Resistance of Human Immunodeficiency Virus Type 1 to a Third-Generation Fusion Inhibitor Requires Multiple Mutations in gp41 and Is Accompanied by a Dramatic Loss of gp41 Function[▽]

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HIV-1 entry into target cells requires the fusion of viral and cellular membranes. This process is an attractive target for therapeutic intervention, and a first-generation fusion inhibitor, T20 (Enfuvirtide; Fuzeon), was approved for clinical use in 2003. Second-generation (T1249) and third-generation (T2635) fusion inhibitors with improved stability and potency were developed. Resistance to T20 and T1249 usually requires one or two amino acid changes within the binding site. We studied the *in vitro* evolution of resistance against T2635. After 6 months of culturing, a multitude of resistance mutations was identified in all gp41 subdomains, but no single mutation provided meaningful T2635 resistance. In contrast, multiple mutations within gp41 were required for resistance, and this was accompanied by a dramatic loss of viral infectivity. Because most of the escape mutations were situated outside the T2635 binding site, a decrease in drug target affinity cannot account for most of the resistance. T2635 resistance is likely to depend on altered kinetics of six-helix bundle formation, thus limiting the time window for T2635 to interfere with membrane fusion. Interestingly, the loss of virus infectivity caused by T2635 resistance mutations in gp41 was partially compensated for by a mutation at the base of the V3 domain in gp120. Thus, escape from the third-generation HIV-1 fusion inhibitor T2635 is mechanistically distinct from resistance against its predecessors T20 and T1249. It requires the accumulation of multiple mutations in gp41, is accompanied with a dramatic loss of gp41 function, and induces compensatory mutations in gp120.

The human immunodeficiency virus 1 (HIV-1) envelope glycoprotein complex (Env) is responsible for viral attachment to target cells and the subsequent fusion of viral and target cell membranes. Env consists of a trimer containing three gp120 subunits and three gp41 subunits. The gp120 surface subunits are responsible for binding to the CD4 receptor and the CXCR4 coreceptor. Receptor- and coreceptor-mediated conformational changes within the trimeric complex cause exposure of the fusion peptides (FP) of gp41 and formation of the α -helical heptad repeat domains 1 and 2 (HR1 and HR2). After insertion of the FP into the target cell plasma membrane, HR2 folds onto the trimeric coiled coil of HR1 to form a stable six-helix bundle that ultimately results in the merger of the viral and plasma membranes and delivery of the viral core into the target cell. HR2-based peptides can bind to HR1 and block HR1-HR2 association, thereby preventing membrane fusion both *in vitro* and *in vivo* (70, 71). Since many viruses share a

similar fusion mechanism, HR2-based peptide drugs can be designed against a variety of viruses (14, 52, 54, 66, 73).

T20 (Enfuvirtide; Fuzeon) is the first and only FDA-approved fusion inhibitor, and it targets the HR1 domain of HIV-1. The amino acid sequence of T20 is identical to the wild-type (wt) HR2 sequence of the HIV-1 HXB2 strain, but modified second- and third-generation HR2-based peptides that include T1249 and T2635 have been developed with improved stability and potency (Fig. 1A) (21, 23, 25, 28, 32). Third-generation T2635 contains several substitutions that stabilize the peptide in its helical form by formation of intrahelical salt bridges.

Resistance against T20 and T1249 can be acquired rather easily *in vitro* and *in vivo*. A single mutation within gp41 is sufficient to cause a dramatic loss of sensitivity to T20. Almost all resistance mutations appear within the binding site of the peptide on HR1, with substitutions G36D/V/S, V38A/E/M, Q40H, N43D, and L45M predominating (4, 5, 43, 57, 69). These escape mutations directly affect the docking and/or binding energy of the peptide. Resistance to T1249 can be obtained by a combination of multiple T20 resistance mutations (17, 38). Alternatively, more dramatic substitutions at the same positions involved in escape from T20 (V38D/E/R/K and N43K) can cause T1249 resistance. Previous studies indicate that the binding energy of T1249 is higher than that of T20, explaining the need for more dramatic HR1 amino acid sub-

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stitutions, for example, to charged residues, to obstruct peptide binding (21, 22, 24, 29, 38, 42).

Infrequently, mutations outside HR1 have been found to confer resistance to fusion inhibitors. Mutations N126K and S138A in the HR2 domain have been reported to cause resistance to T20 but usually in combination with substitutions in HR1 (7, 42, 55, 64). We have previously reported that mutations Q79E and K90E in the loop domain of gp41 confer a low level of resistance to both T20 and T1249 (22). T20 and T1249 resistance mutations generally do not cause cross-resistance to T2635, and high-level resistance to T2635 has not yet been described, although Q79E and K90E do confer modest resistance (22).

In this study, we performed *in vitro* HIV-1 evolution experiments to select and characterize resistance against the third-generation fusion inhibitor T2635. First, we wanted to investigate whether the generation of resistance against T2635 was as easy as that for T20 and T1249, which both require only a single amino acid change. Second, the impact of T2635 resistance mutations on Env function was assessed. Third, the resistance mutations were mapped, and we studied the underlying molecular mechanisms. In order to obtain multiple T2635-resistant viruses, we started 34 evolution cultures with either wt or T20- or T1249-resistant variants. We found that resistance against T2635 was much more difficult to acquire than resistance against T20 and T1249, as multiple mutations within gp41 were required for high-level resistance. The accumulation of these resistance mutations resulted in a dramatic loss of gp41 function and viral replication capacity, necessitating the emergence of compensatory changes in gp120. Furthermore, the spread of resistance mutations in many regions of the gp41 ectodomain suggests that resistance mechanisms other than those previously described for T20 and T1249 are at play.

MATERIALS AND METHODS

Peptide synthesis. Peptides T20, T1249, and T2635 were synthesized by solid-phase peptide synthesis using a 4-(2,4-dimethoxyphenyl-fluorenylmethoxycarbonyl-aminomethyl)-phenoxy (Rink amide) resin (Bachem Biochemica, Heidel-

FIG. 1. HIV-1 escape from T2635. (A) Schematic of the gp41 ectodomain. The various gp41 subdomains (fusion peptide [FP], heptad repeats 1 and 2 [HR1&2], membrane proximal external region [MPER], transmembrane domain [TM], and cytoplasmic tail [CT]) and the positions selected during evolution studies are indicated. Open circles indicate mutations present at start of the culture, and filled circles indicate mutations obtained during the course of the experiment. Numbering is based on the sequence of HXB2 gp41. The sequences of the first-generation (T20), second-generation (T1249), and third-generation (T2635) fusion inhibitors are aligned to the sequence of the HXB2 HR2. (B) Increase of T2635 pressure. Cultures were passaged twice a week for 6 months with increasing T2635 concentrations as indicated. When HIV-induced cytopathic effects or increased CA-p24 production was apparent, the virus was passaged cell free onto uninfected cells. (C) Replication of the viral quasiespecies of 5 evolution cultures in SupT1 cells in the presence of no T2635 or 20, 100, and 500 ng/ml T2635. (D) T2635 escape mutations mapped onto the model of the gp41 postfusion structure (Protein Data Bank [PDB] accession no. 1IF3 [15]) using PyMOL Molecular Viewer (W. L. DeLano, Schrödinger, LLC, Portland, OR [http://www.pymol.org]). Substitutions that were present in some of the starting viruses are indicated in red, while changes that emerged over the course of the experiment are in yellow. One gp41 protomer is in color, and the other two are gray.

berg, Germany) on a Syro synthesizer (MultiSynTech, Witten, Germany) as described previously (22). All amino acids were purchased from Bachem Biochemica and used as N- α -(fluorenylmethoxycarbonyl) protected with side-chain functionalities protected as N-tert-butoxycarbonyl (KW), O-tert-butyl (DESTY), N-trityl (HNO), S-trityl (C), S-S-tert-butyl (C), or N-2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (R) groups. A coupling protocol using a 6.5-fold excess of 2-(1H-benzotriazole-1-yl)-0-1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole/amino acid/N,N-diisopropylethylamine (1:1:1:2) in N-methyl-2-pyrrolidone (NMP) with a 30-min activation time using double couplings was employed. Peptides were cleaved from the resin by generating a reaction with trifluoroacetic acid (TFA) (15 ml g⁻¹ resin) containing 13.3% (wt) phenol, 5% (vol) thioanisole, 2.5% (vol) 1,2-ethanedithiol, and 5% (vol) milliQ-H₂O for 2 to 4 h at room temperature. The crude peptides were purified by reverse-phase high-performance liquid chromatography (RPC), either on a Delta-Pak (25- or 40-mm inner diameter by 100-mm length, 15- μ m particle size, 100-Å pore size; Waters, Milford, MA) or on an XTerra (50- by 4.6-mm inner diameter, 2.5- μ m particle size; Waters, Milford, MA) RP-18 preparative C₁₈ column with a linear A-B gradient of 1 to 2% B min⁻¹. Solvent A is 0.05% TFA in water, and solvent B is 0.05% TFA in acetonitrile (ACN). The correct primary ion molecular weights of the peptides were confirmed by electrospray ionization-mass spectrometry on a Micromass ZQ (Micromass, Almere, the Netherlands) or a VG Quattro II (VG Organic, Cheshire, United Kingdom) mass spectrometer.

Selection of T2635-resistant HIV-1 variants. For the selection of T2635-resistant viruses, SupT1 cells were transfected with 1 μ g DNA of either the wild-type (wt) HIV-1_{LAI} molecular clone or several T20- and/or T1249-resistant variants thereof, as follows: Q79E, K90E, V38A/N126S/N126K, and a mixture of plasmids containing a codon for all possible amino acids at position 38 of gp41, except for the wt valine (7, 22). Transfected cells were split into 6 or 8 separate cultures, and fresh SupT1 cells were added to initiate the evolution cultures. The selections were started with a concentration of 2 ng/ml T2635, which corresponds to about half the IC₅₀ for wt virus. Cultures were split twice weekly, and when HIV-induced cytopathic effects and/or increased CA-p24 production were apparent, virus-containing supernatant was passaged cell-free onto uninfected SupT1 cells while doubling the T2635 concentration. Viruses were cultured for 6 months, although 4 cultures were stopped after 1 or 2 months because no virus replication was ever observed. Cells and supernatant samples were taken at regular time points and stored at -80°C. Cell culturing, transfections, and CA-p24 determination were performed as previously described (7, 35). At day 180, DNA was extracted from infected cells using the QIAamp DNA minikit (Qiagen, Valencia, CA), and the complete proviral *env* gene was PCR amplified using primers 1 (5'-ATAAGCTTAGCAGAAGACAGTGGCAATG-3') and 2 (5'-GCAAAATCCTTTCAGAGCCC-3'). The *env* gene was sequenced as described previously (13).

Construction of HIV-1_{LAI} molecular clones. The full-length molecular clone of HIV-1_{LAI} (pLAI) (53) was used to produce wt and mutant viruses. The plasmid pRS1 was used to introduce mutations as described previously (22, 58), and the complete *env* genes were verified by DNA sequencing. Mutant *env* genes in pRS1 were cloned back into pLAI as SalI-BamHI fragments. Each virus variant was transiently transfected in C33A cells by calcium phosphate precipitation as previously described (18). The virus-containing supernatant was harvested at 3 days posttransfection, filtered, and stored at -80°C, and the virus concentration was quantified by capsid CA-p24 enzyme-linked immunosorbent assay (ELISA). All virus mutants were produced with similar efficiency and CA-p24 levels.

Virus replication. A total of 100 pg CA-p24 virus harvested from the evolution culture or 500 ng virus harvested from transfection was added to 5 $\times$ 10⁵ SupT1 cells in the absence or presence of 20, 100, or 500 ng/ml T2635. Supernatant samples were taken at days 3, 4, 5, 6, and 7, and virus replication was measured by CA-p24 production.

IC₅₀ and infectivity determination. The TZM-bl reporter cell line (19, 69) stably expresses high levels of CD4, CCR5, and CXCR4 and contains the luciferase and β -galactosidase genes under the control of the HIV-1 long-terminal-repeat promoter and was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (J. C. Kappes, X. Wu, and Tranzyme Inc., Durham, NC). One day prior to infection, 17 $\times$ 10³ TZM-bl cells per well were plated on a 96-well plate in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1 $\times$ minimum essential medium nonessential amino acids, and penicillin-streptomycin (both at 100 units/ml) and incubated at 37°C with 5% CO₂. A fixed amount of virus (5 ng CA-p24) was preincubated for 30 min at room temperature with 11 serial 1-in-3 dilutions of inhibitor, starting at 3,000 ng/ml for T20, 1,000 ng/ml for T2635, and 10 μ g/ml for 2F5 (Polymun, Vienna, Austria). This mixture was added to the cells in the presence of 400 nM protease inhibitor saquinavir to block secondary rounds of

infection (Roche, Mannheim, Germany) and 40 μ g/ml DEAE in a total volume of 200 μ l. Two days postinfection, the medium was removed and cells were washed once with phosphate-buffered saline (PBS; 50 mM sodium phosphate, pH 7.0, 150 mM NaCl) and lysed in reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using a luciferase assay kit (Promega, Madison, WI) and a GloMax luminometer according to the manufacturer's instructions (Turner BioSystems, Sunnyvale, CA).

The relative infectivity of mutants was established by measuring their luciferase activities on TZM-bl cells, subtracting the background luciferase activity, and setting the luciferase of wt HIV-1_{LAI} at 100%. The data shown in the figures represent the mean infectivity of at least two, but typically more, independent infection experiments performed in quadruplicate and performed with at least two independent virus stocks. Virus infectivity at 3-fold dilutions of the inhibitors was used to obtain nonlinear regression curves using Prism software version 5.0. Typically the *R* values of the regressions were >0.90. The regressions were used to calculate the 50% inhibitory concentration (IC₅₀). The data in the tables represent the mean IC₅₀s from two or three independent experiments, performed in duplicate, and performed with at least two independent virus stocks. The standard deviations of the IC₅₀s were typically <0.1 log. Data from two or three experiments were combined to calculate the mean IC₅₀. Based on this, we considered 2-fold resistance as statistically meaningful. Throughout the paper, we consider mutations that show >3-fold resistance compared to that of the wt virus.

RESULTS

HIV-1 escapes from T2635 pressure. We set out to investigate if and how HIV-1 could escape from T2635 pressure. HIV-1_{LAI} was passaged on SupT1 T cells under increasing drug pressure, starting with a dose of about half the IC₅₀ (2 ng/ml; 0.45 nM). In addition to the wt virus, we used a number of viruses that harbored resistance mutations to T20 or T1249. The rationale was that acquisition of resistance to the third-generation T2635 peptide may be easier for viruses harboring mutations that confer resistance to the first- and second-generation peptides T20 and T1249, either by accumulation of substitutions or by lowering the genetic threshold for the required codon at already mutated residues (17, 22, 29, 38, 42). Indeed, previous work showed that T20 resistance favored the escape from T1249 (22). Thus, we started evolution cultures with the wt, the T20-resistant variant V38A/N125S/N126K (7), and two T1249-resistant variants, Q79E and K90E (22). Since residue 38 is extremely important in conferring resistance to both T20 and T1249, we were interested to test all amino acids at position 38 for their abilities to contribute to T2635 escape. We therefore also started evolution cultures with a virus library with all 19 possible substitutions, but excluding the wt valine. Six to eight independent evolution cultures were started for every condition, resulting in 34 cultures, increasing the change of escape and identification of the required mutations.

Infected SupT1 T-cell cultures were passaged for 6 months with escalating concentrations of T2635. Inhibitor concentrations were increased up to 1,024 ng/ml, corresponding to ~250 times the IC₅₀ on wt LAI (Fig. 1B). To verify whether the viruses in these cultures had become resistant to T2635, the viral quasiespecies of selected cultures after 6 months of evolution was tested in a replication assay on SupT1 T cells in the absence and presence of T2635 (Fig. 1C). Viruses from all cultures tested were able to replicate in the absence of inhibitor, but in contrast to wt virus, all viruses from the evolution cultures were also able to replicate in the presence of up to 100 ng/ml T2635. The evolved viruses from cultures 1 and 29 were even able to replicate in the presence of higher doses of T2635 (500 ng/ml), although with decreased efficiency. Thus, these

TABLE 1. Amino acid substitutions selected in gp41 during T2635 escape

		gp41 substitution(s) for consensus amino acid at indicated position observed in ≥ 3 cultures ^a									
Culture	Starting virus	HR1									
		6 A	33 L	38 V	66 Q	77 K	79 Q	90 K	94 T	100 N	113 N
1	wt					E					
2	wt					K/E					
3	wt	V	S		R						
4	wt	V				E					
5	wt		S			N		N			
6	wt	V			L					D	
7	Q79E/N125S									D	
8	Q79E/N125S					N				D	D
9	Q79E/N125S										
10	Q79E/N125S		S			N			N		
11	Q79E/N125S		S								
12	Q79E/N125S										
14	K90E/N125S					N				D	
16	K90E/N125S							K			
17	K90E/N125S							E			
18	K90E/N125S							E			
19	V38X			V							
20	V38X			V					N		
21	V38X			V							
22	V38X			mix	R	E					
23	V38X			V		N					
24	V38X			mix	R						D
26	V38A/N125S/N126K			A	R						
27	V38A/N125S/N126K			V	R						
28	V38A/N125S/N126K	V		V	R					D	D
29	V38A/N125S/N126K		S	V	R						D
30	V38A/N125S/N126K			V			E				
31	V38A/N125S/N126K	V		V	R	E					
33	V38A/N125S/N126K	V			H						
34	V38A/N125S/N126K			V							
	wt LAI										

^a Mutations present in the starting virus are indicated in bold. When a mixture of 2 amino acids was present, both are shown. When the sequences could not identify 1 or 2 specific amino acids, the residues are represented as "mix."

^b IC₅₀ (ng/ml) values were derived from single-cycle infection assays as described in Material and Methods. ND, not determined.

^c Fold increase in resistance is shown compared to the IC₅₀ of the wt.

selected cultures harbored T2635-resistant viruses, but the level of resistance varied. To more precisely quantify the level of resistance, the viral populations from all escape cultures were tested in a single cycle infection assay using TZM-bl reporter cells in the absence or presence of escalating T2635 doses. The IC₅₀s for T2635 were calculated and ranged from 22 up to 393 ng/ml, corresponding to 4-fold to 63-fold resistance compared to that of the wt (Table 1), confirming the emergence of T2635 escape variants in the evolution cultures.

T2635 pressure triggers the emergence of substitutions in all gp41 subdomains. The *env* gene from the proviral DNA of all cell cultures was amplified and the complete gp41 part was sequenced, revealing a considerable number of amino acid changes throughout the gp41 ectodomain (Table 1). The cultures contained at least 2 substitutions in gp41, but some cultures harbored as many as 9. We focused on mutations that

were observed in at least three independent cultures because of their apparently preferred selection during T2635 escape.

The mutations map to different gp41 subdomains (Fig. 1). First, a substitution in the FP domain emerged in 6/30 cultures (A6V). Second, we observed two recurring mutations in HR1, which contains the binding site for T2635 (L33S, 5/30 cultures; Q66R/L/H, 10/30 cultures). We noted that in cultures initiated with viruses containing substitutions at position 38 (V38A or V38X, where X refers to the library encoding all amino acids except the wt valine), the virus almost invariably reverted back to the wt valine (12/14 cultures). In contrast, substitutions Q79E, K90E, and N126K that were present in a subset of the starting cultures were generally preserved during the 6 months of evolution in the presence of T2635. Third, mutations emerged in the central part of HR2, the binding partner for HR1 in the postfusion six-helix bundle structure (N126K/H,

TABLE 1—Continued

gp41 substitution(s) for consensus amino acid at indicated position observed in ≥ 3 cultures ^a								T2635		
HR2								gp41 substitutions observed <3 times	IC ₅₀ ^b	n-fold ^c
125	126	132	136	137	151	154	210			
N	N	H	E	E	E	K	L			
N	K		G					N96S, D121E, D248G	393.0	63.0
	K		G					E280E/K	283.3	45.4
	K	Q/H	E/G					V182I/V	120.4	19.3
	K	Q	G						162.9	26.1
	K				G			S251N	72.0	11.5
	K								106.5	17.1
N	K								41.1	6.6
N	K		G					S133S/T, E280K	136.9	22.0
S	K								38.2	6.1
N	K				G				99.9	16.0
S	K							I69K, N89E, K144 M, V178V/I	95.5	15.3
S									29.4	4.7
N										
N	K							E148K	90.0	14.4
	K								ND	
									ND	
	S								24.2	3.9
D	K						F/L	Q40R	>200	
	K					E	F	I69L, D121E, S251N	137.8	22.1
	K		G					S104N,	210.7	33.8
	K							M176I	59.7	9.6
	H		G				L/F	L252M	75.1	12.0
	K						F		371.7	59.6
N	K								182.4	29.3
	K			G	F			I124V, N160D, R231K/R	114.2	18.3
	K	Q				E		L191F	ND	
	K	Q							262.5	42.1
	K								227.5	36.5
	K	Q							22.3	3.6
	K			V		E		N163D	ND	
	K			G				V72M	134.7	21.6
								6.24	1.0	

19/22 cultures, as N126K was present in the starting virus of the other 8 cultures; H132Q, 5/30 cultures; E136G, 7/30 cultures; and E137G/V, 3/30 cultures). In contrast, we observed reversion S125N/D in 9/18 cultures in which N125S was originally present. Fourth, mutations were observed in the loop domain between the two heptad repeats (T94N, 3/30 cultures; N100D, 5/30 cultures; and N113D, 4/30 cultures). Note that N110D, like N126K in HR2, involves the loss of a potential glycosylation site. Finally, two substitutions repeatedly emerged in the membrane proximal region (MPER) (E151G/F, 3/30; and K154E/Q, 3/30) and one in the cytoplasmic tail of Env (CT) (L210F, 4/30). A number of other substitutions in the various gp41 subdomains were found in one or two cultures (Table 1). Although these changes may represent bona fide resistance mutations, we focused the follow-up analyses on the preferred viral escape routes.

T2635 resistance requires multiple substitutions in gp41. To investigate the importance of the specific substitutions found in the evolution cultures, and combinations thereof, we constructed a series of mutants in a wt HIV-1_{LAI} background. All

mutations that were observed in at least 3 independent cultures were constructed as single mutants and in combination with the N126K mutation that was either already present or selected in most cultures. A selected set of triple mutants was also generated. Finally, we recreated a number of hypermutants based on evolution cultures 2 (H2A), 3 (H3B, H3C), 5 (H5B, H5C), 8 (H8B), 28 (H28B, H28C), and 29 (H29B, H29C) (Table 1). Infectious virus was produced and used to infect TZM-bl reporter cells in the absence or presence of various T2635 concentrations to determine the IC₅₀ (Table 2).

Only a few single substitutions cause modest T2635 resistance (>3 -fold compared to wt; A6V, Q79E, K90E, N126K, and K154Q). Addition of the N126K mutation in double and triple mutants increased the resistance levels considerably, with Q66R/N126K (12-fold), Q79E/N126K (13-fold), and K90E/N126K (16-fold) providing the highest resistance. The accumulation of multiple mutations in the hypermutants resulted in a dramatic increase in resistance (for example, A6V/L33S/Q66R/N126K/H132Q/E136G [H3C], 53-fold resistance compared to that of the wt; and L33S/Q66R/N113D/N125S/

TABLE 2. Resistance profiles of gp41 mutants^a

Mutation(s) in LAI (gp41 numbering)	T2635		T20		2F5	
	IC ₅₀ (ng/ml)	<i>n</i> -fold resistance	IC ₅₀ (ng/ml)	<i>n</i> -fold resistance	IC ₅₀ (μg/ml)	<i>n</i> -fold resistance
A6V	21.32	6.5	54.58	1.0	3.34	1.2
L33S	6.30	1.9	2391	45.1	3.39	1.2
Q66R	7.80	2.4	46.83	0.9	0.52	0.2
K77E	9.40	2.9	170.02	3.2	0.73	0.3
K77N	6.24	1.9	129.58	2.4	2.02	0.7
Q79E	15.99	4.9	258.93	4.9	0.72	0.3
K90E	21.30	6.5	166.89	3.2	1.83	0.6
T94N	7.39	2.2	61.94	1.2	3.46	1.2
N100D	4.72	1.4	39.26	0.7	2.35	0.8
N113D	3.85	1.2	37.78	0.7	2.28	0.8
N125S	2.46	0.7				
N126K	15.90	4.8	143.90	2.7	3.12	1.1
H132Q	4.56	1.4	68.14	1.3	2.78	1.0
E136G	6.23	1.9	72.69	1.4	3.23	1.1
E137G	4.84	1.5				
E151G	4.33	1.3				
K154E	3.73	1.1				
K154Q	16.52	5.0	69.48	1.3	>30.0	>10
L210F	3.89	1.2	85.99	1.6	2.86	1.0
A6V/N126K	ND	ND				
L33S/N126K	16.67	5.1	2185	41.2	2.56	0.9
Q66R/N126K	39.35	11.9	303.43	5.7	1.67	0.6
K77E/N126K	5.41	1.6	498.69	9.4	2.53	0.9
K77N/N126K	23.62	7.2	915.12	17.3	4.03	1.4
Q79E/N126K	41.90	12.7	1317	24.9	5.67	2.0
K90E/N126K	51.06	15.5	2970	56.1		
T94N/N126K	17.45	5.3	356.92	6.7	4.15	1.4
N100D/N126K	9.79	3.0	200.15	3.8	5.84	2.0
N113D/N126K	19.99	6.1	110.93	2.1	4.03	1.4
N125S/N126K	9.46	2.9				
H132Q/N126K	15.52	4.7	397.46	7.5	4.30	1.5
E136G/N126K	14.70	4.5	375.44	7.1	4.69	1.6
E137G/N126K	13.94	4.2				
E151G/N126K	12.06	3.7				
K154E/N126K	11.96	3.6				
K154Q/N126K	6.20	1.9				
L210F/N126K	10.71	3.3	227.80	4.3	3.61	1.3
V38A/N125S/N126K	6.60	2.0				
Q66R/N125S/N126K	27.50	8.3				
Q79E/N125S/N126K	30.44	9.2				
K90E/N125S/N126K	32.94	10.0				
A6V/N126K/H132Q	16.23	4.9				
H2A (K77E/N126K/E136G)	34.22	10.4				
H3B (A6V/Q66R/N126K/H132Q/E136G)	25.13	7.6				
H3C (A6V/L33S/Q66R/N126K/H132Q/E136G)	172.4	52.3	>9,000	>150	1.28	0.4
H5B (K77N/T94N/N126K/E151G)	44.95	13.6				
H5C (L33S/K77N/T94N/N126K/E151G)	56.76	17.2	>9,000	>150	>3.0	
H8B (K77N/N100D/N113D/N126K/E136G)	ND	ND				
H28B (Q66R/N125S/N126K/H132Q/K154Q)	86.97	26.4				
H28C (Q66R/N100D/N125S/N126K/H132Q/K154Q)	81.08	24.6	286.85	5.4	>30.0	>10
H29B (Q66R/N113D/N125S/N126K/H132Q)	90.64	27.5				
H29C (L33S/Q66R/N113D/N125S/N126K/H132Q)	280.8	85.2	>9,000	>150	0.47	0.2
wt LAI	3.29		52.98		2.88	

^a IC₅₀s were calculated as described in Material and Methods and are represented in ng/ml or μg/ml. Fold resistance is shown compared to the IC₅₀ of the wt. Bold indicates >2-fold resistance compared to wt virus. ND, not determined.

N126K/H132Q [H29C], 85-fold resistance). A correlation was apparent between the number of selected resistance mutations and the level of resistance ($R^2 = 0.64$; $P < 0.0001$) (Fig. 2). Apparently, the emergence of high-level T2635 resistance has

a high genetic barrier and requires multiple mutations in various gp41 subdomains.

Substitutions Q66R and N113D mediate T2635 resistance in a synergistic manner. The N126K substitution, which provided

FIG. 2. Correlation between the number of escape mutations and resistance to T2635. The correlation was calculated using GraphPad Prism software v5.

5-fold resistance, augmented resistance in combination with several other substitutions (e.g., Q66R, Q79E, K90E) in an additive manner. The N113D substitution, which we observed in 4 cultures, did not confer resistance to T2635 (Table 2), but since it appeared frequently in combination with the Q66R substitution, we were intrigued by a possible synergistic effect of these two substitutions. We therefore constructed a virus with the two substitutions combined. While the N113D and Q66R single substitutions provided no or low-level (2.4-fold) resistance, respectively, the resistance of the Q66R/N113D double mutant was enhanced (5.4-fold) (Table 3).

The molecular structure of the postfusion six-helix bundle may provide the clue for the synergistic effect in T2635 resistance of substitutions Q66R and N113D (Fig. 3). N113D is situated in HR2 and in close contact with residue 66 in HR1 (Fig. 3A, top panels). The introduction of the negatively charged Asp at position 113 may allow for an electrostatic interaction between N113D and Q66R (Fig. 3A, bottom panels). In contrast, the corresponding salt bridge between Q66R and E4 on T2635 would require an unfavorable rotation of the T2635 helix (Fig. 3B). Thus, the Q66R-N113D combination may favor the association of HR1 with HR2, while disfavoring the binding of T2635. In addition, the favored HR1-HR2 interaction could result in faster kinetics of the six-helix bundle formation, decreasing the time window for T2635 to act.

To test this hypothesis, we generated an additional set of viruses with substitutions at position N113. Introduction of

FIG. 3. Molecular interactions of position 66 with T2635 and HR2. Molecular models showing the interactions of residue 66 in HR1 with residues 113 and 117 in HR2 or E4 and W3 in T2635. The models of HR1 with HR2 and T2635 were previously generated (24). Amino acid substitutions were introduced using PyMOL. (A) Q66 can form polar interactions with N113 and W117 (top panels) but also electrostatic interactions with N113D (bottom panels). A putative salt bridge between Q66R and N113D is indicated. (B) Q66 can form a hydrogen bond with the nitrogen of W3 (top panels), while the Q66R substitution probably favors an electrostatic interaction with E4, displacing W3 and resulting in an unfavorable rotation of the T2635 helix (bottom panels). HR1 is depicted in green and T2635 in magenta.

another negatively charged residue (N113E) would be expected to have a similar synergistic effect in combination with Q66R, while substitution with a positively charged amino acid (N113K, N113R) would not be predicted to have such an effect. The single mutations N113E, N113K, and N113R provided minimal levels of resistance (2.5-fold, 1.6-fold, and 1.8-fold, respectively). The Q66R/N113E double mutant virus showed markedly enhanced resistance compared to the two single mutants (7.5-fold resistance), similar to Q66R/N113D. The Q66R/N113K and Q66R/N113R viruses, however, did not show a large increase in resistance (3.5-fold and 2.5-fold resistance, respectively). Thus, negatively charged amino acids at position 113 synergistically augment resistance of Q66R, while positively charged residues do not.

Acquisition of T2535 resistance is accompanied by a loss of gp41 function. Resistance to drugs often results in a partial loss of function of the target protein, as described for HIV-1 reverse transcriptase and protease. However, this effect is frequently counterbalanced by the emergence of compensatory changes (34, 49). To address whether the acquisition of T2635 resistance coincided with a loss of gp41 function, we evaluated the efficiency of viral entry into susceptible target cells in a quantitative single-cycle reporter assay (Fig. 4). Most of the T2635 resistance mutations caused a substantial decrease in virus infectivity, with mutants K79E and K90E showing the

TABLE 3. T2635 resistance profiles of N113 mutants

Mutation(s) in LAI (gp41 numbering)	T2635 ^a	
	IC ₅₀ (ng/ml)	<i>n</i> -fold resistance
None (wt)	3.46	1.0
N113D	2.82	0.8
N113E	8.75	2.5
N113K	5.68	1.6
N113R	6.12	1.8
Q66R	8.31	2.4
Q66R/N113D	18.57	5.4
Q66R/N113E	26.02	7.5
Q66R/N113K	12.22	3.5
Q66R/N113R	8.63	2.5

^a IC₅₀s were calculated as described in Material and Methods. Fold resistance is shown compared to the IC₅₀ of the wt.

FIG. 4. Functional characterization of T2635 escape mutants. (A) Single-cycle infection assays in TZM-bl cells were performed with a fixed amount (5 ng CA-p24) of wt HIV-1_{LAI} or recombinant virus variants with selected substitutions and combinations of substitutions based on the sequences T2635 escape viruses. Infections were performed 4-fold. Luciferase activity was measured and corrected for background luciferase activity. The luciferase activity of wt was set at 100%, and the relative infectivity of the mutant virus variants was normalized accordingly. (B) Correlation between T2635 resistance and infectivity. (C) Correlation between the number of mutations and viral infectivity.

lowest infectivity. Interestingly, the accumulation of mutations further decreased the infectivity, with all hypermutants showing minimal infectivity as low as 0.6% of wt. A strong inverse correlation was observed between the level of T2635 resistance and viral infectivity ($R^2 = 0.74$, $P < 0.0001$) (Fig. 4B). The number of T2635 resistance mutations was also correlated inversely with viral infectivity ($R^2 = 0.62$, $P < 0.0001$) (Fig. 4C). These findings were corroborated in virus replication experiments, showing minimal replication capacity of the hypermutants (data not shown). Thus, the accumulation of mutations in gp41 to acquire high-level T2635 resistance caused a progressive and dramatic loss of Env function. Apparently, with some exceptions, the combination of mutations primarily served the goal of increasing resistance and not the restoration of Env function, as expected from compensatory mutations.

T2635 pressure triggers the emergence of mutations in gp120. The replication capacity of the viral quasiespecies from the evolution cultures was not as dramatically reduced as that of molecularly cloned viruses containing multiple substitutions in gp41 (Fig. 1C and 4A and data not shown). We therefore wanted to investigate whether T2635 pressure would trigger the emergence of substitutions in gp120 that might compensate for the loss of gp41 function caused by the acquisition of multiple resistance mutations. The gp120 region of the *env* gene in 27 of the evolution cultures was sequenced, revealing several mutations in gp120 (Table 4). Mutations in gp120 were much less frequently selected than mutations in gp41 (2.7 mu-

tations in gp120 versus 4.9 mutations in gp41 on average, corresponding to 5.3×10^{-3} versus 13.7×10^{-3} mutations per residue, respectively), indicating that the T2635 selective pressure is higher on the gp41 subunit than on gp120, which is consistent with gp41 being the target of T2635. Only 7 gp120 substitutions were selected in at least 3 evolution cultures. Of these, the R298K substitution at the stem of the V3 loop was by far the most abundant (found in 16/27 cultures). Three other mutations that were frequently selected cause the shift of a potential N-glycosylation site in the V2 loop (D185N, N186K, and T188N). Other frequently observed substitutions were L25S in the leader peptide, S148E/I/R in V1, and A316T in V3. Of note is that we did not observe any substitutions in the putative interactive site of gp120 and gp41, that is, the C1 and C5 domains (26, 33).

R298K substitution in gp120 can compensate for the loss of gp41 function. To investigate the importance of the R298K substitution on T2635 resistance and/or compensation for Env function, it was introduced into the wt virus and a poorly infectious virus variant containing a combination of T2635 resistance mutations in gp41 (hypermutant H28C). R298K as an individual substitution did not cause any resistance to T2635 (0.8-fold) and slightly enhanced T2635 resistance of the H28C hypermutant from 14.90- to 17.8-fold (Table 5). However, the R298K substitution increased the replication capacity of hypermutant H28C in both the absence and presence of T2635 (Fig. 5). Although the replication levels were not restored to

TABLE 4. Amino acid substitutions in gp120 selected during T2635 escape

Culture	Starting virus	gp120 substitution(s) for consensus amino acid at indicated position observed in ≥ 3 cultures ^a							gp120 mutations observed in <3 cultures
		25	148	185	186	188	298	316	
		L	S	D	N	T	R	A	
1	wt	S			K	N	K		Δ FNSTW (391–395)
2	wt	S			K	N	K		
3	wt						K		
4	wt								Δ NTNSS (139–143), L34 M E429K
5	wt			N			K		
6	wt					N			
7	Q79E/N125S								M147 M/I
8	Q79E/N125S	S			K	N	K		
9	Q79E/N125S						K		
10	Q79E/N125S			N			K		D133N/D, S164G
11	Q79E/N125S			N			K		
12	Q79E/N125S								
14	K90E/N125S	S			K	N			G145R
16	K90E/N125S		I						
17	K90E/N125S						K		
18	K90E/N125S								M20I, S164 (mix) ^a
19	V38X		E				K		
20	V38X						K		
21	V38X							T	A329T G404K
22	V38X					T/N			
23	V38X								
27	V38A/N125S/N126K						K		S143G, S398N
28	V38A/N125S/N126K						K		
29	V38A/N125S/N126K		R				K	T	
30	V38A/N125S/N126K							T	S128R, V488I
31	V38A/N125S/N126K						K		
34	V38A/N125S/N126K						K		

^a When the sequences could not identify 1 or 2 specific amino acids, the residues are represented as “mix.”

the wt level, the results clearly show that the R298K substitution in gp120 can compensate at least partially for the loss of Env function caused by resistance mutations in gp41. Similar results were obtained for R298K when combined with other gp41 mutations (data not shown).

Interestingly, two viruses that did not select the R298K substitution acquired another mutation in the V3 domain, namely, at position 329 (Table 4), which is opposite residue 298 in the stem of the V3 loop. Another substitution in the V3, A316T, was selected in 3 cultures. The selection of V3 mutations in 20 of the 27 sequenced cultures suggests that V3 is a hot spot for compensatory changes under T2635 pressure. Considering the importance of the V3 in coreceptor binding, we can speculate

that these viruses alter and/or improve their interaction with the coreceptor. The finding may be related to the observation that 3 substitutions in gp41 can confer resistance to a coreceptor antagonist (2, 9). In summary, HIV-1 is able to partially restore the loss of Env function caused by resistance mutations in gp41 by introducing compensatory changes in gp120.

T2635 resistance mutations can provide cross-resistance to T20. Drug resistance mutations often confer cross-resistance to inhibitors within the same drug class (reviewed in reference 59). We therefore explored whether the identified T2635 resistance mutations provided cross-resistance to the first-generation fusion inhibitor T20 (Table 2). Indeed, some of the single mutations that provided resistance to T2635 also caused some level of resistance to T20 (for example, Q79E and K90E). L33S alone did not cause significant resistance to T2635, but caused 45-fold resistance to T20. High-level T20 resistance of the hypermutants also appeared to be dependent on the presence of L33S since H28C, lacking L33S, was only modestly resistant to T20 (5-fold compared to that for the wt as opposed to >150-fold for H3C, H5C, and H29C). In contrast, A6V and K154Q caused resistance to T2635 but not to T20. Thus, we identified mutations that conferred resistance to T2635, to T20, and to both T20 and T2635. These data suggest that there are common as well as distinct paths to resistance for these two fusion inhibitors.

TABLE 5. T2635 resistance profiles of gp120 R298K mutants

Mutation(s) in LAI (gp41 numbering)	T2635 ^a	
	IC ₅₀ (ng/ml)	n-fold resistance
None (wt)	4.56	1
R298K	3.70	0.8
H28C	68.23	14.9
H28C + R298K	81.28	17.8

^a IC₅₀s were calculated as described in Material and Methods. Fold resistance is shown compared to the IC₅₀ of the wt.

FIG. 5. Replication of viruses containing the R298K substitution. Replication capacities of wt HIV-1_{LAI} and hypermutant 28C were compared in the presence or absence of gp120 substitution R298K. Addition of 298K to wt HIV-1_{LAI} had no effect on replication, but in the background of hypermutant 28C, 298K improved replication capacity both in the absence of inhibitor and in the presence of 100 n/ml T2635.

T2635-resistant HIV-1 is sensitive to the MPER-directed antibody 2F5. 2F5 is a neutralizing antibody directed against the membrane proximal external region (MPER) of gp41 that, similar to fusion inhibitors, appears to inhibit a late step in Env-mediated viral entry (10, 12). To evaluate whether the mutations that we identified also affected sensitivity to 2F5, we determined IC_{50} s for a selected set of T2635 resistance mutants (Table 2). Mutant K154Q provided strong resistance to 2F5, which is not surprising as this residue is part of the core epitope of 2F5 (46) and mutation of this residue has been reported to cause 2F5 resistance. E151G is also located in the epitope for 2F5 and probably caused the 2F5 resistance of the H5C hypermutant. None of the other mutations conferred significant resistance to 2F5. In contrast, many mutants became more sensitive to 2F5. These results imply that T2635 resistance does not necessarily coincide with resistance to other compounds targeting late steps of the viral entry process.

DISCUSSION

We investigated the evolution of resistance to the third-generation HIV-1 fusion inhibitor T2635 by culturing the virus under increasing pressure of T2635. It was previously shown that common T20 and T1249 resistance mutations do not cause high-level cross-resistance to T2635 (21, 22). We demonstrate that the accumulation of numerous mutations within multiple gp41 subdomains is needed to obtain high-level resistance to T2635 and that the number of mutations was strongly correlated with the level of resistance (Fig. 2). Furthermore, this accumulation of mutations was accompanied by a dramatic loss of Env function. We did not find evidence that any of the gp41 mutations were compensatory, i.e., served to improve Env function in the context of initial resistance mutations. In contrast, the data suggest that many of the observed changes within gp41, if not all, contribute to resistance and do not restore Env function. We did, however, find evidence that compensatory changes were selected in gp120. We have investigated the R298K substitution, which was selected in the majority of evolution cultures and which partially restored Env function without contributing to T2635 resistance. In the subsequent paragraphs, we discuss the mechanistic implications of the observations reported in this study.

Some T20 and T1249 resistance mutations present in the starting cultures contribute to T2635 resistance and were

therefore preserved during T2635 escape. In particular, substitution N126K, which was present in 8 starting cultures, was always preserved and selected for in an additional 18 cultures, in addition to the selection of N126H in a single culture. In fact, at the end of the 6-month evolution experiment, only 3/30 cultures did not have a substitution at position 126. The IC_{50} measurements explain the selection of N126K: it causes modest T2635 resistance on its own, and in combination with other substitutions its effect can be additive (e.g., with Q79E) or synergistic (with Q66R). N126K does not cause significant resistance to T20 (Table 2), and its emergence during T20 escape compensates for loss of Env function (slower fusion kinetics) caused by resistance mutations in HR1 (6, 56).

Mutations involved in resistance to T20 and T1249 at position 38 rapidly reverted back to the wt valine. Apparently, they do not contribute to T2635 resistance and are lost because they negatively affect Env function (22, 24). In addition, they may disturb the RNA structure of the underlying Rev response element and only the wt codon creates an optimal RNA structure (48, 63). Mutations Q79E and K90E cause modest T2635 resistance (Table 2) and have a large negative effect on virus infectivity. These mutations were therefore preserved only when no other resistance mutations were selected (Table 1).

Surprisingly few substitutions occurred in the assumed binding site of T2635 in HR1. Only substitutions L33S and Q66R were detected in multiple independent cultures. This is in sharp contrast to escape from first- and second-generation peptides, which is predominantly induced by one or multiple changes in the peptide binding site (particularly positions 36, 38, and 43 [19, 40, 44, 45, 60, 69]). The binding energy of T2635 for HR1 is high compared to that of T20 and T1249 (21, 24). We have previously shown that substitutions at position 38 had a considerable effect on the drug-HR1 binding energy. This was sufficient to cause resistance to T20 but not to T2635 because the relative contribution of residue 38 to peptide binding is much less (24). Apparently, single amino acid substitutions in the binding site are not sufficient to prevent T2635 binding. Multiple mutations in the T2635 binding site may be necessary to sufficiently lower the T2635-HR1 binding energy to cause resistance, but this escape route has a high genetic barrier and therefore is more difficult and may be accompanied by an insurmountable loss of Env function and viral fitness. Therefore, other escape mechanisms are apparently preferred.

FIG. 6. Mechanism of T20 and T2635 docking onto HR1 and escape. The T20 and T1249 resistance “hotspots” at positions 38 and 43 are mapped onto the structures of the T20-HR1 complexes (A), as are positions 33 and 66 on the structure of the T2635-HR1 complex (B). The models of T20-HR1 and T2635-HR1 were generated previously (24). A hypothetical model of the docking of T20 and T2635 onto HR1 is shown. T20 is relatively unstructured in solution and needs to bind to HR1 to acquire a helical structure (41, 65). In contrast, T2635 is mostly helical in solution. See text for further explanations of the model.

The virtually exclusive selection of HR1 substitutions at positions 33 and 66 on the outer edges of the T2635 binding site leads us to speculate that T2635 has another mechanism for initial binding other than T20 and T1249. The T20 and T1249 peptides lack secondary structure in solution, and they assume a secondary structure upon binding to HR1 (21, 41). T20 and probably T1249 are thought to dock onto HR1 around residues 36 to 43 (65), which implies that they “zipper up” bidirectionally to acquire their helical structure, using the HR1 grooves as a template (Fig. 6A). Our results suggest that T2635, which is stably helical in solution (21), does not dock onto the center of HR1 but at either side, and then snaps into the HR1 grooves unidirectionally (Fig. 6B). T2635 binding may therefore depend more on residues near the ends of its binding site. What are the atomic interactions that underlie the interactions of residues 33 and 66 with T2635? The HR1-T2635 model shown in Fig. 6 reveals that L33 engages in hydrophobic interactions with L35 of T2635 (corresponding to L149 of HR2). The L33S substitution will eliminate these interactions and probably decrease docking efficiency of T2635 at this end. Q66 can form a hydrogen bond with the nitrogen of the pyrrole ring of W3 of T2635 (corresponding to W117 in HR2) (Fig. 3) (27). W3 is the most N-terminal residue of T2635 that interacts with HR1 and packs in the hydrophobic groove formed by residues W60, I62, and L65 (30, 36, 39). Between all these hydrophobic interactions, the polar interaction with Q66 may properly orient W3. The positively charged R side chain of Q66R will likely engage in a salt bridge with E4 on T2635 (Fig. 3). As mentioned

before, establishing such a salt bridge would require a slight counterclockwise rotation of the T2635 helix, which could interfere with the proper packing of W3 into the HR1 groove. We have previously shown that adverse salt bridges can interfere with peptide packing onto HR1 and result in resistance to T20 and T1249 (24). This theory is supported by the observation that a variant of T20, containing helix-stabilizing salt bridges similar to those of T2635, is no longer sensitive to resistance mutations at positions 38, 38, or 43 (50, 51). Furthermore, resistance to the stabilized SC29EK peptide occurs at position 37 (I37K), which is located at the end of its binding site (47).

As we found many mutations in regions outside the HR1 binding site, T2635 resistance mechanisms other than direct effects on peptide binding should account for the resistance observed in most cultures. Several mutations were repeatedly selected in the central part of the HR2 domain (N126K, H132Q, E136G, E137G/V). Substitution N126K has been reported several times before (6, 62, 64, 72). It has been implicated in the appearance of a T20-dependent virus (7) and is thought to speed up six-helix bundle formation, thereby limiting the window of opportunity for fusion inhibitors to act. Several studies have described an improved thermal stability and/or improved free energy of the six-helix bundle with substitutions at position 121, 126, 137, or 138 (7, 20, 31, 68). Furthermore, the removal of the glycan at position 126 enhances the thermal stability of the HR1-HR2 complex (67). Ray et al. showed delayed fusion for resistance mutations N43D and Q66R in HR1, while the addition of mutations in HR2 can restore fusion kinetics (55). Similarly, residue E154K is thought to improve fusogenicity (61). Combined, these studies imply that substitutions in HR2 can favor the formation of a stable postfusion six-helix bundle, thereby accelerating fusion kinetics and decreasing the time frame for T2635 interference. We suggest a similar mechanism for the Q66R/N113D double mutant. An electrostatic interaction between N113D and Q66R may favor HR1 and HR2 association while disfavoring the binding of T2635 by an adverse salt bridge, resulting in a preferred binding of HR2 and accelerated fusion kinetics.

The kinetics of the six-helix bundle formation may also be affected at an earlier step. Premature interactions between HR1 and HR2 are prevented by the presence of gp120 domains, and before HR2 can fold onto HR1, the gp120 domains have to relax their grip, or even completely dissociate from gp41 (10). This is accomplished only after engagement of the receptor and coreceptor, allowing the next steps in the fusion process (37). Substitutions that weaken the gp120-gp41 interactions may accelerate six-helix bundle formation and cause T2635 resistance. Interestingly, several of the changes we observed (K77N/E, Q79E, K90E, T94N, N100D) are located in the gp41 loop domain that is known to interact with gp120 (11, 16). Possibly, these changes weaken the gp120-gp41 interactions, thus allowing faster six-helix bundle formation and leaving less time for T2635 to act. Premature six-helix bundle formation, i.e., in the absence of a target cell, will result in Env inactivation that may account for the loss of Env function.

This leaves us with a few less frequently observed non-HR1 substitutions. The A6V substitution, which as a single mutant provides the strongest T2635 resistance (7-fold), is located in

the FP and may therefore alter the gp41 interactions with lipids. It may affect the speed and/or efficiency of the FP insertion in the target membrane which occurs prior to six-helix bundle formation (1) or the transition from membrane hemifusion to full membrane merger. Alternatively, the FP substitution may affect the interaction with gp120. Interestingly, substitutions at positions 5, 7, and 8 in the FP were shown to confer resistance to the CCR5 inhibitor vicriviroc (3, 9), although the mechanism remains unclear. These findings reveal that the hydrophobic FP contributes to the entry process in ways we do not understand yet. Furthermore, we repeatedly observed the L210F change in the cytoplasmic tail of gp41. Since it does not cause resistance (Table 4), we guess that it may be a compensatory change. L210F was present in a virus isolate that showed enhanced Env incorporation into virions (8). Possibly, the loss of Env function is compensated by incorporation of more Env molecules onto virions.

In summary, we present an escape from the third-generation peptide fusion inhibitor T2635 that requires multiple mutations in various gp41 subdomains and that comes at the expense of Env function. The mechanism of resistance is very distinct from escape routes observed for the first- and second-generation fusion inhibitors T20 and T1249. The emerging characteristics of third-generation fusion inhibitors like T2635, including enhanced stability, enhanced potency, and escape that requires multiple mutations and comes with a loss of function, warrant further studies on this class of inhibitors.

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