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Influence of Codon Pair Usage in the Evolvability of HIV-1

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BACKGROUND

HIV-1 populations, like other RNA viruses, are described as a closely related mutant spectra or mutant clouds termed viral quasispecies. Mutant cloud composition can impact virus evolvability, fitness and virulence (1). The extraordinarily large number of possible encodings in natural genes is to some extent restricted by two encoding biases referred to as codon bias and codon pair bias. An unexplored aspect of the genetic architecture of HIV-1 is how codon choice influences population diversity and evolvability.

OBJECTIVE

In this study we compare the development of HIV-1 resistance to protease inhibitors (PIs) of WT and a synthetic virus (MAX) carrying a codon-pair re-engineered protease sequence with 38 (13%) synonymous mutations. WT and MAX viruses replicated indistinguishably in MT-4 cells or PBMCs (2). We aimed to know whether synonymous sequence space influences the development of PIs resistance and consequently to know whether HIV-1 protease natural position in sequence space facilitates its evolvability.

METHODS

Protease re-design and Virus Production. The re-coded protease gene segment (MAX) was synthesized *de novo* and recombined co-transfecting by electroporation in MT-4 cells with a protease-deleted HXB2D infectious clone previously linearized with BstEII. The obtained viruses were named Pwt_P1 (for virus containing the wild-type protease) and Pmax_P1 (for virus containing the re-optimized protease) (2).

Serial Culture Passages. WT virus and the re-optimized variant MAX were serially passaged in MT-4 cells every 3-4 days. After 15 passages, supernatants were collected to obtain the protease of WT virus at passage 15, namely Pwt_P15, and the protease of the re-optimized virus at passage 15, namely Pmax_P15.

cDNA Endpoint Dilution. cDNA obtained from the viral RNA was serially 10-fold diluted. From the last protease positive dilution 2-fold serial dilutions were performed and assayed by PCR. Positive clones were purified and sequenced.

Protease Inhibitor Resistants. WT and MAX viruses were subjected to the selective pressure of PIs [atazanavir (ATV) and darunavir (DRV)] by serial culture passages in MT-4 cells. In each case, two independent replicates were maintained.

Drug Susceptibility Assays. Susceptibility to ATV and DRV was determined in MT-4 cells using a multiplicity of infection of 0,003 and viable cells at different drug concentrations were quantified by a tetrazolium-based colorimetric method (3). After passages in presence or absence of Pis, virus RNA was RT-PCR amplified, cloned and sequenced; frequencies of resistant mutations were compared by a Chi-square test.

RESULTS

Clones obtained by cDNA endpoint dilution. Mutant spectra of WT and MAX proteases after 1 and 15 passages in MT-4 cells were first defined . Sequence analysis of the clones obtained by viral RNA endpoint dilution demonstrated that the protease quasispecies diversity (p-distance) was higher in the case of the re-optimized protease Pmax_P1 (p=0.0027, unpaired t-test). In contrast, Pmax_P15 displayed a lower quasispecies diversity than Pwt_P15 (p<0.0001, unpaired t-test) (Table1), suggesting that synonymous Pmax recoding affects protease diversification. Interestingly, these diferences between WT and MAX virus were not observed when non-protease sequences were compared (data not shown).

Table1. Protease quasispecies diversity calculated after analyze 103 clones in the case of viruses after one passage in MT-4 cells (P1) and 109 clones in the case of viruses after fifteen passages in MT-4 cells (P15).

	p-distance nt	±SE	p-value	Sn_nt
Pwt_P1	0.001245	0.000040		0.16
Pwt_P15	0.002925	0.000057		0.29
Pmax_P1	0.001396	0.000030	0.0027	0.19
Pmax_P15	0.002111	0.000033	p<0.0001	0.33

Sn: Shannon's entropy

Evolvability of the codon pair re-coded protease. Sequence analysis of the clones obtained after thirty two serial passages in MT-4 cells and without PIs in the medium confirmed a higher diversity in the Pmax_P1 than in the Pwt_P1 (p<0.0001, unpaired t-test) at a nucleotide level. These data are according with the results obtained with the cDNA endpoint dilution assay. At an amino acid level, the diversity was also significantly higher in Pmax_P1 clones (p<0.0001). Similarly, a reduction in quasispecies diversity was observed in the Pmax_P15 clones versus Pmax_P1, suggesting again differences between WT and MAX proteases after serial passages in MT-4 cells (Table 2).

Table2. Protease quasispecies diversity after thirty two passages in MT-4 cells in the absence of drug.

	Sn_nt	Sn_aa	Hn(%)	Ha(%)	p-distance (nt)	±SE	p-distance (aa)	±SE
Pwt_P1	0,332	0,279	30,43	26,09	0,002635	0,000223	0,007027	0,000661
Pwt_P15	0,538	0,437	47,83	39,13	0,003780	0,000224	0,007905	0,000559
Pmax_P1	0,566	0,566	45,83	45,83	0,005599	0,000341	0,014420	0,000837
Pmax_P15	0,436	0,382	36,36	31,82	0,003032	0,000218	0,005478	0,000401

Sn: Shannon's entropy
H: Heterogeneity (%)

When WT viruses (Pwt_P1 and Pwt_P15) and the MAX viruses (Pmax_P1 and Pmax_P15) were subjected to the selective pressure of ATV and DRV during thirty two passages, all of them developed phenotypic resistance to PIs (Table 3). Pmax_P1 showed higher IC_{50} values for DRV than Pwt_P1 (p=0,0017, unpaired t-test) which indicate that re-coded protease maintains its evolutionary capacity.

Table3. Susceptibility of the WT viruses (Pwt_P1 and Pwt_P15) and the MAX viruses (Pmax_P1 and Pmax_P15) to ATV and DRV after 32 passages . The IC_{50} values shown are means ± standard deviations of at least three tests of each replicate. Fold increase in IC_{50} relative to the wild type virus is shown in parentheses.

		Pwt_P1	Pwt_P15	Pmax_P1	Pmax_P15
IC_{50}(nM)	ATV	14,63±5,39 (4,76)	17,78±6,92 (5,79)	21,26±8,67 (6,93)	26,62±11,75 (8,67)
	DRV	5,69±1,01 (6,62)	5,80±0,64 (6,75)	9,35±1,85 (10,89)	5,56±0,75 (6,48)

Resistance mutations. Sequence clonal analysis showed the presence, in both viruses (WT and MAX), of previously described resistance mutations to ATV and DRV. However, a different resistance variant repertoire appeared in the MAX virus proteases when compared to WT (Table 4 and 5). Specifically, the G16E substitution was only observed in the WT protease (the WT protease only needed a transition to develop this substitution whereas the MAX protease needed two substitution: a transition and a transversion). In addition, the L10F, L33F, K45I, G48L and L89I substitutions were only detected in the re-coded MAX protease population. Interestingly, some resistance mutations (i.e. K45I, G48L and L89I) selected by the MAX protease are extremely rare non-polymorphic PI-selected mutations.

Table 4. Frequency (%) at which resistance mutations appear after serial passages of the WT virus and re-coded MAX protease virus at passage 1 (A) and at passage 15 (B) in the presence of ATV. Chi-square analysis was performed to asses the differences in the sequences repertoire.

A

	P1wt_P1	P1max_P1	p-value*
L10F	-	14,30	0.0132
V32A	52,18	21,43	0.0424
V32I	4,35	23,81	0.0206
G48L	-	21,43	0.0028
A71V	91,30	40,48	0.0218
L89I	-	14,30	0.0132

*Chi-square test

B

	P1wt_P15	P1max_P15	p-value*
V32A	-	36,36	0.0002
G48V	18,18	-	0.0066
I50L	-	22,73	0.0026
I84V	15,90	-	0.0107

*Chi-square test

Table 5. Frequency (%) at which resistance mutations appear after serial passages of the WT virus and re-coded MAX protease virus at passage 1 (A) and at passage 15 (B) in the presence of DRV. Chi-square analysis was performed to asses the differences in the sequences repertoire.

A

	P1wt_P1	P1max_P1	p-value*
L10F	-	10,87	0.0276
G16E	38,30	-	<0.0001
L33F	-	91,30	<0.0001
K45I	-	10,87	0.0276
M46I	46,81	15,22	0.0163
A71V	-	26,09	0.0009
V82I	59,57	10,87	0.0006

*Chi-square test

B

	P1wt_P15	P1max_P15	p-value*
L33F	-	41,86	0.0002
M46I	26,09	-	0.0066
A71V	-	81,39	0.0026
V82I	36,96	-	0.0107

*Chi-square test

CONCLUSIONS

The differences in the mutation pattern that emerged after PIs treatment suggested that WT and MAX virus proteases occupy different sequence spaces although both virus proteases were able to develop PIs resistance. Further studies will required to elucidate whether HIV-1 sequences have evolved to optimize not only the protein coding sequence but also the DNA/RNA sequences.

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