The Role of Individual Carbohydrate-Binding Sites in the Function of the Potent Anti-HIV Lectin Griffithsin

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S Supporting Information

ABSTRACT: Griffithsin (GRFT) is a lectin that has been shown to inhibit HIV infection by binding to high mannose glycan structures on the surface of gp120, and it is among the most potent HIV entry inhibitors reported so far. However, important biochemical details on the antiviral mechanism of GRFT action remain unexplored. In order to understand the role of the three individual carbohydrate-binding sites (CBS) in GRFT, mutations were made at each site (D30A, D70A, and D112A), and the resulting mutants were investigated. NMR studies revealed that each GRFT variant was folded but showed significant peak movement on the carbohydrate-binding face of the protein. The wild-type and each point mutant protein appeared as tight dimers with a K_d below 4.2 μ M. Mutation of any individual CBS on GRFT reduced binding of the protein to mannose, and ELISA assays revealed a partial loss of ability of each GRFT point mutant to bind gp120, with a near-complete loss of binding by the triple mutant D30A/D70A/D112A GRFT. A more quantitative surface plasmon resonance (SPR) examination showed a rather small loss of binding to gp120 for the individual GRFT point mutants (K_D : 123 to 245 pM range versus 73 pM for wild-type GRFT), but dramatic loss of the triple mutant to bind gp120 derived from R5 and X4 strains (K_D > 12 nM). In contrast to the 2- to 3-fold loss of binding to gp120, the single CBS point mutants of GRFT were significantly less able to inhibit viral infection, exhibiting a 26- to 1900-fold loss of potency, while the triple mutant was at least 875-fold less effective against HIV-1 infection. The disparity between HIV-1 gp120 binding ability and HIV inhibitory potency for these GRFT variants indicates that gp120 binding and virus neutralization do not necessarily correlate, and suggests a mechanism that is not based on simple gp120 binding.

KEYWORDS: griffithsin (GRFT), lectin, HIV entry inhibitor, microbicide, carbohydrate binding site (CBS), nuclear magnetic resonance (NMR), mannose titration, analytical ultracentrifugation, surface plasmon resonance (SPR)

INTRODUCTION

In the fight against HIV/AIDS, HIV entry inhibition is increasingly important. To inhibit entry of the virus, general strategies include the development of drugs that interact with the HIV envelope proteins gp120 and gp41, or that interact with human cell surface proteins that are the targets of the virus, such as the coreceptor CCR5.^{1–3} One important class of potent HIV entry inhibitors is the lectin class, which bind to the glycans that cover the surface of HIV gp120. In particular, the branched mannose oligomers Man-8 and Man-9 on HIV-1 gp120 are the likely target of inhibitors such as cyanovirin-N and griffithsin (GRFT),^{4–9} each of which are highly potent HIV inhibitors in cell culture.

GRFT is a lectin derived from a red alga and is among the most efficient anti-HIV lectins, showing low nanomolar inhibition in cell fusion assays and subnanomolar inhibition against single round and replication-competent HIV.³ GRFT has been shown to be produced easily in gram quantities,¹⁰ and

to be nonirritating and largely unable to activate cellular receptors under conditions necessary for clinical use.^{11,12} Therefore, this protein is considered as a leading drug candidate to prevent the sexual spread of HIV.

GRFT has been crystallized as a domain-swapped dimer, and several high resolution structures have shown three putative carbohydrate-binding sites (CBS) on each monomer (Figure 1).^{4–6,13} Each site appears to be able to bind only one glycan unit, but affinity studies with monosaccharides show only a micromolar binding potential. Therefore, some combination of glycan binding by the multiple sites on GRFT, rather than by individual sites, seems to be necessary to achieve high levels of virus inhibition. Mechanistic work on the particular sites of

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Figure 1. Left: The structure of the GRFT dimer in complex with mannose.⁵ The arrows denote the three mannose binding sites of one monomer. Right: The structure of Man-9,⁹ an oligosaccharide on the surface of gp120 that is likely bound by GRFT. The protein structure was made with the program Chimera³⁴ using PDB structure 2gud.

glycosylation of gp120 reveals the likely importance of N-linked carbohydrates at N295, N234, and N448 in the action of GRFT.^{14–16} Calculations have been carried out suggesting that all three sites on a GRFT subunit could indeed be used simultaneously to bind the three terminal mannose residues of the branched Man-9 of gp120.⁶ However, more recent structural work by these research groups shows that an engineered GRFT monomer “1GS-S” in complex with nona-mannoside does not appear to have all three sites bound simultaneously to the three terminal mannose groups of a high-mannose glycan. In this structure, two of the terminal mannoses bind in two sites on GRFT (pocket 1 and pocket 3, using the nomenclature from Ziolkowska 2006^{4,5}), while the pocket 2 site was bound by a mannose from another symmetry related nona-mannoside.⁴ This work indicated that this pocket 2 site (with key residue Asp30) was not equivalent to the other two sites within the monomeric subunit, and emphasized the role of the GRFT dimer by showing that a monomeric variant was greatly diminished in its ability to inhibit HIV.⁴

Therefore, an outstanding question regarding the mechanism of HIV inhibition by GRFT is the role each of the three putative carbohydrate-binding sites plays in the affinity of the protein for its mannose target and subsequent anti-HIV function. Here, we show the effect on the structure and function of GRFT when each individual CBS is mutated. We also present NMR chemical shift assignments of wild-type GRFT and describe further biochemical investigations of the wild-type protein and its mutants, including analytical ultracentrifugation, surface plasmon resonance and assessment of carbohydrate binding and anti-HIV function of wild-type and mutant GRFT.

■ EXPERIMENTAL METHODS

DNA Construction. Single site mutations of GRFT (D30A, D70A, D112A) were made using Stratagene QuikChange Site-Directed Mutagenesis or using a standard thermocycling strategy. Genes of GRFT variants were cloned into the pET-15b expression vector (Novagen) between the *Nco*I and *Bam*HI cleavage sites. The triple mutation of GRFT (D30A/D70A/D112A) was made using a standard thermocycling strategy, and all mutants were confirmed by DNA sequencing.

Protein Production and Purification. Plasmids with an N-terminal histidine-tag were transformed to *Escherichia coli* BL21(DE3) (Novagen) competent cells and expressed in minimal media with ¹⁵NH₄Cl as the sole nitrogen source. Each mutant was produced using the following procedure. Protein production was induced upon addition of isopropyl β-D-1-

thiogalactopyranoside (IPTG) with further incubation at 37 °C for 6 h. Cells were harvested by centrifugation at 6000g for 10 min, and the pellet was resuspended in 5 M guanidine hydrochloride, 500 mM NaCl, 10 mM benzimidazole, and 20 mM Tris pH 8; this allowed complete solubilization of proteins from both the inclusion body and the supernatant upon cell disruption. The solution was French pressed twice at 16,000 psi, and then centrifuged at 15000g for 1 h. The soluble portion was loaded onto a Ni chelating column (Qiagen) equilibrated with the same resuspension buffer. Proteins that bind nonspecifically were first eluted in the same buffer in the presence of 50 mM imidazole. These were discarded. Finally, GRFT or its variants were then eluted using 500 mM imidazole, 5 M guanidine hydrochloride, 500 mM NaCl and 20 mM Tris pH 8 and refolded by adding dropwise to low salt refolding buffer (50 mM NaCl, 20 mM Tris pH 8) over the course of 30 min. The solution was dialyzed against in the same refolding buffer at 4 °C overnight. The protein solution was then centrifuged at 15000g for 1 h to remove precipitated material, and purified on a C4 reversed-phase chromatography column (Vydac, Hesperia, CA). The fractions were analyzed on a SDS–PAGE gel to confirm the size and then lyophilized in a Labconco freeze-dry system (Labconco Corporation).

For the D30A/D70A/D112A triple mutation, in some preps a slight variation was used. The cell pellet was resuspended in high salt cracking buffer (500 mM NaCl, 20 mM Tris pH 8) without the presence of guanidinium so that only the supernatant was used without further refolding. The purification continued as described above, in buffers lacking guanidinium using a nickel chelating column, followed by dialysis and C4 column purification.

Concentration of protein was determined using absorbance at 280 nm with an extinction coefficient of GRFT subunit (11920 cm^{−1} M^{−1}, from the ExPASy program located at <http://web.expasy.org/protparam/>) except as described for analytical ultracentrifugation, which also used A₂₃₀. Results indicate the concentration of GRFT subunits (monomers), except for the surface plasmon resonance, which show the concentration of dimers, since those were established here to be the relevant binding unit.

GRFT Binding to D-Mannose-agarose Column. The D-mannose-agarose column was obtained from Sigma (St. Louis, MO) and is composed of a single mannose saccharide bound to the agarose bead, likely through the C6 hydroxyl. GRFT was made to 15 μM in 50 mM Tris pH 7.4 and bound to the column. Elution was carried out with a gradient up to 200 mM mannose, 50 mM Tris pH 7.4.

ELISA Studies of GRFT-gp120 Interactions. To test the binding of each GRFT mutant to HIV gp120, ELISA binding assays were carried out as described previously.^{3,17} In brief, 100 ng of HIV gp120_{ADA} (ImmunoDiagnostic) was coated on each well in a 96 well plate (Maxisorp Immunoplate; Nalge NUNC) overnight at 4 °C. After the plate was washed with 0.5% Tween20 in PBS and blocked with 1% bovine serum albumin for 1 h, serial dilutions of wild-type GRFT or its mutants were added to triplicate wells and incubated for 2 h at 37 °C. After the plate was washed, a 1:1000-fold dilution of horseradish-peroxidase-conjugated nickel-NTA (Qiagen) (which detects the N-terminal his-tag of GRFT and its mutants) was added according to the company's instructions. Then the substrate for horseradish-peroxidase (2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid; ABTS) (Fisher Scientific) was added and signal was measured by absorbance at 405 nm.

Virus Isolates. HIV virions deleted in *vpr* and *env* were used to make pseudo viral particles as follows. Viral plasmids containing the *env* gene from HIV-1 were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, as follows: HIV-1 ADA-M (referred to as HIV-1 ADA, R5) was from Dr. Howard Gendelman.^{18–21} Plasmid pSV-JRFL (R5) was a kind gift from Nathaniel Landau.²² pCAGGS-SF162-gp160 (R5) was from Leonidas Stamatatos and Dr. Cecilia Cheng-Mayer.^{23–25} 293FT cells were kind gifts from Dr. Jennifer Manilay and were originally obtained from Invitrogen. For single round virus production, plasmids with deleted *vpr* and *env* (pNL-luc3-R⁺E⁺ containing the firefly luciferase gene; a kind gift from Dr. Nathaniel Landau²²) were cotransfected into 293FT cells with different HIV strain *env* plasmids using Roche X-treme HP transfection reagent according to the manufacturer's instructions. Supernatant medium containing virions was harvested 48 h post transfection, centrifuged at 1000g, filtered through a 0.45 μ M syringe (Millipore) and stored at –80 °C for later experiments. HIV-1(III_B) was provided by R. C. Gallo, at that time at the National Institutes of Health (Bethesda, MD).

Single-Round Infection Assays. TZM-bl cells stably expressing CD4, CCR5 and CXCR4 coreceptors were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). 10⁴ cells per well were first seeded into a 96-well plate for one day. The medium was then changed 3 h before the assay and made to a volume of 50 μ L per well, and then a serial dilution of GRFT variants was added from the top row to the bottom row, as follows: 20 μ L of GRFT variants of different concentration was added into the first row and mixed well with culture medium. Then 20 μ L of medium was removed and added into the second row, and so on. Virus was then added into each well containing different GRFT variants. The medium was changed in 24 h, and cells were incubated for another 24 h. PBS containing 0.5% NP-40 was used to lyse the cells, and substrate chlorophenol red- β -galactopyranoside [CPRG] (Calbiochem) was added. Signal was measured at 570 nm and 630 nm (absorbance). The ratio of 570/630 for each well was calculated. EC₅₀ values were determined using a linear equation fitted between two data points surrounding 50% inhibition. For presentation purposes, data shown in the figures were plotted and fitted as curves using a four-parameter logistic equation in Kaleidagraph (Synergy Software, Reading, PA).

Anti-HIV Assays in CEM Cell Cultures. CEM cells (5 $\times$ 10⁵ cells per mL) were suspended in fresh culture medium and infected with HIV-1(III_B) at 100 times the CCID₅₀ (50% cell

culture infective dose) per mL of cell suspension, of which 100 μ L was then mixed with 100 μ L of the appropriate dilutions of the test compounds. After 4 to 5 days at 37 °C, HIV-1-induced syncytia formation was recorded microscopically in the cell cultures. The 50% effective concentration (EC₅₀) corresponds to the compound concentration required to prevent syncytium formation by 50% in the virus-infected CEM cell cultures.

Cocultivation Assay. Persistently HIV-1(III_B)-infected HuT-78 cells were washed to remove free virus from the culture medium, and 5 $\times$ 10⁴ cells (50 μ L) were transferred to 96-well microtiter plates. Next, a similar amount of Sup-T1 cells (50 μ L) and appropriate concentrations of test compound (100 μ L) were added to each well. After 2 days of coculturing at 37 °C, the EC₅₀s were quantified by microscopic inspection on the basis of the appearance of syncytia.

NMR Spectroscopy. GRFT variants were expressed in minimal media with ¹⁵NH₄Cl as the only nitrogen source or with ¹⁵NH₄Cl as well as ¹³C labeled glucose as the sole nitrogen and carbon source. These mutants were produced and purified as described above. To dissolve the protein powder after purification, 20 mM sodium phosphate buffer (pH 7.0) with 5% D₂O was used and 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) was added for calibration. Spectra were collected at 25 °C on a four-channel 600 MHz Bruker Avance III spectrometer. The backbone resonances were assigned using standard spectra: 3D HNCO, HN(CA)CO, HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA.^{26–29} Data was processed using NMRPipe.³⁰ The programs PIPP, Sparky and NMRVIEW^{31–33} were used for visualization and chemical shift assignment. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41RR001081).³⁴ Changes in backbone chemical shift ($\Delta\delta_{\text{obs}}$) upon mutation or titration with mannose were determined using the weighted average of changes in ¹H and ¹⁵N, with the equation^{35–37}

$$\Delta\delta_{\text{obs}} = \{[\Delta\delta_{\text{HN}}^2 + (\Delta\delta_{\text{N}}/5)^2]/2\}^{1/2}$$

Surface Plasmon Resonance (SPR) Analysis. Recombinant gp120 proteins from HIV-1(III_B) strain (ImmunoDiagnostics Inc., Woburn, MA), from HIV-1(ADA) strain (ImmunoDiagnostics) and recombinant gp41 protein from HIV-1(HxB2) (Acris Antibodies GmbH, Herford, Germany) were covalently immobilized on the carboxymethylated dextran matrix of a CM5 sensor chip in 10 mM sodium acetate, pH 4.0, using standard amine coupling chemistry. The exact chip densities are given in the Results. A reference flow cell was used as a control for nonspecific binding and refractive index changes. All interaction studies were performed at 25 °C on a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden). The test compounds were serially diluted in HBS-P (10 mM HEPES, 150 mM NaCl and 0.05% surfactant P20; pH 7.4) covering a concentration range between 1.51 and 12.05 nM by using 2-fold dilution steps. Samples were injected for 2 min at a flow rate of 45 μ L/min and the dissociation was followed for 8 min. One duplicate sample and several buffer blanks were used as a positive control and for double referencing, respectively. The CM5 sensor chip surface was regenerated with 1 injection of glycine-HCl pH 1.5. The experimental data were fit using the 1:1 binding model (Biacore T200 Evaluation software 1.0) to determine the binding kinetics. In a separate experiment, both monomeric gp120 and trimeric gp140 derived from HIV-1/JR-

Figure 3. (A) ^{15}N HSQC of mannose titration of GRFT. Resonances for unliganded wild-type protein are shown in black. Orange peaks represent GRFT in the presence of 3 mM mannose; green peaks show the presence of 10 mM mannose; red peaks show the presence of 23 mM mannose (saturated). (B) Structure of GRFT showing regions from one subunit of the protein affected by titration with mannose, as determined by NMR spectroscopy. Yellow indicates residues whose chemical shift changed by 1 standard deviation from the average upon addition of mannose, while red indicates residues whose chemical shift changed by 2 standard deviations, and magenta indicates residues that became impossible to follow upon addition of mannose. These residues are listed in Table S1 in the Supporting Information. Mannose monosaccharides are shown in green. Residues G8 and G9 are from the domain-swapped other subunit. Structure is PDB 2gud obtained from ref 5.

mutant, having the Asp at each of the three CBS mutated to Ala (Figure S3 in the Supporting Information). When this triple mutant D30A/D70A/D112A was titrated with mannose, the HSQC spectra showed essentially no change for any concentration tested, up to 23 mM mannose, indicating no binding (Figure S6 in the Supporting Information).

To determine whether the mutations at each CBS in GRFT altered the dimer affinity, analytical ultracentrifugation was carried out on the wild-type protein and each variant using

sedimentation velocity analysis. To better ascertain whether any mass action effects were detectable, wild-type and mutant GRFT were measured over a wide concentration range. In each case vertical sedimentation coefficient $G(s)$ distributions were obtained with the enhanced van Holde–Weischet analysis,⁴⁴ and had nearly identical sedimentation coefficients (see Figure 5 and Figure S4 in the Supporting Information). Vertical $G(s)$ distributions indicate that all samples were homogeneous.⁴⁹ 2-Dimensional spectrum analysis^{42,43} combined with Monte Carlo analysis⁴⁷ resulted in a molecular weight consistent only with the dimer (see Table 1), even at the lowest concentration tested ($4.2 \mu\text{M}$).

Mutations in the CBS of GRFT Reduce the Ability of GRFT To Bind Mannose and gp120. The ability of wild-type GRFT and its variants to bind mannose was first assessed by using a commercial mannose column, having mannose monosaccharide bound to a standard bead. This column is a crude method of determining mannose binding, since the attachment of each carbohydrate to the bead at C6 precludes protein binding from that direction. Each protein was loaded onto the column and eluted using a mannose gradient. GRFT bound to the column, requiring 50 mM mannose for elution, while mutation at any single CBS resulted in a protein that flowed through without binding the column (data not shown). This indicates that carbohydrate binding was diminished for each mutant, but since the column is composed of a single carbohydrate ring that is not freely available at all sites for binding, further interpretation is not possible.

The most relevant binding interaction for GRFT in terms of HIV inhibition is its ability to bind HIV envelope protein gp120. A straightforward way to measure this interaction is the ELISA assay, in which gp120 is bound to a surface, and varying amounts of GRFT or its mutants are exposed to the gp120. The level of binding between GRFT and gp120 is determined by addition of a peroxidase-conjugated protein that is specific for the N-terminal His-tag on GRFT. (We and others have shown that an N-terminal His-tag does not impede the activity of GRFT^{3,48}). As shown in Figure 6A, wild-type GRFT binds very well to gp120 derived from HIV-1 strain ADA, with clear binding between 10 nM and 100 nM. In contrast, each point mutant binds gp120 less well, requiring 100 nM to 1,000 nM. In general, the GRFT point mutant D70A, having a mutation at the so-called pocket 3,⁴ repeatedly appeared to bind the worst in these experiments under the same conditions, although ELISA experiments are not quantitative. The triple GRFT mutant D30A/D70A/D112A binds very poorly to gp120, showing only a low signal increase at the high-tested amount of $1.28 \mu\text{M}$. Therefore, overall the single point mutants show an approximate 10-fold reduced ability to bind, while the triple mutant binds much more weakly to gp120.

A more quantitative way to determine binding to the HIV-1 envelope is the use of surface plasmon resonance (SPR).^{50,51} In this experiment, binding properties of GRFT and its mutants were evaluated against monomeric gp120 III_B (X4) and gp120 ADA (R5), and also against the glycosylated HIV-1 (HxB2) gp41 fusion protein. All ligands were covalently immobilized on CM5 sensor chips, and 140 RU ($\sim 1.17 \text{ fmol}$), 90 RU ($\sim 0.75 \text{ fmol}$) and 240 RU ($\sim 5.85 \text{ fmol}$) of gp120 density were obtained, respectively. Such low gp120 density chips are required to enable reliable determination of K_D values, since high gp120-density chips may easily allow rebinding of GRFT to another gp120 molecule on the same sensor chip resulting in erroneous k_{off} rate determinations. The binding and release of

Figure 4. (A) The three putative mannose binding sites of GRFT, as determined by ref 5. Mannose saccharide is shown in green; selected residues from griffithsin are shown in blue and labeled. (B) ^{15}N HSQC spectrum of GRFT D30A (red) overlaid with the spectrum of wild-type griffithsin. Some peaks with significant movement upon mutation are labeled. (C) Chemical shift changes of GRFT upon mutation of residues at the mannose binding sites. Some residues broaden or move and cannot be visualized upon mutation of one of the sites so they are not shown. Changes for D30A are shown in red; D70A, purple; D112A, blue. (D) Structure of GRFT showing regions that have chemical shift changes upon mutation of the mannose site at D30. Yellow indicates chemical shift change that is 1 standard deviation greater than average, red indicates chemical shift change that is 2 standard deviations greater than average, and magenta indicates peaks that broaden or otherwise have moved too much to assign upon mutation. These residues are listed in Table S1 in the Supporting Information. Mannose residues are shown in green. Structure is 2gud from ref 5.

GRFT (concentration expressed in dimer units) can be measured in real time to provide on- and off-rates as well as affinity constants (K_D values). As shown in Figure 6B and Table 2, wild-type GRFT binds very tightly to gp120 derived from the X4 strain III_B as well as the R5 strain ADA, with K_D values of 72.7 ± 1.7 pM and 73.2 ± 2.0 pM, respectively. The basis for

the tight binding appears to be a fast on-rate (k_{on}) (about $6.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and a slow off-rate (k_{off}) (about $5 \times 10^{-4} \text{ s}^{-1}$). The binding kinetics for gp120 derived from HIV-1(III_B) and HIV-1(ADA) were very similar regarding the K_D as well as the k_{on} and k_{off} rates (Table 2 and Figure S5A–C in the Supporting Information). Since the native form of gp120 is a trimer in the

Figure 5. Van Holde–Weischet $G(s)$ sedimentation coefficient distributions of GRFT at 4 concentrations: (A) 0.3 OD 230 nm; (B) 0.9 OD 230 nm; (C) 0.3 OD 280 nm; (D) 0.9 OD 280 nm. Filled circles: wild-type. Open squares: D30A. Open triangles: D112A. Open diamonds: D70A. Vertical distributions indicate homogeneity.

viral envelope, the binding of GRFT to trimeric gp140 has also been evaluated side by side, but no significant difference in the K_D value was observed (K_D : 0.111 nM and 0.152 nM, respectively). GRFT also binds well to gp41 with a similar affinity constant compared to gp120, although this viral fusion protein is only exposed at a later stage of the entry process^{52,53} and most likely does not play a relevant role in the eventual anti-HIV activity of GRFT.

While each point mutant of GRFT showed reduced binding to gp120 derived from both X4 and R5 HIV-1 strains, the effect was small, with D30A and D112A generally exhibiting an as little as 2-fold reduction in binding compared to the wild-type protein, while the point mutant D70A showed only a 3-fold reduction in affinity. The triple mutant D30A/D70A/D112A did not show a binding signal when exposed to gp120 at 12 nM (Table 2). However, higher concentrations (i.e., 24 and 48 nM) start to show an interaction signal for binding to gp120 and gp41 (Figure S5D in the Supporting Information).

In an attempt to reveal the binding capacity of wild-type GRFT and its mutants to specific sites of gp120 other than the N-glycans, *E. coli*-expressed nonglycosylated gp160 was bound to the sensor chip, and binding of the GRFTs but also the carbohydrate-recognizing mAb 2G12, the α 1,3/ α 1,6-mannose-recognizing plant lectin HHA and sCD₄ was examined in comparison with their binding to the glycosylated gp120. Whereas binding of GRFT, mutant GRFT, 2G12, HHA and sCD₄ to glycosylated gp120 was observed, no significant

binding for GRFT, mutant GRFT, 2G12 and HHA was found for nonglycosylated gp160 (Figure 6C). Instead, sCD₄ that binds to gp120 without the requirement of the presence of N-glycans could still bind to nonglycosylated gp160 (Figure 6C, middle panel). These data indicate that the interactions of GRFT (and also 2G12 and HHA) are predominantly N-glycan-dependent.

Mutation of the Individual CBS Markedly Reduces the Anti-HIV Activity of GRFT. GRFT and its variants were tested against several strains of HIV using single-round viral assays. As shown in Table 3 and Figure 6D, while wild-type GRFT inhibited HIV strain ADA with an EC_{50} of 10.5 pM, the D30A mutant and D112A mutant were about 1,000-fold less potent (20.3 and 6.57 nM, respectively). The D70A mutant was somewhat a better inhibitor than the other point mutants, with an EC_{50} of 2.63 nM. This is in contrast to the ELISA and SPR results, which showed only a very moderate effect of the mutations for the ability of these GRFT variants to bind gp120 derived from the HIV-1 strain ADA. However, since this set of gp120 binding and antiviral data only includes the ADA strain, it is premature to assume that the superior antiviral activity of the D70A mutant versus the other mutants is a general phenomenon.

Similar functional results were obtained using R5 HIV-1 strain JR-FL, where wild-type GRFT inhibited single round virus infection with an EC_{50} of 72.5 pM. The D30A and D112A variants were approximately 300-fold lower in anti-HIV potency than wild-type GRFT, and the D70A mutant exhibited about a 10-fold greater antiviral potency than the other two point mutants. Again for R5 HIV-1 strain SF162, both D30A and D112A variants were reduced in potency compared to the wild-type protein by several hundred fold, while the D70A mutant was worse than the wild-type protein, but better than the other point mutants. When viral inhibition assays using X4 HIV-1 strain III_B in CEM cell cultures were performed in which inhibition of virus-induced cytopathicity was recorded at day 4 post infection and post addition of the compounds, wild-type GRFT inhibited the virus-induced cytopathicity at an EC_{50} of 0.109 nM, while the point mutants were much worse, ranging from 4.8 to 6.8 nM (Table 3). In this case the D70A variant had quite similar antiviral activity as the other point mutants. In all cases, the triple mutant D30A/D70A/D112A was a very poor antiviral inhibitor, with an EC_{50} of 95.3 nM for the III_B strain, and EC_{50} values being higher than the maximum concentration

Table 1. Molecular Weight of the Major Species Determined with the 2-Dimensional Spectrum Analysis⁴² Combined with Monte Carlo Analysis⁴ for Wild-Type GRFT and Mutant at Four Different Loading Concentrations^a

sample/concn	230 nm		280 nm	
	0.3 OD	0.9 OD	0.3 OD	0.9 OD
wild type	2.71×10^4 (1.584×10^4 , 3.83×10^4)	2.73×10^4 (2.39×10^4 , 3.27×10^4)	2.83×10^4 (2.15×10^4 , 3.11×10^4)	2.98×10^4 (2.70×10^4 , 3.10×10^4)
D30A	3.15×10^4 (3.03×10^4 , 3.20×10^4)	3.04×10^4 (2.81×10^4 , 3.28×10^4)	3.15×10^4 (2.35×10^4 , 3.54×10^4)	3.00×10^4 (2.73×10^4 , 3.22×10^4)
D112A	3.00×10^4 (2.88×10^4 , 3.04×10^4)	2.79×10^4 (2.57×10^4 , 3.01×10^4)	3.07×10^4 (2.91×10^4 , 3.23×10^4)	3.13×10^4 (2.13×10^4 , 4.11×10^4)
D70A	not measd	not measd	3.01×10^4 (2.81×10^4 , 3.13×10^4)	3.01×10^4 (2.89×10^4 , 3.27×10^4)
GRFT D30A/D70A/D112A	not measd ^b	2.84×10^4 (2.72×10^4 , 2.97×10^4)	not measd ^b	not measd ^b

^aValues in parentheses refer to the 95% confidence intervals for each fit. In all cases the molecular weight closely matches the molecular weight of the GRFT dimer. ^bFor the triple mutant GRFT D30A/D70A/D112A, other concentrations were also tested and consistent with this data (Figure S4 in the Supporting Information).

Figure 6. (A) ELISA assay indicates the ability of GRFT and its variants to bind to gp120_{ADA}. Wild-type GRFT (black) binds better than any single point mutant (D30A, red; D70A, purple; D112A, blue). The triple mutation D30A/D70A/D112A (magenta) binds poorly to gp120_{ADA}. Typical results are shown from an experiment in triplicate. Each experiment was repeated at least 3 times. Error bars indicate standard deviation from a typical triplicate experiment. (B) Surface plasmon resonance sensorgrams of GRFT binding to gp120_{ADA} immobilized on the surface. Kinetic analysis of the interactions of GRFT (left panel), GRFT D30A (middle panel) and GRFT triple mutant (right panel) with immobilized HIV-1 ADA gp120. Serial 2-fold analyte dilutions (covering a concentration range from 1.51 to 12.05 nM) were injected over the surface of the immobilized gp120. The experimental data (colored curves) were fit using the 1:1 binding model (black lines) to determine the kinetic parameters. The biosensor chip density was 90 RU. Concentration shown here is in dimer units. (C) Surface plasmon resonance binding assay of GRFT binding to immobilized glycosylated HIV-1/III_B gp120 (expressed in CHO) and nonglycosylated HIV-1/III_B gp160 (expressed in *E. coli*). A protein density of 647 RU and 667 RU were obtained for the glycosylated HIV-1/III_B gp120 and the nonglycosylated HIV-1/III_B gp160. Binding signals for the interactions of 10 nM GRFT triple mutant and 200 nM 2G12 (left panel); 500 nM sCD4 and 50 nM HHA (middle panel); and GRFT WT and its single mutants (all at 10 nM) (right panel) with immobilized glycosylated HIV-1 III_B gp120 (red curves) and nonglycosylated HIV-1 III_B gp160 (green curves). (D) Results of inhibition by GRFT mutants in single-round virus assays. Typical results are shown from an experiment in triplicate. Wild-type GRFT is shown in black; D30A, red; D70A, purple; D112A, blue; triple mutation D30A/D70A/D112A, magenta). Each experiment was repeated at least 3 times. Error bars indicate standard deviation from a triplicate experiment.

tested (500 nM) against the RS HIV-1 strains (Figure 6D and Table 3).

The much poorer antiviral activity of the point mutants and the triple GRFT mutant was also confirmed in cocultivation experiments in which the compounds were exposed to a coculture of persistently HIV-1(III_B)-infected HuT-78 cells and uninfected Sup T1 CD4⁺ T-lymphocytes. Whereas the wild-type GRFT potently prevented syncytia formation in the cocultures at an EC₅₀ of 1.91 ± 1.23 nM, the D30A, D70A and D112A point mutants showed EC₅₀ values of 67.4 ± 39.7 nM, 88.6 ± 23.8 nM and 36.1 ± 14.3 nM, respectively. The triple GRFT mutant was only inhibitory in this assay at 340 nM.

GRFT neutralizes HIV-1 by binding to specific N-glycans on HIV-1 gp120 rather than those on cells. Therefore, a preincubation experiment was performed in which MT-4 cells were exposed to GRFT for 1 h prior to infection of the cell cultures. In these experiments, an increased antiviral (5- to 10-fold decreased EC₅₀) efficacy of the compound was observed (data not shown). A similar phenomenon was previously also reported for several plant lectins,⁵⁴ and might be important in view of the potential of GRFT as an anti-HIV microbicide drug.

DISCUSSION

GRFT is a protein lectin that potently inhibits HIV entry and infection by binding to gp120.³ Since its discovery in 2005, GRFT has been considered a leading candidate for an anti-HIV microbicide, due to its high potency, low toxicity, and good stability to a variety of pH conditions and temperature.^{3,11,12} This lectin has been shown to be easily produced in gram quantities, leading to the possibility of simple and inexpensive production.^{10,55} It proved also able to inhibit hepatitis C virus infection and dissemination both in vitro and in vivo by acting on the entry step of the viral life cycle.⁵⁶

Several high quality X-ray structures and thermodynamic analyses of GRFT have been carried out, showing a protein dimer and indicating three carbohydrate-binding sites per monomer of protein;^{4-6,13} Figure 4A. Competition data and further structural work^{3,6} indicate that each binding site binds one mannose monosaccharide, which is likely the key to GRFT's ability to inhibit HIV. The HIV envelope protein gp120 contains between 18 and 33 N-linked glycosylation sites (depending on the virus strain), approximately a dozen of which are high-mannose type glycans.⁵⁷⁻⁶⁰ These high-mannose glycans, in particular their highly clustered appearance on the HIV envelope gp120, are rarely found on mammalian

Table 2. Kinetic Data for the Interaction of GRFT and Its Mutants with Immobilized HIV-1 Envelope Proteins gp120 and gp41^a

	gp120 III _B (X4)			gp120 ADA (R5)			gp41 HxB2		
	K_D (pM)	k_{on} (1/M·s)	k_{off} (1/s)	K_D (pM)	k_{on} (1/M·s)	k_{off} (1/s)	K_D (pM)	k_{on} (1/M·s)	k_{off} (1/s)
GRFT WT	72.7 ± 1.7	(6.30 ± 0.94) × 10 ⁶	(4.57 ± 0.58) × 10 ⁻⁴	73.2 ± 2.0	(8.45 ± 0.68) × 10 ⁶	(6.17 ± 0.33) × 10 ⁻⁴	44.9 ± 1.5	(8.01 ± 1.46) × 10 ⁶	(3.61 ± 0.78) × 10 ⁻⁴
GRFT D30A	155.6 ± 11.2	(7.89 ± 1.26) × 10 ⁶	(1.24 ± 0.29) × 10 ⁻³	122.7 ± 7.3	(9.08 ± 0.74) × 10 ⁶	(1.12 ± 0.16) × 10 ⁻³	137.5 ± 18.0	(1.26 ± 0.07) × 10 ⁷	(1.72 ± 0.14) × 10 ⁻³
GRFT D70A	244.9 ± 88.7	(4.15 ± 2.59) × 10 ⁶	(8.58 ± 3.29) × 10 ⁻³	194.3 ± 26.3	(4.44 ± 2.10) × 10 ⁶	(8.35 ± 2.91) × 10 ⁻⁴	164.9 ± 37.8	(7.87 ± 0.21) × 10 ⁶	(1.29 ± 0.26) × 10 ⁻³
GRFT D112A	141.8 ± 9.7	(8.43 ± 3.20) × 10 ⁶	(1.21 ± 0.54) × 10 ⁻³	122.5 ± 3.6	(9.27 ± 3.37) × 10 ⁶	(1.14 ± 0.45) × 10 ⁻³	130.9 ± 22.2	(1.41 ± 0.51) × 10 ⁷	(1.90 ± 0.98) × 10 ⁻³
GRFT D30A/D70A/D112A ^b	no specific binding observed at 12.05 nM								
	no specific binding observed at 12.05 nM								

^aConcentration shown here is in dimer units. The p values for interactions with gp120_{III_B} strains are 0.0280 (wild-type GRFT: GRFT D30A); 0.0207 (wild-type GRFT: GRFT D70A) and 0.0282 (wild-type GRFT: GRFT D112A). K_D , dissociation equilibrium constant; k_{on} , association rate constant; k_{off} , dissociation rate constant. ^bAt concentrations higher than 12.05 nM (i.e., 24.1 and 48.2 nM), a weak binding signal was observed.

cells, reducing the possibility of adverse host interactions⁶¹ although one study indicates that GRFT can bind to the surface of some cells.¹² High-mannose glycans contain several branched carbohydrate chains, having three terminal (α 1,2) mannose residues (Figure 1). It is therefore generally assumed that GRFT inhibits viral entry by binding tightly to the mannose on the surface gp120.

Given its relatively high profile and potential for clinical use, it is important that the action of GRFT is biochemically well understood. However, many biochemical details of the role of GRFT in HIV inhibition have not yet been elucidated. For example, while modeling studies suggested three equivalent CBS for each monomer subunit of GRFT, a recent X-ray analysis indicates a subtle lack of equivalence between these sites.⁴ Recent studies have used a GRFT mutant with all three CBS mutated to asparagine, and this mutant GRFT showed no activity in biological assays against several viruses, including *Coronaviridae* and hepatitis C.^{12,56,62} While this triple Asp-to-Asn mutant is likely quite similar to the triple D30A/D70A/D112A mutant reported here, we do see some minimal activity in D30A/D70A/D112A, while none has yet been reported for the triple Asn construct. It appears that our triple mutant retains some weaker binding activity to gp120 as shown by SPR technology, while these assays were not performed for the triple-Asn mutation. Also, the lack of activity for the triple-Asn mutant was reported for viruses other than HIV, which makes direct comparison to our findings not obvious.

In the present work, we have individually mutated each CBS in GRFT, and analyzed the resulting mutants for their ability to dimerize, to bind to mannose, to bind to gp120, and to inhibit HIV. We found that GRFT and its point mutants form a high-affinity dimer that does not dissociate at the lowest level of detection using analytical ultracentrifugation. Further, while the wild-type protein binds well to a mannose monosaccharide column, mutation at any individual site results in the lack of ability to bind this commercial column. NMR spectroscopy of the wild-type protein in the presence of mannose reveals chemical shift changes across the entire face of the protein containing the mannose binding sites. Single site mutants at each binding site show localized changes in the chemical shifts at those sites, as well as some changes at more distal locations, but generally on the mannose binding face of the protein rather than in more buried residues.

Disruption of any single CBS in GRFT results in a moderate loss of ability to bind HIV gp120 as shown by ELISA. These assays show a reduction in ability to bind gp120 from strain ADA, requiring concentrations about 10-fold higher from each mutant GRFT compared to the wild-type protein, with the D70A variant appearing to perform several fold worse than D30A or D112A. In more quantitative binding assays using SPR, GRFT and its mutants were tested for binding to immobilized gp120 (from R5 strain ADA and X4 strain III_B) as well as to gp41. These experiments showed picomolar binding by wild-type GRFT and only a 2–3-fold reduction in binding by the point mutants D30A, D112A, and D70A, the latter showing slightly worse binding than the other two mutants.

However, when these mutants were tested in single-round HIV infection assays as well as HIV cytopathicity assays (read-out after 4 days) and cocultivation assays (24 h read-out), it was found that their ability to inhibit HIV was much more drastically affected than would be predicted based on their ability to bind gp120 (or gp41). For each virus strain tested, the point mutants showed at least a 26-fold or higher increase in

Table 3. Inhibition of GRFT Variants in Single-Round Viral Assays or Antiretroviral Assays^a

compd	inhibition of							
	ADA strain (R5)		JR-FL strain (R5)		SF 162 strain (R5)		III _B strain (X4)	
	EC ₅₀ (nM)	fold decrease	EC ₅₀ (nM)	fold decrease	EC ₅₀ (nM)	fold decrease	EC ₅₀ (nM)	fold decrease
GRFT	0.011 ± 0.008	1	0.073 ± 0.030	1	0.032 ± 0.015	1	0.109 ± 0.0	1
GRFT D30A	20 ± 14	1933	23 ± 23	322	13 ± 2.1	417	6.8 ± 0.6	63
GRFT D70A	2.6 ± 2.2	250	3.0 ± 1.7	41	0.8 ± 0.6	26	6.7 ± 2.2	61
GRFT D112A	6.6 ± 6.1	625	21 ± 9	284	8.7 ± 3.1	276	4.8 ± 0.5	44
GRFT triple mutant D30A/D70A/D112A	>500	>47600	>500	>6900	>500	>15800	95 ± 33	875

^aEach experiment was repeated at least 3 times in triplicate, and the values shown are ± the standard deviation of the EC₅₀ from all experiments. The experiments with R5-tropic strains were carried out with TZM-bl target cells. The EC₅₀ determination is described in the Experimental Methods section and is defined as the compound concentration required to inhibit HIV-induced syncytium formation by 50%. The experiments with X4-tropic strain III_B were carried out with CEM target cells. In this assay system, HIV-induced syncytia formation was examined microscopically at day 4 post infection. "Fold decrease" indicates decrease in potency compared to wild-type GRFT.

EC₅₀ compared to the wild-type protein (although it should be noted that in the context of a dimer, each point mutant results in an overall loss of two CBSs). It was also found that the D70A mutant, while showing lower binding to gp120 in both ELISA and SPR assays, showed better HIV inhibition than the other point mutants in some strains, again indicating a lack of direct correlation between binding to HIV gp120 and viral inhibition.

While the three mannose binding sites of GRFT each use similar amino acid side chains and backbone for carbohydrate binding, a recent crystallography study suggests that two of the sites bind equivalently (pocket 1, including D112, and pocket 3, including D70), while the third site binds differently and may have a less optimal binding environment (pocket 2 including D30);^{4,6} notation introduced in refs 4 and 5. The present work directly investigated each CBS, and our data indicate that the D30 and D112 site are largely identical in terms of contribution to binding of gp120 and inhibition of HIV. The loss of the D70 CBS site appears to have a somewhat different outcome, with consistently better HIV inhibition than the other single mutants. Therefore, the present experiments are not completely in agreement with the previous work, so further experiments may be necessary to resolve this issue.

Our results have implications for the mechanism of action of GRFT. Although mutants lacking one of the three mannose binding sites perform well in binding gp120, all three sites are apparently important for efficient antiviral activity. This strongly implies that GRFT does not function simply by binding gp120 and causing a steric blockade, but rather uses multiple binding interactions with glycans to cause an allosteric shift or a structural shift that inhibits infection by compromising the ability of gp120 to efficiently interact with the host cell during infection. In support of this, recent work has shown that binding of GRFT to gp120 enhances the exposure of the CD4 binding site in gp120, while apparently decreasing the ability of the intact virus to bind the coreceptor.⁶³ In fact, it is well-known from antibody research that binding to gp120 does not guarantee an antiviral effect; binding to gp120 can result in potent neutralization of the virus but may also have poor, if any, effect on viral infectivity, depending on the epitope that is recognized on gp120 by these antibodies.^{64,65} Thus, it seems that the GRFT mutants bind nearly as efficiently to gp120 as wild-type GRFT (as found in our ELISA and SPR experiments) but are not necessarily equally efficiently neutralizing the virus. The efficient capacity of cross-linking of glycans or glycan parts on gp120 by wild-type GRFT may have been markedly

decreased by the mutants, explaining the substantially decreased antiviral potential of the mutants versus wild-type protein. Although this hypothesis has still to be proven by direct experimental evidence, these observations suggest that binding as such to gp120 is not necessarily predictive for eventual antiviral activity of the agents and cross-linking of glycans on gp120 may be a key event to efficiently inhibit virus infectivity. The importance of glycan cross-linking for eventual potent antiviral activity by carbohydrate-binding agents has also been suggested earlier by Moulai et al.⁴ for GRFT, who showed that a monomeric form of the protein is able to bind two different carbohydrate moieties, and also that the monomer itself is not sufficient for anti-HIV function. The possibility of lectin function based on glycan cross-linking has also been raised for cyanovirin-N, which can bind multiple carbohydrates and requires two active sites for its antiviral function.^{9,66}

The tight GRFT dimer is also likely critical for antiviral function, leading to a total of 6 mannose binding sites. Indeed, as mentioned, an engineered GRFT monomer was recently shown to have low antiviral potency despite retaining binding ability to gp120.⁴ We are currently investigating the role of the GRFT dimer in HIV inhibition, where it is possible that the protein uses both arms to alter the conformation of a gp120 subunit or to bind two separate gp120s. The commercially obtained gp120 used in our ELISA and for most SPR experiments is a monomer, while the viral surface contains trimeric gp120.^{67,68} However, when binding of wild-type GRFT (and its individual point mutants) to trimeric gp120 was compared with monomeric gp120, no significant differences were observed. Nevertheless, these findings leave open the possibility that the antiviral activity of GRFT involves cross-linking of separate gp120 subunits on the virus particle.

CONCLUSIONS

The potent HIV inhibitor GRFT has been shown to be a tight dimer, with three nearly equivalent mannose binding sites that are each critical for full potency of the anti-HIV activity of the protein. Mutation of any of the three sites results in a loss of at least 26-fold in ability to inhibit HIV, even though these single mutations only moderately affect the ability of GRFT to bind gp120 or gp41. Overall, this suggests that the antiviral mechanism of GRFT action is not simply to bind and sterically block the action of gp120, but rather to use the binding of mannose residues on gp120 to affect the gp120 structure or its

oligomeric state, possibly by cross-linking gp120 glycans, resulting in a loss of viral infectivity.

■ ASSOCIATED CONTENT

■ Supporting Information

Additional figures and tables as discussed in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

GRFT, griffithsin; CBS, carbohydrate binding site; SPR, surface plasmon resonance; ELISA, enzyme-linked immunosorbent assay; RU, resonance unit; IPTG, isopropyl β -D-1-thiogalactopyranoside; CPRG, chlorophenol red-D-galactopyranoside; DSS, 2,2-dimethyl-2-silapentane-5-sulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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