Emergence and Evolution of Enfuvirtide Resistance following Long-Term Therapy Involves Heptad Repeat 2 Mutations within gp41

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The objective of this study was to track the evolution of sequence changes in both the heptad region 1 (HR1) and HR2 domains of gp41 associated with resistance to enfuvirtide (ENF) in a patient cohort receiving long-term ENF treatment. We studied 17 highly antiretroviral agent-experienced patients receiving long-term ENF treatment with virological rebound or a lack of suppression. Sixty-two samples obtained after between 5 and 107 weeks of ENF therapy were analyzed. Baseline samples from 15 of these 17 patients were available for analysis. Viruses from five samples from four patients were also sequenced after the cessation of ENF therapy. Drug susceptibilities were assessed by a pseudotype virus reporter assay. We identified HR1 and HR2 sequence changes over time in relation to the baseline sequences. Mutations in HR1 (amino acids 36 to 45) were noted in all cases, including previously unreported changes N42Q/H and N43Q. In addition to a range of HR2 sequence changes at polymorphic sites, isolates from 6 of 17 (35%) patients developed an S138A substitution in the HR2 domain at least 8 weeks after the start of ENF treatment and also subsequent to the first emergence of HR1 mutations. In most, but not all, cases the S138A mutation accompanied HR1 mutations at position 43. Molecular modeling demonstrates the close proximity of S138A with amino acids 40 and 45 in HR1. Of note, isolates in samples available from four patients demonstrated the loss of both the HR1 and the S138A HR2 mutations following the cessation of therapy. We show that the S138A HR2 mutation increased the level of resistance by approximately threefold over that conferred by the HR1 mutation N43D. Continual evolution of HR1 in the domain from amino acids 36 to 45 was observed during long-term ENF therapy. We have identified, for the first time, an ENF resistance-associated HR2 mutation, S138A, which appeared in isolates from 6 of 17 patients with virological failure and demonstrated its potential to contribute to drug resistance. We propose that this represents a possible secondary and/or compensatory mutation, particularly when it coexists with mutations at position 43 in HR-1.

The envelope glycoprotein (*env*) of human immunodeficiency virus type 1 (HIV-1) plays a key role in viral entry into the host cell (11, 31). *Env* contains two noncovalently associated subunits, gp120 and gp41. The binding of gp120 to the cellular receptor CD4 and a chemokine coreceptor triggers a series of complex conformational changes in gp41 that lead to the fusion of viral and cellular membranes. There are three functional regions in the ectodomain of gp41: the fusion peptide, heptad repeat 1 (HR1) and HR2, and the transmembrane region. Crystallographic studies have demonstrated that the fusion-active (fusogenic) conformation of gp41 is a six-helix bundle structure in which three N-terminal helices (HR1) form a central trimeric coiled coil and three C-terminal helices (HR2) pack in an antiparallel manner into hydrophobic grooves of the coiled core. The hairpin interactions between the HR1 and HR2 domains of gp41 bring the viral and host cell membranes together for fusion (4, 32, 34).

Blocking of the conformational change in gp41 that is crucial

for fusion should inhibit viral entry into host cells. Synthetic peptides derived from the HR2 domain (C peptide) have been shown to effectively block both virus-mediated cell-cell fusion and cell-free virus infection (14, 21, 26, 33, 35). The fusion inhibitor enfuvirtide (ENF; T-20) is a synthetic 36-amino acid peptide that corresponds to residues 127 to 162 of the HR2 (C-helix) domain of gp41. Its mechanism of inhibition is proposed to block HIV-1 entry, mediated by competitive interaction with the HR1 (N-helix) domain in a dominant negative manner and prevention of the formation of the fusogenic conformation of gp41 (3, 5, 17, 35).

Phase III clinical trials of ENF added to an optimized background (OB) in treatment-experienced patients have demonstrated significant improvement in the virologic and immunologic responses of patients receiving ENF plus OB, with a median reduction of the viral load of 1.48 log₁₀ at 48 weeks compared to a reduction of 0.63 log₁₀ in those given OB alone (19, 20, 29). The evolution of resistance has been demonstrated in vitro and in clinical trials. The glycine-isoleucine-valine (GIV) motif (residues 36 to 38) within HR1 of gp41 was initially identified as a genotypic marker of ENF resistance, with two mutations (G36S and V38M) being shown to confer

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reduced drug susceptibility in vitro (24). More recent data have shown further mutations in a wider region of HR1 (residues 36 to 45) (9, 28). Since the fusion process requires the interaction between the HR1 and HR2 regions, it was anticipated that compensatory changes in the HR2 domain might arise in resistant virus isolates in order to restore or improve contacts with the HR1 region in the six-helix bundle fusogenic confirmation of gp41. However, rather surprisingly, previously reported sequencing of ENF-resistant mutants generated in vitro (mutations within the GIV motif) failed to identify any compensatory changes in the HR2 domain. In addition, sequencing of viruses from ENF-treated patient samples has also rarely revealed any amino acid changes in the HR2 domain (16, 18, 23, 30). ENF is the first member of a new class of antiretroviral agents referred to as fusion inhibitors. Information on the extent and nature of the resistance mutation pathways used by HIV-1 in response to this drug is critical for understanding the complex mechanisms by which resistance emerges and for providing guidance for treatment decisions. This study was designed to track the evolution of sequence changes in both the HR1 and the HR2 domains of gp41 associated with resistance in a patient cohort in whom viral rebound or a lack of suppression was identified during long-term ENF therapy.

MATERIALS AND METHODS

Patient samples. We studied 17 highly antiretroviral agent-experienced patients receiving ENF in whom virological rebound or nonsuppression was identified during follow-up. Sixty-two samples obtained between 5 and 107 weeks after the start of ENF therapy were analyzed. Baseline (pre-ENF treatment) samples from 15 of these 17 patients were available for analysis. The isolates in five samples from four patients were also sequenced between weeks 1 and 22 after the cessation of ENF therapy.

PCR amplification and sequencing. Viral RNA was extracted from plasma by use of the QIAmp RNA kit (Qiagen). A 486-bp region of gp41 covering both the HR1 and HR2 domains was amplified by reverse transcription-PCR (RT-PCR). The following primer pairs were used for sequencing in a nested PCR: primers L5'/gp41 (TTCAGACCTGGAGGAGGAGATA) and L3'/Env (GGTGGTAGC TGAAGAGGCACAGG) as the outer primers and primers L5'/HR1 (AGAAG AGTGGTGCAGAGAGAAAA) and L3'/HR2 (GGTGAGTATCCCTGCCTA ACTCT) as the inner primers. Population sequencing was carried out with the PCR amplicon in both directions by using a Beckman Coulter CEQ2000XL sequencer.

Preparation and characterization of pseudotype reporter viruses. The *env* genes from the HIV-1 isolates were amplified from proviral DNA by PCR or from virus RNA extracts by RT-PCR. HIV-1 *env* genes from plasma samples were amplified by RT-PCR after isolation of RNA. Full-length envelope amplicons were cloned into a pCR3.1-derived expression vector and used for production of pseudotype reporter viruses (12, 13). The captured envelope clones served as the starting point for the site-directed mutagenesis constructs.

Coreceptor tropism and sensitivity to ENF inhibition of reporter viruses pseudotyped with envelope clones (and site-directed mutant envelope clones) were determined for reporter viruses produced following cotransfection of envelope expression vectors and an *env*-deficient strain NL4-3-based reporter virus construct into 293T cells. The pseudotyped reporter viruses were evaluated with U87 cells expressing CD4 and either CCR5 or CXCR4 (12, 13). Assessment of susceptibility to ENF infection of U87 cells was carried out in the presence or the absence of various concentrations of ENF, and the concentration of ENF required to inhibit infection (assessed with the luciferase reporter) by 50% (IC_{50}) was determined.

Site-directed mutagenesis. Site-directed mutagenesis was performed with a Qiagen Quick Change kit with parental envelopes at gp41 amino acids 43 (within HR1) and 138 (within HR2) to further investigate the roles of these specific residues and the HR1 and HR2 regions as possible contributors to susceptibility to ENF.

Nucleotide sequence accession numbers. The GenBank accession numbers of the PCR amplicon sequences are AY768582 to AY768660.

RESULTS

Patient characteristics. All patients were highly experienced with antiretroviral therapy and prior to ENF therapy had a median viral load 24,029 copies/ml and a median CD4 cell count of 43×10^6 cells/liter. A variety of other antiretroviral drugs were coprescribed, according to the baseline resistance patterns.

Sequence diversity in HR1 domain of gp41. Sequence analysis of the HR1 domain from the baseline samples from 17 patients showed that the wild-type GIV motif (amino acids 36 to 38), initially identified as the genotypic marker of ENF resistance in vitro, was completely conserved. Examination of a larger region of the HR1 domain (aa 36 to 45), which in subsequent clinical trials was found to contain single or multiple amino acid substitutions (28), again revealed a high degree of conservation, with the exception of an N42S polymorphism in viruses from patients 1, 11, and 17 (Fig. 1).

Following virological rebound or nonsuppression during ENF treatment, viruses in all samples ($n = 62$) from 17 patients showed mutations in the region from aa 36 to 45 of the HR1 domain (Fig. 1). Of the total of 119 substitutions identified, 69 (58%) were found as a mixture with the wild-type sequence. Table 1 summarizes the number of patients harboring viruses with single, double, and multiple mutations within HR1 at some point in time. The V38A single substitution occurred at the highest frequency, being observed in viruses from 7 of 17 (41%) patients. Viruses from four of these seven patients contained only the mutant sequence, while viruses from the other three patients contained mutant sequences mixed with the wild-type sequence.

Viruses from three patients developed double mutations (G36V and V38A) within the GIV ENF resistance motif. For two of the three patients (patients 1 and 4), this double mutation was identified in viruses from the earliest samples (after 8 weeks of ENF treatment), but only a single mutation (G36V or V38A) was present in the virus from subsequent samples (taken 4 weeks after the previous sample was obtained). The virus in the first sample from the third patient (patient 7) carried a triple mutation (G36V, V38A, and N43D), with the G36V and V38A substitutions emerging in the second sample (19 weeks later). The viruses in the last two samples from this patient (taken 21 and 37 weeks after the second sample was obtained) possessed only a single substitution (V38A).

Five independent sets of double mutations (G36D and N43D, G36S and N43D, G36S and N43Q, V38A and Q40H, and Q40H and L45M) were each found in two patients. Several other double substitutions (G36D and V38M, G36D and N42Q, G36V and N43D, G36S and N43Q, G36V and N43D, and G36D and L44M) were each detected in one patient.

ENF-containing therapy for more than 5 to 24 months resulted in variants with multiple substitutions. Of the five patient samples containing viruses harboring multiple mutations, the virus from patient 10 developed a V38A and Q40H double mutation after 6 weeks of treatment and a Q40H and L45M double mutation at 20 and 23 weeks of treatment, before a triple mutation (G36D, Q40H, and L45M) emerged in the sample obtained at 24 weeks after the start of treatment. Q40H, N42H, and L45M triple mutations were detected at the 96-week follow-up. Four substitutions (G36S, V38M, N42T,

Subject	Time (weeks)	36-45	HR1	HR2	138
Hxb2		QARQLLE	GVQQQNLL	RAIEAQQLHLLQTVWGKQLQARILAVERYLKDQQLL	WMWEWDREINNYTSLIHSLEEESQNQQEKNEQELLELDKWASLWNWF
1	B**				
1	12	V*A*			
1	16	V.....			
1	22	V.....			
1	-22	M.....			
2	B				
2	8	D*	D*		
2	18				
2	25	D*			
2	-1				
2	-6				
3	B				
3	8	D*	H*		
3	16	D*	D*		
3	25	D*	D*		
3	34	D*	D*		
3	47		D*		
3	62		D*		
3	77		D*		
3	98		D*		
3	107		D*		
4	B				
4	17	V*A*			
4	21	A*			
5	B				
5	5	A*			
5	12	D*M*			
5	20	D*	M*		
5	26	D*	M*		
6	B				
6	16	A*H*			
6	20	A*			
6	-7				
7	B	T.....			
7	8	T.....			
7	27	T.....			
7	48	T.....			
7	64	T.....			
8	B				
8	82	S*	D*		
9	B				
9	8	S*	D*		
9	20	S*	Q*		
9	24	S*	Q*		
9	32	S*	Q*		
9	42	S*	Q*		
9	54	S*	Q*		
9	67	S*	Q*		
9	-10	S*	Q*		
10	6	A*H*			
10	20	H*	M*		
10	23	H*	M*		
10	24	D*	H*		
10	57	H*	M*		
10	64	H*	M*		
10	74	H*	M*		
10	96	H*	M*		
11	B	V.....			
11	12	V.....			
11	24	V.....			
11	38	V.....			
11	64	V.....			
11	73	V.....			
11	90	V.....			
12	B	L.....			
12	24	L.....			
12	36	L.....			
13	B				
13	31	A.....			
13	82	A.....			
14	8	D*	QS*		
14	20	D*	QS*		
14	36	D*	Q*		
14	43	D*	Q*		
14	49	D*	Q*		
15	B				
15	5	A.....			
15	8	A.....			
16	B				
16	24	S*M*	T*M*		
17	B	S.....			
17	14	D.....			

FIG. 1. Sequence analysis of the HR1 and HR2 domains of viruses from ENF-treated patients. The boxed area in the HR1 (aa 36 to 45) domain has previously been associated with clinical resistance. The arrow indicates amino acid 138 in the HR2 domain, with the S-to-A change observed in viruses from patients 1, 2, 3, 6, 9, and 14. The second column shows the time (in weeks) following treatment initiation (+) and discontinuation (-). *, the viruses in the samples showed mixtures of mutations; B, baseline samples.

TABLE 1. Genotypic changes^a in viruses from sequential samples from 17 ENF-treated patients

Substitution(s)	No. of patients
G36V.....	1
V38A.....	7
N43D.....	3
G36V, V38A.....	3
G36D, V38M.....	1
G36D, N42Q.....	1
G36D, N43D.....	2
G36V, N43D.....	1
G36S, N43D.....	2
G36S, N43Q.....	1
G36D, L44M.....	1
V38A, Q40H.....	2
Q40H, L45M.....	2
G36V, V38A, N43D.....	1
G36D, Q40H, L45M.....	1
G36S, N42T, N43Q.....	1
G36D, N42Q, N43S.....	1
Q40H, N42H, L45M.....	1
G36S, V38M, N42T, L44M.....	1

^a Changes at aa 36 to 45.

and L44M) were detected in the virus from one patient (patient 16) at the 24-week follow-up.

Sequence diversity in HR2 domain of gp41. In addition to a range of HR2 mutations at polymorphic sites, viruses from 35% of the patients (6 of 17) developed a S138A substitution (aa 649 in gp160) in the HR2 domain at least 8 weeks after the start of ENF treatment and also subsequent to the first emergence of HR1 mutations (Fig. 1). Viral sequences from these six patients obtained at 22 time points were analyzed. At 20 of these 22 time points a mutation at residue 43 (N43/D/Q/S) within the HR1 domain was also observed, and at 14 time points, a G36V, G36D, or G36S mutation was detected. Table 2 summarizes the genotypic profiles of residues 36 to 45 in the HR1 domain from these time points for the six patients whose viruses acquired the S138A substitution in the HR2 domain.

HR1-HR2 interaction. Viruses from five of six patients that developed the S138A substitution in the HR2 domain had mutations at codon 36 or 43 in the HR1 domain. S138A lies in an *a* position of the heptad coil within the ENF peptide sequence, and both codons 36 and 43 are located in *c* positions of the heptad coil (Fig. 2a). A structural model of the interaction between HR1 and HR2 (ENF) (Fig. 2b) based on the crystal structure of the six-helix bundle (4) demonstrates that there is antiparallel packing between the α helices, with the serine at position 138 (*a* position) on the C helix (HR2) forming a close contact with the leucine at position 45 (*e* position) of the N helix (HR1). Serine 138 also forms a close contact with the glutamine at position 40 (*g* position) of the N helix (HR1) (data not shown) and at its closest point is less than 5 Å from the asparagine at position 43 (*c* position) in HR1.

Sequence changes on cessation of ENF treatment. Following the cessation of therapy with ENF, the viruses in five samples available from four of six patients whose viruses had the HR2 S138A mutation demonstrated the loss of this mutation, as well as HR1 mutations, suggesting the outgrowth of fitter virus (Fig. 1).

Phenotypic impact of S138A within HR2. We used a plasma sample to recover a full-length viral envelope clone with the N43D and the S138A mutations. The ENF IC₅₀ for this envelope clone was 41.1 µg/ml by a pseudotyped reporter virus assay. We then constructed three site-directed mutant envelopes, derived from the original envelope clone in the plasma sample. These included reversion of the 43 position back to N in one construct and reversion of the 138 position back to S in another construct. In addition, reversion of both mutations to generate the wild type was undertaken. Within our assay system, reversion of N43D back to N43 increased the sensitivity to ENF nearly 20-fold (a drop in the IC₅₀ from 41.1 to 2.62 µg/ml). Reversion of position 138 back to an S in the N43D background led to a threefold increase in sensitivity to ENF (a drop in the IC₅₀ from 41.1 to 13.0 µg/ml). When position 138 was reverted back to an S in the context of the wild-type N43, a similar threefold increase in ENF sensitivity was observed (the IC₅₀ dropped from 2.62 to 0.875 µg/ml) (Table 3).

DISCUSSION

Resistance to ENF is conferred by mutations in the HR1 region of gp41, classically, within the GIV motif. We now demonstrate that the evolution of resistance passes through a number of mutations and sets of mutations. This suggests a continual selective process during a period of viremia in the presence of the drug.

ENF is a synthetic amino acid corresponding to the last 36 aa of the HR2 domain, and it achieves its inhibition of fusion by interacting with the HR1 domain. Resistance initially develops within this HR1 domain; it might also be expected that resistant variants may develop compensatory changes in the HR2 domain, which encodes the ENF peptide and the region of the virus gp41 that needs to interact productively with HR1

TABLE 2. Substitutions in HR1 for viruses that developed the S138A mutation in the HR2 domain of gp41

Subject no.	Time on ENF treatment (wk)	Substitution(s) in HR1 ^a
1	22	G36V
2	18	N43D
2	25	G36D, N43D
3	25	G36D, N43D
3	34	N43D
3	47	N43D
3	62	N43D
3	77	N43D
3	98	N43D
3	107	N43D
6	20	V38A
9	20	G36S, N43Q
9	24	G36S, N43Q
9	32	G36S, N42T, N43Q
9	42	G36S, N43Q
9	54	G36S, N43Q
9	67	G36S, N43Q
14	8	G36S, N42Q, N43S
14	20	G36S, N42Q, N43S
14	36	G36S, N42Q, N43S
14	43	G36S, N42Q, N43S
14	49	G36S, N42Q, N43S

^a Substitution in HR1 aa 36 to 45.

FIG. 2. (a) Schematic representation of the HIV-1 gp41 showing three functional domains and amino acid sequences of HR1, HR2, and ENF (T-20). The positions of the amino acids according to the heptad repeat assignment are shown under the amino acid by italicized lowercase letters. The *c* position of codons 36 and 43 in the HR1 domain and the *a* position of S138 in the HR2 domain are shown in gray. (b) An X-ray crystallographic structure illustrating the interaction between the HR1 and HR2 regions of HIV-1 gp41. The structure has been modified such that residues 29-79 of HIV-1 HR1 (1-51 PDB numbering) and residues 125-152 of HIV-1 HR2 (125-152 PDB numbering) are shown. The position of serine 138 (HR2) is shown in black. The region of HR1 that contains previously described enfuvirtide mutations is also marked in black. The side chain of amino acid leucine 45 HR1 is shown, as its spatial arrangement brings it into close contact with amino acid 138 (HR2).

for fusion to occur. However, the previously reported sequencing of in vitro-generated ENF-resistant mutants (with mutations within the GIV motif) failed to identify any such HR2 mutations. It was speculated that mutations in the GIV motif within the HR1 domain which prevent interaction or binding by the free HR2 (ENF) may not have a significant impact on binding to the covalently attached HR2-interacting site within gp41. It is also possible that other sites outside the GIV motif in HR1 may play a more dominant role in the formation of a fusogenic structure of gp41 during the fusion process (6, 24); indeed, HR2 may affect drug susceptibility, in addition to co-receptor tropism (6).

We identified a range of mutations in HR1 that emerged outside of the classical GIV motif during therapy, including mutations at positions 40, 42, and 45. These have also been observed in more detailed analyses of data from a clinical trial of ENF and contribute to reduced drug susceptibility (28). Interestingly, although the V38A mutational change remains the most prevalent resistance-associated mutation in HR1 in this (41%) and other studies (27, 28, 30), viruses from five of

six patients who developed an S138A substitution in the HR2 domain harbored mutations at residue 36 (G36V/D/S) or 43 (N43/D/Q/S), or both residues, in the HR1 domain; and virus from only one patient had a V38A mutation. The genetic background of individual isolates may determine different genetic routes to resistance. The HR1 domain is highly conserved across group M subtypes (10, 25, 36, 38). By comparison, studies of the genetic diversity of the HR2 domain reveal greater sequence variability (7, 10). Nevertheless, position S138 appears to be highly conserved in subtype B viruses from ENF-naïve patients. Hanna et al. (10) found that subtype B virus in 3 of 68 samples had an S138A substitution. Similarly, Dorn et al. (7) found the same S-to-A substitution in 3 of 62 subtype B virus-infected samples and 1 of 16 subtype A virus-infected samples. In the present study, all viruses were of subtype B. Viruses from 6 of 17 patients (35%) developed the S138A substitution while the patient was receiving ENF therapy. This prevalence is significantly higher ($P < 0.0003$) than the natural sequence variation found among subtype B viruses from the ENF-naïve patient population, as discussed above.

Gallagher et al. (8) and Chamber et al. (2) were the first to predict that heptad repeats with an α -helical coiled coil conformation represent the structural basis for membrane fusion by enveloped viruses. Classical coiled coil proteins have a characteristic heptad repeat sequence labeled *a-b-c-d-e-f-g*, with preference for the hydrophobic residues at positions *a* and *d* (22), and protein-protein interactions are largely dependent on hydrophobic contacts (15, 37). Crystal structure studies of two interacting peptides derived from the HR1 and HR2 domains of gp41 have confirmed that in the fusogenic six-helix bundle structure of gp41, three N helices (HR1) form a trimeric coiled coil with three hydrophobic grooves on the surface (4). Three C helices (HR2) pack in an antiparallel orientation into these grooves. The interactions between the *a* and *d* positions of the N helices are important for maintenance of the stabilization of the trimeric central core. The *a* and *d* positions in the C helices generally interact with the *e* and *g* positions of N helices, although contacts with other positions have also been observed (4, 32, 34). The mutation S138A identified and characterized in this study lies in an *a* position. This mutation may have an impact on the fusogenic HR1-HR2 structure of gp41 and therefore interferes with its fusion property, in addition to influencing the inhibitory activity of ENF. Site-directed mutagenesis studies of structure-function demonstrated that a

TABLE 3. ENF susceptibility of pseudotype reporter virus derived from a clinical plasma sample containing virus with N43D and S138A substitutions^a

Virus	Mutation		ENF susceptibility	
	HR1 position 43	HR2 position 138	IC ₅₀ (μg/ml) ^u ^b	Fold resistance
Patient derived	D	A	41.1	47
Patient derived	D	S	13.0	15
Patient derived	N	A	2.6	3
Wild type	N	S	0.8	1

^a Site-directed mutagenesis was undertaken to alter the *env* gene derived from a virus from a clinical plasma sample at positions 43 of HR1 and 138 in HR2. All envelope clones were subjected to phenotypic analysis.
^b IC₅₀ are the means of three observations.

single mutation at another α position (N145L, aa 656 in gp160) within the ENF-coding sequence caused substantial defects in membrane fusion, while other mutations in the same C-helix region resulted in enhanced fusion (1). Although the c positions in the coiled coils of gp41 are also highly conserved, little is known about their functional role in the six-helix bundle structure. In our study, viruses from five of six patients that developed the S138A substitution had mutations at codon 36 or 43. Of note, both codon 36 and codon 43 are located in c positions of the heptad coil (Fig. 2a). Furthermore, structural modeling of the interaction between HR1 and HR2 (ENF) demonstrates that there is antiparallel packing between the α helices, with the serine at position 138 (a position) on the C helix (HR2) forming a close contact with the leucine at position 45 (e position) of the N helix (HR1) (Fig. 2b). The mutation from serine to alanine at position 138 (HR2) increases hydrophobicity without altering the steric properties of that position and may help to stabilize the interaction between the α helices. Most heptad repeat structures feature hydrophobic residues at positions a and d , which, when they are buried within an antiparallel helix bundle, promote the stability of that bundle.

In this study, interestingly, viruses from three patients (patients 10, 11, and 16) developed an L45M mutation (a change of leucine to methionine reduces the hydrophobicity value of position 45) during the course of ENF therapy. The viruses from none of these patients developed the S138A mutation. Hypothetically, a stable interaction between the 138 position (HR2) and the 45 position (HR1) may be advantageous, and that stability can be attained by increasing the hydrophobicity at both positions (138A and 45L) or by reducing the hydrophobicity between both positions (138S and 45M), allowing a polar interaction.

In support of the structural data, we now demonstrate that the S138A mutation in HR2 can contribute to resistance when it is present with N43D in HR1 within an envelope gene derived from an ENF-experienced patient. Even in the absence of HR1 mutations, the HR2 change conferred a modest reduction in drug susceptibility (threefold), although in no case did we identify HR2 mutations without HR1 mutations. These data provide evidence that the S138A mutation represents a secondary and/or compensatory ENF mutation. Whether the S138A mutation has any substantive effects on viral replication kinetics or whether the effects of this substitution on ENF susceptibility are dependent on the envelope context in which it arises will require further study.

In summary, we identified a mutation in HR2 that comigrates with HR1 resistance mutations in viruses from 35% of patients receiving an ENF-containing regimen with virological treatment failure. We showed that the mutation in HR2 contributes to drug resistance in the context of HR1 mutations, particularly mutation at position 43. This study shows the importance of examining changes in gp41 to further understand the mechanisms used by HIV to overcome inhibition of fusion.

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