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Novel Ring Structure in the gp41 Trimer of Human Immunodeficiency Virus Type 1 That Modulates Sensitivity and Resistance to Broadly Neutralizing Antibodies▽†

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The identification of the determinants of sensitivity and resistance to broadly neutralizing antibodies is a high priority for human immunodeficiency virus (HIV) research. An analysis of the swarm of closely related envelope protein variants in an HIV-infected individual revealed a mutation that markedly affected sensitivity to neutralization by antibodies and antiviral entry inhibitors targeting both gp41 and gp120. This mutation mapped to the C34 helix of gp41 and disrupted an unexplored structural feature consisting of a ring of hydrogen bonds in the gp41 trimer. This mutation appeared to affect the assembly of the six-helix bundle required for virus fusion and to alter the conformational equilibria so as to favor the prehairpin intermediate conformation required for the binding of the membrane proximal external region-specific neutralizing antibodies 2F5 and 4E10 and the antiviral drug enfuvirtide (Fuzeon). The "swarm analysis" method we describe furthers our understanding of the relationships among the structure, function, and antigenicity of the HIV envelope protein and represents a new approach to the identification of vaccine antigens.

A major goal in human immunodeficiency virus (HIV) vaccine research is the identification of antigens able to elicit broadly neutralizing antibodies (bNAbs) effective against primary isolates of HIV. Despite more than 20 years of effort, antigens able to elicit robust broadly neutralizing activity have yet to be described (9, 18). In order to understand this problem, we have begun to investigate the molecular features of the HIV type 1 (HIV-1) envelope glycoproteins gp120 and gp41 that confer the sensitivity and resistance of viruses to neutralization by bNAbs. In this paper, we have used a new method (swarm analysis) to identify mutations that confer sensitivity and resistance to neutralization by bNAbs in polyclonal HIV-positive sera with broad neutralizing activity. This method takes advantage of the swarm of closely related virus variants that occur in each HIV-infected individual to establish panels of envelope proteins that differ from each other by a limited number of mutations causing amino acid substitutions (0.2 to 2%). By studying the effect of these mutations in swarms of viruses from the same individual, we can identify specific amino acids that affect sensitivity and resistance to neutralization by HIV-positive sera. We have used this method to identify a novel structural element in the gp41 fragment of the HIV envelope glycoprotein that appears to stabilize the oligomeric six-helix bundle in the HIV-1 fusion apparatus. Mutations that affect this structure confer sensitivity or resistance to virus neutralization.

The studies described made use of a large collection of clinical specimens from new and recent HIV infection collected in the course of a phase 3 clinical trial (VAX004) of a candidate HIV-1 vaccine, AIDSVAX B/B (20). These specimens were obtained within 6 months of infection and are representative of viruses currently circulating throughout North America. The transmission of HIV-1 involves a genetic bottleneck where, out of the myriad of genetic variants in each HIV-infected donor, only a single homogeneous variant of HIV-1 successfully replicates in the recipient (25, 31, 55). This variant replicates to very high titers in the first days and weeks after HIV-1 infection and continuously mutates in response to error-prone reverse transcription to generate a swarm of closely related variants (40, 49). The swarm of viruses further diversifies in response to selective pressures imposed by both cellular and humoral antiviral immune responses. Virus variation, driven by errors in reverse transcription and selection by the immune system, occurs throughout the course of HIV infection and is perhaps the greatest challenge in the development of vaccines and therapeutic products. We reasoned that by studying viruses from early infections, sequence variation would be limited compared to that of sequences collected at later times. The analysis we describe is made possible by high-throughput, automated methods for virus infectivity and neutralization assays as well as systems for the construction and analysis of pseudotype viruses (43) with defined amino acid sequences. This technology allows for the accurate and efficient analysis of thousands of individual envelope glycoproteins for sensitivity/resistance to neutralization by panels of HIV-positive sera. These analyses provide particular insight

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into the strategies employed by HIV to evade the immune response and can guide the development of a new generation of HIV vaccine antigens.

MATERIALS AND METHODS

Sera and plasma. Cryopreserved plasma used to clone full-length envelope glycoproteins were collected in the course of a phase 3 clinical trial (20) of a candidate HIV vaccine (AIDSVAX B/B) sponsored by VaxGen, Inc. (S. San Francisco, CA). Deidentified specimens and data required for these studies were provided by Global Solutions for Infectious Diseases (GSID; S. San Francisco, CA). All of the viruses used in this study were obtained from patient plasma collected within 6 months of the initial infection. HIV-positive sera containing bNAbs (Z23, Z1679, Z1684, and N16) were provided by Monogram Biosciences, Inc. (S. San Francisco, CA), and are known from previous studies (17, 43) to neutralize a variety of primary clinical isolates of HIV. The monoclonal antibodies (MAbs) used in these studies were obtained from two different sources. The broadly neutralizing MAbs b12, 2F5, and 4E10 were obtained from the NIH AIDS Reagent Repository and Polymun A.G. (Vienna, Austria). The antiviral compound CD4-immunoglobulin G (IgG) was described previously (3, 11) and provided by GSID.

Construction of envelope gene libraries and pseudoviruses. Libraries of envelope glycoproteins were created from each subject by the PCR amplification of full-length envelope genes from cryopreserved plasma using the method described previously (43) (see Fig. S1 in the supplemental material). The swarm of PCR products was cloned into a plasmid expression vector, eETV, useful for the construction of pseudoviruses. The vector was specifically designed to permit the construction of pseudovirus libraries for use in a well-established and validated virus neutralization assay (17, 43). However, instead of pooling all of the clones together and carrying out neutralization assays or drug sensitivity assays with an entire library of cloned genes from each infected individual as had been done previously, we plated the plasmid library on agar plates and picked 24 to 48 clones from each individual for infectivity studies. The plasmid DNA was isolated from each clone and used to create a stock of pseudovirus particles that were then screened for infectivity and chemokine receptor usage (43, 52). After verifying infectivity and receptor usage, we then selected approximately 10 CCR5-dependent pseudotype viruses with good infectivity for virus neutralization assays. The virus neutralization assays were carried out as described by Schweighardt et al. (43).

Sequencing and mutagenesis. Plasmids containing cloned envelope glycoproteins were sequenced using fluorescently labeled dideoxynucleotides at either Monogram Biosciences or the University of California Sequencing Facility (Berkeley, CA) using capillary electrophoresis sequencing devices (Applied Biosystems, Foster City, CA). HIV envelope glycoprotein sequences were mutagenized by a mismatched primer method using a QuikChange mutagenesis kit (Stratagene, San Diego). All mutations were confirmed by DNA sequencing. The numbering of amino acids is made with reference to the sequence of gp160 from clone 022 from subject 108060. Position 655 corresponds to position 653 of the HXB2 reference strain of HIV-1.

Virus neutralization assay. The automated virus neutralization assay described in this study has been described previously (40, 43). This assay involved the preincubation of pseudovirions with inhibitors of infection (antibodies or fusion inhibitors), followed by the transfer of the mixture to an indicator cell line coexpressing CD4 and chemokine receptors. Virus neutralization, resulting in a reduction in relative light units, can result from inhibitors interacting directly with pseudovirions in solution, or with cell-associated pseudovirions, where the virus envelope assumes the receptor-engaged conformation. The neutralization data reported represent 50% inhibitory concentration values calculated from serum dilution curves. This assay employs multiple assay controls, including a positive pseudotype virus control panel and a negative pseudotype virus control panel. Assay acceptability criteria have been established to minimize interassay variability and assure the comparability of data from different experiments. The positive virus control panel includes the pseudotypes from the neutralization-sensitive isolate NL43 and the less neutralization-sensitive primary isolate JRcsf. The negative virus (specificity) control consists of pseudotype viruses prepared from the envelope of the amphitropic murine leukemia virus. Previous studies (T. Wrin, D. Montefiori, and F. Sinangil, unpublished data) have shown that the Monogram virus neutralization assay yields results comparable to those of the TZM-BL pseudotype virus neutralization assay (37) when tested on standard panels of HIV-1 isolates distributed by the NIH (33).

Molecular modeling. Although the complete gp41 HIV-1 glycoprotein structure is currently unavailable, a crystal structure comprising the fusion-active structure of the N36 and C34 helices of the gp41 antiparallel helical core is available (Protein Data Bank accession code 1AIK). This structure duplicates the essential intramolecular as well as intermolecular packing interactions in which the crystallographic threefold axis corresponds to the natural gp41 trimer threefold axis. The intramolecular and intermolecular hydrogen bonding contacts involving Q655 were identified in the context of the gp41 trimeric structure in the PyMOL molecular visualization software package (16). The potential effects of the various Q655 mutations upon both sets of packing interactions were then analyzed by in silico mutagenesis in PyMOL combined with crystallographic symmetry-constrained energy minimization molecular modeling (using the crystallographic software package Phenix [2]) to enforce the gp41 trimeric symmetry. The results of the crystal-structure-based molecular modeling efforts were subsequently analyzed in PyMOL.

RESULTS

Cryopreserved plasma was obtained from 28 individuals who became infected with HIV during the course of the VAX004 clinical trial. The specimens were all collected from the first postdiagnosis blood draw, with a mean estimated time postinfection of 109 ± 58 days. Based on the estimated time of infection, as well as serological markers, the specimens selected for analysis were all derived from stage V and VI infections according to the classification system of Fiebig et al. (19). Envelope proteins recovered from these stages of infection are distinct from viruses recovered from acute infections (stages I to III) that exhibit low sequence diversity and are therefore not ideal for the method we describe that depends on significant but limited intrapatient sequence variation. Populations of gp160 genes were amplified from each patient plasma sample by reverse transcriptase PCR and ligated into a plasmid expression vector to create libraries of envelope genes (43). A diagram that describes the swarm analysis strategy is provided in Fig. S1 in the supplemental material. The plasmid libraries from each individual were then used to create pseudoviruses for neutralization assays. Because HIV infection is known to result in a high frequency of defective envelope genes, it was necessary to screen individual clones for infectivity prior to performing virus neutralization assays. For this purpose, 24 to 48 individual colonies were selected from each library and the plasmids from each used to construct pseudotype viruses for initial screening in infectivity and receptor tropism assays (52). Data from these infectivity studies on a cell line (CCR5/CD4/U89) expressing CD4 and CCR5 are provided in Fig. S2 in the supplemental material. Based on the results of this assay, sets of 10 pseudotype viruses with robust infectivity were selected from each individual for use in a pseudotype virus neutralization assay (43). These 280 pseudotype viruses were then tested for sensitivity/resistance to neutralization by a panel of four standard HIV-positive sera (Z23, Z1679, Z1684, and N16) known from previous studies to possess bNAbs (17, 43). The results of these studies provided insights into both virus variation and variation in the specificities of bNAbs in different HIV-positive sera. Overall, three different neutralization phenotypes were observed in the viruses. We found that one individual (1/28) possessed viruses that were extremely resistant to neutralization, such that none of the 10 clones were sensitive to neutralization by any of the HIV-positive sera. Conversely, we found that some individuals (3/28) possessed viruses that were extremely sensitive to neutralization, such that almost all of the clones were sensitive to neutralization by all four HIV-positive sera. However, in the majority of the individuals (24/

TABLE 1. Neutralization in subjects 108059 and 108060[a]

Subject and clone	Neutralization titer of indicated HIV-positive serum			
	Z1679	Z1684	N16	Z23
108059				
002	<40	<40	<40	**251**
005	<40	<40	<40	**234**
008	<40	<40	<40	**244**
010	<40	<40	<40	**238**
013	<40	<40	<40	**196**
014	<40	<40	<40	**436**
016	44	50	49	**490**
018	<40	<40	<40	**167**
021	<40	<40	<40	**278**
023	<40	<40	<40	**258**
108060				
022	53	58	51	117
024	**804**	**609**	**612**	**1,667**
002	**303**	**160**	**195**	**379**
003	69	57	67	**151**
011	136	130	177	**222**
012	62	57	70	**241**
013	53	50	58	**158**
018	**428**	**243**	**388**	**1,378**
019	44	<40	40	**145**
021	47	47	70	**157**

[a] The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent neutralization titers that are at least three times greater than those observed against the negative control (amphitropic murine leukemia virus). All clones tested were CCR5 tropic. Clones indicate gp160 envelope proteins.

28), we found a mixture of neutralization-sensitive and -resistant clones.

When the activities of the four HIV-positive sera were compared, differences in the apparent potency and specificity of the bNAbs were observed. For example, in some cases (e.g., subject 108059), only one of the four sera was able to neutralize the clones from this individual (Table 1). This result suggested that serum Z23 possessed at least one population of neutralizing antibodies that was missing or underrepresented in the antibodies from the other HIV-positive sera. One particularly interesting pattern of neutralization was found for subject 108060 (Table 1), for which all four HIV-positive sera neutralized 3 of the 10 clones. These results raised the possibility of a mutational difference between clones that affected a population of neutralizing antibodies common to all four HIV-positive sera. Because we expected sequence variation between clones from the same individual to be minimal, we reasoned that comparison of the sequences between the neutralization-sensitive and -resistant variants would allow us to identify the mutation that conferred neutralization sensitivity. Further examination of the data set revealed that 7/28 individuals exhibited a similar pattern of neutralization sensitivity, as at least one clone was sensitive to neutralization by all four HIV-positive sera and at least one clone was resistant to all four HIV-positive sera. Based on this observation, we selected pairs of viruses (one neutralization sensitive and the other neutralization resistant) from 7 of the 28 individuals with the largest differences in neutralization titers for further analysis.

We next sequenced the envelope glycoproteins from each neutralization-sensitive and -resistant pair and compared the sequences. As expected, we found that sequence variation was limited between neutralization-sensitive and neutralization-resistant clones from the same individual. Pairs of envelope genes with limited sequence variation allowed for the possibility of in vitro mutagenesis to localize the amino acids responsible for conferring sensitivity or resistance to neutralization by HIV-positive sera. To test this possibility, we selected the viruses from subject 108060 (Table 1) for further analysis.

Identification of a mutation in gp160 from subject 108060 that confers sensitivity to neutralization by HIV-positive sera. It can be seen (Table 1) that 3 of the 10 clones from subject 108060 (clones 002, 018, and 024) were sensitive to neutralization by all four HIV-positive sera, and of the remaining 7 clones, most were resistant to neutralization by HIV-positive sera Z1679, Z1684, and N16 but somewhat sensitive to HIV-positive serum Z23. Based on the fact that there was at least a 10-fold difference in neutralization sensitivity with all four HIV-positive sera, clones 022 and 024 were selected for further study. When the gp160 sequences of the neutralization-resistant variant (clone 022 wtR) and a neutralization-sensitive variant (clone 024 wtS) were compared (Fig. 1A), it was found that they differed at only seven positions. Two of the amino acid differences were in gp120, two amino acid differences were in the gp41 ectodomain, and the remaining three differences were in the cytoplasmic tail of gp41. To determine which amino acids were responsible for the difference in sensitivity to neutralization between clone 022 and clone 024, a series of mutant envelope proteins were constructed and used to create pseudovirions where polymorphisms from the neutralization-sensitive variant (clone 024) were introduced into the neutralization-resistant background (clone 022) (Fig. 1A).

We found (Table 2) that the substitution of asparagine (N) for serine (S) at position 323 (N323S) in the V3 domain of gp120 had no effect on sensitivity to neutralization. Similarly, the substitution of N for glycine (G) at position 530 in the C5 domain (N530G) of gp120 had no effect. The replacement of lysine (K) at position 634 of the second heptad repeat domain (C34 helix) of gp41 with glutamic acid (E) in the mutant K634E also failed to show a significant difference in neutralization sensitivity. However, the substitution of glutamine (Q) for arginine (R) at position 655 (Q655R) resulted in a remarkable increase (>30-fold) in neutralization sensitivity for all four of the HIV-positive sera. Mutations in the cytoplasmic tail region (832/833 and 827/832/833) were also examined and had no significant effect. An example of the primary data used to generate the results shown in Table 2 for HIV-positive serum Z23 is presented in Fig. S3 in the supplemental material.

Localization of residue 655 on the linear sequence and three-dimensional structure of gp41. To better understand the impact of this mutation on the structure and function of subject 108060 envelope glycoprotein, we located residue 655 on the linear sequence and three-dimensional structure of gp41 (13, 34, 51). An examination of the linear sequence (Fig. 1B) revealed that position 655 was located in the conserved second heptad repeat of gp41 in a region also known as the C34 helix. This part of the molecule is known to play an integral role in virus fusion and forms an essential component of the six-helix bundle in the trimeric structure of gp41 that mediates the fusion of the viral membranes with cellular membranes in the course of HIV infection (13, 28, 51). Position 655 is also lo-

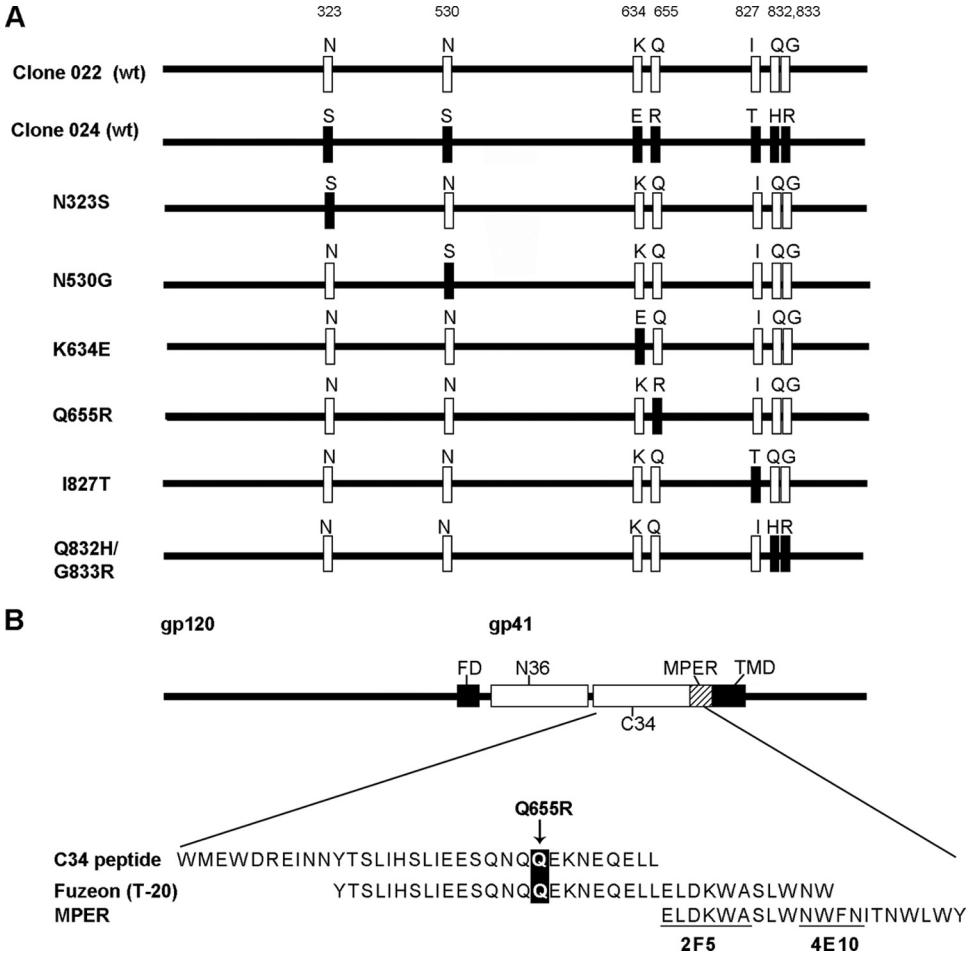

FIG. 1. Mutation of neutralization-resistant clone 022 from subject 108060. (A) Amino acids from neutralization-resistant clone 022 are shown as unfilled rectangles. Amino acids from neutralization-sensitive clone 024 were inserted by in vitro mutagenesis and are shown as filled rectangles. (B) Schematic showing the position of the Q655R mutation (filled box) in relation to the entry inhibitor enfuvirtide (or the T-20 peptide), the MPER, and peptides recognized by the broadly neutralizing MAbs 2F5 and 4E10. The locations of gp41 structural elements are shown as follows: filled boxes for the hydrophobic fusion domain (FD) and the transmembrane domain (TMD), unfilled rectangles for the sequences defining the C34 and N36 helices, and a cross-hatched box for the MPER.

cated within the T20 peptide (53) that serves as the basis for the antiviral drug enfuvirtide (Fuzeon) that inhibits HIV infectivity by inhibiting virus fusion and entry (30). Finally, the location of this mutation is only eight amino acids from a distinct structural region of gp41, termed the membrane proximal external region (MPER), that is known to contain distinct epitopes recognized by the broadly neutralizing MAbs 2F5, 4E10, and Z13 (12, 56, 57). Taken together, these results suggest that this mutation occurs in a region important for virus fusion and close to, but structurally distinct from, a region known to contain other epitopes recognized by bNAbs. Interestingly, while the Q655R mutation in the C34 helix of gp41 had a marked effect on virus neutralization, the K634E mutation also in the C34 helix had no significant effect. These results demonstrated that some amino acid substitutions in the C34 helix, but not others, can cause a significant change in the sensitivity and/or resistance to neutralization by antibodies in HIV-positive sera.

The availability of the Protein Data Bank coordinates of the gp41 fusion domain allowed us to evaluate the impact of the substitution of R for Q at position 655 upon the structure and function of gp41. Using the structure of Chan et al. (13), we were able to determine that in the postfusion, activated form of the gp41 trimer, Q at position 655 is located two turns from the terminus of the C34 helix (Fig. 2A and B) and is subject to both intramolecular interactions with the N36 helix of the same monomer and intermolecular interactions with the N36 helix of adjacent monomers. The N36 and C34 helices within a gp41 monomer pack together in a fairly standard antiparallel coiled-coil hairpin structure. The threefold symmetric packing interface of the gp41 trimer is mediated almost exclusively by a set of parallel three-helical bundle contacts between the N36 helices of each gp41 monomer. One of the few exceptions to this is the set of contacts mediated by Q655. Although Q655 resides in the C34 helix, its side chain accepts an intramolecular hydrogen bond from Q553 of the N36 helix within the gp41 monomer, and it donates an intermolecular hydrogen bond to the backbone carbonyl oxygen of V551 in the N36 helix of an adjacent gp41 monomer (Fig. 2C). The gp41 trimeric structure is thus stabilized by a ring of amino acids Q655-Q553-V551 in

TABLE 2. Neutralization of wild-type and mutated clones from subject 108060 by HIV-positive sera possessing broadly neutralizing antibodies[a]

Clone	Mutation	Neutralization titer of indicated HIV-positive serum			
		Z1679	Z1684	N16	Z23
022	wtR	**75**	**104**	**76**	**384**
024	wtS	**728**	**1,086**	**982**	**1,926**
022	N323S	**73**	**95**	**54**	**382**
022	N530G	**37**	**42**	**41**	**308**
022	K634E	**67**	**73**	**72**	**346**
022	Q655R	**2,165**	**2,562**	**4,472**	**8,290**
022	I827T	**39**	**<20**	**113**	**<100**
022	Q832H/G833R	**104**	**50**	**63**	**404**
022	I827T/Q832H/G833R	**72**	**53**	**81**	**279**

[a] The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent neutralization titers that are significantly higher than the background value (see Materials and Methods). All clones tested were CCR5 tropic. Clones indicate gp160 envelope proteins. wtR and wtS indicate wild-type neutralization-resistant and -sensitive clones, respectively.

a threefold symmetric repeat (Fig. 2B and 3A). Hence, the three copies of Q655 contribute six hydrogen bonds that specifically stabilize the trimeric structure through intramolecular as well as intermolecular hydrogen bonding contacts. Mutations of Q655 clearly possess a significant potential to disrupt the stability of the tertiary gp41 structure as well as the quaternary structure of the gp41 trimer. Molecular modeling suggested that the replacement of Q with R might impact the structure of the six-coiled-coil bundle in two ways. First, the longer R side chain may have a steric effect that disrupts the close packing of the C34 helix with the N36 helix on the adjacent monomers (Fig. 2D). A second mechanism by which this substitution at position 655 could confer neutralization sensitivity is by the disruption of the intramolecular hydrogen bond with position 553 (Fig. 2D and 3B), as there is no longer a keto oxygen to act as a hydrogen bond acceptor. Hence, the effect of the mutation is predicted to destabilize each of the gp41 monomers in the trimeric structure. However, the potential to form the intermolecular hydrogen bond with V551 remains, so that if the gp41 monomer can still fold correctly, a partially stable trimer should be able to form.

Role of inter- and intramolecular hydrogen bonds. To further investigate the role of R655 in conferring sensitivity to virus neutralization, we used in vitro mutagenesis to replace Q at position 655 with other residues predicted to affect inter- and intramolecular interactions in the hydrogen-bonded ring structure and examined their affect on neutralization sensitivity (Table 3). Some of the replacements, such as threonine (T), failed to yield infectious viruses. We found that the conservative substitution of Q for asparagine (N) at position 655 resulted in a small but significant increase in neutralization sensitivity to sera Z1679 and Z23. Glutamine and asparagine share the same side chain amide functionality, but asparagine has one fewer side chain carbon atom than glutamine. Hence, the Q655N mutation is unique in that it retains the potential to form both the intramolecular hydrogen bond and the intermolecular hydrogen bond (Fig. 3A), providing that a local distortion of the helical backbone can compensate for the shortening of the side chain by one carbon atom. This observation explains the relative sensitivity of HIV-1 to the Q655N mutation.

We next examined the replacement of Q at position 655 with lysine (K). The side chain of lysine is shorter than that of arginine and has reduced potential to interfere with the interhelix packing structure than arginine. Modeling suggested that the Q655K mutation, like the Q655R mutation, was unable to form the intramolecular hydrogen bond with Q553 but preserved the intermolecular hydrogen bond with V551 similar to that shown in Fig. 3B. We found the Q655K mutation resulted in a highly neutralization-sensitive phenotype. This result suggested that the destruction of the hydrogen bond was a more important factor in conferring neutralization sensitivity than the steric hindrance provided by the longer side chain of arginine. This conclusion was confirmed by the next two mutants examined, in which serine (S) replaced glutamine at position 655 (Q655S) and where glutamic acid (E) replaces glutamine (Q655E). We found that these substitutions also resulted in a significant increase in neutralization sensitivity (Table 3), albeit not as high as the Q655K mutation. The effect of S or E at position 655 is predicted to differ from that of the Q655R mutation in that S and E preserve the intramolecular hydrogen bond but are unable to form the intermolecular hydrogen bonds (Fig. 3C). Together, these results suggest that both the

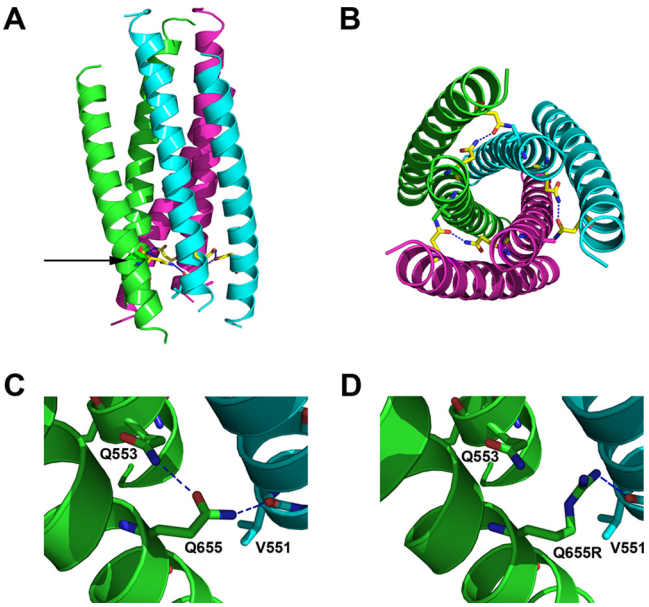

FIG. 2. Location of position 655 in the fusion-activated helical structures of the gp41 trimer. (A and B) The fusion-active six-helix bundle of gp41 consists of a trimer formed from three hairpin shaped coiled-coil monomers (green, cyan, and magenta). The three N36 helices form a parallel coiled-coil structure at the interior of the six-helix bundle, and the three C34 helices are packed in an antiparallel manner on the outside. The locations of the glutamine 655 side chains are shown in yellow (arrow), and hydrogen bonds are indicated by dashed blue lines. (C and D) The interhelical packing of neutralization-sensitive and -resistant variants of the gp41 envelope glycoprotein from subject 108060. The two hydrogen bonds (intra- and intermolecular) formed by glutamine 655 (Q655), glutamine 553 (Q553), and valine 551 (V551) in the neutralization-resistant clone 022 are shown in panel C as dashed blue lines. The single hydrogen bond (intermolecular) formed between arginine 655 and valine 551 by the neutralization-sensitive clone 024 is shown in panel D (dashed blue line).

TABLE 3. Mutagenesis to investigate the role of intra- and intermolecular hydrogen bonds at position 655[a]

Clone	Mutation	Neutralization titer of indicated HIV-positive sera			
		Z1679	Z1684	N16	Z23
022	wtR	**40**	<20	**36**	**281**
024	wtS	**1,099**	**1,193**	**545**	**4,167**
022	Q655R	**14,276**	**2,876**	**2,610**	**8,422**
022	Q655K	**5,486**	**8,590**	**4,276**	**19,476**
022	Q655E	**564**	**132**	**366**	**2,424**
022	Q655S	**1,565**	**472**	**674**	**2,650**
022	Q655N	**148**	**24**	**57**	**820**
024	R655Q	**50**	<20	**39**	**372**

[a] The neutralizing antibody titer (IC_{50}) is defined as the reciprocal of the plasma dilution that produces a 50% inhibition in target cell infection. Values in bold represent neutralization titers that are significantly higher than the background value (see Materials and Methods). All clones tested were CCR5 tropic. Clones indicates gp160 envelope genes. wtR and wtS indicate wild type neutralization-resistant and -sensitive clones, respectively.

intermolecular and intramolecular hydrogen bonds are important for stabilizing the ring structure and that the disruption of either the set of three intramolecular hydrogen bonds or the set of three intermolecular hydrogen bonds results in increased sensitivity to neutralization.

Sensitivity to neutralization by MAbs and fusion inhibitors. While the structural analysis provided insight into the functional consequences of mutations at position 655, two alternate hypotheses can account for a mechanism by which this mutation increases sensitivity to antibody-mediated neutralization. One possibility is that this mutation is located at or near an antibody binding site and that the Q655R mutation restores an epitope recognized by a population of neutralizing antibodies present in all four HIV-positive sera. Alternatively, it is possible that this mutation results in a significant conformational change that is transmitted to other parts of gp41 such as the adjacent MPER or the gp120/gp41 trimer complex in such a way as to increase exposure or access to antibodies at other locations on the molecule.

To explore these possibilities, antibody neutralization studies were carried out with a panel of neutralizing MAbs to epitopes in gp120 and gp41 as well as fusion inhibitors targeting either the gp120 or the gp41 portion of the HIV envelope glycoprotein. In these studies, we examined two broadly gp41-neutralizing MAbs, 2F5 and 4E10 (39, 45, 56); the broadly neutralizing b12 antibody able to block CD4 binding to gp120 (8, 10); and 2G12, an antibody that binds to a carbohydrate epitope in gp120 (47). In addition, we tested the antiviral entry inhibitor CD4-IgG (11), which binds to sequences in gp120 and is able to neutralize lab-adapted CXCR4-dependent clinical isolates at low concentrations (0.01 to 0.1 μg/ml), and primary clinical isolates of HIV (15) at high concentrations (10 to 100 μg/ml). We also examined the sensitivity of envelope mutants to enfuvirtide, a peptide virus entry inhibitor (30, 53) that consists of a gp41-derived peptide that includes sequences from the C34 helix containing Q655. The results of these studies are shown in Table 4, in which the sensitivities of clone 022 and clone 024 from subject 108060 to neutralizing MAbs were compared. It can be seen that the neutralization-resistant clone 022 is moderately sensitive to the 2F5 and 4E10 MAbs specific for the MPER of gp41 but resistant to neutralization by the b12 and 2G12 MAbs reactive with gp120. This virus was also sensitive to enfuvirtide and resistant to CD4-IgG. The high CD4-IgG concentration required for the neutralization of this

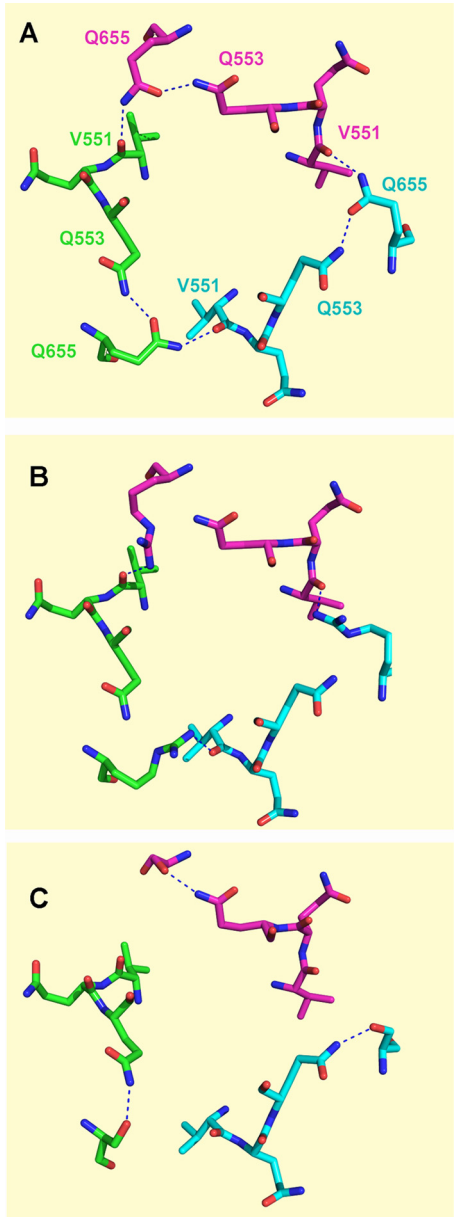

FIG. 3. Effect of amino acid substitutions at position 655 in the C34 helix of gp41 on intra- and intermolecular hydrogen-bonded ring structure. (A) Carbon backbone diagram of key amino acids and hydrogen bonds in the predicted ring structure for the gp41 trimer of the neutralization-resistant 108060_022 envelope protein containing Q at position 655. Individual gp41 monomers are indicated by the colors green, cyan, and magenta. Hydrogen bonds are indicated as dashed blue lines. (B) The occurrence of arginine at position 655 in the neutralization-sensitive 108060_024 envelope protein is predicted to preserve the intermolecular hydrogen bonds but not the intramolecular bonds required for the hydrogen-bonded ring structure. (C) The substitution of serine for glutamine at position 655 is predicted to preserve the intramolecular hydrogen bonds but destroy the intermolecular hydrogen bonds required for the hydrogen-bonded ring structure.

TABLE 4. Sensitivity to neutralizing monoclonal antibodies and entry inhibitors in 108060 clones and unrelated viruses[a]

Clone	Mutation	IC_{50} (μg/ml) of indicated MAb or fusion inhibitor					
		2F5	4E10	b12	2G12	Enfuvirtide	CD4-IgG
108060_022	wtR	**3.250**	**5.201**	>20	>20	**0.068**	>20
108060_022	Q655R	**0.093**	**0.156**	>20	>20	**0.004**	**0.161**
108060_024	wtS	**0.151**	**0.333**	>20	>20	**0.019**	**0.798**
108060_024	R655Q	**3.434**	**6.546**	>20	>20	**0.130**	>20
108069_005	wtR	**1.129**	**3.556**	>20	>20	**0.071**	>20
108069_011	wtS	**0.043**	**0.040**	>20	>20	**0.145**	>20
108069_005	Q655R[b]	**0.052**	**0.044**	>20	>20	**0.011**	**1.080**
108051_005	wtR	>20	>20	>20	>20	**0.088**	>20
108051_006	wtS	**1.176**	**1.369**	>20	>20	**0.008**	**0.231**
108051_005	Q655R[b]	**0.343**	**1.314**	>20	>20	**0.036**	**5.209**

[a] The neutralizing antibody titer (IC_{50}) is defined as the concentration (μg/ml) of an MAb or entry inhibitor that produces a 50% inhibition in target cell infection. Values in bold represent neutralization titers that are significantly above the background (see Materials and Methods). All clones tested were CCR5 tropic. Clones indicate gp160 envelope proteins. wtR and wtS indicate wild-type neutralization-resistant and -sensitive clones, respectively.

[b] Numbering with reference to subject 108060 protein.

virus is consistent with the concentration required to neutralize other primary, CCR5-dependent viruses (15). We next examined the neutralization-sensitive clone 024 that differs from the neutralization-resistant clone 022 at only seven amino acid positions. We found that this clone was 15- to 20-fold more sensitive to the MPER-specific MAbs (2F5 and 4E10) than the 022 clone. Similarly, the neutralization-sensitive clone 024 was more than 20-fold more sensitive to CD4-IgG and 3.5-fold more sensitive to neutralization by enfuvirtide (Table 4). Thus, clone 024 exhibited significantly increased sensitivity to neutralization by MAbs and antiviral entry inhibitors as well as antibodies in HIV-positive sera. We then mutated the neutralization-sensitive clone 024 so as to replace R with Q at position 655. We found that the resulting mutant (108060_024 R655Q) became resistant to neutralization and showed a pattern of neutralization sensitivity closely resembling that of the neutralization-resistant clone 022. Conversely, when we mutated the neutralization-resistant clone 022 to replace Q at position 655 with R, the resulting mutant (108060_022 Q655R), which differed from the parental neutralization-resistant clone by a single amino acid, exhibited an extraordinary increase in neutralization sensitivity (Table 3). We observed a >125-fold increase in sensitivity to CD4-IgG compared to that of the wild-type clone 022 and a 30- to 35-fold increase in sensitivity to the MPER-reactive antibodies 2F5 and 4E10. We also noted a 17-fold increase in sensitivity to the antiviral drug enfuvirtide. These results highlight the importance of glutamine at position 655 and suggest that epistatic mutations at other sites in clone 024 moderate sensitivity to neutralization. The results of these studies are remarkable in that they show that a single amino acid substitution in gp41 not only confers sensitivity to neutralization by MAbs and entry inhibitors directed to gp41 but also increases sensitivity to CD4-IgG, a molecule that binds to gp120, an entirely different protein. Thus, the Q655R mutation appears to cause a conformational change in gp41 that affects not only the binding of antibodies and entry inhibitors (2F5, 4E10, and enfuvirtide) that bind close to the site of the mutation but also the binding of another inhibitor (CD4-IgG) that binds to a site on gp120 located a considerable distance from the mutation.

Transfer of the Q655R mutation to related and unrelated viruses. In order to determine whether the Q655R mutation could confer neutralization sensitivity and resistance to other viruses, this mutation was introduced into two unrelated viruses highly resistant to neutralization (from subjects 108069 and 108051) that normally possessed a Q at a position corresponding to 655 of the virus from subject 108060 (the 108060 virus). The results of these experiments are shown in Table 3. Interestingly, we found that the replacement of Q655 with R had little or no effect on neutralization by any of the HIV-positive sera. However, these mutations significantly increased the sensitivity to neutralization by the 2F5 and 4E10 MAbs (25- to 35-fold). These mutations also increased the sensitivities to neutralization by the entry inhibitors enfuvirtide and CD4-IgG. Thus, the mutation of Q to R at a position corresponding to 655 in the 108069 virus increased the sensitivity to enfuvirtide by more than 17-fold and increased the sensitivity to CD4-IgG by more than 20-fold. The 108069 mutant with the Q655R mutation seemed to be somewhat more sensitive to enfuvirtide and possibly CD4-IgG than the corresponding mutant of the 108051 virus. Together, these results demonstrate that the mutation of Q to R at positions corresponding to 655 of the 108060 virus confers sensitivity to neutralizing MAbs to the MPER and antiviral compounds targeted to the C34 helix and the MPER of gp41. However, it was interesting that these mutations failed to increase the sensitivity to bNAbs in HIV-positive sera. We do not know whether neutralizing activity in HIV-positive sera is attributable to antibodies binding to the C34 region, the MPER, or other parts of the molecule. It has been recently reported (42, 44) that antibodies with specificities similar to 2F5 and 4E10 are rare in HIV-positive sera, which might account for the lack of effect. Alternatively, the failure of the Q655R mutation to increase neutralization sensitivity by HIV-positive sera might be attributable to polymorphisms outside of the MPER and the C34 region that preclude the binding of otherwise bNAbs. This may well be the case since the 108069 and 108051 viruses were selected because of their resistance to neutralization by the HIV-positive sera selected for use in these studies.

DISCUSSION

These studies describe a novel method for the identification and mapping of mutations that affect the sensitivity/resistance of viruses to neutralization by HIV-positive sera and antiviral entry inhibitors. This method enabled the identification of a previously unexplored ring structure in the gp41 trimer that appears to stabilize the six-helix bundle required for virus fusion and can affect sensitivity and resistance to virus neutralization by modulating the exposure of epitopes recognized by bNAbs. This approach to HIV envelope structure/function studies differs from previously described methods of mutational analysis in that it relies on naturally occurring mutations in the swarm of closely related viruses that evolve during the course of HIV infection. The use of intrapatient sequence variation to map epitopes was suggested in previous studies (4), for which we noted a difference in the binding of a MAb between two clones of the HIV-1 gp120 envelope protein obtained from a high-risk volunteer who participated in a phase I trial of a candidate HIV vaccine. However, at that time, the technology did not exist to reintroduce the mutant back into the viruses with assurance that the sequences were stable in vitro. The advent of pseudotype virus neutralization assays (37, 40, 43) utilizing HIV envelope genes incorporated into a stable DNA plasmid vector provided the opportunity to take advantage of naturally occurring mutations in HIV envelope genes without the fear of reversion or further mutation. A somewhat similar approach and result was recently reported by Blish et al. (6), who studied virus variation in a patient soon after HIV infection. They reported that a pair of mutations in gp41, one in the MPER and one in the N36 helix, conferred a neutralization-sensitive phenotype to viruses collected from the same individual soon after HIV infection. The MPER mutation was located in a sequence known to contain epitopes recognized by the broadly neutralizing 4E10 and 2F5 MAbs and was structurally distinct from the C34 helix containing the Q655R mutation described above. However, the MPER mutation alone was not sufficient to cause neutralization sensitivity, and another mutation (T569A) was required to achieve the neutralization-sensitive phenotype. An examination of the nature and location of this mutation suggested a distinct structural basis for the increase in neutralization sensitivity and indicated that the T569A mutation appears to destabilize the N36-C34 interface by interfering with a classical "knob in hole" structure (48). Thus, several distinct types of mutations in gp41 appear to be able to induce structural changes that affect sensitivity and resistance to bNAbs.

Identification of a mutation at position 655 in gp41 that confers sensitivity to neutralization by bNAbs. In this study, we identified a naturally occurring mutation (Q655R) that affects the sensitivity/resistance of viruses to neutralization by bNAbs. X-ray crystallography studies showed that glutamine at position 655 is located close to the C terminus of the C34 helix and contributes to two hydrogen bonds: one mediating an intramolecular interaction with the N36 helix on the same monomer and the other mediating an intermolecular interaction with the N36 helix on an adjacent monomer. These two hydrogen bonds appear to stabilize the fusion-active conformation of the six-helix bundle in trimeric gp41 in such a way as to increase infectivity and confer resistance to neutralization. Our data suggest that naturally occurring mutations (e.g., Q655R and Q655K) and experimental mutations (e.g., Q655S or Q655E) that interfere with either the intramolecular or intermolecular hydrogen bonds normally provided by Q655 confer sensitivity to neutralization by interfering with the formation of the hydrogen-bonded ring. In this regard, the function of this ring structure appears to be twofold: (i) to stabilize interactions between the backbones of adjacent N36 helices in the core of the six-helix bundle and (ii) to stabilize the ends of the coiled-coil hairpin structures in each gp41 monomer. This latter interaction may serve a function analogous to that of a fibular clasp on a brooch or a badge.

Possible mechanism by which the Q655R mutation confers sensitivity and resistance to neutralization. HIV fusion is thought to be a stepwise process (14, 21) that begins with the binding of CD4 and a suitable chemokine receptor (CXCR4 or CCR5) to gp120. This triggers a conformational change in gp41, resulting in the formation of the prehairpin fusion intermediate complex via rearrangement of the amphipathic helices in the external domains of gp41. The N36 helices pack in a parallel three-helical bundle. The prehairpin is characterized by the exposure of the N-terminal hydrophobic fusion domain and the C-terminal MPER of gp41, which are normally folded inside the gp41 trimer and not exposed to circulating antibodies (21). Further molecular rearrangements result in the closure of the hairpin structure, resulting in antiparallel packing of each C34 helix into the grooves on the outside of each N-helix in the gp41 trimer. Ultimately, a highly thermostable six-helix bundle is formed, which is thought to provide the energy required to fuse viral membranes with cellular membranes. We hypothesize that the Q655R mutation alters the conformational equilibria so as to favor the prehairpin fusion intermediate structure, where both the N-terminal fusion domain and MPER are exposed. The prolongation of the half-life of the transient fusion intermediate structure would explain the increased sensitivity to the 2F5 and 4E10 MAbs which recognize the exposed MPER (see Fig. S4 in the supplemental material). Previous studies have suggested that the transition of the fusion intermediate to the fusion-active conformation is the rate-limiting step in virus infection and is estimated to be as long as 15 min based on T-20 (enfuvirtide) sensitivity (38). An interesting possibility is that HIV envelope glycoproteins that are "trapped" in the fusion intermediate conformation might represent superior HIV vaccine antigens, since they would expose epitopes normally hidden and only exposed during virus fusion. The results obtained with swarm analysis are consistent with the possibility that mutations at position 655 in the 108060 virus, such as Q655R and Q655K, alter the conformational equilibria to favor the gp41 trimer in the fusion intermediate conformation. While this hypothesis fits the available structural information and is consistent with the models of Chan and Kim (14) and Frey et al. (21), alternate hypotheses are possible. Three-dimensional structures do not exist for the resting, prefusion conformation of the gp41 trimer alone, or in association with gp120, and we do not know whether the bNAbs in HIV-positive sera react with the resting state or the short-lived, fusion-activated envelope protein. It is possible that the mutation at position 655 in some way destabilizes the interaction between gp120 and gp41 in such a way as to increase the exposure of the MPER or other yet-to-be-defined

epitopes recognized by bNAbs in HIV-positive sera. However, this explanation seems less likely since position 655 appears to be buried at the base of the gp41 trimer close to the viral membrane in an area that is remote from the known CD4 binding regions and gp120/gp41 contact regions (5, 29, 54). Another hypothesis relates to the recent data suggesting that the portion of the MPER recognized by the broadly neutralizing 4E10 and 2F5 MAbs has a kinked structure and is partially buried in the virus membrane bilayer (46). Thus, it is also possible that the Q655 mutation in some way affects the kinked structure or causes the exposure of sites that are normally buried in lipid. Additional studies will be needed to distinguish between these hypotheses and to clarify the specific mechanism by which this mutation increases sensitivity to neutralization by MAbs and polyclonal antibodies as well as the virus entry inhibitors CD4 and enfuvirtide.

Sera from early infections may represent an opportunity to identify rare mutations that confer sensitivity to bNAbs. Based on the examination of sequence data in the Los Alamos HIV Sequence Database, it appears that the mutation of glutamine at position 655 is rare and occurred with an observed frequency of 8/1,242 (0.64%) independent isolates from all stages of HIV infection. An examination of the GenBank database, which included acute sequence data from Keele et al. (25) and Abrahams et al. (1), revealed six published sequences with the Q655R mutation and a single sequence with the Q655K mutation. The sequences were found in a clade A cohort (27) (GenBank accession no. FJ1610700.1), clade B (7, 26, 35), clade C (41), and clade D (36) viruses. Interestingly, a mutation homologous to Q655R was also found in simian immunodeficiency virus (23).

How is it then that we were able to find such a rare mutation within the first seven viruses examined? One possible explanation relates to the fact that the viruses analyzed in this study were all collected close to the time of infection and may possess antigenic structures that are uncommon in viruses recovered from later infections due to the kinetics of the development of the neutralizing antibody response. Several studies have shown that bNAbs do not occur until 6 to 12 months after infection (22, 40, 49). It could well be the case that viruses recovered from early infections possess a broader range of antigenic features because they are being selected primarily for infectivity rather than neutralization resistance. Once effective neutralizing antibodies are present, neutralization-sensitive variants, such as Q655R, would be selected against and rapidly disappear from plasma. The possibility that viruses from early infections may contain mutations resulting in unusual structures is consistent with a previous study (24) which found that viruses recovered from the same clinical cohort as the 108060 virus had an unexpectedly high frequency of mutations that affected the disulfide structure of gp120.

Envelope proteins from early infections with rare mutations such as Q655R may represent a new source of vaccine antigens. How are mutations that occur with such low frequencies useful for HIV vaccine development? The results obtained for the Q655R mutation suggest that mutations of this type significantly alter the antigenic structure of the envelope protein in such a way as to expose important epitopes that are normally shielded from contact with the immune system. Frey et al. (21) have hypothesized that immunization with a gp41 trimer locked into the prehairpin fusion intermediate conformation might be an effective way to elicit bNAbs to the MPER with activities similar to those of 2F5 and 4E10. We believe that the Q655R and other mutations that we have described may have "trapped" the gp41 trimer into this prehairpin intermediate conformation (see Fig. S4 in the supplemental material) and might be more effective in inducing bNAbs than the immunogens tested to date. The immunogenicity of Q655 variants has not yet been explored; however, studies are under way to examine their immunogenic potential.

Virus fusion is a delicately balanced process that involves major conformational transitions triggered by ligand binding. These transitions are no doubt aided, and stabilized, by a variety of cooperative interactions. The studies described highlight a set of novel interactions mediated by hydrogen bonds that appear to facilitate the fusion of viruses with cellular membranes. The six-helix bundle structure and fusion mechanism are conserved throughout evolution and are essential for the infectivity of most enveloped viruses (28, 32, 50). It may well be that the infectivity of other enveloped viruses also depends on stabilizing interactions from hydrogen-bonded structures of the type that we have observed in gp41. Knowledge of these stabilizing interactions may be useful in understanding the details of the fusion process and may provide a new approach to the development of vaccines and therapeutic products, for which the alteration of these interactions may provide a functional benefit.

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REFERENCES

1. **Abrahams, M. R., J. A. Anderson, E. E. Giorgi, C. Seoighe, K. Mlisana, L. H. Ping, G. S. Athreya, F. K. Treurnicht, B. F. Keele, N. Wood, J. F. Salazar-Gonzalez, T. Bhattacharya, H. Chu, I. Hoffman, S. Galvin, C. Mapanje, P. Kazembe, R. Thebus, S. Fiscus, W. Hide, M. S. Cohen, S. A. Karim, B. F. Haynes, G. M. Shaw, B. H. Hahn, B. T. Korber, R. Swanstrom, and C. Williamson.** 2009. Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-Poisson distribution of transmitted variants. J. Virol. **83:**3556–3567.
2. **Adams, P. D., R. W. Grosse-Kunstleve, L.-W. Hung, T. R. Ioerger, A. J. McCoy, N. W. Moriarty, R. J. Read, J. C. Sacchettini, N. K. Sauter, and T. C. Terwilliger.** 2002. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr. D **58:**1948–1954.
3. **Ashkenazi, A., D. H. Smith, S. A. Marsters, L. Riddle, T. J. Gregory, D. D. Ho, and D. J. Capon.** 1991. Resistance of primary isolates of human immunodeficiency virus type 1 to soluble CD4 is independent of CD4-rgp120 binding affinity. Proc. Natl. Acad. Sci. USA **88:**7056–7060.
4. **Berman, P. W., A. M. Gray, T. Wrin, J. C. Vennari, D. J. Eastman, G. R. Nakamura, D. P. Francis, G. Gorse, and D. H. Schwartz.** 1997. Genetic and immunologic characterization of viruses infecting MN-rgp120-vaccinated volunteers. J. Infect. Dis. **176:**384–397.
5. **Binley, J. M., R. W. Sanders, B. Clas, N. Schuelke, A. Master, Y. Guo, F. Kajumo, D. J. Anselma, P. J. Maddon, W. C. Olson, and J. P. Moore.** 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. J. Virol. **74:**627–643.
6. **Blish, C. A., M. A. Nguyen, and J. Overbaugh.** 2008. Enhancing exposure of HIV-1 neutralization epitopes through mutations in gp41. PLoS Med. **5:**e9.
7. **Boutonnet, N., W. Janssens, C. Boutton, J. L. Verschelde, L. Heyndrickx, E. Beirnaert, G. van der Groen, and I. Lasters.** 2002. Comparison of predicted scaffold-compatible sequence variation in the triple-hairpin structure of human immunodeficiency virus type 1 gp41 with patient data. J. Virol. **76:**7595–7606.

8. **Burton, D. R., and C. F. Barbas III.** 1994. Human antibodies from combinatorial libraries. Adv. Immunol. **57:**191–280.
9. **Burton, D. R., R. C. Desrosiers, R. W. Doms, W. C. Koff, P. D. Kwong, J. P. Moore, G. J. Nabel, J. Sodroski, I. A. Wilson, and R. T. Wyatt.** 2004. HIV vaccine design and the neutralizing antibody problem. Nat. Immunol. **5:**233–236.
10. **Burton, D. R., J. Pyati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Nara, M. Lamacchia, E. Garratty, E. R. Stiehm, Y. J. Bryson, Y. Cao, J. Moore, D. D. Ho, and C. F. Barbas.** 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science **266:**1024–1027.
11. **Capon, D. J., S. M. Chamow, J. Mordenti, S. A. Marsters, T. Gregory, H. Mitsuya, R. A. Byrn, C. Lucas, F. M. Wurm, J. E. Groopman, S. Broder, and D. H. Smith.** 1989. Designing CD4 immunoadhesins for AIDS therapy. Nature **337:**525–531.
12. **Cardoso, R. M., M. B. Zwick, R. L. Stanfield, R. Kunert, J. M. Binley, H. Katinger, D. R. Burton, and I. A. Wilson.** 2005. Broadly neutralizing anti-HIV antibody 4E10 recognizes a helical conformation of a highly conserved fusion-associated motif in gp41. Immunity **22:**163–173.
13. **Chan, D. C., D. Fass, J. M. Berger, and P. S. Kim.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:**263–273.
14. **Chan, D. C., and P. S. Kim.** 1998. HIV entry and its inhibition. Cell **93:**681–684.
15. **Daar, E. S., X. L. Li, T. Moudgil, and D. D. Ho.** 1990. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. Proc. Natl. Acad. Sci. USA **87:**6574–6578.
16. **DeLano, W. L.** 2007. MacPyMOL: a PyMOL-based molecular graphics application for MacOS X. DeLano Scientific LLC, Palo Alto, CA.
17. **Dhillon, A. K., H. Donners, R. Pantophlet, W. E. Johnson, J. M. Decker, G. M. Shaw, F. H. Lee, D. D. Richman, R. W. Doms, G. Vanham, and D. R. Burton.** 2007. Dissecting the neutralizing antibody specificities of broadly neutralizing sera from human immunodeficiency virus type 1-infected donors. J. Virol. **81:**6548–6562.
18. **Fauci, A. S., M. I. Johnston, C. W. Dieffenbach, D. R. Burton, S. M. Hammer, J. A. Hoxie, M. Martin, J. Overbaugh, D. I. Watkins, A. Mahmoud, and W. C. Greene.** 2008. HIV vaccine research: the way forward. Science **321:**530–532.
19. **Fiebig, E. W., D. J. Wright, B. D. Rawal, P. E. Garrett, R. T. Schumacher, L. Peddada, C. Heldebrant, R. Smith, A. Conrad, S. H. Kleinman, and M. P. Busch.** 2003. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS **17:**1871–1879.
20. **Flynn, N. M., D. N. Forthal, C. D. Harro, F. N. Judson, K. H. Mayer, and M. F. Para.** 2005. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J. Infect. Dis. **191:**654–665.
21. **Frey, G., H. Peng, S. Rits-Volloch, M. Morelli, Y. Cheng, and B. Chen.** 2008. A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. Proc. Natl. Acad. Sci. USA **105:**3739–3744.
22. **Gray, E. S., P. L. Moore, I. A. Choge, J. M. Decker, F. Bibollet-Ruche, H. Li, N. Leseka, F. Treurnicht, K. Mlisana, G. M. Shaw, S. S. Karim, C. Williamson, and L. Morris.** 2007. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. J. Virol. **81:**6187–6196.
23. **Jayaraman, P., T. Zhu, L. Misher, D. Mohan, L. Kuller, P. Polacino, B. A. Richardson, H. Bielefeldt-Ohmann, D. Anderson, S. L. Hu, and N. L. Haigwood.** 2007. Evidence for persistent, occult infection in neonatal macaques following perinatal transmission of simian-human immunodeficiency virus SF162P3. J. Virol. **81:**822–834.
24. **Jobes, D. V., M. Daoust, V. Nguyen, A. Padua, S. Michele, M. D. Lock, A. Chen, F. Sinangil, and P. W. Berman.** 2006. High incidence of unusual cysteine variants in gp120 envelope proteins from early HIV type 1 infections from a phase 3 vaccine efficacy trial. AIDS Res. Hum. Retrovir. **22:**1014–1021.
25. **Keele, B. F., E. E. Giorgi, J. F. Salazar-Gonzalez, J. M. Decker, K. T. Pham, M. G. Salazar, C. Sun, T. Grayson, S. Wang, H. Li, X. Wei, C. Jiang, J. L. Kirchherr, F. Gao, J. A. Anderson, L. H. Ping, R. Swanstrom, G. D. Tomaras, W. A. Blattner, P. A. Goepfert, J. M. Kilby, M. S. Saag, E. L. Delwart, M. P. Busch, M. S. Cohen, D. C. Montefiori, B. F. Haynes, B. Gaschen, G. S. Athreya, H. Y. Lee, N. Wood, C. Seoighe, A. S. Perelson, T. Bhattacharya, B. T. Korber, B. H. Hahn, and G. M. Shaw.** 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc. Natl. Acad. Sci. USA **105:**7552–7557.
26. **Keele, B. F., L. Tazi, S. Gartner, Y. Liu, T. B. Burgon, J. D. Estes, T. C. Thacker, K. A. Crandall, J. C. McArthur, and G. F. Burton.** 2008. Characterization of the follicular dendritic cell reservoir of human immunodeficiency virus type 1. J. Virol. **82:**5548–5561.
27. **Khamadi, S. A., R. W. Lihana, D. L. Mwaniki, J. Kinyua, N. Lagat, J. Y. Carter, H. Ichimura, I. Oishi, F. A. Okoth, and W. Ochieng.** 2008. HIV type 1 genetic diversity in Moyale, Mandera, and Turkana based on env-C2-V3 sequences. AIDS Res. Hum. Retrovir. **24:**1561–1564.
28. **Kielian, M., and F. A. Rey.** 2006. Virus membrane-fusion proteins: more than one way to make a hairpin. Nat. Rev. Microbiol. **4:**67–76.
29. **Kim, S., H. B. Pang, and M. S. Kay.** 2008. Peptide mimic of the HIV envelope gp120-gp41 interface. J. Mol. Biol. **376:**786–797.
30. **Lazzarin, A., B. Clotet, D. Cooper, J. Reynes, K. Arasteh, M. Nelson, C. Katlama, H. J. Stellbrink, J. F. Delfraissy, J. Lange, L. Huson, R. DeMasi, C. Wat, J. Delehanty, C. Drobnes, and M. Salgo.** 2003. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. N. Engl. J. Med. **348:**2186–2195.
31. **Learn, G. H., D. Muthui, S. J. Brodie, T. Zhu, K. Diem, J. I. Mullins, and L. Corey.** 2002. Virus population homogenization following acute human immunodeficiency virus type 1 infection. J. Virol. **76:**11953–11959.
32. **LeDuc, D. L., and Y. K. Shin.** 2000. Insights into a structure-based mechanism of viral membrane fusion. Biosci. Rep. **20:**557–570.
33. **Li, M., F. Gao, J. R. Mascola, L. Stamatatos, V. R. Polonis, M. Koutsoukos, G. Voss, P. Goepfert, P. Gilbert, K. M. Greene, M. Bilska, D. L. Kothe, J. F. Salazar-Gonzalez, X. Wei, J. M. Decker, B. H. Hahn, and D. C. Montefiori.** 2005. Human immunodeficiency virus type 1 *env* clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. **79:**10108–10125.
34. **Liu, S., W. Jing, B. Cheung, H. Lu, J. Sun, X. Yan, J. Niu, J. Farmar, S. Wu, and S. Jiang.** 2007. HIV gp41 C-terminal heptad repeat contains multifunctional domains. Relation to mechanisms of action of anti-HIV peptides. J. Biol. Chem. **282:**9612–9620.
35. **Liu, Y., J. McNevin, J. Cao, H. Zhao, I. Genowati, K. Wong, S. McLaughlin, M. D. McSweyn, K. Diem, C. E. Stevens, J. Maenza, H. He, D. C. Nickle, D. Shriner, S. E. Holte, A. C. Collier, L. Corey, M. J. McElrath, and J. I. Mullins.** 2006. Selection on the human immunodeficiency virus type 1 proteome following primary infection. J. Virol. **80:**9519–9529.
36. **McCutchan, F. E., M. Hoelscher, S. Tovanabutra, S. Piyasirisilp, E. Sanders-Buell, G. Ramos, L. Jagodzinski, V. Polonis, L. Maboko, D. Mmbando, O. Hoffmann, G. Riedner, F. von Sonnenburg, M. Robb, and D. L. Birx.** 2005. In-depth analysis of a heterosexually acquired human immunodeficiency virus type 1 superinfection: evolution, temporal fluctuation, and intercompartment dynamics from the seronegative window period through 30 months postinfection. J. Virol. **79:**11693–11704.
37. **Montefiori, D.** 2004. Evaluating neutralizing antibodies against HIV, SIV and SHIV in luciferase reporter gene assays. *In* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, W. Strober, and R. Coico (ed.), Current protocols in immunology, vol. 12. John Wiley and Sons, New York, NY.
38. **Muñoz-Barroso, I., S. Durell, K. Sakaguchi, E. Appella, and R. Blumenthal.** 1998. Dilation of the human immunodeficiency virus-1 envelope glycoprotein fusion pore revealed by the inhibitory action of a synthetic peptide from gp41. J. Cell Biol. **140:**315–323.
39. **Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, and H. Katinger.** 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J. Virol. **67:**6642–6647.
40. **Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos.** 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc. Natl. Acad. Sci. USA **100:**4144–4149.
41. **Rousseau, C. M., B. A. Birditt, A. R. McKay, J. N. Stoddard, T. C. Lee, S. McLaughlin, S. W. Moore, N. Shindo, G. H. Learn, B. T. Korber, C. Brander, P. J. Goulder, P. Kiepiela, B. D. Walker, and J. I. Mullins.** 2006. Large-scale amplification, cloning and sequencing of near full-length HIV-1 subtype C genomes. J. Virol. Methods **136:**118–125.
42. **Sather, D. N., J. Armann, L. K. Ching, A. Mavrantoni, G. Sellhorn, Z. Caldwell, X. Yu, B. Wood, S. Self, S. Kalams, and L. Stamatatos.** 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. J. Virol. **83:**757–769.
43. **Schweighardt, B., Y. Liu, W. Huang, C. Chappey, Y. S. Lie, C. J. Petropoulos, and T. Wrin.** 2007. Development of an HIV-1 reference panel of subtype B envelope clones isolated from the plasma of recently infected individuals. J. Acquir. Immune Defic. Syndr. **46:**1–11.
44. **Shen, X., R. J. Parks, D. C. Montefiori, J. L. Kirchherr, B. F. Keele, J. M. Decker, W. A. Blattner, F. Gao, K. J. Weinhold, C. B. Hicks, M. L. Greenberg, B. H. Hahn, G. M. Shaw, B. F. Haynes, and G. D. Tomaras.** 2009. In vivo gp41 antibodies targeting the 2F5 monoclonal antibody epitope mediate human immunodeficiency virus type 1 neutralization breadth. J. Virol. **83:**3617–3625.
45. **Stiegler, G., R. Kunert, M. Purtscher, S. Wolbank, R. Voglauer, F. Steindl, and H. Katinger.** 2001. A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. AIDS Res. Hum. Retrovir. **17:**1757–1765.
46. **Sun, Z. Y., K. J. Oh, M. Kim, J. Yu, V. Brusic, L. Song, Z. Qiao, J. H. Wang, G. Wagner, and E. L. Reinherz.** 2008. HIV-1 broadly neutralizing antibody extracts its epitope from a kinked gp41 ectodomain region on the viral membrane. Immunity **28:**52–63.
47. **Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger.** 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the

gp120 glycoprotein of human immunodeficiency virus type 1. J. Virol. **70:** 1100–1108.
48. **Walshaw, J., and D. N. Woolfson.** 2003. Extended knobs-into-holes packing in classical and complex coiled-coil assemblies. J. Struct. Biol. **144:**349–361.
49. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312.
50. **Weissenhorn, W., L. J. Calder, S. A. Wharton, J. J. Skehel, and D. C. Wiley.** 1998. The central structural feature of the membrane fusion protein subunit from the Ebola virus glycoprotein is a long triple-stranded coiled coil. Proc. Natl. Acad. Sci. USA **95:**6032–6036.
51. **Weissenhorn, W., A. Dessen, S. C. Harrison, J. J. Skehel, and D. C. Wiley.** 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature **387:** 426–430.
52. **Whitcomb, J. M., W. Huang, S. Fransen, K. Limoli, J. Toma, T. Wrin, C. Chappey, L. D. Kiss, E. E. Paxinos, and C. J. Petropoulos.** 2007. Development and characterization of a novel single-cycle recombinant-virus assay to determine human immunodeficiency virus type 1 coreceptor tropism. Antimicrob. Agents Chemother. **51:**566–575.
53. **Wild, C., T. Oas, C. McDanal, D. Bolognesi, and T. Matthews.** 1992. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc. Natl. Acad. Sci. USA **89:**10537–10541.
54. **Zhou, T., L. Xu, B. Dey, A. J. Hessell, D. Van Ryk, S. H. Xiang, X. Yang, M. Y. Zhang, M. B. Zwick, J. Arthos, D. R. Burton, D. S. Dimitrov, J. Sodroski, R. Wyatt, G. J. Nabel, and P. D. Kwong.** 2007. Structural definition of a conserved neutralization epitope on HIV-1 gp120. Nature **445:**732–737.
55. **Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho.** 1993. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science **261:**1179–1181.
56. **Zwick, M. B., R. Jensen, S. Church, M. Wang, G. Stiegler, R. Kunert, H. Katinger, and D. R. Burton.** 2005. Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1. J. Virol. **79:**1252–1261.
57. **Zwick, M. B., A. F. Labrijn, M. Wang, C. Spenlehauer, E. O. Saphire, J. M. Binley, J. P. Moore, G. Stiegler, H. Katinger, D. R. Burton, and P. W. Parren.** 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J. Virol. **75:**10892–10905.