Crystal Structure of a Human Rhinovirus that Displays Part of the HIV-1 V3 Loop and Induces Neutralizing Antibodies against HIV-1

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Summary

We report the 2.7 Å resolution structure of a chimeric human rhinovirus (HRV14):HIV-1 virus that displays part of the HIV-1 gp120 V3 loop and elicits HIV-neutralizing antibodies. The V3 loop insert is dominated by two type I β turns. The structures of two adjacent tripeptides resemble those of analogous segments in three Fab/V3 loop peptide complexes. Although two of the three corresponding antibodies bind and neutralize MN-III-2 well, only one of the three can bind without significant rearrangement. These results suggest that the V3 loop insert: (1) can share some local conformational similarity to V3 loop sequences presented on different structural frameworks; (2) must be able to adopt multiple conformations, even in a relatively constrained environment; and (3) may mimic the conformational variability of the epitope on HIV-1, increasing the likelihood of eliciting appropriate neutralizing immune responses.

Introduction

The third variable (V3) loop of the HIV-1 envelope glycoprotein gp120 plays an essential role in HIV-1 infection and tropism. The V3 loop appears to undergo a critical conformational change when gp120 binds to the primary cellular receptor, CD4, resulting in and specifying the formation of one or more cellular chemokine coreceptors, particularly the CCR5 [1–5] and CXCR4 [1, 6] coreceptors. Coreceptor formation is thought to be derived from the juxtaposition of parts of the V3 loop with the so-called bridging sheet at the base of the V1/V2 loops [7, 8], promoting the tripartite association of gp120, CD4, and coreceptor. This association is presumed to cause the dissociation of the transmembrane gp41, promoting the insertion of the gp41 fusion peptide into the target cell membrane and subsequent entry of the virus core into the cytoplasm (reviewed in [9]).

Interest in targeting the V3 loop for AIDS vaccine development arose from its ability to elicit antibodies capable of neutralizing tissue culture laboratory-adapted (TCLA) strains of HIV in animals [10–13] and the majority of HIV-1-infected people [14]. In addition, passive immunization studies in animals demonstrated that monoclonal antibodies (mAbs) directed against the V3 loop could provide protection from challenge with HIV [15–18]. However, the immune responses to the V3 loop proved to be quite type specific (e.g., [19, 20]), and primary isolates of HIV-1 were unexpectedly less sensitive to neutralization by V3 loop antibodies (as well as by antibodies directed toward other epitopes [21, 22]). The apparent insensitivity of primary isolates to neutralization by V3 loop and other antibodies remains poorly understood, but it is encouraging to note that a number of new V3 loop-directed mAbs can sensitively neutralize diverse primary isolates of HIV-1 (M.K. Gorny and S. Zolla-Pazner, personal communication) suggesting that it should be possible to elicit effective neutralizing antibodies in vivo using appropriate immunogens.

There is no experimental structure of the V3 loop in its entirety. The crystallographic structures of two gp120 “core” proteins (one from a TCLA isolate and one from a primary isolate, both lacking the V3 loop) complexed with the two N-terminal domains of CD4 and an Fab fragment of human mAb 17b revealed that the V3 loop emanates from two antiparallel β strands of a β sheet on the outer surface of gp120 [7, 23]. Numerous structural studies have been performed on the apical 10–20 residues of this 30 residue loop, including linear and constrained presentations, some of which were bound to Fab fragments. While solution NMR studies have shown that V3 loop peptides by themselves are largely disordered in solution, there are some reports of ordered conformations (e.g., [24–31]). More consistent structures have been observed crystallographically using linear and constrained peptides complexed with Fab fragments [32–35]. The combination of these results suggests that the V3 loop is endowed with inherent conformational flexibility.

We have been engineering the cold-causing human rhinovirus type 14 (HRV14) to display immunogens from foreign pathogens on their surface to serve as potent and effective stimulators of immune responses against the corresponding pathogens. As with other picornaviruses, the 300 Å diameter capsids of rhinoviruses consist of 60 symmetry-related protomers, each comprising one of each of the viral coat proteins (VP1-VP4) [36]. Due to the roles of the V3 loop in viral infection and immunity, we have generated HRV14 chimeras that display V3 loop sequences on their surfaces. Large libraries of chimeric HRV14:HIV-1 viruses have been generated

Key words: AIDS; antibody recognition; chimeric virus; engineered vaccine; virus structure
Table 1. Summary of Diffraction Data and Structure Refinement

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Statistics of refinement and model

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* $R_{merge} = \Sigma ||I_o|| - \angle <I_o,I_i> / \Sigma <I_o,I_i>$

$R$ factor = $\Sigma |F_o| - |F_c| / \Sigma |F_o|$

using combinatorial mutagenesis, whereby the foreign sequences are inserted into surface loops flanked by linkers of variable length and sequence. A library of such viruses will display the foreign sequence with diverse presentations, some of which may resemble those found on HIV. Chimeric viruses that display the foreign sequence in appropriate conformations are immunoselected using anti-HIV-1 V3 loop antibodies. This methodology has been used to identify numerous HRV14:HIV-1 V3 loop chimeric viruses capable of eliciting the production of antisera with anti-HIV-1 neutralizing activity [37, 38] and may ultimately prove useful for generating AIDS vaccine components.

To gain insights into the structural basis of the antigenicity and immunogenicity of the V3 loop of HIV-1, we have been using X-ray crystallography to determine the structures of HRV14:HIV-1 V3 loop chimeric viruses. We present here the crystal structure of an immunogenic HRV14:HIV-1 V3 loop chimeric virus, designated MN-III-2, at 2.7 Å resolution. Determination of this structure revealed regions of local conformational similarity among V3 loop sequences presented in different contexts. This similarity is striking in light of the numerous observations of different conformations of the V3 loop region seen to date. Antibody binding and neutralization studies, combined with attempts at docking the structure of the MN-III-2 virus with the Fab fragments of three V3 loop peptide/Fab complexes, revealed that the inserted V3 loop segment of the chimeric virus must undergo significant conformational changes to interact with two of the three antibodies.

Results and Discussion

Structure Overview

The HRV14:HIV-1 V3 loop chimeric virus MN-III-2 consists of the entire HRV14 structure plus an insertion of 12 amino acids of the immunogenic gp120 V3 loop of HIV-1$\text{Hin}$ [GPGRAFYTTKN] flanked on the N-terminal side by the randomized linker ADT [38]. The insertion was made between Ala159 and Asn160 of the VP2 puff [39] of the neutralizing immunogenic site II (Nim-II). This region is highly solvent exposed and capable of eliciting neutralizing antibodies directed against HRV14. Due to the symmetry of the viral capsid, the insertion is displayed in 60 copies on the surface of HRV.
The crystal structure of the HRV14:HIV-1 V3 loop MN-III-2 chimeric virus has been determined at 2.7 Å resolution using the molecular replacement method with the wild-type HRV14 structure (PDB entry 4RHV) as the starting model. Structure refinement of the final atomic model converged to an R factor of 0.216 for 494,360 reflections (89.0% completeness) with \( F_o \approx 2 \sigma (F_o) \) between 50.0 and 2.7 Å resolution and an R factor of 0.220 for all 513,239 reflections with \( F_o \approx 2 \sigma (F_o) \) (92.4% completeness). All atoms were refined with restrained individual isotropic temperature factors and full occupancy except for the water molecules located at the icosahedral 2-fold, 3-fold, and 5-fold axes. Regions with the highest B factors are on the surface and solvent exposed. The mean error in atomic positions was estimated by the Luzzati method [40] to be 0.25 Å. Protein stereochemistry was analyzed using the programs X-PLOR [41] and PROCHECK [42]. The RMS deviations of bond lengths and angles for the final model are 0.01 Å and 1.0°, respectively. Analysis of protein main chain conformation shows 99.5% of amino acid residues in the most favored or allowed regions of the Ramachandran plot; no amino acid residues are located in the disallowed region of the Ramachandran diagram. A summary of the diffraction data collection and structure refinement statistics is given in Table 1. Figure 1 shows a representative portion of an averaged SIGMAA-weighted 2mFo-Fc map at 2.7 Å resolution in the region of the insertion in the VP2 puff.

The overall structure of the MN-III-2 chimeric virus is similar to that of the wild-type HRV14 structure. Superposition based on all Cα atoms reveals RMS deviations of 1.2 Å for VP1 (273 Cα atoms), 0.6 Å for VP2 (255 Cα atoms, excluding the V3 loop insert), 0.7 Å for VP3 (236 Cα atoms), and 0.7 Å for VP4 (40 Cα atoms), respectively. The most significant structural differences occur: (1) at the site of the V3 loop insertion, between residues 2155 and 2165 of the VP2 puff, and (2) in a loop of VP1 on the surface in the canyon including residues 1213–1224 (for which the numbering scheme of the viral proteins and amino acid residues is of the form VNNN, where “V” represents the number of the viral protein, 1–4 for VP1–VP4, respectively, and “NNN” represents the number of the amino acid residue in the viral protein). The conformation of the region affected in VP1 is similar to that observed when antiviral agents are bound to HRV14 [43]. This would suggest that the observed changes in VP1 are likely to be associated with the unexpected presence of a bound sphingosine molecule (described below in Structure of the Sphingosine Pocket Factor).

Electron density was evident at the N terminus of VP1, allowing the visualization of 9 amino acid residues (1007–1015) that had not previously been observed in the HRV14 structure. The first 6 residues of VP1 remain disordered. Otherwise, modifications of main and side chain conformations were made only in a few solvent-exposed surface loop regions based on electron density maps, including residues 1207–1212 of VP1 and 3178–3184 of VP3, and the N-terminal residues 4029–4031 of VP4. The loop regions of the viral proteins are not as tightly constrained as the core β barrels of the viral proteins and can tolerate greater structural variation and flexibility.

As in the structures of HRV14 [44], HRV1A [45], HRV16 [46], poliovirus type 2 Lansing (PV2L) [47], and bovine enterovirus (BEV) [48], the structure of MN-III-2 has significant residual electron density at several locations in the channel along the 5-fold axis, and the N-terminal residues 4029–4031 of VP4. The loop regions of the viral proteins are not as tightly constrained as the core β barrels of the viral proteins and can tolerate greater structural variation and flexibility.

There is also a flattened, planar piece of residual electron density near the side chain of Trp2038. Analogous
Figure 2. A Ribbon Diagram Showing the Structure of the HRV14-HIV-1 V3 Loop Chimeric Virus MN-III-2 Protomeric Unit (Consisting of One Copy Each of the Four Viral Coat Proteins)

VP1, VP2, VP3, and VP4 are shown in blue, green, red, and yellow ribbons, respectively, and the V3 loop insert is highlighted in gold with side chains [85]. The V3 loop insertion is characterized by three type I $\beta$ turn structures that fold along the virus surface and interact with the bulk of the viral protein. A sphingosine molecule can be seen (in red) in a pocket below the receptor binding canyon.

density has been observed in VP2 in the structures of many other picornaviruses and has been suggested to belong to a nucleotide base of the genomic RNA [44, 47, 51, 52].

Structure of the V3 Loop Insert
The N-terminal ADT linker and V3 loop sequence IGP GRAFYTKKN inserted between Ala2159 and Asn2160 have been numbered Ala-Ins1 to Asn-Ins15, and the residue numbering of the C-terminal portion of VP2 beyond the insert has retained the original numbering of Asn2160 and so on. The initial averaged SIGMAA-weighted 2mFo-Fc maps (using phases computed from coordinates of the wild-type HRV14 structure) showed clear and continuous electron density for most of the inserted residues. However, there was a discontinuity of the density between Gly-Ins5 and Pro-Ins6, and most of the side chains had relatively poor density. Iterations of structure refinement, modifications of some main chain and side chain conformations, and inclusion of more than 600 water molecules greatly improved the structural model. In the averaged electron density maps calculated using the refined model, the density corresponding to the backbone for the entire inserted region is continuous (including between Gly-Ins5 and Pro-Ins6), and most side chains are well defined (Figure 1). Analysis of the main chain conformations reveals that all of the inserted residues occupy favored or allowed regions of the Ramachandran plot. With the exception of residue Gly-Ins5, which is located in the left-handed $\alpha_R$ region of the Ramachandran plot (a region primarily available to Gly residues), all other residues are located either in the right-handed $\alpha_R$ region or the $\beta$ region.

The entire insert is located on the outer surface of the virion and interacts largely with the surface of VP2 and a small portion of VP3 (Figures 2 and 3). At the N-terminal junction of HRV14 and the inserted linker, the SSAA residues form a helix-like turn. The DT (from the linker) and IG (from the V3 loop) residues form the first type I $\beta$ turn, which is nestled in a depression on the virus surface. The conserved V3 loop residues, GRAF, constitute the second type I $\beta$ turn, which protrudes from the virus surface and is relatively solvent exposed. The C-terminal residues, TTKN of the V3 loop insert, make the third type I $\beta$ turn that directs the overall insert back to the position where it connects with the wild-type HRV14 structure. The adjacent HRV14 residues, NEVG, are displaced outward relative to their location in the wild-type structure to accommodate the insert. For each protomer, the insert has a solvent-accessible surface area [53] of 964 Å² (roughly 5% of that of the protomer [54]), which increases the solvent-accessible surface of each protomer by 672 Å². The conformation of the V3 loop insert appears essentially unaffected by the crystal packing forces.

The amino acid residues that constitute the three type I $\beta$ turns in the V3 loop insert are commonly observed in their respective positions in type I $\beta$ turns in protein structures [55], with the exception of the Ile at the third position of the first type I $\beta$ turn (DTIG). Statistical analyses of type I $\beta$ turns in protein structures [55] indicate that at the first position of a type I $\beta$ turn, Asp is the most likely residue, followed in likelihood by either Gly or Thr. At the i+1 position, Thr is significantly preferred, followed by Arg. At the i+2 position, Ala and Lys occur with typical frequencies; the preference for Ile is rather unusual. At the i+3 position, the preference for a Gly residue is strong and the preference for Asn or Phe is also above average. Thus, the three type I $\beta$ turns observed in the V3 loop insert are composed almost exclusively of residues preferred at type I $\beta$ turns. This suggests that the three type I $\beta$ turns observed in MN-III-2 could be characteristic of the structure of this region in its natural context on the surface of HIV-1.

Most residues of the V3 loop insert make van der Waals contacts with residues of the viral proteins VP2 (predominantly) and VP3. In addition, several residues of the V3 loop insert make hydrogen-bonding interactions with residues of VP2 and VP3, other residues of the insert, and water molecules (Table 2). At the N terminus of the V3 loop insert, the main chain amide group of
**Structure of an HRV14-HIV-1 V3 Loop Chimeric Virus**

**Figure 3. Structural Comparison of Wild-Type HRV14 (Green) and the MN-III-2 Chimeric Virus (Gold) in the /H9252 B-/H9252 C Loop of VP2**

The insert in the MN-III-2 chimeric virus (N-terminal linker ADT and HIV-1 MN V3 loop sequence IGPGRAFYTTKN) is bordered by black bars (joined as a double thickness black bar in the native HRV14 structure) between Ala2159 and Asn2160.

**Conformational Flexibility of the V3 Loop**

Prior to any structural studies, sequence analysis of the V3 loop predicted a type II β turn conformation [56] for the GPGR motif. However, the same sequence in other solved protein structures has been seen to adopt alternative conformations, such as a β strand [57], an extended conformation [58], or an undefined turn [59].

NMR studies of V3 loop peptides whose lengths, sequences, and linearity or cyclization are varied have revealed that the V3 loop peptides by themselves are generally disordered in solution, but in some cases preferentially adopt specific conformations, including a type II β turn [60, 61], a combination of type I and II β turns [24, 26], a helical structure [62], a nonspecific β turn [63], and a double turn [28] similar to the first two turns seen in the crystal structure of the Fab 59.1/peptide complex [33]. NMR studies have also suggested that glycosylation of the V3 loop peptide may affect its conformation [27, 30] and that sequence changes can also affect its conformation [25]. Structural studies of a V3 loop peptide RP135 in complex with a 16 amino acid V3 loop peptide, the N-terminal 8 residues are ordered and adopt an extended conformation [32]. The conserved GPG residues appear to initiate a β turn; the residues C-terminal to the GPG sequence are disordered. In two structures of the Fab fragment of murine mAb 59.1 [29, 31, 64, 65] and in solid-state NMR [30] and are also different from those determined in crystallographic studies [32–35]. Furthermore, solid-state NMR studies suggest that the GPGR motif adopts an antibody-dependent conformation in the antibody-bound state and may be conformationally heterogeneous in the unbound state [30].

Crystal structures of V3 loop peptides, either linear or cyclic, bound to Fab fragments of three anti-V3 loop-neutralizing murine mAbs, have been reported [32–35]. In a structure of the Fab fragment of murine mAb 50.1 complexed with a 16 amino acid V3 loop peptide, the N-terminal 8 residues are ordered and adopt an extended β conformation [32]. The conserved GPG residues appear to initiate a β turn; the residues C-terminal to the GPG sequence are disordered. In two structures of the Fab fragment of murine mAb 59.1 complexed with either of two 24 amino acid V3 loop peptides that differ...
The main chain torsion angles for the central residue Ala are typically between 90° and 120°, and the hydrogen position. If the donor is an oxygen atom, the V3 Loop Insert in the MN-III-2 Chimeric Virus and Surrounding Structures Amino Acid Residues or Water Molecules complexes indicate that the V3 loop insert in the MN-III-2 chimeric virus adopts a different conformation from that seen in the V3 loop peptide/Fab complexes. However, the GPG and RAF of the V3 loop can be superimposed as two separate segments among these structures (Figure 4). Superposition of the V3 loop insert of the MN-III-2 chimeric virus onto a variety of V3 loop peptides in complexes with Fab 50.1 (PDB entry 1GGI), Fab 59.1 (PDB entry 1ACY), and Fab 58.2 (PDB entries 1F58 and 2F58) reveal rms deviations for the C
atoms of residues GPG equal to 0.1, 0.4, 1.0, and 1.0 Å, respectively, and rms deviations for the C
atoms of residues RAF that were not determined (disordered in the case of the V3 loop/Fab 50.1 complex), 0.2, 0.2, and 0.2 Å, respectively. The GPG residues in the MN-III-2 chimeric virus structure adopt an extended conformation. For the RAF segment, the side chain orientations for all three amino acids and the main chain torsion angles for the central residue Ala are similar in all of these structures (Table 3).

Although the V3 loop has a flexible structure and can adopt different conformations, it appears that the major conformational differences are localized to the junction of the Gly and Arg residues, yielding two conserved structural modules consisting of residues GPG and residues RAF, respectively. These two structural modules could change their relative orientations by pivoting about the hinging Gly residue, resulting in different overall conformations for the V3 loop and possibly explaining a great deal of the structural variation seen in this region. Thus, superimposition of the GPG residues of the V3 loop peptides in its complex with Fab 58.2 onto the corresponding segment in the MN-III-2 structure followed by rotation of the RAF sequence (along the axis vertical to the PGR plane by approximately 180°) results in superimposition of the RAF residues onto the corresponding segment of the MN-III-2 structure (Figure 4A).

Likewise, superimposition of the GPG segment of the V3 loop peptide in its complexes with Fab 59.1 onto the corresponding segment in the MN-III-2 structure followed by rotation of the segment RAF in the V3 loop peptide/Fab complexes (along the Pro-Gly peptide bond by approximately 180°) leads to the RAF portion of the peptide superimposing well onto the corresponding segment in the MN-III-2 structure (Figure 4A). The V3 peptide in complex with Fab 50.1 superimposed
Figure 4. Structural Comparisons of the V3 Loop Conformations of the MN-III-2 Chimeric Virus and the Three V3 Loop Peptide/Fab Complexes

The major point of structural departure appears to be localized to the junction of the Gly and Arg residues.

(A) Superposition of the GPG structural module.

(B) Superposition of the RAF structural module. The relative conformations of these two structural modules could be adjusted by rotation about the Gly-Arg junction as a pivot point.

Table 3. Main Chain Dihedral Angles (°) of the V3 Loop Insert in the MN-III-2 Chimeric Virus and Comparisons of the V3 Loop Conformation with V3 Loop Peptides Bound to Fabs

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tations may provide the structural basis for the ability of this antibody to bind to and potently neutralize the infectivity of MN-III-2 in cell culture.

Docking experiments were carried out to assess whether the conformation of the V3 loop insert in MN-III-2 could be bound by Fab 59.1, Fab 58.2, and Fab 50.1, whose structures in complexes with various V3 loop peptides are known [32–35]. The structures of the V3 loop peptide/Fab complexes were superimposed onto either the GPG or RAF segment of the MN-III-2 chimeric virus. For the V3 loop/Fab 59.1 complex, simple superimpositions based on either the GPG or RAF segment resulted in some steric conflicts between the antibody and the viral protein. However, slight conformational adjustment between the GPG and RAF segments along the GR pivot point yielded an MN-III-2/Fab 59.1 complex model in which no significant steric conflicts were observed. In contrast, for the other two Fab fragments, 58.2 and 50.1, superimposition of either the GPG or the RAF segment created more serious structural overlaps between the Fabs and the virus that could not be relieved by only adjusting the relative orientations of the GPG and RAF segments.

To see if the structural superimposition differences corresponded to comparable biological differences in how MN-III-2 interacts with mAbs 59.1, 58.2, and 50.1, we performed binding and neutralization assays. To assess binding, sandwich ELISAs were performed in which MN-III-2 was captured by immobilized anti-HRV14 antiserum and then allowed to bind to each of the three mAbs. As can be seen in Table 4, the tightest binding at 0.1 μg/ml antibody was observed with mAb 59.1 (relative OD250 = 1.0), followed by 58.2 (relative OD250 = 0.87), and poorly by 50.1 (relative OD250 = 0.13). These differences in affinity were paralleled by the differences in the number of V3 loop residues recognized by the antibodies that are also present on MN-III-2 (Table 4). The ability of the mAbs to neutralize MN-III-2 was found to follow the same pattern, with mAb 59.1 neutralizing MN-III-2 infection by 50% at 1.7 ng/ml, 58.2 at 5.7 ng/ml, and 50.1 at >8000 ng/ml (Table 4). These results suggest that, to some extent, the ability of the V3 loop mAbs to bind to and neutralize the chimeras must be a function of the ability of the V3 loop sequences to accommodate to the structure necessary for favorable interactions with the antibodies, possibly in part via rearrangement of the relative orientations of the GPG and RAF segments. This accommodation appears to be favored by the presence of lengthier V3 loop epitopes to bind to the complementarity-determining regions of the antibodies.

The determination of additional structures of varied chimeric viruses, free and complexed with neutralizing anti-HIV antibodies, may reveal important relationships between structure and immunological attributes. An understanding of these relationships might ultimately allow for the design or selection for chimeric viruses capable of eliciting more effective and cross-reactive neutralizing antibodies.

### Table 4. Binding and Neutralization of MN-III-2 by V3 Loop-Directed mAbs

<table>
<thead>
<tr>
<th>mAb</th>
<th>Shared Epitope</th>
<th>Relative Binding</th>
<th>TCID&lt;sub&gt;50&lt;/sub&gt; (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>59.1</td>
<td>IGPGRAFY</td>
<td>1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>58.2</td>
<td>IGPGRAFY</td>
<td>0.87</td>
<td>5.7</td>
</tr>
<tr>
<td>50.1</td>
<td>IGPG</td>
<td>0.13</td>
<td>&gt;8000</td>
</tr>
</tbody>
</table>

*Portion of sequence of native HIV-1 V3 loop recognized by antibody (as defined from structural studies) that is also present on MN-III-2.

* Determined by ELISAs, in duplicate for values corresponding to 0.1 μg/ml mAb.

* Determined by microtiter neutralization assays, in triplicate for values corresponding to the number of ng/ml of antibody capable of neutralizing 50% of the tissue culture infectious doses (TCID<sub>50</sub>).

### Structure of the Sphingosine Pocket Factor and Its Interactions with Protein

Pocket factors have been observed in all of the known HRV structures except for wild-type HRV14 [39] and HRV3 [86], which, for unknown reasons, have empty pockets. However, the work reported here led to the appearance of sphingosine in a virus derived from HRV14. The presence of sphingosine could be attributed to the addition of 15 residues to the virus, differences in the virus preparation and purification procedures, or differences in the crystallization conditions. It might also imply that the chimeric virus is less stable than the wild-type virus and thereby requires a stabilizing molecule for virus growth.

The electron density corresponding to the natural pocket factor, sphingosine, in the hydrophobic pocket within the antiparallel eight-stranded β barrel of VP1 was clear in the initial difference Fourier maps and in the averaged SIGMAA-weighted 2mFo-Fc maps. The binding pocket is approximately 26 Å in length and has a volume of approximately 182 Å³. Sphingosine, which itself is approximately 20.5 Å in length, lies in the middle of the pocket and occupies most of the pocket space (Figure 5). The solvent-accessible surface of the virus that is buried after the binding of sphingosine is 982 Å², which is within the range of most stable protein-protein and protein-inhibitor interactions (600–1000 Å²) [70, 71]. More than 97% of the total 645 Å² solvent-accessible surface of the sphingosine is buried in the complex.

Upon the binding of sphingosine, there are significant conformational differences in the inhibitor binding pocket in the region of residues 1213–1224 of VP1. No other major main chain movements were observed. This is consistent with the observations of the other structures of HRV that contain sphingosine or antiviral compounds. The orientation of sphingosine in the binding pocket is similar to that observed in the other HRV and poliovirus structures. However, the internal geometry of sphingosine is slightly different, especially with poliovirus type 1 Mahoney, due to differences of the surrounding amino acids forming the pocket.

The interactions between sphingosine and the surrounding protein residues are primarily hydrophobic in nature (Figure 5). Among 12 contacts with distances of less than 3.6 Å, there are only two weak hydrogen bonding interactions. The hydroxyl O1 atom of sphingosine forms weak hydrogen bonds with the S<sub>γ</sub> atom of Cys1199 (d = 3.4 Å and the S-H...N angle = 118°) and the O<sub>γ</sub> atom of Ser1107 (d = 3.6 Å and the O-H...O angle = 148°, respectively). In addition, the hydroxyl O1 atom forms hydrogen bonds with two
water molecules (W6128 \(d = 3.6 \AA\)) and W6234 \(d = 3.4 \AA\). The hydroxyl O3 atom of sphingosine also forms a hydrogen bond with water molecule W6128 \(d = 3.1 \AA\).

### Biological Implications

Vaccines have successfully helped prevent and control a wide variety of diseases. A disease for which there is a glaring need for a vaccine is AIDS. The AIDS epidemic is a problem of enormous dimensions, with some 40 million people infected with HIV worldwide. Some progress has been made in developing an AIDS vaccine, but greater understanding of the basic principles of vaccinology and immunology may be required. A better understanding of the structural determinants of immunogenicity may ultimately help enable structure-based vaccine design in a manner analogous to the proven paradigm of structure-based drug design. We have been engineering human rhinoviruses, common cold viruses, to display immunogenic segments from more dangerous pathogens, including HIV-1, for the purpose of developing vaccine components against these pathogens. We have generated combinatorial libraries of HRV14 containing HIV immunogens that are linked to the HRV14 coat protein sequences via adapters of randomized sequences and lengths, leading to vast arrays of presentations of the HIV immunogen. Immunoselection techniques have been used to identify immunogens that can mimic corresponding immunogens of HIV. The structure of an HRV14:HIV-1 chimeric virus, designated MN-III-2, containing an immunogenic insert from the gp120 V3 loop of HIV-1, has been solved at 2.7 Å resolution. The virus was isolated from a previously described library of HRV14:HIV chimeras using anti-HIV-1 V3 loop monoclonal antibodies as selecting agents. The MN-III-2 chimera displays on its surface the HIV-1MN V3 loop sequence, IGPGRAFYTTKN, flanked at its N terminus by a three residue linker, ADT. The foreign sequence was inserted into the Nm-II site of HRV14 between VP2 residues Ala159 and Asn160. This chimeric virus elicits the production of guinea pig antisera capable of potently neutralizing HIV-1 strains MN and ALA-1 in cell culture. The overall structure of MN-III-2 is similar to that of wild-type HRV14. A sphingosine molecule has been identified in a hydrophobic pocket in VP1 that has been shown in many picornaviruses to bind to either small cellular molecules ("pocket factors") or antiviral drugs; this contrasts with wild-type HRV14, which does not have a bound pocket factor. The structure of the HIV-1 V3 loop insert is dominated by two type I β turns. The first type I β turn consists of residues GRAF and the second, residues TTKN. Comparisons with the previously reported structures of V3 loop peptides in complexes with the Fab fragments of three neutralizing anti-HIV-1 antibodies indicate that the conserved GPGGRAF motif of the V3 loop insert in the MN-III-2 chimeric virus adopts a different conformation as a whole from that seen in several Fab/peptide complexes. The major conformational differences are localized to the junction of the GR residues of the first β turn. As individual units, however, the GPG and the RAF residues in the chimeric virus superimpose well upon the corresponding segments in the Fab/peptide complexes. The results suggest that the V3 loop has inherent conformational flexibility and can adopt multiple conformations. The ability of this loop to accommodate many different amino acid substitutions indicates that conformational flexibility within this region is compatible with, and perhaps essential to, the functionality of HIV. The relatively conserved GPGGRAF motif of the V3 loop appears to consist of two well-defined structural modules whose relative orientations can be altered, resulting in distinct V3 loop conformations. Such distinct conformations could best enable the diverse set of interactions between the V3 loop and its various biological partners. Two of the three antibodies (59.1 and 58.2) corresponding to the Fab/V3 loop peptide crystal structures efficiently neutralize the
MN-III-2 chimeric virus; all three antibodies were shown to bind to MN-III-2 using an ELISA, with 50.1 binding the weakest, consistent with its inability to neutralize the chimera. Docking experiments indicate that one of the three Fabs (59.1) could interact well with the V3 loop of MN-III-2 to block the entry of the GPGRFA segment, but the other two did not. These results imply that the inserted segment in the chimeric virus structure is likely to adopt a different structure when it interacts with neutralizing antibodies and antibody-like molecules (e.g., B cell receptors) in the immune system. It is possible that the structural variability of the V3 loop segment presented on the surface of human rhinovirus could mimic its conformational variability on HIV, enhancing its potential value as an immunogen.

Experimental Procedures

Virus Propagation, Purification, and Crystallization

The generation, propagation, and purification of the HRV14:HIV-1 V3 loop chimeric virus MN-III-2 have been described previously [58]. Crystals of the MN-III-2 chimera were grown at room temperature using the hanging-drop vapor diffusion method. The crystallization solution consisted of 1.5 M ammonium formate and 0.15 M sodium HEPES, pH 7.5. A broad-spectrum antiviral agent, SDZ 880061 [72], was dissolved in DMSO and diluted to 0.12 M and mixed with the virus sample (5 mg/ml in 10 mM Tris-HCl, 0.1 M NaCl, pH 7.2) at a volume ratio of 1:20 corresponding to a molar ratio of 10:1. However, in the experiments that led to the structure determination, the drug was unable to displace the resident sphingosine molecule in the drug binding pocket and appears to have had no bearing on the structure. Hanging drops were created by mixing 3–5 μl of this virus solution with an equal volume of the crystallization solution on a plastic coverslip and placing the inverted coverslips over reservoirs containing 1 ml of crystallization solution. Plate-shaped crystals began to appear after 1–2 days and grew to approximately 0.1 × 0.4 × 0.4 mm within a week.

Diffraction Data Collection

X-ray diffraction data were collected from crystals that were mounted on a nylon loop, dried in the air for about 8–10 s, and then flash cooled directly in a cold gaseous stream of N2 (−165°C) [73, 74]. The air drying step should lead to higher ammonium formate concentrations in the environment of the loop, which apparently provided cryoprotection (the air drying time varied depending on the size of the loop and the humidity and temperature of the environment). Experiments with flash cooling of ammonium formate solutions not containing crystals showed that a concentration of 4 M ammonium formate can prevent ice formation and hence can serve as a cryoprotectant. A total of 265 oscillation images with 0.3° oscillation were collected from a frozen crystal at the Brookhaven National Synchrotron Light Source (NSLS) using the highly intense X25 beamline with a wavelength of 1.10 Å. The diffraction data were recorded on a Brandeis B4 CCD detector mounted on a Nonius CAD4-style diffractometer. Another 122 images with 0.3° oscillation were collected from a frozen crystal at the Cornell High Energy Synchrotron Source (CHESS) using the F1 beamline with a wavelength of 0.92 Å and recorded on a Quad4 2k × 2k CCD detector (Area Detector System Corporation, Poway, CA). Crystals of the chimeric virus diffracted to at least 2.5 Å resolution and decayed gradually from radiation damage. Diffraction data were processed and scaled together using DENZO [75] and SCALEPACK [76]. The final dataset contained 513,239 unique reflections (I/σ(I) > 0) to 2.7 Å resolution with an Rmerge (I) of 0.114 and a completeness of 92.4%. The diffraction data statistics are summarized in Table 1.

Structure Determination and Refinement

The chimeric virus crystals used for the structure determination were of the I222 space group with unit cell dimensions a = 318.9, b = 349.3, and c = 368.4 Å. With this space group, the virus particle must be situated at the origin with its three 2-fold icosahedral symmetry axes coincident with the three crystallographic 2-fold symmetry axes. Given that the asymmetric unit contains a quarter of a virus particle (i.e., 15 protomers, each consisting of the 4 viral coat proteins), the corresponding Matthew’s coefficient (Vd) would be 2.40 Å3/Da with a solvent content of 49.0% (assuming a standard partial specific volume for protein of 0.74 ml/g [77]).

The structure of the chimeric virus MN-III-2 was solved using the molecular replacement method with a recomplied version of the X-PLOR program that makes use of an increased number of strict NCS constraints [41]. A rotation function search and a translation function search using the wild-type HRV14 structure coordinates (PDB entry 4RVH [54]) as a starting model yielded only one solution. The virus particle was located at the origin without any skewing, which is consistent with the particular I222 space group of this crystal form. The initial phases were calculated from the wild-type HRV14 model, giving an initial R factor of 46.5%. The R factor decreased to 31.5% after 100 cycles of energy minimization using X-PLOR.

Structure refinement was carried out using the X-PLOR conjugate-gradient energy minimization and molecular dynamics simulated annealing protocols with a bulk-solvent correction, using the Engh and Huber parameters to restrain the protein structure [78]. Strict 15-fold NCS constraints were imposed throughout the refinement procedure. This increased the bias-free R-factor by a factor of 15, corresponding to more than 17 observations per parameter refined. Molecular modeling was guided by difference Fourier maps and averaged SIGMAA-weighted 2mFo–Fc maps. The free R factor [79] was calculated in early stages of model building and refinement to monitor the progress. Due to the strong interdependency of structure factors in the presence of high NCS, the free R factor did not provide any useful information. Therefore, in the later stages of structure refinement, all data were included and no free R factor was calculated.

To improve the phase quality and reduce model bias, real-space electron density map averaging was performed with the 15-fold NCS present in the asymmetric unit using the program RAVE [80]. The averaged difference Fourier maps were of good quality and helped resolve ambiguities in the backbone and side chain placement in a few surface regions exposed to solvent. Upon the convergence of the electron density averaging, the overall agreement between the experimental diffraction data and those computed from back-transformation of the averaged electron density map (averaging R factor) was 17.4% with an overall correlation coefficient of 0.917.

In the initial difference Fourier maps and the averaged SIGMAA-weighted 2mFo–Fc maps, clear electron density was evident corresponding to the natural pocket factor, sphingosine, nine amino acid residues at the N-terminal of VP1 that were disordered in the HRV14 structure, and many well-ordered water molecules. The electron density showed clear indications of protein conformational changes in the vicinity of the inhibitor binding pocket. Water molecules, the sphingosine pocket factor, and the V3 loop insert were not included in the model until the R factor decreased to approximately 28% after a few cycles of model building and refinement. Electron density corresponding to the inserted amino acid residues at the Nm-II site of VP2 was also present in the initial difference Fourier maps and in the averaged maps for all 15 NCS-related protomers, and was gradually improved from refinement and the inclusion of the pocket factor and water molecules. Electron density peaks in difference Fourier maps at a height of at least 2σ level were assigned as water molecules if they had reasonable geometry in relation to hydrogen bond donors or acceptors from viral proteins or other water molecules. The atomic model for the sphingosine pocket factor was modified from the coordinates of sphingosine in the structure of its complex with the poliovirus type 1 Mahony (PVM1; PDB entry 1POV). The parameter and topology files were generated using the program XPLOR2D [80]. The asymmetric nature of the well-defined electron density permitted the sphingosine to be uniquely oriented in the binding pocket. The configuration of individual moieties of sphingosine was interpreted without ambiguity based on the difference Fourier maps and the averaged electron density maps. Structural interpretation of the inserted sequence at the Nm-II site
of VP2 was based on the averaged electron density maps. The atomic model was built using the graphics program O [81].

**Antibodies**

Three V3 loop-directed antibodies were used for binding and neutralization assessment. Murine mAb 59.1 (derived from immunization with a cyclic 40 amino acid peptide of the HIV-1 Env sequence [66]; mapped serologically to GPGRA FYT [33, 34]) and murine mAb 50.1 (derived from immunization with the same peptide; mapped serologically to RIHIGG [32]) were gifts from Al Proby at Repligen Corporation. Murine mAb 58.2 (derived from immunization with the same peptide; mapped serologically to HIGPGRAFY [66]) and structurally to RIHIGPGRAFT [55] was a gift of Ian Wilson at the Scripps Research Institute.

**ELISAs**

Nunc-immuno MaxiSorp 96-well ELISA plates were coated with rabbit anti-HRV14 antiserum, blocked with PBS containing 3% gelatin, and then treated with 6 \times 10^8 plaque-forming units of the MN-III-2 chimeric virus. The anti-V3 loop murine mAbs (59.1, 58.2, and 50.1) were subsequently added by serial dilution, followed by the addition of horseradish peroxidase-conjugated goat anti-mouse IgG (ICN Pharmaceuticals, Inc., Costa Mesa, CA). Visualization of the complexes at 450 nm was achieved by the addition of 3,3',5,5'-tetramethyl benzidine. Under these conditions, wild-type HRV14 was unreactive with mAb 58.2, and a non-V3 loop, non-HRV14-directed mAb (2F5, [82]) was unreactive with MN-III-2.

**Microtiter Neutralization Assays**

This assay has been described [38]. In brief, 50 µl containing 10^4 plaque-forming units of chimeric viruses was added to wells in triplicate containing 50 µl of serially diluted anti-V3 loop mAbs (59.1, 58.2, and 50.1). After 1 hr at 37°C, 50 µl containing 10^4 H1-HeLa cells [83] was added, and the plates were incubated at 34.5°C and 2.5% CO_2. Virus neutralization was assessed by a cytotoxicity assay [84] once all of the cells in the antibody-free wells displayed cytopathic effects. 15 µl of a 5 mg/ml solution of 3,4,5-dimethlythiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT; Sigma, Saint Louis, MO) in PBS was added to all wells. After 1.5 hr at 34.5°C, 150 µl of 20% SDS in 50% N,N-dimethylformamide was added to all wells. The absorbance at 570 nm was then determined and expressed as a percentage of the average absorbance from wells that received cells only (corresponding to 100% viability). Titors are expressed as the concentrations of antibodies (in ng/ml) that reduced cell death (measured as the reduced number of tissue culture infectious doses) by 50% (TCID_50).

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Accession Numbers

The full coordinates and structure factors of the HRV14-HIV-1 V3 loop chimeric virus MN-III-2 have been deposited with the Protein Data Bank (entry 1K5M) to become available at the time of publication.