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Impact of Human Immunodeficiency Virus Type 1 gp41 Amino Acid Substitutions Selected during Enfuvirtide Treatment on gp41 Binding and Antiviral Potency of Enfuvirtide In Vitro

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Enfuvirtide (ENF), a novel human immunodeficiency virus type 1 (HIV-1) fusion inhibitor, has potent antiviral activity against HIV-1 both in vitro and in vivo. Resistance to ENF observed after in vitro passaging was associated with changes in a three-amino-acid (aa) motif, GIV, at positions 36 to 38 of gp41. Patients with ongoing viral replication while receiving ENF during clinical trials acquired substitutions within gp41 aa 36 to 45 in the first heptad repeat (HR-1) of gp41 in both population-based plasma virus sequences and proviral DNA sequences from isolates showing reduced susceptibilities to ENF. To investigate their impact on ENF susceptibility, substitutions were introduced into a modified pNL4-3 strain by site-directed mutagenesis, and the susceptibilities of mutant viruses and patient-derived isolates to ENF were tested. In general, susceptibility decreases for single substitutions were lower than those for double substitutions, and the levels of ENF resistance seen for clinical isolates were higher than those observed for the site-directed mutant viruses. The mechanism of ENF resistance was explored for a subset of the substitutions by expressing them in the context of a maltose binding protein chimera containing a portion of the gp41 ectodomain and measuring their binding affinity to fluorescein-labeled ENF. Changes in binding affinity for the mutant gp41 fusion proteins correlated with the ENF susceptibilities of viruses containing the same substitutions. The combined results support the key role of gp41 aa 36 to 45 in the development of resistance to ENF and illustrate that additional envelope regions contribute to the ENF susceptibility of fusion inhibitor-naïve viruses and resistance to ENF.

The emergence of drug-resistant viruses remains one of the most serious impediments to successful antiretroviral therapy for human immunodeficiency virus (HIV)-infected individuals. All currently available antiretrovirals belong to one of four mechanistic classes: nucleoside/nucleotide reverse transcriptase inhibitors, nonnucleoside reverse transcriptase inhibitors, protease inhibitors, and the newest class, fusion inhibitors. Cross-resistance within the nucleoside/nucleotide reverse transcriptase inhibitor, nonnucleoside reverse transcriptase inhibitor, and protease inhibitor classes is extensive, and treatment options for multidrug-experienced patients are often severely limited. Consequently, there is a need for new antiretrovirals that act against novel targets and will not be affected by viral resistance to earlier classes of drugs; the process of viral entry represents one such target.

HIV type 1 (HIV-1) entry occurs via a multistep process involving the interaction of trimeric envelope (Env) glycoprotein complexes with cellular receptors and subsequently with the target cell membrane. The process ultimately brings the viral and target cell membranes into contact, leading to the formation of a fusion pore and viral entry. In the first part of the process, HIV-1 glycoprotein 120 (gp120) attaches to the cellular CD4 receptor, resulting in conformational changes that expose the coreceptor-binding domain and the trimeric coiled coil formed by the heptad repeat 1 (HR-1) regions of gp41 (26). Binding to cellular coreceptors (CCR5 or CXCR4) then triggers the release of gp120 from the viral envelope glycoprotein complex. In the second phase of entry, a putative hydrophobic fusion peptide at the N terminus of gp41 exposed by the release of gp120 inserts into the target cell membrane. At this stage, the HR-1 regions of the three gp41 molecules are thought to exist as a trimeric coiled-coil structure, and three cognate HR-2 domains bind along the grooves in the HR-1 coiled coil to form a six-helix bundle, thereby facilitating contact between the viral and target cell membranes. Finally, a fusion pore between the viral and target cell membranes that is permissive for viral entry is thought to be created by the aggregation of multiple gp41 trimers that have converted to the six-helix bundle conformation (9, 30).

Several entry inhibitors have been described in the literature and have entered clinical evaluation, including the CD4 binding antagonists PRO 542 (11) and BMS 806 (19), the CXCR4 binding inhibitor AMD3100 (7), the CCR5 binding inhibitors SCH-C and -D (28), UK-427,857 (25), and AK602/GW873140 (20), and the peptide fusion inhibitors enfuvirtide (ENF, formerly T-20) and T-1249 (8, 13). ENF, which has been approved by a number of health authorities worldwide, is a 36-amino-acid (aa) synthetic peptide whose primary sequence was derived from the gp41 HR-2 region of HIV-1_{LAI} (32). ENF has potent antiviral activity against HIV-1 in vitro (32) and during clinical trials has demonstrated significant virological and immunological benefits when used in combination with other antiretrovirals in treatment-experienced patients (15–18).

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An understanding of mechanisms of resistance and the potential for intra- and interclass cross-resistance is an important aspect of antiviral drug development. In vitro passaging of HIV-1 IIIB and NL4-3 viruses in the presence of increasing concentrations of ENF resulted in the selection of ENF-resistant viruses carrying mutations in the N-terminal HR-1 region of HIV-1 gp41 (24). Clones derived from these viral populations revealed that aa substitutions at positions 36 to 38 of gp41 were responsible for the reduction in susceptibility to ENF. The involvement of this three-aa motif in conferring viral resistance to ENF was also confirmed by site-directed mutagenesis experiments. These studies suggested that the 36S, 36D, 37T, and 38M mutations could all contribute to ENF resistance but that these mutations had to be present in pairs in order to generate a resistant phenotype. These findings were later confirmed by Derdeyn et al. (5, 6). They constructed mutant viruses based on HIV-1 NL4-3 with mutations to aa 36 to 38 and showed that viruses carrying 36D or 36S plus 38M had significant resistance to ENF. The selection of resistance mutations in aa 36 to 38 of the HR-1 region of gp41 is consistent with the hypothesis that ENF exerts its anti-HIV activity by binding to this region (4). This hypothesis is further supported by the fact that radiolabeled ENF could bind a peptide representing residues 29 to 75 of wild-type HR-1 but not to the equivalent peptide carrying the SIM sequence at positions 36 to 38. In addition, the 29- to 75-residue wild-type peptide could competitively block the antiviral activity of ENF (24), consistent with earlier studies (4).

Studies of viral resistance to ENF carried out during early clinical trials have suggested that changes in the gp41 genotype conferring resistance to ENF may cover a wider aa region than the aa-36-to-38 motif identified in the initial in vitro selection experiments (24, 27, 29; M. Mink, M. L. Greenberg, S. Mosier, et al., Abstr. 11th Int. Drug Resistance Workshop, abstr. 22, 2002). The aim of the present study was to further investigate this possibility by examining the effects of mutations arising in vivo in response to therapy with ENF on ENF susceptibility in vitro and on the binding of ENF to HR-1.

MATERIALS AND METHODS

Samples. Peripheral blood mononuclear cell (PBMC)-derived virus isolates used for these studies were obtained from samples from 14 patients participating in the T20-205 phase II trial (16) and from 3 patients participating in the T20-208 phase II trial (31). Patients in trial T20-205 received subcutaneous (s.c.) injections of ENF (45 mg, twice daily) in addition to an individualized background of two or more antiretrovirals (16), while patients in trial T20-208 received 67.5 or 90 mg of s.c. ENF twice daily for a period of 48 weeks (31). All virus isolates that were recovered at baseline and at weeks 4, 16, 24, 32, and 48 were examined for susceptibility to ENF (27). Isolates recovered from patients receiving treatment (on-treatment isolates) exhibiting either a 10-fold decrease in ENF susceptibility or mutations arising in the HR-1 region of gp41 were selected for study along with their paired baseline isolates.

Population sequencing of plasma HIV-1 RNA. Viral RNAs were extracted from plasma samples from patients participating in phase II trials of ENF (12, 13, 16, 31) with a QIAamp viral RNA kit (QIAGEN), using a slight modification of the manufacturer's instructions. The gp41-encoding region was amplified by reverse transcription-PCR (RT-PCR) using random hexamers for RT and the forward primer E45 (CCTGCCTAACTCTATTCAC), with either SK68i (TTC TTiGGAGCAGCiGGAAGCACiATGG; "i" denotes deoxyinosine) or E170 (A GCAGGAAGCACTATGGGCG) as the reverse primer, for PCR. A nested PCR step was included prior to sequencing. The sequence of plasma virus gp41 aa 1 to 177 was determined using SRA GenoScan technology at SRA Life Sciences Inc. (Rockville, MD).

Virus isolation from patient PBMC and expansion. Virus isolates were recovered from patient PBMC via coculture with healthy donor PBMC at SRA Life Sciences Inc. (Rockville, MD), as follows. Approximately 3×10^6 patient PBMC were suspended with 2×10^6 healthy donor PBMC in 1 ml of RPMI 1640, 15% fetal bovine serum, 10% interleukin-2, 1% penicillin-streptomycin, and 2 mM L-glutamine. Phytohemagglutinin (PHA) was added to give a concentration of 5 μg/ml, and cultures were incubated for 24 h at 37°C in 5% CO_2 in a humidified incubator for 30 min, after which 4 ml of the medium (described above) was added. Cultures were incubated for an additional 20 to 24 h before the cells were washed and resuspended in 2.5 ml of the same medium as that described above, but containing 2 μg/ml Polybrene. On day 3 or 4, 2 ml of culture supernatant was removed, discarded, and replaced with 2×10^6 PHA-stimulated donor PBMC and 2 ml of culture medium containing 2 μg/ml Polybrene. On days 7, 14, 21, and 28, 2 ml of culture supernatant was removed and assayed for p24 content to assess virus production, and 2×10^6 PHA-stimulated donor PBMC and 2 ml of culture medium containing 2 μg/ml Polybrene were added to refeed the cultures. Virus isolations were expanded when the p24 assay yielded an optical density reading of 2.0 or above for two consecutive assays. The contents of cultures for expansion were transferred to T25 flasks, and 4×10^6 PHA-stimulated donor PBMC and 3 ml of culture medium containing 2 μg/ml Polybrene were added. On days 4 and 7, 2 ml of culture supernatant was removed, and 4×10^6 PHA-stimulated donor PBMC and 4 ml of culture medium containing 2 μg/ml Polybrene were added. On day 11, 2 ml of culture supernatant was removed, 4×10^6 PHA-stimulated donor PBMC and 4.5 ml of culture medium containing 2 μg/ml Polybrene were added, and the cultures were incubated as described above until day 14, when the virus stocks were harvested and clarified by centrifugation. Virus stocks were tested in a cMAGI cell assay (see below) to ascertain whether they were of sufficient titers for antiviral studies (generally at least 10^4 infectious centers/ml).

Proviral DNA gp41 sequencing. Genomic DNAs were isolated from infected PBMC using a genomic DNA purification kit (Gentra Systems) according to the manufacturer's protocol. The entire gp41 region was amplified from proviral DNA using the primers TO-702 (GGCGATATGAGGGACAATTGGAGA) and TO-701 (CTGTATTGCTACTTGTGATTGTGATTGCTCC). Nested PCR was performed with TO-698 (GCACCCTCTAGAGCAAAGAGAAGAGTGG TGCAGAGA) and TO-699 (CTAGGTCTCGAGATACTGCTCCCACCCCAT CTGCT). The sequence of the gp41 ectodomain (aa 1 to 177) was determined in both directions using dye terminator chemistry with a CEQ 2000XL DNA analysis system (Beckman Coulter) and DNAStar software in the research laboratories at Trimeris Inc. (Morrisville, NC).

Measurement of ENF binding to mutant HR-1. A Stratagene QuikChange site-directed mutagenesis kit was used to introduce mutations into the ectodomain of HIV gp41 (lacking HR-2) cloned into the expression vector pMal-p2 (New England Biolabs). The site-directed mutants were transformed into *Escherichia coli*, and the truncated ectodomain was expressed as a maltose binding protein chimera, M41Δ178, as previously described (4).

ENF was synthesized to contain fluorescein at its amino terminus. Equilibrium saturation binding parameters were determined using a constant amount of fluorescein-labeled ENF mixed with increasing amounts of mutant protein. Binding was measured by fluorescence polarization. Data were first calculated in polarization units and then normalized to the percentage of maximum binding to account for subtle differences in microplate background fluorescence.

Generation of mutant viruses by site-directed mutagenesis. Substitutions were introduced into a 3′NL4-3 plasmid template using synthetic oligonucleotide primers containing the relevant mutations and a Stratagene QuikChange site-directed mutagenesis kit as previously described. The mutated 3′NL4-3 plasmid (10 μg) was combined with a 5′NL4-3 plasmid (10 μg), and both were then linearized with EcoRI prior to transfection. The two plasmids comprise the complete HIV-1 pNL4-3 proviral clone (1). This laboratory strain of HIV-1 has an unusual aspartic acid (D) at position 36 of gp41 instead of the highly conserved glycine (G) observed in almost all fusion inhibitor-naïve viruses (24, 27). Therefore, a D36G substitution was engineered into the original NL4-3 virus to produce NL4-3/D36G, which was then used as the parental strain to produce the series of mutants to be tested. NL4-3/D36G was considered the wild type for all comparative analyses. All site-directed mutant constructs were confirmed by DNA sequencing as described above. Wild-type and mutant virus stocks were generated by transfection of CEM-x174 cells followed by infection of CEM4 cells with cell-free viral supernatants from the transfected cultures.

Phenotypic analysis of ENF susceptibility of NL4-3 mutants and clinical isolates. The susceptibility of NL4-3 mutants and clinical isolates was determined with a MAGI or cMAGI cell assay, respectively. HeLa cells express the CXCR4 chemokine receptor. MAGI cells are HeLa cells stably transfected to express the CD4 receptor and contain a modified β-galactosidase gene under the control of

the HIV-1 long terminal repeat region and are susceptible to infection by HIV-1 strains that use the CXCR4 coreceptor (14). cMAGI cells have been further modified to also express the CCR5 coreceptor and are susceptible to infection by HIV-1 strains that can use the CCR5 coreceptor (3). The infection of either MAGI or cMAGI cells with HIV-1 and the resultant expression of the *tat* gene product lead to the production of β-galactosidase and its accumulation in the nucleus (14). Infected cells can be visualized by staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and quantified using charge-coupled device microscopy.

Viruses to be tested were pretitrated to determine the virus dilution yielding approximately 1,000 to 2,000 infectious centers per 0.1 ml. The day prior to ENF susceptibility testing, 2.2×10^4 MAGI or cMAGI cells were plated in 0.3 ml of complete medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% penicillin-streptomycin, 2 mM L-glutamine, 0.1% amphotericin B [Fungizone]). On the day of the drug susceptibility assay, subconfluent MAGI or cMAGI cells in 48-well plates were incubated at 37°C in 5% CO_2 in complete medium containing 20 μg/ml DEAE with duplicate serial dilutions of ENF. Viruses to be tested were added to the cells at 0.1 ml/well. After 20 to 24 h of incubation, ENF or T-1249 (for ENF-resistant viruses) was added to a final concentration of 5 μg/ml in order to prevent subsequent rounds of infection, limiting the analysis to a single round of infection. The cells were incubated for a further 48 h before being fixed and stained for counting. The concentration of ENF required to inhibit viral replication by 50% (IC_{50}) was determined according to the method of Reed and Muench (22), using a Microsoft Excel macro, from a plot of the log drug concentration against the number of infected centers.

Preparation of reporter viruses pseudotyped with wild-type or mutant envelopes and characterization of their susceptibility to ENF. Full-length viral envelope genes were amplified from viral DNA by PCR (for NL4-3/D36G) or from viral RNA by RT-PCR with virus stocks of the primary isolates 030, 060, and 098. The envelope amplicons were cloned into a modified pCR3.1 vector in a similar manner to that previously described (10, 33), and the captured envelope clones served as the starting point for site-directed mutant constructs. Site-directed mutants were made using a Stratagene QuikChange site-directed mutagenesis kit and confirmed by DNA sequencing as described above. Stocks of reporter viruses pseudotyped with parental or mutant envelopes were prepared essentially as follows. 293T cells (a gift from Robert Doms) were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum and 1% penicillin-streptomycin. 293T cells were seeded at 6×10^5 cells per well in a six-well dish 1 day prior to transfection. Cells were cotransfected with 1.33 μg of an *env* expression vector and 0.67 μg of plasmid pNL4-3.Luc.R^-E^- (obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute for Allergy and Infectious Diseases, National Institutes of Health, from Nathaniel Landau) using PolyFect (QIAGEN) according to the manufacturer's recommendations. Virus-containing supernatants were harvested at 48 h posttransfection, filtered, mixed with heat-inactivated fetal calf serum to bring the concentration to 20% (vol/vol), and stored at −80°C until used for infection. The ability of cloned *env* to mediate infection via the CCR5 and/or CXCR4 coreceptor was assessed in U87 astroglioma cells stably expressing the CD4 receptor and either the CCR5 or CXCR4 coreceptor. U87 cells were seeded at 7.5×10^3 cells per well in a 96-well plate and infected with 100 μl of pseudotyped virus-containing supernatant. Cells were harvested at 72 h postinfection and assayed with the Promega luciferase assay system. The sensitivities to ENF inhibition of reporter viruses pseudotyped with envelope clones and site-directed mutant envelopes were determined with U87 cells expressing CD4 and CXCR4. To assess susceptibility to ENF, the infection of U87 cells was carried out in the presence or absence of various concentrations of ENF, and the IC_{50} (assessed via the luciferase reporter) was determined according to the method of Reed and Muench, as described above (22).

RESULTS

Effects of mutations in HR-1 region of gp41 on ENF binding and antiviral activity. The binding of ENF to recombinant maltose binding fusion proteins either containing the wild-type HR-1 region of the gp41 ectodomain or carrying a subset of amino acid substitutions in the region between aa position 29 and position 43 observed in clinical samples is shown in Table 1. This region was chosen to extend seven aa (or one heptad repeat) on either side of the GIV motif that was previously shown to be involved in reducing susceptibility to ENF (24).

TABLE 1. Effects of amino acid substitutions at positions 29 to 43 of recombinant HIV-1 gp41 on ENF binding to the first heptad repeat and on the antiviral activity of ENF

Amino acid sequence[a]	Binding (B_{50} [μg/ml]) (fold change)[b]	Antiviral activity (IC_{50} [μg/ml]) (fold change)[b]
QARQLLSGIVQQQNN	0.10	0.009
RARQLLSGIVQQQNN	0.24 (2.4)	ND
QAR**L**LLSGIVQQQNN	0.30 (3.0)	0.005 (<1)
QARQLLS**D**IVQQQNN	0.70 (7.0)	0.091 (10.1)
QARQLLS**S**IVQQQNN	0.95 (9.5)	0.063 (7.0)
QARQLLSGI**A**QQQNN	ND	0.160 (17.8)
QARQLLSGIV**H**QQNN	0.25 (2.5)	0.011 (1.2)
QARQLLSGIVQ**H**QNN	4.33 (43.3)	0.256 (28.4)
QARQLLSGIVQQQN**D**	4.96 (49.6)	0.210 (23.3)

[a] Positions 29 to 43 of gp41. Substituted amino acids are given in bold.
[b] Spearman nonparametric correlation analysis indicated a significant correlation between the measured binding affinities and antiviral activities (Spearman $r = 0.82143$; $P = 0.0234$). ND, not determined.

The mutations investigated were identified based on those that were initially observed during treatment with ENF in clinical studies (12, 13, 16). Recombinant NL4-3-based virus constructs carrying the same mutations in gp41 were also tested in the MAGI assay for their susceptibility to ENF. This allowed us to explore the potential mechanism(s) of resistance by assessing whether a correlation existed between the effects of HR-1 mutations on ENF binding to the mutated target and their effects on susceptibility to inhibition by ENF. The results of these studies performed with a subset of the HR-1 mutations are shown in Table 1. There was a good correlation between the two assays in terms of the reduction in ENF binding or susceptibility conferred by the aa substitutions studied (Spearman nonparametric correlation analysis; $r = 0.82143$; $P = 0.0234$). Other mutations in HR-1 (or HR-2) were not assessed in these studies, and their effects on ENF binding are unknown. Of the mutations examined, only substitutions in the region of aa positions 36 to 43 resulted in a >4-fold reduction, and the largest reductions in ENF binding (>40-fold) and susceptibility (>20-fold) were seen for the substitutions Q40H and N43D. A recent report by Nameki et al. (21) also showed that an HR-1 substitution (I37K) decreased binding of a related fusion inhibitor peptide (C34) to the mutated HR-1 target and that an HR-2 substitution in the fusion inhibitor peptide sequence (N126K) enhanced the binding of the fusion inhibitor peptide to the mutated HR-1 target.

ENF susceptibility of site-directed mutants with substitutions in gp41 aa 36 to 45 derived from clinical samples. The results of the initial studies presented above and the patterns of mutations selected during phase II trials (27, 29) prompted us to focus specifically on the region from aa 36 to 45 of gp41. Population sequencing was used to identify substitutions in this region in plasma HIV-1 from patients enrolled in ENF phase II clinical trials. In addition, virus isolates were obtained from patient PBMC at baseline and during ENF therapy. The ENF susceptibilities of virus isolates recovered from patients undergoing treatment were compared with those of the paired baseline isolates in a MAGI and/or cMAGI infectious center assay, and proviral DNAs of the baseline and on-treatment isolates were sequenced to identify substitutions occurring in gp41 aa 36 to 45 in on-treatment isolates exhibiting reduced suscepti-

TABLE 2. ENF susceptibilities of NL4-3/D36G mutants and clinical isolates carrying single substitutions in gp41 amino acid residues 36 to 45

Substitution(s)[c]	Data for NL4-3/D36G based viruses[a]		Data for primary clinical isolates[b]		
	ENF IC_{50} (μg/ml)	Fold change compared to NL4-3/D36G (parental virus)	Baseline ENF IC_{50} (μg/ml)	Treatment ENF IC_{50} (μg/ml)	Fold change compared to baseline virus
NL4-3/D36G (parental virus)	0.012				
Q40H	0.256	21	0.028	0.536	19
N43D	0.210	18	0.004	0.996	249
N43D			0.013	1.593	123
V38A	0.188	16	0.018	1.079	60
V38E	13.2	1,100		—[d]	
G36D	0.091	7.6	0.006	2.701[f]	450
G36D			0.014	0.242	17
G36D			0.003	0.013	4.3
G36E	0.471	39.3	0.019	0.811	43
G36S	0.088	7.3	0.014	0.41	29
G36S			0.041	0.499	12
N43S	0.067	5.6		—[d]	
N43K	0.063	5.3	0.027	0.085	3.1
N42T	0.045	3.8		—[d]	
L44M	0.021	1.8		—[d]	
L45M	0.017	1.4		—[d]	
N42S[e]	0.006	0.5			

[a] Produced by site-directed mutagenesis of NL4-3, altered to match the consensus sequence at amino acid position 36 (aspartic acid replaced by glycine).
[b] On-treatment clinical isolates from trials T20-205 and T20-208 that acquired single amino acid substitutions between amino acids 36 and 45 of gp41 and their paired baseline isolates, which had the consensus sequence indicated in footnote *c*.
[c] Relative to the consensus wild-type sequence GIVQQQNNLL.
[d] Mutation only observed in recovered on-treatment isolates in combination with another mutation at positions 36 to 45.
[e] N42S is a polymorphism observed in ≈16% of fusion inhibitor-naïve baseline isolates and was not seen as a change during treatment.
[f] The isolate also contained an N-K mixture at position 126 in HR-2 of gp41.

bility to ENF. Substitutions were also introduced into a modified pNL4-3 parental laboratory HIV-1 strain (NL4-3/D36G) by site-directed mutagenesis. The effects on ENF susceptibility of the NL4-3/D36G mutants were ascertained in MAGI and cMAGI assays in comparison to the parental virus, and the results are shown in Table 2 and Table 3 for single and double mutations, respectively. Changes in viral susceptibility for single substitutions introduced into the modified NL4-3 virus ranged from an 1,100-fold decrease in susceptibility for the V38E substitution in the modified NL4-3 background to a 2-fold increase in susceptibility for the N42S polymorphism (Table 2). Melby et al. have noted that the N42S polymorphism is seen in approximately 16% of fusion inhibitor-naïve viruses in patients participating in the phase III trials of ENF, and they found an association between the presence of the N42S polymorphism in baseline isolates and a lower IC_{50} for ENF (submitted for publication). Other individual substitutions in HR-1 led to decreases in ENF susceptibility in NL4-3 of either con-

TABLE 3. ENF susceptibilities of NL4-3/D36G mutants and clinical isolates carrying double substitutions in gp41 amino acid residues 36 to 45

Substitution(s)	Data for NL4-3/D36G mutants[a]		Data for primary clinical isolates[b]		
	ENF IC_{50} (μg/ml)	Fold change compared to NL4-3/D36G (parental virus)	Baseline ENF IC_{50} (μg/ml)	Treatment ENF IC_{50} (μg/ml)	Fold change compared to baseline virus
NL4-3/D36G (parental virus)	0.012				
G36S + L44M	0.181	15	0.006	3.791	632
N42T + N43K	0.388	32	0.007	1.762	252
N42T + N43S	0.727	61	0.006	2.031[f]	339
V38A + N42D	1.685	140	0.022	1.074[d]	49
V38A + N42T	1.782	149		—[e]	
V38E + N42S	6.156	513		—[e]	
N42T + L45M[c]	0.162	14	0.168	5.042	30
Q40H + L45M	0.805	67	0.033	10.776	327

[a] Produced by site-directed mutagenesis of NL4-3, altered to match the consensus sequence at amino acid position 36 (aspartic acid replaced by glycine).
[b] On-treatment clinical isolates that carried double amino acid substitutions between amino acids 36 and 45 of gp41 and their paired baseline isolates that exhibited the consensus sequence, except as noted in footnote *c*.
[c] The L44M mutation was observed in the baseline isolate cultured in vitro. The sequence derived from plasma RNA had the consensus wild-type Leu at gp41 position 44. This patient developed N42T and L45M mutations upon treatment, but the L44M mutation was no longer present.
[d] The cultured on-treatment isolate contains a mixture of the consensus and the mutant amino acids at positions 38 and 42.
[e] Cultured isolates containing these mutations were not obtained from patients.
[f] The isolate also contained an S138A substitution in HR-2 of gp41 as a change from baseline.

siderable magnitude (>10-fold) for the Q40H, N43D, V38A, and G36E mutations or more modest magnitude (<10-fold) for the remaining substitutions examined (Table 2). In general, susceptibility changes for single substitutions were lower in the NL4-3 background than those seen for double substitutions. One notable exception was the V38E mutation assessed in combination with the N42S polymorphism (Table 3). In the NL4-3 background, this combination led to an approximately 500-fold decrease in ENF susceptibility (Table 3), consistent with the individual contributions of the 1,100-fold decrease conferred by the V38E mutation alone and the 2-fold enhancement of ENF susceptibility seen when the N42S was inserted into the NL4-3 test virus. Additionally, we found that some combinations of mutations caused greater reductions in ENF susceptibility in NL4-3 than might be expected from the effects of the individual mutations assessed alone in this background. For example, the combination of N42T and N43S led to a 61-fold reduction in ENF susceptibility (Table 3), in contrast to the 3.8-fold and 5.6-fold reductions seen for these individual substitutions, respectively (Table 2). Similarly, the 149-fold reduction in ENF susceptibility found for the V38A and N42T combination or the 67-fold reduction seen with the Q40H and L45M combination appeared to be larger than expected based on the effects seen with the individual substitutions (compare Tables 2 and 3).

ENF susceptibility of primary virus isolates with substitutions in gp41 aa 36 to 45. PBMC-derived virus isolates from patients receiving chronic ENF treatment in phase II clinical trials were also tested for their susceptibility to ENF. Such analyses permit a comparison of the observed effects of mutations engineered into the NL4-3/D36G constructs with those occurring in the context of primary isolates as a result of in vivo selection during treatment with ENF. The sensitivities to ENF of virus isolates recovered from on-treatment samples in which either a single or double mutation in the region of aa 36 to 45 of gp41 was detected are shown in Tables 2 and 3, respectively. A comparison of the ENF susceptibilities of on-treatment viruses with mutations to those of their matched pretreatment isolates allowed us to derive the degree of change in the ENF IC_{50} from the baseline. The changes (representing increases in the IC_{50}) observed for viruses carrying single mutations ranged from 3-fold (N43K) to 450-fold (G36D) (Table 2). It is noteworthy that viruses recovered from three individual patients had acquired the same single G36D mutation in HR-1 during treatment but exhibited very different changes in ENF susceptibility, ranging from approximately 4-fold to 450-fold (Table 2). The isolate with a G36D HR-1 mutation exhibiting a 450-fold change also had an N-K mixture at position 126 in HR-2, which could have contributed to the observed change (2, 21). We also observed another isolate with an on-treatment change in HR-2. This isolate is shown in Table 3 with the N42T-plus-N43S combination and contained an S138A substitution which may have contributed to the degree of change seen (33). These results suggest that on-treatment changes occurring in regions of the virus envelope outside of gp41 aa 36 to 45 may contribute to reductions in ENF susceptibility or that the reduction in ENF susceptibility conferred by a specific mutation can be modulated by the envelope background in which it emerges. Four of the six viruses carrying double mutations exhibited a >250-fold change in the IC_{50}, with a maximum change of 632-fold (G36S plus L44M). The N42T-plus-L45M mutant resulted in a 30-fold change in the IC_{50}, but the cultured pretreatment virus for this patient contained L44M, which may have contributed to the elevated baseline IC_{50} (Table 3). The cultured on-treatment virus isolate with the V38A and N42D double mutation contained a mixture with the wild-type (baseline) sequence. Therefore, the 49-fold decrease in ENF susceptibility may have been moderated by the presence of wild-type virus. Overall, the levels of ENF resistance seen for the clinical isolates tended to be higher than those seen for the NL4-3/D36G mutant viruses. For example, the introduction of N43D into NL4-3/D36G resulted in an 18-fold increase in IC_{50}, while the two clinical isolates carrying this single mutation had changes in the ENF IC_{50} of 249- and 123-fold compared with their corresponding pretreatment isolates. When independent isolates harboring the same HR-1 mutation were tested, different susceptibilities to ENF were observed, as illustrated with the on-treatment isolates containing the G36D mutation and also those with the N43D mutation (Table 2). These results suggest that the phenotypic penetration of ENF-selected mutations may be influenced by the genetic context of the virus envelope in which they arise.

TABLE 4. ENF susceptibilities of four envelopes bearing substitutions in gp41 amino acids 36 to 45 in a pseudotype reporter virus assay

Env[a]	ENF IC_{50} (μg/ml) (fold change from wild type) for indicated background[a]			
	GIV	030	060	098
Wild type	0.093	3.38	0.265	0.836
G36D	0.372 (4.0)	8.696 (2.6)	ND	1.639 (2.0)
V38E	17.858 (192)	>100 (>30)	ND	40.06 (48)
N42T + N43S	0.276 (3.0)	ND	91.08 (344)	22.493 (27)

[a] GIV was derived from NL4-3/D36G; 030, 060, and 098 were derived from baseline virus isolates from three patients in the T20-205 trial (16). ND, not determined.

ENF susceptibility of primary isolate envelopes and NL4-3/G36D envelopes with substitutions in gp41 aa 36 to 45, as assessed in a pseudotype reporter virus system. To specifically examine whether the effect of ENF-selected substitutions could be moderated by the envelope background, we cloned envelopes from three baseline virus isolates as well as the NL4-3/D36G virus into a modified pCR3.1 expression vector and inserted a subset of the mutations examined into each of the cloned envelopes by site-directed mutagenesis. The parental and mutant envelopes were used to generate pseudotype reporter viruses which were assessed on U87 cells expressing CD4 and CXCR4 for their susceptibility to ENF (Table 4). As shown in Table 4, the starting parental envelopes each displayed distinct sensitivities to ENF. The G36D mutation conferred relatively similar decreases in ENF susceptibility when assessed in three different envelopes in this system. We were unable to analyze the G36D mutation in the 060 envelope due to low infectivity. We did observe that the V38E single mutant and the N42T-plus-N43S combination conferred different extents of reduction in ENF susceptibility in each of the three envelopes tested (Table 4). The most dramatic difference in the effects on ENF susceptibility observed with different envelopes was seen for the N42T-plus-N43S double mutation,

where the changes ranged from a low of 3-fold for the NL4-3/D36G envelope to a high of 344-fold for the 060 envelope. These data indicate the potential for regions of the envelope outside of gp41 aa 36 to 45 to modulate the extent of decreases in ENF susceptibility conferred by ENF-selected mutations and caution against attempts to arrive at a generalized rank order for the effect of ENF-selected mutations based solely on an analysis of their effects in any one envelope background.

DISCUSSION

This study examined the effect of ENF-selected amino acid substitutions in gp41 on the susceptibility of viruses to inhibition by ENF and the binding of ENF to gp41-derived HR-1 regions containing several of the same substitutions. Such an approach allowed us to evaluate a mechanistic basis for alterations in ENF susceptibility conferred by these substitutions. The effects of substitutions in gp41 amino acids 29 to 45 on ENF sensitivity were assessed in the following three ways: (i) through site-directed mutagenesis of a modified laboratory strain of HIV-1 (NL4-3/G36D), (ii) through an evaluation of primary isolates recovered from patients undergoing chronic treatment with ENF during phase II clinical studies, and (iii) through site-directed mutagenesis of virus envelopes using a pseudotype reporter virus assay. NL4-3-based viruses were engineered to harbor many of the substitutions in the HR-1 region of HIV gp41 that had been observed in the plasma viruses of patients enrolled in ENF phase I/II and phase II clinical trials and in their recovered virus isolates displaying reduced susceptibilities to ENF. We observed that several single amino acid changes in gp41 aa 36 to 45 conferred a >10-fold reduction in NL4-3 susceptibility to ENF (G36E, V38A, V38E, Q40H, and N43D). Reductions in NL4-3 susceptibility conferred by single substitutions ranged as high as 1,100-fold for the V38E mutation. In addition, we found that the N42S polymorphism, which occurs in approximately 16% of fusion inhibitor-naïve viruses, increased NL4-3 sensitivity to ENF inhibition about twofold, consistent with the observations of Melby et al., who found that this polymorphism was associated with lower ENF IC_{50}s for baseline virus envelopes from patients participating in ENF phase III trials (submitted). Substitutions at two positions in aa 36 to 45 generally caused greater reductions in NL4-3 susceptibility to ENF than did single substitutions. Similarly, substitutions at two positions in gp41 were generally associated with larger changes in the ENF IC_{50} for on-treatment virus isolates than those for on-treatment isolates carrying only one of the changes. The effects of mutations within gp41 aa 36 to 45 on ENF susceptibility and their appearance in the vast majority of viruses from patients experiencing virological failure during ENF therapy confirm the key role of this region in the development of resistance to ENF.

Initial studies of the mechanism of ENF action indicated that ENF acts through binding to the HR-1 region of gp41 (4, 24). The binding affinities of ENF for HR-1-containing fusion proteins engineered to carry a subset of ENF resistance-associated substitutions correlated in rank order with the ENF susceptibilities of mutant NL4-3 viruses with the same substitutions. This observation suggests a partial mechanistic basis for ENF resistance. A decreased susceptibility to ENF may in part be due to a decreased binding affinity of ENF for the altered target HR-1 regions of gp41. This inference is consistent with the recent studies of Nameki et al. (21), who found that an I37K mutation reduced the binding of a related fusion inhibitor peptide, C34, to an HR-1 target. In addition to that study, the present study and other recent reports (2, 33) indicate that the mechanism of ENF resistance is not fully accounted for by decreased binding of ENF to the HR-1 target nor restricted to substitutions in gp41 aa 36 to 45.

The levels of resistance seen for NL4-3-based mutants were generally lower than those for clinical isolates bearing the same mutations. There was also a wide variation in the ENF susceptibilities of clinical isolates bearing a given mutation. These observations suggest several alternatives that could influence resistance to ENF. Among them are the following: (i) the envelope background may affect the magnitude of the shift in susceptibility to ENF conferred by substitutions within gp41 aa 36 to 45, (ii) changes occurring in the HR-2 region of gp41 may contribute to ENF resistance, and (iii) changes occurring in gp120 in response to ENF could influence ENF resistance. In this study, gp120 sequences were not obtained from plasma viruses or from the initial baseline or on-treatment clinical isolates, and thus we cannot directly address their potential impact on the results reported here. However, a series of statistical and modeling analyses of entire gp160 sequences and ENF susceptibilities of patients participating in the phase III clinical trials of ENF have been conducted by C. Su and colleagues (unpublished data) to identify changes occurring in the viral envelope during ENF therapy that are significantly associated with alterations in ENF susceptibility. In their study, analysis-of-variance models indicated that approximately 90% of the variations in phenotypic sensitivity were accounted for by changes in gp41 aa 36 to 45. Nevertheless, they did observe that changes at three positions in gp120 and five positions in gp41 outside of aa 36 to 45 were associated with significant reductions in ENF susceptibility, leaving open the possibility that changes in gp120 could influence resistance to ENF.

Derdeyn et al. (5, 6) have suggested that coreceptor specificity defined by the V3 loop may modulate HIV's susceptibility to ENF. However, their findings were not confirmed in a separate study analyzing the ENF susceptibilities of 111 virus isolates using a variety of cell-based assay systems (M. L. Greenberg, C. B. McDanal, S. Stanfield-Oakley, et al., Abstr. 8th Conf. Retrovir. Opportunistic Infect., abstr. 473, 2001). No differences in susceptibility were seen between X4-, R5-, or dual-tropic viruses from patients or between serially obtained R5 and X4 isolates from the same patient. In the phase III studies of ENF, virologic responses were equivalent for patients with baseline isolates exhibiting either R5, X4, or dual tropism (Melby et al., submitted), indicating that the coreceptor tropism at baseline is not a significant determinant of the therapeutic response to ENF.

Studies by Heil et al. and Stanfield-Oakley et al. suggested that both the HR-1 and HR-2 regions of the viral envelope may contribute to ENF susceptibility (M. Heil, J. M. Decker, J. Chen, et al., Abstr. 2nd Int. AIDS Soc. Conf. HIV Pathogenesis Treatment, abstr. 801, 2003; S. A. Stanfield-Oakley, J. Jeffrey, C. B. McDanal, et al., abstr. 12th Int. HIV Drug Resistance Workshop, abstr. 56, 2003). Those findings were extended by Xu et al. (33), who found that an S138A HR-2

mutation could enhance resistance to ENF, and by Baldwin et al., who reported that an N-to-K mutation in HR-2 (equivalent to N126K in HXB2) contributed to ENF resistance and conferred enhanced fusogenicity on the virus (2). Nameki et al. (21) found that the N126K HR-2 mutation led to tighter binding between an HR-2 peptide and an HR-1 target. Taken together, these studies suggest another mechanism of resistance to ENF involving HR-2. In this case, alterations in the HR-2 region may provide a competitive advantage over ENF for binding to the mutant HR-1 coiled coil, either through increases in binding affinities or by shortening the kinetic window for six-helix bundle formation, as posited by Reeves et al. (23).

Finally, our studies also demonstrate that the envelope background in which an ENF-selected mutation arises can influence the degree of resistance to ENF that is conferred. This finding adds to our previous observation that the envelope background of fusion inhibitor-naïve viruses influences the sensitivity to ENF (27). Previous reports have documented that fusion inhibitor-naïve isolates display a wide range of sensitivities to ENF, despite the fact that the vast majority have the consensus sequence GIVQQQNNLL for gp41 aa 36 to 45. Those findings indicate that regions outside of this motif can influence the sensitivity to ENF. The current study extends the influence of the envelope context to the effects of HR-1 mutations on ENF resistance. The data presented here clearly demonstrate that substitutions in the region of aa 36 to 45 of gp41 are primary determinants for the development of viral resistance to ENF. Overall, these results lend strong support to the mechanism of ENF action targeting the HR-1 region as a competitive inhibitor that acts to block the interactions of the viral HR-1 and HR-2 regions required for the fusion of viral and target cell membranes. These studies also indicate that an important mechanism of resistance to ENF involves decreases in ENF binding to the HR-1 target sequences that are altered during exposure to ENF. In addition, there are other mechanisms contributing to ENF resistance. These include alterations in HR-2 that may increase binding to HR-1 or shorten the kinetic window of six-helix bundle formation, changes in gp120 that act in ways that are unclear to affect ENF susceptibility, and influences of the envelope background on resistance to ENF. Further studies will be required to more fully understand these mechanisms and the influence of the envelope's genetic context.

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