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Characterization of HIV-1 resistance to a fusion inhibitor, N36, derived from the gp41 amino-terminal heptad repeat

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ABSTRACT

A transmembrane glycoprotein of HIV-1, gp41, plays a central role in membrane fusion of HIV-1 and host cells. Peptides derived from the amino- and carboxyl-terminal heptad repeat (N-HR and C-HR, respectively) of gp41 inhibit this fusion. The mechanism of resistance to enfuvirtide, a C-HR-derived peptide, is well defined; however the mechanism of resistance to N-HR-derived peptides remains unclear. We characterized an HIV-1 isolate resistant to the N-HR-derived peptide, N36. This HIV-1 acquired a total of four amino acid substitutions, D36G, N126K and E137Q in gp41, and P183Q in gp120. Among these substitutions, N126K and/or E137Q conferred resistance to not only N36, but also C34, which is the corresponding C-HR-derived peptide fusion inhibitor. We performed crystallographic and biochemical analysis of the 6-helix bundle formed by synthetic gp41-derived peptides containing the N126K/E137Q substitutions. The structure of the 6-helix bundle with N126K/E137Q was identical to that in wild-type HIV-1 except for the presence of a new hydrogen bond. Denaturing experiments revealed that the stability of the 6-helix bundle of N126K/E137Q is greater than in the wild-type. These results suggest that the stabilizing effect of N126K/E137Q provides resistance to N36 and C34.

1. Introduction

An envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1), gp120, interacts with CD4 and co-receptors, such as CCR5. This induces conformational changes of gp120 to activate gp41, which mediates viral membrane fusion to the host membrane (Eckert and Kim, 2001b). Briefly, after the fusion domain located in the N-terminal end of gp41 penetrates into the host cell membrane, the interaction of a trimer of the amino-terminal heptad repeat (N-HR) with the carboxyl-terminal HR (C-HR) of gp41, results in the formation of a 6-helix bundle in which the N- and C-HRs are arranged in a three-hairpin structure. Alternatively, the three N-HRs form a coiled-coil, and the three C-HRs are packed in an antiparallel manner into highly conserved, hydrophobic grooves on the surface of the coiled-coil (Chan et al., 1997). Peptides derived from the N- and C-HR regions inhibit fusion by blocking the interaction between the N- and C-HRs and preventing the formation of the 6-helix bundle fusogenic state of gp41 (Chan et al., 1998; Wild et al., 1993, 1992). One of the C-HR-derived peptides, enfuvirtide (T-20), effectively suppresses HIV-1 replication *in vivo* (Kilby et al., 1998; Lalezari et al., 2003; Lazzarin et al., 2003). Another C-HR-derived peptide, C34, which contains the four amino acids, W117, W120, D121, and I124, required to dock into a hydrophobic pocket termed the "deep pocket" of the trimer of the N-HR also exerts strong inhibition of HIV-1 fusion *in vitro* (Chan et al., 1997). In addition to peptides derived from the HIV-1 gp41 consensus amino acid sequence, several modified peptides have also been developed, including T-1249 (Eron et al., 2004), T-2635 (Dwyer et al., 2007), SC34EK (Nishikawa et al., 2009) and T-20$_{S138A}$ (Izumi et al., 2009).

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To date, resistant variants to C-HR-derived peptides, such as T-20 and C34, have been extensively investigated. Resistant variants to T-20 have emerged with mutations in the N-HR region, especially from L33 to L45, which is thought to be the binding site of T-20, both *in vitro* (Fikkert et al., 2002; Rimsky et al., 1998) and *in vivo* (Aquaro et al., 2006; Bienvenu et al., 2006; Cabrera et al., 2006; Labrosse et al., 2006; Menzo et al., 2004; Perez-Alvarez et al., 2006; Ray et al., 2007; Wei et al., 2002), suggesting that substitutions in the N-HR directly interfere with T-20 binding. Variants resistant to C34 also emerged in vitro with amino acid mutations in the N-HR region (Armand-Ugon et al., 2003; Lohrengel et al., 2005; Nameki et al., 2005). Taken together, mutations in the N-HR region play a key role in resistance to C-HR-derived peptides.

An N-HR-derived peptide N36 corresponding to the leucine/isoleucine zipper sequence of gp41 has anti-HIV-1 activity to a lesser extent when compared to T-20 and C34 (Dubay et al., 1992; Wild et al., 1994; Wild et al., 1992). It is believed that N36 easily aggregates in physiological solutions, resulting in reduced potency (Lu et al., 1995; Lu and Kim, 1997). Meanwhile, a stabilized and trimeric coiled-coil N peptide, IZN17, was developed and displayed 100-fold greater potency compared with N36 (Eckert and Kim, 2001a). Moreover, $(CCIZN17)_3$, a covalently stabilized trimer of IZN17s, represented 30-fold increase in potency compared with IZN17 (Bianchi et al., 2005). These studies demonstrated that appropriate engineering of N-HR-derived peptides could increase their inhibitory effect, suggesting that development of N-HR-derived peptides is one of the novel candidates for effective HIV-1 inhibitors.

In this study, we selected for and characterized HIV-1 variants resistant to N36 by virological, biochemical and X-ray crystallographic analyses, with the aim of elucidating further information regarding HIV-1 fusion.

2. Materials and methods

2.1. Cells and viruses

MT-2 and 293T cells were grown in RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM), respectively. HeLa-CD4-LTR-β-gal cells were kindly provided by M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (Bethesda, MD, USA), and used for the drug susceptibility assay as described previously (Nameki et al., 2005). Recombinant infectious HIV-1 clones carrying various mutations were generated by pNL4-3 plasmid with site-directed mutagenesis as described previously (Nameki et al., 2005). Each molecular clone was transfected into 293T cells with *Trans*IT® (Mirus Bio LLC, Madison, WI, USA). After 48 h, the supernatants were harvested and stored at −80 °C until required.

2.2. Antiviral agents

The peptides used in this study were synthesized as described previously (Otaka et al., 2002). A reverse transcriptase inhibitor, 2′,3′-dideoxycytidine (ddC), and an adsorption inhibitor, dextran sulfate (DS5000) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Determination of drug susceptibility

The peptide sensitivity of infectious clones was determined by the multinuclear activation of galactosidase indicator (MAGI) assay as described previously (Nameki et al., 2005). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in 96-well flat-bottomed microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI unit/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after viral exposure, cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and blue cells were counted in each well. The activity of test compounds was determined as the effective concentration that blocked HIV-1 replication by 50% (EC_{50}).

2.4. Induction of HIV-1 variants resistant to N36

MT-2 cells were exposed to wild-type HIV-1 (HIV-1_{WT}) and cultured in the presence of N36 at an initial concentration of 0.1 μM. Cultures were incubated at 37 °C until extensive cytopathic effect (CPE) was observed. The culture supernatants were used for further passage of MT-2 cells in the presence of increasing concentrations of N36 until massive CPE was seen at earlier stages of culture. Such dose-escalating culture was performed until resistant variants were obtained. This selection was carried out for a total of 25 passages. At the indicated passages, 10, 20, and 22, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from the infected MT-2 cells.

2.5. Viral replication kinetics assay

MT-2 cells (10^5 cells/mL) were infected with each virus preparation (500 MAGI unit) for 16 h. The infected cells were then washed and cultured in a final volume of 3 mL. The culture supernatants were collected from days 2–7 after infection, and the amount of p24 antigen was determined.

2.6. Circular dichroism (CD) spectroscopy

Each peptide was incubated at 37 °C for 30 min, with the final concentration of peptides were 10 μM in phosphate buffered saline (PBS) pH 7.4. CD spectra were recorded on a AVIV model 202 spectropolarimeter (AVIV) with a 1 mm path-length cuvette at 25 °C from an average of eight scans. The thermal stability was assessed by monitoring the change in the CD signal at 222 nm. The midpoint of the thermal unfolding transition, the melting temperature (T_m) of each complex was determined as described previously (Otaka et al., 2002).

2.7. Crystallization, data collection and refinement

Crystallization was performed using the hanging-drop vapor-diffusion method at 4 °C. The solution for crystallization was prepared by mixing 2 μL peptide solution (10 mg/mL each of N36 and $C34_{KQ}$ peptides) with 2 μL of mother liquor. The triangular prism-shaped crystals of the N36/$C34_{KQ}$ complex were grown in 80 mM ammonium chloride, 16% 2-methylpentan-2,4-diol and 25% isopropanol, which diffract to beyond 1.7 Å resolution and belong to space group *C*2 with unit-cell parameters $a = 88.63$, $b = 50.48$, $c = 56.15$ Å, $\beta = 90.88°$. X-ray diffraction data were collected at 100 K on a rotating copper-anode home X-ray source (MicroMax-007, Rigaku, Japan) equipped with an imaging plate detector (*R*-axis IV^{++}, Rigaku). The structure was solved by molecular replacement using the program MOLREP (Vagin and Teplyakov, 1997) with the model of a wild-type 6-helical bundle structure which was generated by symmetry operations from the PDB coordinate file 1AIK. Structure refinement was performed with the programs CNS (Brunger et al., 1998) and XtalView (McRee, 1999).

3. Results

3.1. Selection of N36-resistant HIV-1

In order to induce HIV-1 variants resistant to N36, escalating doses of N36 (from 0.1 μM) were applied to HIV-1_{WT}-infected MT-2 cells. At passage 11 (P-11), P-20 and P-22, when the concentration of N36 was 6.4, 12.8 and 25.6 μM, respectively, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from MT-2 cells as described previously (Fig. 1) (Nameki et al., 2005). Sequence analysis of HIV-1 at P-11 revealed that the aspartic acid residue at position 36 (D36) and the asparagine at position 126 (N126) of gp41 had been substituted for glycine (D36G) and lysine (N126K), respectively. At P-20 and P-22, E137Q in gp41 and P183Q in gp120 had emerged, respectively. Both N126K and E137Q substitutions were located in the C-HR which is thought to be the interactive site of N36 (Fig. 1A) (Chan et al., 1997). An N126K mutation was also induced in resistant viruses to C34 (Nameki et al., 2005), T-20 (Baldwin et al., 2004), modified C-HR-derived peptide, T-1249 (Eggink et al., 2008), and cell membrane-anchored C-peptide, maC46 (Hermann et al., 2009). In the bulk-sequencing, we found mixed substitution at N126K, AAG and AAA. Therefore, we cloned PCR products to a cloning vector pSL301 (Invitrogen, Carlsbad, CA, USA) and revealed that 6 and 3 clones were AAG and AAA, respectively, and that no other substitutions in the both HRs were observed.

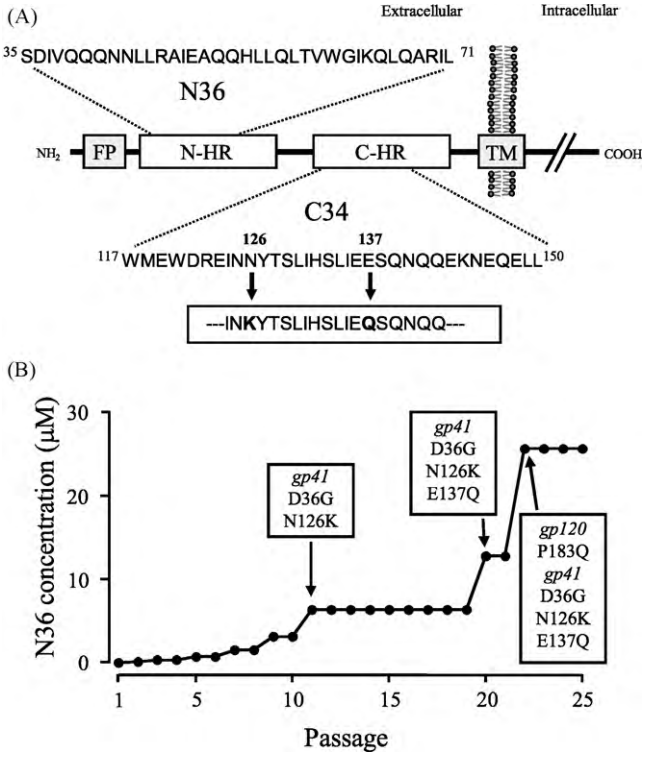

Fig. 1. Schematic view of HIV-1 gp41 (A) and induction of N36-resistant HIV-1 (B). The locations of the fusion peptide (FP), N-terminal heptad repeat region (N-HR), C-terminal heptad repeat region (C-HR), transmembrane domain (TM), and the gp41-derived peptides, N36 and C34, are shown (A). The residue numbers of each peptide correspond to their positions in gp41. The bold underlined letters in the box indicate the novel mutations that were observed in the C-HR of N36-resistant HIV-1 variants. (B) HIV-1_{WT} was passaged in MT-2 cells with increasing concentrations of N36. The dose-escalating selection was carried out for a total of 25 passages, with compound concentrations ranging from 0.1 to 25.6 μM. At the indicated passages, proviral DNA from the lysates of infected cells were sequenced.

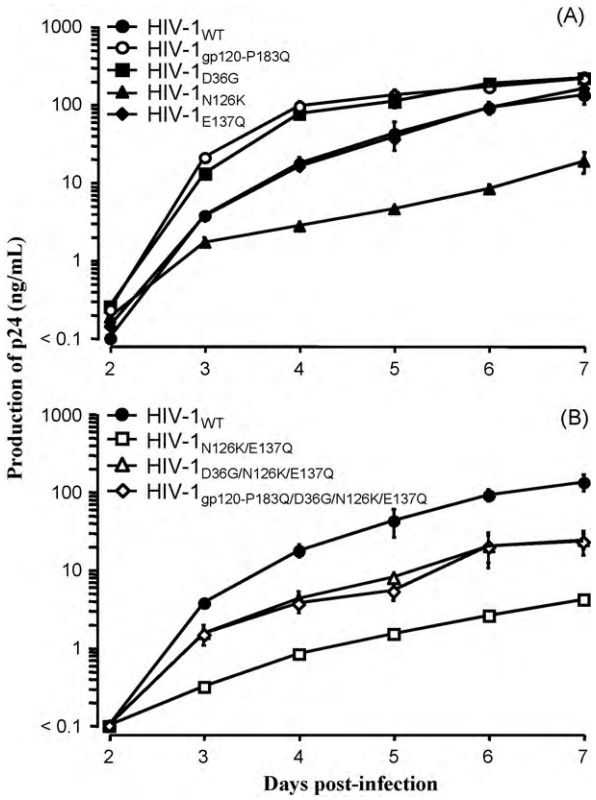

Fig. 2. Replication kinetics of N36-resistant variants. Replication kinetics of N36-resistant HIV-1 variants with a single (A) or combination of mutations (B). Supernatants were collected on days 2–7 from infected MT-2 cells and were subjected to determination of p24 production. Representative results show the mean and standard deviation of experiments performed independently three times.

3.2. Susceptibility of HIV-1 variants to N36

To identify which substitutions were responsible for N36 resistance, we generated seven recombinant viruses, and examined the susceptibility of N36 against these viruses with the MAGI assay. HIV-1_{N126K}, HIV-1_{E137Q}, HIV-$1_{N126K/E137Q}$, HIV-$1_{D36G/N126K/E137Q}$, and HIV-$1_{gp120\text{-}P183Q/D36G/N126K/E137Q}$ showed reduced susceptibility to N36 (Table 2). Since N126K emerged in HIV-1 variants resistant to C-HR-derived peptides (Baldwin et al., 2004; Nameki et al., 2005), all N126K containing viruses also demonstrated a 4–5-fold reduction in susceptibility to C34. The D36G substitution, observed in the majority of HIV-1 strains (Kuiken et al., 2009), and P183Q in gp120 demonstrated no resistance to all the inhibitors tested. P183Q which is located in the gp120 V2 region was observed in a wide range of HIV-1 subtypes including subtypes A, B, and C as well as the wild-type, indicating that P183Q is a polymorphism (Kuiken et al., 2009). These results indicate that the single mutations, N126K and E137Q, are involved in resistance to N36, but when both mutations were present, only a weak effect on resistance was observed.

3.3. Replication kinetics of N36-resistant variants

To address effects of the mutations on HIV-1 replication, we examined replication kinetics of HIV-1 variants through p24 production in culture supernatants. The N126K substitution had an adverse effect on replication kinetics, while E137Q exhibited no effect on replication kinetics compared to HIV-1_{WT} (Fig. 2A). The variant, HIV-$1_{N126K/E137Q}$ had markedly reduced replication kinetics, however the D36G mutation moderately restored these kinetics

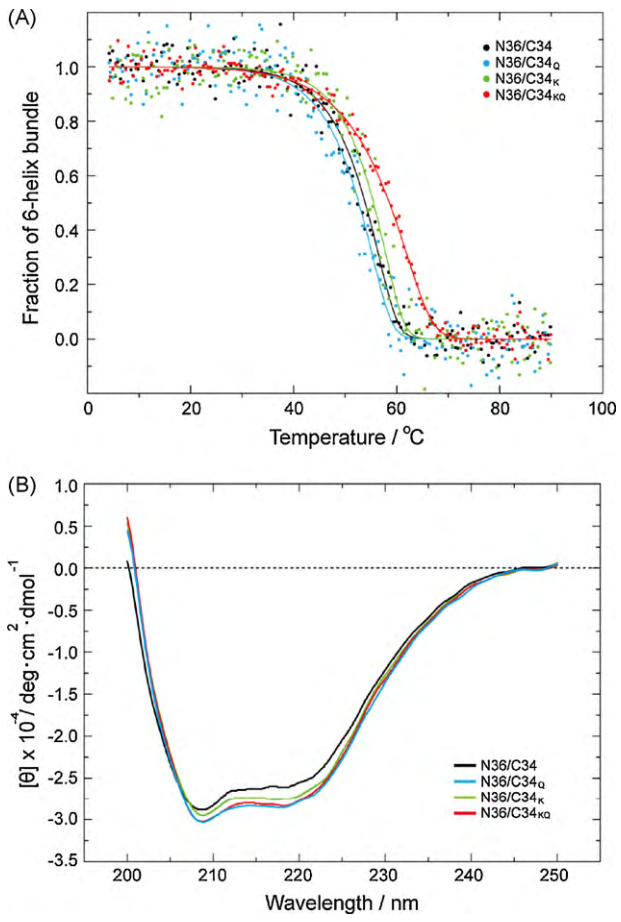

Fig. 3. Affinity of N36 and C34 with or without mutation. Melting temperature (T_m) and CD spectra of the N36/C34 (wild-type), N36/C34$_K$, N36/C34$_Q$ and N36/C34$_{KQ}$ complexes are shown (A). CD spectra profile of N36/C34 and N36/C34$_{KQ}$ complexes are shown (B).

(Fig. 2B). These results indicate that the primary mutations, N126K and E137Q, are strongly and weakly associated with a reduction in HIV-1 replication kinetics, respectively. In contrast, P183Q in gp120 solely enhanced the replication kinetics of HIV-1 (Fig. 2A) but did little to alter the replication kinetics of HIV-1$_{D36G/N126K/E137Q}$ (Fig. 2B). P183Q and D36G appear to be secondary or spontaneous mutations in HIV-1 induced by serial passage in an attempt to adapt to a cell culture environment.

3.4. Thermal stability of the 6-helix bundle

Synthetic peptides representing the N- and C-HRs have been shown to fold into thermally stable 6-helix bundles (Lu et al., 1999; Lu and Kim, 1997), and thus provide a model of gp41 for investigating structural and other biophysical properties of a 6-helix bundle. To determine whether N126K and E137Q contributed to the stability of the 6-helix bundle, we measured the T_m of the complexes, N36/C34 (wild-type) and N36/C34$_{KQ}$ (C34 with N126K and E137Q mutations). As shown in Fig. 3A, T_m values of the complex of N36/C34$_K$ and N36/C34$_Q$ were similar to that of N36/C34 complex, while that of N36/C34$_{KQ}$ was increased. In addition, the CD spectra observed in N36/C34$_{KQ}$ and N36/C34$_Q$ at 25 °C showed a high helix extent compared to that in N36/C34, while N36/C34$_K$ showed intermediate helicity (Fig. 3B). These results indicate that the two mutations, N126K and E137Q, co-operatively provide high thermal stability of the N36/C34$_{KQ}$ complex.

Table 1
Crystallization data, statistics and refinement parameters.

Crystallization	
Peptide solution	
N36	10 mg/mL
C34$_{KQ}$	10 mg/mL
Mother liquor	
Ammonium chloride	80 mM
2-Methylpentan-2,4-diol	16%
Isopropanol	25%
Data collection	
Resolution range/Å	35–1.7
No. of observed reflections	99,054
No. of unique reflections	27,434
Completeness	99.1%
Redundancy	3.61
R_{merge}	7.2%
Space group	*C*2
Unit-cell parameters	
$a = 88.63$, $b = 50.48$, $c = 56.15$ Å, $\beta = 90.88°$	
Refinement	
Resolution range/Å	23.8–1.7
No. of reflections in the working set	25,818
No. of protein atoms	1803
No. of water molecules	146
R/R_{free}	18.8/21.9%

3.5. Crystal structure of the 6-helix bundle

To reveal the structural basis of the resistance and stabilization mechanisms by the double mutations, we solved the crystal structure of the N36/C34$_{KQ}$ complex (PDB code 3AHA). The statistics for data collection and refinement are summarized in Table 1. The solved structure showed a 6-helix bundle structure, which was the same as in the wild-type N36/C34 complex (PDB code 1AIK) (Fig. 4). Their main chain structures are completely identical in helical pitch as well as in twist angle. A careful comparison between the N36/C34 and N36/C34$_{KQ}$ complexes revealed some differences in side-chain interactions. The N36/C34 complex has been solved in the trigonal *P*321 space group so that the asymmetric unit contains one of each N36 and C34 peptide, in which symmetrical side-chain interactions along the 3-fold rotation axis are formed around the bundle. We solved the N36/C34$_{KQ}$ complex in the lower symmetry *C*2 space group, and this asymmetric unit contained one helix bundle structure (Fig. 4).

The N126K mutation does not seem to induce conformational changes in the crystal structure when compared to the N36/C34 complex (Fig. 4C). It is consistent with the location of N126 that the N126K mutation does not directly interact with the binding groove formed by the N-HR trimer (Fig. 4A). In the case of E137Q, local rearrangement of a hydrogen bond was induced (Fig. 4D–G), although the hydrogen bond network formed by E/Q137-N43-R46-N42-Q142′-Q40′ is maintained. These asymmetrical and inter-helical side-chain interactions may contribute to the stability of gp41.

4. Discussion

In this study, we induced N36-resistant variants of HIV-1 *in vitro* and characterized them. The variants contained four mutations, P183Q in gp120 and D36G, N126K, and E137Q in gp41. Among these mutations, N126K and E137Q were directly associated with N36 resistance. The N126K mutation also conferred resistance in HIV-1 to C-HR-derived peptides (Baldwin et al., 2004; Eggink et al., 2008; Nameki et al., 2005) and a cell surface expressed peptide (Hermann et al., 2009), indicating that N126K is a key mutation for acquisition

of resistance to all gp41-derived fusion inhibitors.

E137Q was not observed as a polymorphism (Kuiken et al., 2009) and was not identified in HIV-1 that was resistant to the N-HR-derived peptide, N13ΔTrimer, which had reasonable solubility, high helicity and thermostability (Dwyer et al., 2008). Therefore, E137Q is a novel mutation for N36 resistance. The solved crystal structure revealed that E137Q induces local rearrangement of the hydrogen bond network in gp41. A polymorphism E137K (Kuiken et al., 2009) has been identified as one of resistance associated mutations to N44 in a CCR5 tropic HIV strain, HIV-1_{JR-CSF} (Desmezieres et al., 2005). Recently, Tolstrup et al. (2007) also reported that E137K restored T-20-resistant virus infectivity impaired by the acquisition of the N43D mutation. Since E137 formed hydrogen bonds with N43, it is likely that the E137K mutation partially restores the 6-helix bundle stability (Bai et al., 2008), suggesting that E137 is an important position for stability of the 6-helix bundle and E137Q is a mutation for conferring N36-resistance in HIV-1. On the other hand, the overall structure of the 6-helix bundle with mutations was barely affected, which is consistent with our recent observations. The C34 derivative, SC34EK, which contains 12 hydrophilic and one artificial substitution (Nishikawa et al., 2009) has highly potent anti-HIV activity and maintains its structure in the 6-helix bundle even with these extensive modifications. These results indicate that the basic structure of the 6-helix bundle appears to be crucial for gp41 function, suggesting that agents which disrupt this structure will have inhibitory effects upon fusion.

Other substitutions observed in the N36 selection, P183Q in gp120 and D36G in gp41, enhanced viral replication kinetics (Fig. 2) but little influenced N36 susceptibility (Table 2). It is likely that faster entry kinetics theoretically provides resistance to fusion inhibitors through relatively short period to allow interaction of fusion inhibitors with the target, gp41. Indeed, HIV-1 with the

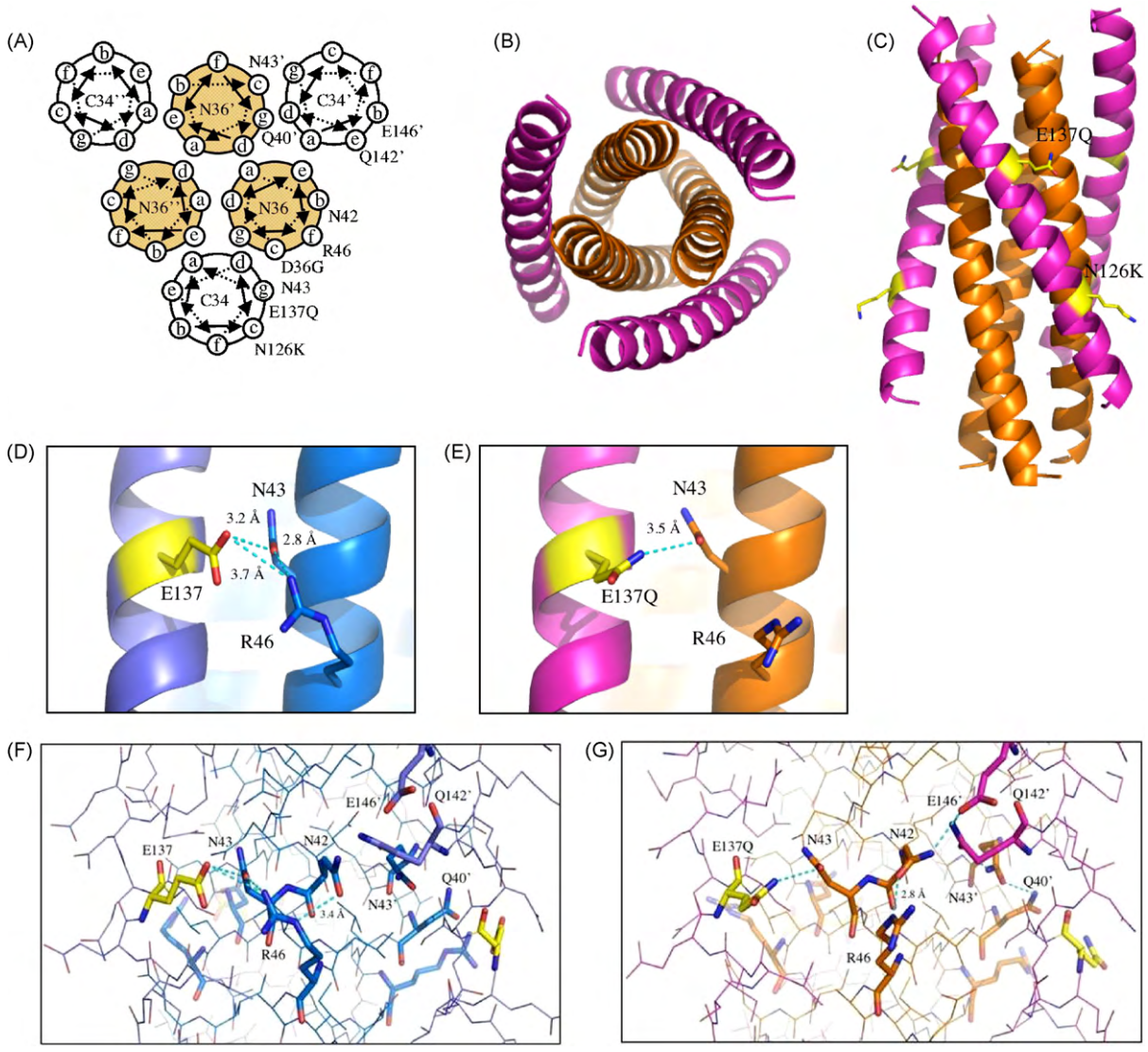

Fig. 4. Helical wheel representation of N36 and C34, and structure of N36-resistant HIV-1 gp41. The 6-helix bundle is represented as helical wheel projections (Chan et al., 1997) and the view is from the top of the complex. N36 and C34, N36′ and C34′, and N36″ and C34″ indicate each pair of a N36- and a C34-helix. The position of amino acid mutations in N36-resistant HIV-1 and the amino acids related to formation of hydrogen bonds is shown (A and B). (C) The entire structure of the 6-helix bundle with N126K and E137Q mutations. The views, focused on binding between an N36 and a C34 (D and F) and an N36 and a $C34_{KQ}$ (E and G), are shown. A hydrogen bond is depicted by the blue dashed line. (D) A hydroxyl group of the E137 side chain formed two hydrogen bonds with the amide oxygen of N43 and the amine of R46. A hydrogen bond also formed between the amide oxygen of N43 and the amine of R46. (E) The amide hydrogen of the E137Q side chain sustains a hydrogen bond with the amide oxygen of N43. The hydrogen bond formed between the amide hydrogen of E137Q and the amine of R46 was lost. The hydrogen bond formed between the amide oxygen of N43 and the amine of R46 in N36 was also lost. The views, focused on the hydrogen bond in the entire 6-helix bundle, formed with N36 and C34 (F) and N36 and $C34_{KQ}$ (G) are shown. A hydrogen bond is depicted by a blue dashed line. An apostrophe, upper right of an amino acid, means the amino acids of a neighboring helix pair as indicated in (A).

Table 2
Antiviral activity of HIV-1 gp41-derived peptides against recombinant viruses.

Viruses	EC_{50} [a] (nM)			
	ddC	DS5000	C34	N36
HIV-1_{WT}	505 ± 15[b]	56 ± 8.2	2.8 ± 1.2	200 ± 13
HIV-1_{D36G}	640 ± 76[c] (1.3)	94 ± 22 (1.7)	2.8 ± 1.2 (1)	250 ± 46 (1.3)
HIV-1_{N126K}	515 ± 86 (1)	88 ± 19 (1.5)	11 ± 3.1 (4)	550 ± 105 (2.8)
HIV-1_{E137Q}	500 ± 90 (1)	84 ± 14 (1.5)	3.0 ± 1.0 (1.1)	636 ± 159 (3.2)
HIV-$1_{N126K/E137Q}$	642 ± 84 (1.3)	74 ± 6.0 (1.3)	12 ± 4.7 (4.4)	731 ± 121 (3.7)
HIV-$1_{D36G/N126K/E137Q}$	728 ± 29 (1.4)	40 ± 4.0 (0.7)	15 ± 1.0 (5.2)	796 ± 144 (4)
HIV-$1_{gp120\text{-}P183Q}$	414 ± 50 (0.8)	58 ± 7.5 (1)	2.7 ± 0.6 (1)	343 ± 76 (1.7)
HIV-$1_{gp120\text{-}P183Q/D36G/N126K/E137Q}$	634 ± 150 (1.2)	43 ± 5.7 (0.8)	15 ± 2.4 (5.2)	573 ± 64 (2.9)

[a] Anti-HIV activity was determined with the MAGI assay.
[b] The data shown are mean value and standard deviation (SD) obtained from the results of at least three independent experiments.
[c] Values in parentheses represent fold-resistance in EC_{50} for recombinant viruses compared to HIV-1_{WT}.

faster entry kinetics shows resistance to T-20 (Reeves et al., 2002; Heredia et al., 2007; Hermann et al., 2009). However, only CCR5 tropic HIV-1s were subjected to be examined the entry kinetics and T-20 susceptibility in these studies, while CXCR4 tropic HIV-1s have been less examined yet. Interestingly, cell surface expression level of CCR5 influenced T-20 susceptibility but those of CD4 did not (Heredia et al., 2007), suggesting that entry kinetics of HIV-1 only through CCR5 influences T-20 susceptibility. In contrast, our study and others also revealed that D36G, the faster virus with CXCR4 tropism, showed high susceptibility to T-20 (Ueno et al., 2009; Mink et al., 2005; Kinomoto et al., 2005). In the present study, the faster viruses with mutations, such as P183Qand D36G little provided N36 resistance (Table 2). Thus, it is likely that there are some unknown differences in fusion inhibitor susceptibility between CXCR4 and CCR5 tropic viruses. As one of possibilities, Kahle et al. (2009) reported interesting data that asymmetric deactivation is observed in the C-HR targeted 5-helix peptide. Activity of the C-HR-derived peptide, C37 is well-correlated with binding affinity to the N-HR (K_D value), while that of 5-helix targeted the C-HR is poorly-correlated. They proposed a novel mechanism of fusion inhibition that peptides targeting N-HR and C-HR have distinctively reversible and irreversible deactivation of gp41 function, respectively.

D36G is one of the characteristic substitutions or polymorphisms for HIV-$1_{NL4\text{-}3}$, since only HIV-$1_{NL4\text{-}3}$ has D36 and others originally contains G36 (Kuiken et al., 2009). Kinomoto et al. (2009) analyzed D36G effect in a structure modeling with SIV gp41 and revealed D36 could induce distortion or incorrect positioning of the N and C helices by misdirected salt bridge with K144. However, less difference between the N36 conformations of wild-type and D36G mutant was expected, since the D36G, the second amino acid from the N-terminal end of N36, is located in the flexible region of N36 sequence. Experiments with the N-HR-derived peptide including expanded N-terminal region of N36 will be needed to reveal structural feature of D36G in the N-HR. In addition to a role in the viral entry, D36G has another role in viral replication kinetics as nucleotides ($D_{GAU}36G_{GGU}$) in the stem II_C of Rev responsive element (Ueno et al., 2009; Nameki et al., 2005). Nameki et al. (2005) demonstrated that $A_{GCC}30V_{GUC}$ located outside of the N-HR but complementally with $D_{GAU}36$ in the stem II_C, also enhanced replication kinetics of HIV-$1_{NL4\text{-}3}$ (underlined nucleotides; complementally located in the stem II_C) through compensation of the RNA structure. As such, D36G has at least 2 advantages, fusion and RNA stability for viral replication. In the present study, we, therefore, focused mainly on mutations in the C-HR region. In the CD analysis, enhanced 6-helix bundle stability of $N36/C34_{KQ}$ was observed, while structural alteration of the 6-helix bundle was not apparent. We hypothesize that the mechanism of resistance to N36 has little association with the binding ability of C-HR. Although the resistance of HIV-1 to C-HR-derived peptides accounts for a correlation with the binding affinity of N-HR, an important factor in the resistance to N36 may exist before completion of 6-helix bundle formation. Steger et al. reported that the 5-helix fusion inhibitor, containing three N-HR and two C-HR segments, targeting the C-HR region in gp41 as well as N36, demonstrated poor correlation between inhibitory potency and interaction affinity with C-HR (Steger and Root, 2006). The IC_{50} values of 5-helix variants with some mutations in the sequence were inversely proportional to their association rate constants. It is consistent with observations by Kahle et al. (2009) mentioned above that mechanism of inhibition of peptides targeting the N- and C-HRs seems to be different, deactivation of gp41 function and dominant negative/decoy effect, respectively. The anti-HIV activity of N36, which has the same target as the 5-helix variants, might be also correlated with association rate constants to C-HR and finally irreversibly deactivate the gp41 function.

In conclusion, non-aggregating and trimeric coiled-coil N-HR-derived peptides such as $(CCIZN17)_3$ have been developed and exhibit more potent anti-HIV-1 activity (Bianchi et al., 2005; Eckert and Kim, 2001a), as well as exerting a strong synergistic effect with T-20 (Bianchi et al., 2005). Therefore, N-HR-derived peptides with appropriate modifications are promising because of possible co-administration with T-20 and modulation of the resistance profile. The mechanism of resistance to N36, described in this paper, provides a role for the N126K and E137Q mutations in 6-helix bundle stability, although N126 does not directly associate with the N36 surface. Further experiments are needed to clarify the role of the common N126K mutation in HIV-1 fusion.

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