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Short communication

Amino acid insertions at position 35 of HIV-1 protease interfere with virus replication without modifying antiviral drug susceptibility

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Abstract

Among 1330 patients undergoing highly active antiretroviral therapy (HAART), 3 showed 1 or 2 amino acid (aa) insertions at position 35 of the HIV-1 protease gene. Protease genes containing aa insertions, either in the presence (ins35G+res.muts, ins35TN+res.muts) or absence (ins35G, ins35TN) of other resistance mutations, were introduced into the wild-type HIV-1 strain NL4-3. The introduction of ins35G and ins35TN in the wild-type protease confirmed that these mutations were per se not responsible of decreased drug susceptibility. The replication rate of mutant recombinant viruses was determined by HIV RNA quantification in supernatants of cell cultures in comparison with a recombinant HIV-1 strain with wild-type protease. Recombinant ins35G and ins35TN HIV-1 strains did not display increased resistance to currently used protease inhibitors (PIs). Comparison of ins35TN+res.muts and ins35G+res.muts with respect to the corresponding recombinant rescue mutants showed that ins35TN decreased the replication rate of the PI-resistant strain, while ins35G had a protective effect.

Keywords: HIV-1; Protease; Insertion; Drug resistance; Viral replication

HIV-1 is characterized by a high degree of genetic diversity (Domingo and Holland, 1997). The genetic variability is caused by the high mutation rate of HIV-1 genome associated with the low fidelity of HIV-1 reverse transcriptase, the lack of proofreading function and the rapid turnover of viral population, which induce the emergence of multiple HIV-1 variants in vivo. It is generally assumed that the wild-type HIV-1 population expresses the maximal viral replicative capacity. Antiretroviral drug pressure selects for viral variants with mutations causing "misrecognition" and processing of enzyme-interfering drugs. Thus, it is well established that such mutations favor virus replication in a drug-containing environment by providing resistance to the inhibitory drug effect. However, it is assumed that drug-resistance-associated mutations may reduce the enzyme activity and eventually impair viral replication (Nijhuis et al., 1999). The effect of specific mutations (primary mutations) in reducing drug susceptibility is largely known, but the impact of secondary mutations or amino acid (aa) polymorfisms in modifying the final level of drug resistance or viral replicative capacity is largely obscure. Aa insertions in the HIV-1 protease gene (Kim et al., 2001; Sturmer et al., 2003) are rarely detected in protease inhibitor (PI)-naive and -treated patients and little is known about their impact on the enzyme activity or viral biology. To date, aa insertions in the protease gene have been reported not to modify the susceptibility to PIs, while it is unclear how they affect viral fitness.

In this study, the effect of aa insertions in HIV-1 protease on antiviral drug susceptibility and viral replication was investigated by generating a panel of HIV-1 recombinant strains.

Patients submitted to highly active antiretroviral therapy (HAART) were routinely monitored by quantification of HIV-1 RNA levels in plasma (Versant HIV-1 RNA 3.0 Assay, Bayer, NY, USA), $CD4^+$ T cell counts (Flow Cytometry, Coulter Epics XL MCL, Fullerton, CA, USA) and determination of drug-resistance-associated mutations in HIV-1 reverse transcriptase and protease genes by direct sequencing of viral genes in plasma samples (Paolucci et al., 2001).

Recombinant viruses were constructed from patient-derived HIV-1 sequences by introducing protease genes containing aa insertions, either in the presence or absence of other resistance mutations, into the vector pNLΔpro carrying the wild-type HIV-1 strain NL4-3 genetic backbone (Menzo et al., 2000). In particular, ins35G+res.muts, ins35TN and ins35TN+res.muts recombinants from HIV-1 strains carrying one (ins35G) or two

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(ins35TN) aa insertions at position 35 of the protease gene were constructed. In addition, aa insertions were suppressed by site-directed mutagenesis in protease genes carrying other mutations (Higuchi, 1990), and the relevant rescue recombinant strains (Δins35G+res.muts and Δins35TN+res.muts) were generated. Finally, two recombinant viruses were constructed by introducing by site-directed mutagenesis ins35G and ins35TN into pNLΔpro (ins35G and ins35TN).

Upon transfection of $CD4^+$ HeLa cells with recombinant pNLΔpro constructs, infectious recombinant viruses were obtained (Paolucci et al., 2004). In detail, 0.5 μg of each plasmid construct DNA were transfected into $CD4^+$ HeLa cells by using Lipofectin according to the Manufacturer's recommendations (Invitrogen, Groningen, The Netherlands). After 3-day incubation at 37 °C, cell supernatants containing reconstituted viable recombinant viruses were collected. The newly produced recombinant strains were quantified by determination of the HIV RNA copy number in cell culture supernatants by a commercially available assay (Versant HIV-1 RNA 3.0 Assay). Since the assay has an upper limit of 500,000 HIV-1 RNA copies/ml, cell culture supernatants scored as containing >500,000 copies/ml of viral RNA were serially diluted before quantification. Results were reported as the HIV-1 RNA copy number scored by the assay multiplied by the dilution factor.

Susceptibilities of ins35G, ins35TN and wild-type HIV-1 recombinant strains to representative protease inhibitors (PI), i.e. saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir, were tested as reported (Paolucci et al., 2003). Briefly, 0.5 μg of each plasmid construct were transfected into 30% confluent HeLa $CD4^+$ cells and the evaluation of drug susceptibility was coincident with the virus reconstitution. In fact, after a 6-h incubation at 37 °C following transfection, cell culture supernatant was removed and replaced with fourfold dilution of antiretroviral drugs. No-drug controls for each drug dilution were included in each assay. After 72 h incubation (the time required to perform a single replication cycle in the newly infected HeLa $CD4^+$ cells), HIV-1 p24 antigen was quantified in the cell culture supernatant. The degree of inhibition of viral replication was measured by determining the HIV-1 p24 antigen level (NEN Research Product, Boston, MA, USA) in the supernatant of cell cultures, and was expressed as fold-increase in IC_{50} values of mutagenized recombinant HIV-1 variants with respect to IC_{50} values of wild-type recombinant virus. Each test was performed in triplicate.

The replication rates of insertion-containing recombinant viruses, the corresponding recombinant viruses lacking the aa insertions and the wild-type recombinant HIV-1 strain were determined in parallel in cell cultures as reported (Paolucci et al., 2004). In detail, a volume of each transfected HeLa $CD4^+$ cell culture supernatant containing 8.5×10^9 RNA copies of each recombinant HIV strain was used to infect aliquots of 5×10^6 phytohemagglutinin-stimulated (PHA) peripheral blood mononuclear cells (PBMC) obtained from HIV-seronegative blood donors. After a 4-h incubation, supernatants were removed, and infected PBMC were incubated at 37 °C in 10 ml RPMI 1640 medium (Eurobio, Les Ulis Cedex B, France) supplemented with 20% fetal calf serum (Life Technologies Ltd., Paisley, Scotland, UK), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% interleukin-2 (ZeptoMetrix Co., Buffalo, NY, USA) and 5 μg/ml hydrocortisone (Sigma Chemical Co., St Louis, MI, USA). Then, at day 7 post-infection, the culture supernatant (10 ml) was used to infect fresh PBMC as reported above (first passage). A second passage was performed 7 days later. The kinetics of viral replication was measured by determining the HIV-1 RNA copy number in cell culture supernatants. In detail, HIV-1 RNA was determined in supernatants collected 3, 7, 10, 14 days post-infection and after the first and second passage.

The HIV-1 protease genes from 1330 HIV-1 infected patients undergoing HAART were sequenced as a part of routine virologic monitoring. On the whole, three patients showed the presence of one (G) or two (TN) aa insertions at position 35 of protease gene. Of note, the G and one of TN insertions were associated with other drug resistance mutations (ins35G+res.muts and ins35TN+res.muts), while the other TN insertion was detected in the protease gene in the absence of drug-resistance-associated mutations (ins35TN).

In detail, patient #1 (ins35TN) had been previously treated with different drug combinations including stavudine, abacavir, and didanosine. At the time of drug-resistance testing, the patient was receiving stavudine and tenofovir. HIV-1 RNA level and $CD4^+$ T cell count were 2762 copies/ml plasma and 143 cells/μl, respectively. The patient had never been exposed to PIs. Patient #2 (ins35TN+res.muts), was treated in the past with zidovudine, stavudine, indinavir, nelfinavir, and amprenavir, while at the time of drug-resistance testing the HAART regimen existed of abacavir, lamivudine, and lopinavir. HIV-1 RNA level and $CD4^+$ T cell count were 21,578 copies/ml plasma and 193 cells/μl, respectively. The pattern of HIV-1 reverse transcriptase mutations was: M41L, E44D, A98S, M184V, L210W, and T215Y, while the protease mutations were: L10V, L24I, L33F, ins35TN, M36T, M46L, I54V, L63P, V82A, and I84V. Finally, patient #3 (ins35G+res.muts) had stavudine, lamivudine, efavirenz, and indinavir treatment, while at the time of drug-resistance testing the patient was receiving didanosine, lamivudine, stavudine, and lopinavir. HIV-1 RNA level and $CD4^+$ T cell count were 101,453 copies/ml plasma and 11 cells/μl, respectively. The pattern of HIV-1 mutations in antiretroviral drug targets was as follows: for reverse transcriptase, M41L, D67N, T69D, K70R, L74I, K103N, V108I, V179I, Y181C, M184V, T215F, and K219Q; and for protease, K20I, ins 35G, M36I, I54V, L63P, A71T, V82A, and L90M.

IC_{50} values for PIs of recombinant HIV-1 strains carrying only ins35G and ins35TN were comparable to those observed in the wild-type recombinant virus (Table 1). Comparison of the replicative capacity of recombinant viruses was performed by infecting cell cultures with comparable amounts of virus (Fig. 1A and D). Following two passages, HIV-1 RNA levels of wild-type, ins35G and ins35G+res.muts recombinant strains were comparable, while HIV-1 RNA level of Δins35G+res.muts progressively decreased (Fig. 1B–C). Kinetics of HIV-1 RNA level of wild-type and ins35TN recombinant strains were overlapping at each time point (Fig. 1E–F). Although showing a similar kinetics, Δins35TN+res.muts consistently yielded lower

Table 1
Evaluation of the susceptibility of HIV-1 recombinant strains to protease inhibitors

HIV-1 recombinant strain	IC_{50}[a] (μM fold increase)					
	RTV	SQV	IDV	APV	NFV	LPV
pNL4-3 (wild-type)	0.024 ± 0.004	0.04 ± 0.011	0.03 ± 0.005	0.03 ± 0.007	0.01 ± 0.002	0.0025 ± 0.0001
ins35G	0.08 ± 0.05 (3.3)	0.01 ± 0.03 (0.25)	0.01 ± 0.02 (0.3)	0.025 ± 0.005 (0.8)	0.01 ± 0.01 (1.0)	0.005 ± 0.002 (2.0)
ins35TN	0.025 ± 0.001 (1.0)	0.02 ± 0.02 (0.5)	0.03 ± 0.01 (1.0)	0.04 ± 0.01 (1.3)	0.02 ± 0.01 (2.0)	0.004 ± 0.001 (1.6)

RTV: ritonavir; SQV: saquinavir; IDV: indinavir; APV: Amprenavir; NFV: nelfinavir;. LPV: lopinavir.
[a] IC_{50} are mean of results of three independent assays.

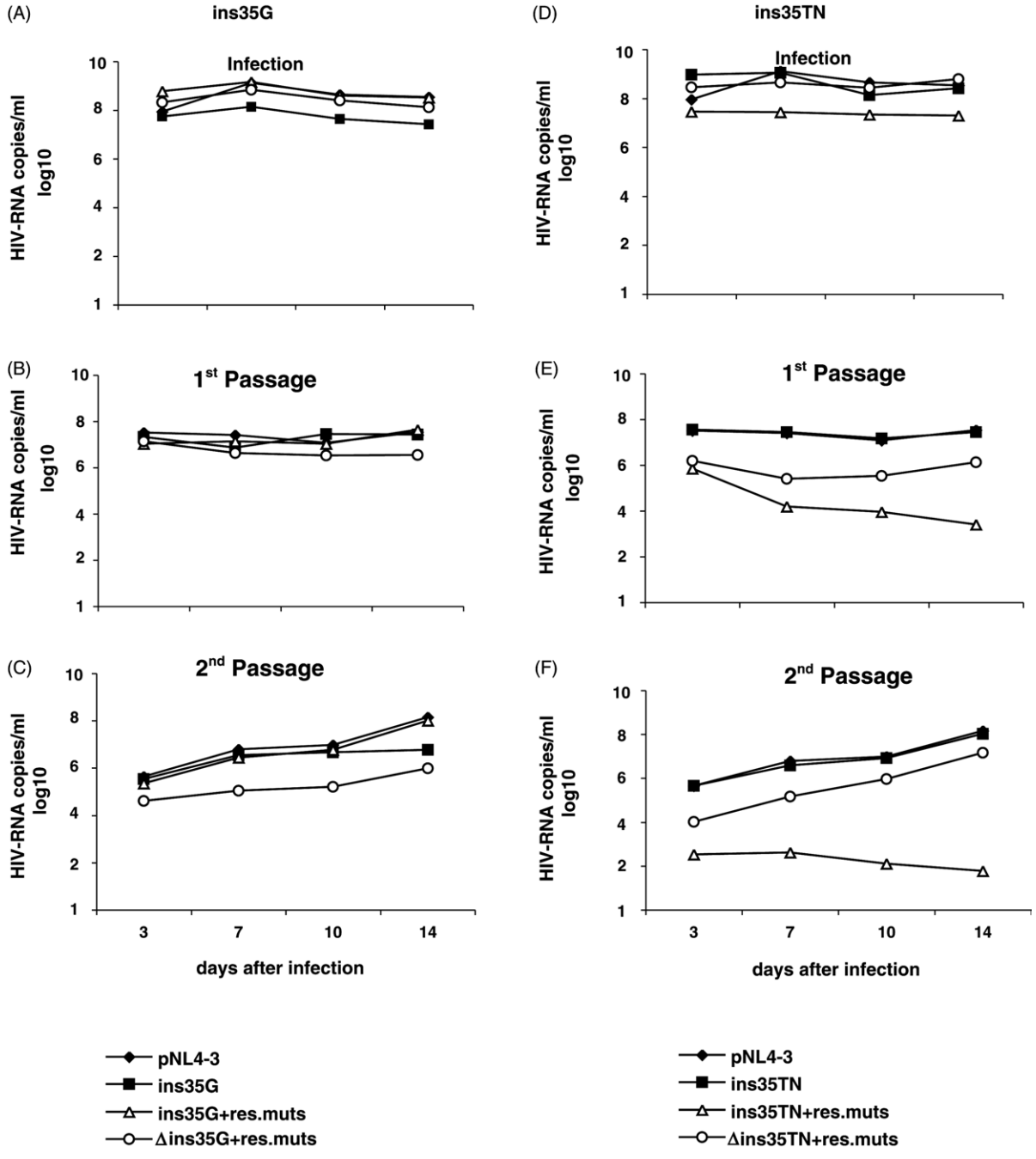

Fig. 1. Effect of single (A–C) or double (D–E) amino acid insertions at position 35 of HIV-1 protease on viral replication.

HIV RNA levels with respect to wild-type recombinant strain (Fig. 1E–F). However, ins35TN+res.muts showed a further important decrease in HIV-1 RNA levels further decreasing over time. All experiments were repeated three times and results showed a variation within 15%. Thus, G and TN insertions in HIV-1 protease showed a differential impact on the replication rate of drug-resistant strains, the former resulting in an enhancement, and the latter in an inhibition of virus replication.

Following emergence of mutations conferring resistance to antiretroviral drugs, different molecular pathways associated with either increase or decrease of HIV-1 replicative capacity were described (Simon et al., 2003). Despite the low prevalence of insertions in the protease gene of HIV-1, results presented in this report confirm that insertions in viral protease may have been selected either before or during HAART (Kim et al., 2001; Sturmer et al., 2003). However, these mutations do not appear to modify susceptibility to PIs. On the other hand, an effect on HIV-1 replication rate was observed. In fact, while replication of strains carrying ins35G and ins35TN was not affected in the absence of drug-resistance-associated mutations, the presence of these mutations in the context of multiple drug-resistance-associated mutations displayed a different effect on viral fitness. In particular, data showed that the presence of ins35TN in association with mutations conferring PI resistance may be greatly detrimental for virus replication. In fact, the striking impairment of viral replication observed in ins35TN+res.muts recombinant strain could be rescued (at least partially) by suppression of ins35TN alone. In this particular mutant (Δins35TN+res.muts), it appears reasonable to attribute the incomplete recovery of the replication capacity to the combined effect of the remaining mutations. On the other hand, the suppression of ins35G in the context of a different pattern of protease mutations led to a reduced replication capacity of the recombinant strain (Δins35G+res.muts), suggesting a protective role of the insertion. Of note, different patterns of PI resistance-associated mutations appeared to induce a similar (and limited) replication impairment in the two recombinant strains lacking insertions at position 35. In contrast, the presence of these insertions in those genetic backgrounds was able to modify the relative replicative capacity by $\geq 4 \log_{10}$. Thus, although aa insertions in HIV-1 protease did not affect drug susceptibility, their impact on viral replication was unpredictable and appeared to depend on both the inserted aa and, possibly, the particular pattern of drug-resistance-associated mutations. Taking into account their presence in PI-naive patients, one could speculate that these mutations may arise to compensate potential mutations in the gag cleavage site, finally resulting in an increase or decrease of the overall protease activity.

Although referring to a very limited number of strains recovered from HAART-treated patients, this report may not be clinically irrelevant. A previous report indicated the possibility of transmission of an insert-containing virus (Grant et al., 2001), thus suggesting that these strains could be encountered in the future at a greater frequency. In this respect, attempts to select for ins35TN-containing virus could be adopted in multi PI-failing patients. Such a strategy has already been utilized in patients with failure to multiple reverse transcriptase inhibitors showing the presence of drug-resistant strains with mutations (M184V/I, K65R) impairing viral replication (Turner et al., 2004; Sharma et al., 2004).

A general conclusion of this study is that assessment of viral fitness should not be inferred from sequencing data but based upon biological assays aimed at obtaining direct evaluation of the viral replication rate.

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