were used for recombinant expression in the BL21(DE3) strain of *Escherichia coli*. Proteins were expressed in bacteria grown in Luria-Bertani broth. Five hours post induction with 1 mM isopropyl β -D-1-thiogalactopyranoside at 37 °C, cells were harvested by centrifugation (6000g). 6H-G2-GRFT and 6H-BanLec were suspended in 20 mM Tris-HCl, 10 mM benzamidine, and 8 M urea, pH 7.4, and lysed by sonication. Following centrifugation (60 min, 20000g), the supernatant containing 6H-2G-GRFT or 6H-BanLec was loaded onto a Ni chelating column equilibrated in 20 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole, and 8 M urea, pH 7.4, washed with 10 column volumes of the same buffer, and eluted with a linear gradient from 20 to 500 mM imidazole. Fractions containing 6H-G2-GRFT or 6H-BanLec were combined and added dropwise to a stirred flask containing PBS overnight to allow for refolding. Each protein was cleaved using 1.5 units of thrombin per milligram of protein at 37 °C overnight. The resulting GRFT contained two glycine residues at the N-terminus that were necessary for removal of the His tag. Buffer was exchanged to 20 mM Tris-HCl, pH 7.4 or 8.0 for GRFT and BanLec, respectively. Proteins were then purified on a monoQ 10/100GL column (GE Healthcare) equilibrated in the same buffer and eluted with a linear gradient from 0 to 1 M NaCl, pH 7.4 or 8.0 for GRFT or BanLec, respectively.

Monomeric GRFT and CV-N purification did not require the unfolding step. mGRFT was purified in a similar manner as GRFT, skipping the unfolding and folding steps. CV-N was purified using a C18 column equilibrated with 0.05% trifluoroacetic acid and eluted with a step gradient of 20% increments of acetonitrile. Fractions were lyophilized, and CV-N was further purified by size exclusion chromatography using a HiLoad 16/60 Superdex 75 (GE Healthcare). Fractions containing lectins were combined and concentrated in a 3500 or 5000 Da molecular weight cutoff concentrator and stored at 4 °C until further use. GNA was obtained from Sigma. Protein concentrations were determined from A_{280} values (11920 cm⁻¹ M⁻¹ per GRFT subunit, 17420 cm⁻¹ M⁻¹ per BanLec subunit, 10220 cm⁻¹ M⁻¹ for CV-N, and 34964 cm⁻¹ M⁻¹ per GNA subunit). Samples were analyzed by AUC (Figure S7) to confirm the oligomerization state of each lectin. For the studies here, results are presented in concentration of monomer lectin subunits unless specified otherwise. Trimeric gp140 clade A HIV-1 92/UG/037 was obtained from Polymun. Alditrithiol-2-inactivated HIV-1_{BAL} virions at an estimated 10¹¹ virions mL⁻¹ were obtained from the AIDS and Cancer Virus Program, Leidos Inc., Frederick National Laboratory of Cancer Research, Frederick, MD, USA.

Single-Cycle Pseudo-HIV-1 Viruses TZM-bl Cell-Line Based Neutralization Assay.

HIV-1 pseudotypes were generated using pSG3^{env} plasmids (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Drs. John C. Kappes and Xiaoyun Wu) co-transfected

with HIV Env expressing plasmid pYU-2, pBaL, or pSF162 into 293 T cells using XtremeGene (Roche).

Single-cycle neutralization assays were performed as described previously.^{28,41} Briefly, serial dilutions of each lectin were added to 40 mL of cDMEM (DMEM augmented with 10% FBS and 1 mg mL⁻¹ penicillin/streptomycin) to individual wells in a 96-well microtiter plate. To each well was added 40 mL of virus followed by 20 mL of TZM-bl cells (5×10^5 cells mL⁻¹). Twenty-four hours after infection, 150 mL of cDMEM was added, and plates were incubated at 37 °C for an additional 24 h. Viral infectivity was visualized by measuring the activity of firefly luciferase. Inhibitory concentrations (IC₅₀) were calculated as the concentration needed to reduce 50% relative light units (RLU) compared to viral controls. All experiments were performed in triplicate.

Virus Stocks and Antibodies.

Alditrithiol-2-inactivated HIV-1_{BAL} virions at an estimated 10^{11} virions mL⁻¹ were obtained from the AIDS and Cancer Virus Program, Leidos Inc., Frederick National Laboratory of Cancer Research. Primary rabbit polyclonal anti-CV-N antibodies were produced by AnaSpec, Inc., and anti-GRFT antibodies were a generous gift from Dr. Barry O'Keefe (NCI Frederick). The secondary gold-labeled anti-rabbit goat antibody was purchased from Nanoprobe.

Surface Plasmon Resonance.

Measurements were made using a Biacore T100 (GE Healthcare) system. In one flow cell recombinant gp140 derived from clade A HIV-1 strain 92/U/037 (Polymun Scientific) was covalently linked to a carboxymethylated dextran matrix (CM5 chip) using EDC/NHS coupling to a final loading of 240 response units (RU). A reference cell was modified with ethanolamine and used as a control for nonspecific binding and refractive index changes. The assays were performed in PBS containing 0.05% surfactant P20, pH 7.4, at 25 °C. All samples were prepared in filtered and degassed buffer. For direct binding assays, 2-fold serial dilutions of lectins ranging from 2 μ M to 0.25 nM were used as analyte. Solutions were injected over the gp140 surface at a flow rate of 20 mL/min for 1 min followed by flowing running buffer through the cell for 3 min. A duplicate injection and several buffer injections (blanks) were used for a positive control and double referencing, respectively. The sensor chip was regenerated by injection of 2 M D-(+)-mannose (Sigma) or 10 mM NaOH for 30 s, followed by running buffer. For capture binding assays, a 100 nM solution of lectin was injected over the chip for 60 s at a flow rate of 20 mL/min. Next, 2-fold serially diluted gp140 solutions ranging from 40 to 0.62 nM were used as analyte to the now immobilized gp140 trimer:lectin complex. Solutions were injected over this surface for 1 min followed by flowing running buffer through the cell for 3 min. Again, a duplicate injection and several buffer injections (blanks) were used for a positive control and double referencing, respectively. The sensor chip was regenerated by injection of 2 M D-(+)-mannose (Sigma) for 30 s, followed by running buffer. Association (k_{on}) and dissociation (k_{off}) rates of the lectins were determined using a 1:1 binding curve. Separate experiments were performed on a higher density chip with gp140 immobilized to 400 RUs (data not shown). Those

sensorgrams were similar to those for the lower density chip, indicating that no rebinding or mass transfer effects were taking place on either chip.

Analytical Ultracentrifugation (AUC).

Sedimentation velocity experiments were conducted at 50,000 rpm and 20.0 °C on a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge. Lectin samples dissolved in PBS at various concentrations were loaded into two-channel, 12 mm path length sector shaped cells and thermally equilibrated at zero speed. Absorbance and interference velocity scans were subsequently acquired at approximately 5 min intervals. Absorbance data were collected in a continuous mode as single measurements at 280 or 230 nm, depending on the sample concentration, using a radial spacing of 0.003 cm. Data were analyzed in SEDFIT 15.01bq⁴² in terms of a continuous $c(s)$ distribution of sedimenting species using an s range of 0–12 with a linear resolution of 240 and a maximum entropy regularization confidence interval of 0.68. In all cases, excellent fits were observed. The partial specific volumes of the various lectins were calculated on the basis of the amino acid composition using SEDNTERP (<http://sednterp.unh.edu/>);⁴³ the solution density and viscosity were also calculated in SEDNTERP. Sedimentation coefficients were corrected to standard conditions at 20.0 °C in water, $s_{20,w}$.

Dynamic Light Scattering Measurement.

Solutions containing 2×10^{10} virions mL⁻¹ and 15 μ M lectin were incubated at room temperature for 30 min. DLS measurements were made on a Brookhaven Instruments Corp. BI-200 goniometer equipped with a BI-9000AT digital autocorrelator and a Spectra Physics Stabilite 2017 argon ion laser operating in the TEM₀₀ mode at 488 nm and constant power (50 mW). DLS autocorrelation functions were accumulated in batch mode for 2–4 min at an angle of 90°, a temperature of 20 °C and a photomultiplier tube aperture of 200 nm. Data were analyzed in terms of a regularized CONTIN distribution of hydrodynamic radii using the Brookhaven Instruments 9KDLSW 2.12 software.

Transmission Electron Microscopy.

Solutions containing 2×10^{10} virions mL⁻¹ and 15 μ M lectin were combined and incubated at room temperature for 30 min. Aliquots (5 μ L) were absorbed onto carbon film applied to lacey carbon supports on 300 mesh copper grids and rinsed with water; primary antibodies (anti-GRFT and anti-CV-N, 1:1000 dilution) were allowed to bind for 1 h and washed three times with PBS; and then donkey anti-rabbit antibodies labeled with 6 nm gold nanoparticles were allowed to bind for 1 h and washed two times with PBS and one time with water. Immunostained virions were exposed to 0.5% uranyl acetate for 1 min for negative staining. Images were acquired with an FEI Morgani transmission electron microscope, operating at 80 kV and equipped with an ATM Advantage camera. Images were recorded at 11,000 $\times$ magnification.

Cryo-electron Microscopy.

Solutions for cryo-EM were prepared identically to those used for DLS. After 30 min of incubation, a 3.5 μ L aliquot was delivered to a plasma-cleaned (Fishione Inc.) Quantifoil

holey carbon EM grid (SPI Supplies), blotted with filter paper, and flash-frozen in liquid ethane using a Leica EM GP (Leica Microsystems). The grids were subsequently stored in liquid nitrogen. The vitrified samples were imaged at liquid nitrogen temperature on a TF20 electron microscope (FEI) operating at 120 kV and recorded with a Gatan 4K × 4K camera. Images were recorded using low-dose settings in Digital Micrograph. Distances between virions (Table 3) were measured using ImageJ software. Totals of 533, 360, and 507 virions were analyzed for control and CV-N- and GRFT-treated viral particles. A cutoff of 5 nm was apparent in a plot of distances as a function of the number of particles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CV-N	cyanovirin-N
GRFT	griffithsin
BanLec	banana lectin
mGRFT	monomeric griffithsin
SPR	surface plasmon resonance
cryo-EM	cryo-electron microscopy

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Figure 1.

Glycosylated HIV-1 Env gp120-gp41: X-ray structure of trimeric gp120-gp41 with Man₉GlcNAc₂ (Man-9) modeled onto known oligomannose-containing Asn residues.⁵ Panels A and B show the trimer from the side and top of the envelope spike with gp120 and gp41 displayed as yellow and gray ribbons and Man-9 as spheres with Man and GlcNAc residues colored green and blue, respectively. The structure and symbolic representation of Man-9 are shown in panels C and D, respectively.

Figure 2.

Structures, valencies, and carbohydrate specificities of mannose-binding lectins. (A) Surface representation of lectins with domains colored blue and yellow. Mannose residues are shown as spheres with C and O atoms colored gray and red, respectively. Oligomeric states of lectins and number of carbohydrate-binding sites per domain are shown in (B) and (C), respectively, with the total number of binding sites in parentheses. (D) Symbolic representations of Man-9 with mannose specificity indicated by red circles and preferred binding fragments denoted with red lines and listed in (E).

Figure 3.

Surface plasmon resonance sensorgrams of lectin binding immobilized trimeric gp140. (A) Schematic representation showing trimeric gp140 (gray with glycans as green sticks) covalently immobilized on the surface of the CM5 chip and lectin (in orange) flowing over the surface. (B) Sensorgrams showing association and dissociation curves for each of the lectins used in this study. All experiments were performed in duplicate, and serial dilutions of 10 different concentrations were run over gp140 for each lectin. The black lines show the theoretical curves for all runs.

Figure 4.

Cryo-EM images of HIV-1 virions in the absence and presence of lectins. (A–C) In the absence of lectins virions appear as individual particles and envelope spikes are observed on the membrane surface, indicated by white arrows. (D–F) In the presence of GRFT virion aggregation is apparent and electron density for proteins is observed between virions (red arrows). (G–H) In the presence of CV-N individual spikes on virions are no longer observed. Instead, dense masses several nanometers in width are observed on virion surfaces, indicated by red arrows in panels H and I. Scale bars = 100 nm. Distances between virions were measured using ImageJ. Quantification of the number of aggregated particles is presented in Table 3.

Figure 5.

Detection of gp140 capture by HIV-1 Env gp140:lectin complexes. (A) Schematic illustrating SPR capture assay. Gp140:lectin complexes were formed on sensor chips. Complexes were then subjected to a separate injection of trimeric gp140. (B) Sensorgrams showing the association and dissociation curves reveal second binding events that correspond to gp140 capture by the gp140:lectin complexes. All experiments were performed in duplicate with eight serial dilutions of gp140 run over each lectin complex. The black lines show the theoretical curves for all runs. Dissociation for GRFT, BanLec, and CV-N is very slow, allowing for qualitative K_d values only. mGRFT and GNA show no binding, indicating that these two lectins are not able to cross-link gp140.

Figure 6.

Schematic showing unique modes of multivalency and binding site geometry lead to differential effects on viral particles. (A) GRFT: the three mannose-binding sites on a GRFT subdomain engage two Man-9 units of gp140. Interactions with another Env spike effectively through an opposing subdomain effectively bridges viral particles. (B) CV-N: binding sites located on the same face of the protein and separated by 36 Å are positioned to engage glycans on neighboring Env trimers, disfavoring aggregation of virions and leading to clustering of viral spikes.

Table 1.IC₅₀ and *K_d* Values for Lectins^a

	IC ₅₀ (nM)				<i>K_d</i> (nM)
	HIV-1 strain SF162	HIV-1 strain BaL	HIV-1 strain YU-2		
GRFT	0.0064 ± 0.0002	0.114 ± 0.05	0.044 ± 0.003	4.3 ± 0.1	
mGRFT	16.3 ± 11.7	49.0 ± 2.7	44.1 ± 5.6	17.5 ± 0.8	
BanLec	0.95 ± 0.07	0.91 ± 0.06	2.6 ± 0.2	5.2 ± 0.1	
CV-N	0.37 ± 0.07	0.73 ± 0.08	1.14 ± 0.09	2.3 ± 0.1	
GNA	6.5 ± 1.7	126 ± 18.5	127.0 ± 17.5	7.2 ± 0.1	

^aIC₅₀ values were measured in single-round neutralization assays against the strains listed (see ref 28 and Materials and Methods).

Table 2.**Aggregation of HIV-1 Virions by Lectins^a**

lectin	mean diameter (nm)	aggregation
no lectin	180 ± 20	no
GRFT	240 ± 110	yes
BanLec	260 ± 80	yes
CV-N	170 ± 10	no
GNA	170 ± 20	no
mGRFT	160 ± 4	no

^aParticle size was determined by DLS (Figure S3, Supporting Information), and solutions were prepared as described under Materials and Methods. Note that DLS provides a mean virion diameter of 161–177 nm in the absence of aggregation, whereas cryo-EM gives an approximate diameter of 100 nm. DLS analyzes the whole solution, whereas EM selects individual particles.

Table 3.

Effects on Viral Particles by Cryo-EM

	control (<i>n</i> = 533)^a	CV-N (<i>n</i> = 360)	GRFT (<i>n</i> = 507)
0–5 nm ^b	20.5	18.6	89.7
>5 nm ^c	79.5	81.4	10.3

^a *n* = number of virions counted manually using ImageJ software. Every particle on a given grid was counted.

^b Percentage of viral particles separated by ≤ 5 nm, defined as aggregated.

^c Percentage of viral particles separated by >5 nm, appearing as individual particles.