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Characterization and Structural Analysis of HIV-1 Integrase Conservation

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Abstract

The HIV-1 integrase, responsible for the chromosomal integration of the newly synthesized double-stranded viral DNA into the host genomic DNA, represents a new and important target of potential clinical relevance. For instance, two integrase inhibitors, raltegravir and elvitegravir, have been shown to be promising in clinical trials, and the first has been recently made available for clinical practice. As is the case for other antiviral drugs, drug resistance to integrase inhibitors occurs both in vitro ***and/or*** in vivo ***through the selection of mutations within the HIV genome. Indeed, many integrase mutations have already been associated with resistance to all the different integrase inhibitors tested in*** in vitro ***and/or*** in vivo ***studies. Among them, about 40 substitutions have been specifically associated with the development of resistance to raltegravir and/or elvitegravir; some of them were also found*** in vivo ***in patients failing such integrase inhibitors.***

The relevance of integrase mutations in clinical practice has yet to be defined, in light of the lack of long-term follow-up of treated patients and the limited data about the prevalence of integrase inhibitor-associated mutations in integrase inhibitor-naive patients (either untreated, or treated with antiretrovirals not containing integrase inhibitors).

Therefore, by structural analysis elaboration and literature discussion, the aim of this review is to characterize the conserved residues and regions of HIV-1 integrase and the prevalence of mutations associated with integrase inhibitor resistance, by matching data originated from a well-defined cohort of HIV-1 B subtype-infected individuals (untreated and antiretroviral-treated) and data originated from the public Los Alamos Database available in the literature (all patients integrase inhibitor-naive by definition).

In integrase inhibitor-naive patients, 180 out of 288 HIV-1 integrase residues (62.5%) are conserved (< 1% variability). Residues involved in protein stability, multimerization, DNA binding, catalytic activity, and in the binding with the human cellular cofactor LEDGF/p75 are fully conserved. Some of these residues clustered into large defined regions of consecutive invariant amino acids, suggesting that consecutive residues in specific structural domains are required for the correct performance of HIV-1 integrase functions.

All primary signature mutations emerging in patients failing raltegravir (Y143R, Q148H/K/R, N155H) or elvitegravir (T66I, E92Q, S147G, Q148H/K/R, N155H), as well as secondary mutations (H51Y, T66A/K, E138K, G140S/A/C, Y143C/H, K160N, R166S, E170A, S230R, D232N, R263K) were completely absent or highly infrequent (< 0.5%) in integrase inhibitor-naive patients, either infected with HIV-1 B subtype (drug-naive or antiretroviral-treated), or non-B subtypes/group N and O.

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Differently, other mutations (L74M, T97A, S119G/R, V151I, K156N, E157Q, G163K/R, V165I, I203M, T206S, S230N) occurred as natural polymorphisms with a different prevalence according to different HIV-1 subtype/circulating recombinant form/group.

In conclusion, the HIV-1 integrase* in vivo *is an enzyme requiring the full preservation of almost two-thirds of its amino acids in the absence of specific integrase inhibitor pressure. Primary mutations associated with resistance to integrase inhibitors clinically relevant today are absent or highly infrequent in integrase inhibitor-naive patients. The characterization of the highly conserved residues (involved in protein stability, multimerization, DNA binding, catalytic activity, LEDGF binding, and some with still poorly understood function) could help in the rational design of new HIV-1 inhibitors with alternative mechanisms of action and more favorable resistance profiles. **(AIDS Rev. 2009;11:17-29)**

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Key words

Integrase. Genotype. Conservation. Antiretroviral therapy. HIV drug resistance. Mutations.

Introduction

In the last ten years, important progress has been made in the development and clinical use of drugs for treating HIV-1 infection. To date, nearly 25 antiretroviral drugs belonging to six drug classes have been licensed for the treatment of HIV-1. Most of them target the viral enzymes reverse transcriptase and protease, others the gp41, CCR5/gp120, and very recently the integrase. The combined use of all these drugs and the increased clinical experience has substantially improved the clinical management of HIV-1 infection in terms of delaying disease progression, prolonging survival, and improving quality of life[1]. Nevertheless, antiretroviral therapy can still fail to be fully suppressive and new viral variants emerge, thus allowing HIV-1 to become resistant to one or more drugs by accumulating mutations, either alone or in multiple and complex patterns[2-12]. Understanding the mechanisms underlying resistance development to both existing and novel drugs is thus essential for a better clinical management of resistant viruses, and to prevent further resistance development and spread.

The HIV-1 integrase represents a new and important target of potential clinical relevance[13-15]. For instance, two integrase inhibitors, raltegravir and elvitegravir, have been shown to be promising in clinical trials, and the former has been recently made available for clinical practice[16-20].

The HIV-1 integrase enzyme is responsible for the chromosomal integration of the newly synthesized double-stranded viral DNA into the host genomic DNA[21,22], enabling HIV-1 to establish a permanent genetic reservoir that can both initiate new virus production and replicate through cellular mitosis. HIV-1 integrase is a 32 kDa protein of 288 amino acids, comprising three functional domains: the N-terminal domain (amino acids 1-49), the catalytic core domain (amino acids 50-212), and the C-terminal domain (amino acids 213-288)[23]. The N-terminal domain contains a highly conserved zinc-binding $H_{12}H_{16}C_{40}C_{43}$ motif[22,24] involved in the stabilization folding and proper multimerization of the integrase subunits[25-27]. The catalytic core domain, which plays a critical role in integrase enzymatic activity, contains the catalytic $D_{64}D_{116}E_{152}$ motif that is conserved in all retroviral integrase, as well as in retro-transposons from plants, animals and fungi and in some bacterial transposases[22,24,28,29]. It also contains other functional domains and residues such as the nuclear localization signal, a critical sequence mediating the nuclear import of the integrase in the context of the preintegration complex[30]; the $K_{186}R_{187}K_{188}$ multimerization motif at the dimer:dimer interface[31,32]; and several important residues (H12, L102, A128, A129, C130, W131, W132, I161, R166, Q168, E170, H171, T174, M178, Q214L) involved in the chemical bond and hydrophobic contacts with the human lens epithelium-derived growth factor (LEDGF/p75), which is an essential cellular cofactor for HIV integration, linking the integrase to chromatin[33-37].

The C-terminal domain has strong but nonspecific DNA-binding activity and is involved in the binding with viral and cellular DNA with the minimal nonspecific DNA binding region (MDBD 220-270 aa)[38-41]. This domain, required for the integration reaction, is involved also in protein oligomerization and interactions with the reverse transcriptase[39].

Following reverse transcription, a multimer form of the integrase enzyme catalyzes two reactions: the first is a cleavage of two conserved nucleotides from the 3' ends of both long terminal repeat (LTR) strands of the viral cDNA (3' processing)[42]. This reaction takes place in the cytoplasm within a nucleoprotein complex, referred to as the pre-integration complex[43]. The pre-integration complex is transported through the nuclear pore to the nucleus where the second step (strand transfer) occurs. This consists of the insertion and covalent ligation of the viral cDNA into the host genome[42]. Gap filling of the interfaces between the viral and host genomic DNA is then completed using the host DNA repair machinery via a mechanism that is not yet fully understood[44,45].

Since there is no human homolog of this enzyme, the HIV integrase represents a rational and important target for treating HIV infection and preventing AIDS. All integration steps can potentially be inhibited and each step can be considered a possible drug target. Multiple integrase inhibitors have been in different phases of development and can be divided into five classes: (i) DNA-binding inhibitors, (ii) 3' processing inhibitors, (iii) nuclear translocation/import inhibitors, (iv) strand transfer inhibitors, and (v) gap repair inhibitors[13-15].

To date, the strand transfer inhibitors have been the most successful class of integrase inhibitors, and the development of two clinically relevant inhibitors (elvitegravir and raltegravir) is a remarkable therapeutic success story[16-20].

As is the case for other antiviral drugs, drug resistance to integrase inhibitors occurs both *in vitro* and/or *in vivo* through the selection of mutations within the HIV genome. So far, more than 60 integrase mutations have already been associated with the resistance to all different integrase inhibitors tested in *in vitro* and/or *in vivo* studies[6,12,17,18,46-64,68]. However, in several cases there is absolutely no phenotypic evidence that these changes contribute to resistance, and in fact it is virtually certain that many of the changes cited are simply polymorphisms that are co-selected with true resistance mutations. Most integrase inhibitor resistance mutations are in the vicinity of the putative integrase inhibitor binding pocket. Some mutations were associated with a specific class of integrase inhibitors, others to various inhibitors within the same strand transfer inhibitor class, others with specific inhibitors within the same strand transfer inhibitor class, with a widely different magnitude of resistance[18,50-52,54,55,58,59,64]. More than 40 integrase substitutions have been associated with the development of resistance to raltegravir and/or elvitegravir; some of them were also found *in vivo* in patients failing such integrase inhibitors[6,17,18,49,53,57-63] (Stanford HIV Drug Resistance Database, http://hivdb.stanford.edu).

For instance, raltegravir failure was associated with integrase mutations in three distinct non-overlapping genetic pathways defined by two or more mutations including: (i) a primary signature mutation at either Q148H/K/R or N155H or Y143R; and (ii) one or more minor mutations unique to each pathway[6,17,49,56,57,60,63]. Secondary mutations described in the Q148H/K/R pathway include L74M, E138A/K, or G140A/S. Secondary mutations described in the N155H pathway include L74M, E92A/Q, T97A, Y143H/C, V151I, G163K/R or D232N[6,17,49,58,63]. The most common mutation pattern was Q148H + G140S, which in fact exhibited the greatest loss of raltegravir susceptibility (> 1,000-fold) and high replication capacity *in vitro*[56,58].

Similarly, in patients failing elvitegravir, the mutations T66I, E92Q, S147G, Q148R/H/K, and N155H have been identified as "signature" resistance mutations, while the mutations H51Y, T66A/K, L68I/V, S119R/G, E138K, G140S/C, E157Q, K160N, R166S, E170A, S230R, and D232N have been found as secondary mutations[18,59].

Generally, all secondary mutations (for both raltegravir and elvitegravir) had little if any effect on drug susceptibility *in vitro* in the absence of a primary "signature" mutation, thus suggesting rather a secondary role for viral fitness rescue and/or increasing resistance[18,49,51,52,55,57-59,64].

Although these first data are available regarding specific mutations and pathways that confer integrase inhibitor resistance, insufficient attention has been given so far as to how integrase inhibitor resistance and cross-resistance are affected by the natural sequence variation in the integrase gene.

The relevance of all integrase mutations in clinical practice has yet to be defined, in light of the lack of long-term follow-up of treated patients, the limited data about the prevalence of integrase inhibitor-associated mutations in integrase inhibitor-naive patients (either untreated, or treated with antiretrovirals not containing integrase inhibitors), and the scattered information about conservation and variability of HIV integrase in clean datasets.

Some studies have recently started to analyze, within the public Los Alamos Database, the prevalence of natural polymorphisms and mutations associated with integrase inhibitor resistance in the HIV-1 integrase either in clade B[50] or from different subtypes of group M, and in N and O viruses[65-67]. In addition, a single study added some information regarding the integrase

variability in drug-naive versus antiretroviral-treated patients with non integrase inhibitor drugs (i.e. reverse transcriptase and protease inhibitors)[68].

Beside its obvious clinical relevance, the identification and characterization of conserved regions/residues within the HIV-1 integrase is of fundamental importance that can help in the design of new therapeutic strategies aimed at driving the virus to mutate at key amino acids that are crucial for the maintenance of sufficient viral fitness.

Therefore, by structural analysis elaboration and literature discussion, the aim of this review is to characterize the conserved residues and regions of HIV-1 integrase and the prevalence of mutations associated with integrase inhibitor resistance, by matching data originated from a well-defined cohort of HIV-1 B subtype-infected individuals, untreated or antiretroviral-treated[68], and data originated from the public Los Alamos Database available in the literature[50,65-67] (all patients integrase inhibitor-naive by definition).

HIV-1 integrase conservation

The HIV-1 integrase conservation *in vivo*, in the absence of integrase inhibitor pressure, was assessed first by evaluating 448 HIV-1 B-subtype protein integrase sequences derived from 134 drug-naive infected individuals and 314 patients failing antiretroviral regimens, all integrase inhibitor-naive, who were enrolled in different clinical centers in Italy or in the Pitie-Salpetriere Hospital in Paris, France[68].

The analysis of sequences showed that in integrase inhibitor-naive patients, 187 out of 288 integrase residues (65%) were conserved (< 1% variability), and some of these residues clustered into six large defined regions of consecutive invariant amino acids (Fig. 1).

A detailed analysis of amino acid conservation shows that residues required for successful HIV-1 integration within the catalytic triad ($D_{64}D_{116}E_{152}$) and the HHCC zinc-binding site ($H_{12}H_{16}C_{40}C_{43}$) are fully conserved (variability ≤ 0.5%). The residues involved in chemical bond and hydrophobic contacts with the cellular cofactor LEDGF/p75 (H12, L102, A128, A129, C130, W131, W132, I161, R166, Q168, E170, T174, M178, Q214) are also fully conserved (variability ≤ 0.25%). A single position, H171, has a variability of 2.7%, yet the mutation H171Y, known to interfere with (or even abolish) the integrase-LEDGF/p75 binding[37], was extremely rare (0.45%). The high conservation of all these residues lends support to the finding of the importance of these sites for surface interaction with LEDGF/p75 and for HIV integration[69].

These results confirm recently published data, where the HIV-1 integrase gene diversity in group M, N, and O viruses within the public Los Alamos Database was evaluated[65-67]. In the first study, analyzing 1,304 isolates of HIV-1 group M, N, and O, 35% (101/288) amino acid positions within integrase had ≥ 1% variability (therefore 65% residues conserved)[65]. Very similarly, Rhee, et al., analyzing more than 1,500 published integrase sequences of group M, reported that 115 out of 288 (39.9%) integrase amino acid positions had at least one polymorphism with ≥ 0.5% variability, and 202/288 residues (70%) were conserved with variability ≤ 1%[67]. Interestingly, among the conserved residues in HIV-1 B subtype integrase (Fig. 1), 180 residues out of 188 (96.5%) were conserved in the integrase sequences belonging to all subtypes of group M, as well as the six largest invariant regions[67]. There were two single exceptions: in region III (K127-G134), the residue G134 was high variable among group M (with variability > 25%); in region IV (Q137-V150), the F139Y mutation occurred in subtype A sequences (with variability of 8%)[67].

Overall, this indicates that in the absence of integrase inhibitor pressure and independent of both virus origin and circulation, HIV-1 integrase amino acid variations (i.e. mutations) are indeed allowed (around 30% of the sequence), but only in restricted and selected regions of the protein.

All studies reported a very high conservation (variability < 0.5%) of the catalytic triad ($D_{64}D_{116}E_{152}$) of the HHCC motif, as well as of all residues involved in the binding with the cellular cofactor LEDGF/p75 (with the exception of H171, where H171Y and H171Q mutations were 1% prevalent), and of residues associated with primary resistance to raltegravir and/or elvitegravir[65,67].

Another recent study[50], analyzing 243 HIV-1 clade B integrase gene sequences within the Los Alamos Database, reported only 36% of integrase residues conserved. This is in apparent contrast with the other (our, Hackett's and Rhee's) data, since the lower conservation is based upon highly stringent criteria of variability; a mutation present in a single sequence out of 243 analyzed was considered as a polymorphism. Nevertheless, by reanalyzing those data[50], using conditions of stringency similar to ours and others (residues conserved with ≤ 1% variability), the prevalence of conservation returns to being very similar to our results, both for primary resistance mutations as well as for those integrase mutations impairing interaction with LEDGF/p75, chromosome tethering, and HIV-1 replication (A128T, C130G, W131A, I161A, V165A, R166A, Q168A/L/P, E170G, L172A, K173A, Q214L, and Q216L, completely

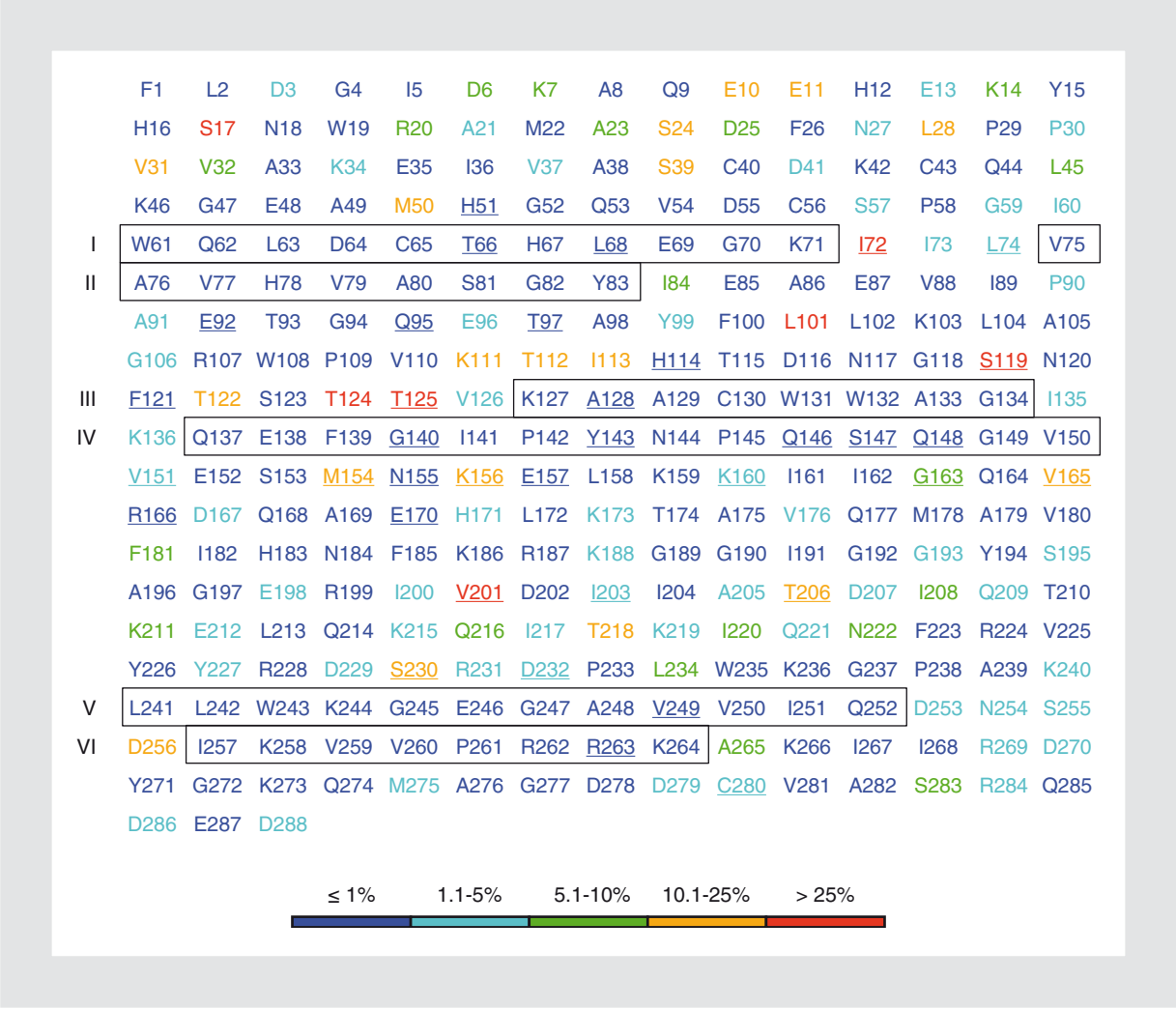

Figure 1. *Conserved regions and residues of HIV-1 integrase in HIV-1-infected patients naive to integrase inhibitors. The amino acid sequence of HIV-1 integrase (288 amino acids) of clade B consensus (shown as a reference) is colored according to the frequency rate of mutations observed in plasma samples from 448 integrase inhibitor-naive patients[68]. Residues associated with integrase inhibitor resistance are underlined. Conserved regions of amino acids are boxed. The bar indicates the frequency rate of mutations (%) relative to the colors used in the figure.*

absent)[35,50,70-74]. Taken together, all these data confirm the high conservation of specific amino acids residues, mandatory for the correct performance of integrase functions.

Functional elaboration of the HIV-1 integrase conserved residues and regions

Performing a more detailed structural and functional elaboration within the three integrase regions (N-terminal, catalytic core, and C-terminal domains), analyzing our B-subtype[68] and Rhee's HIV-1 M group sequences data[67], a different degree of conservation (variability ≤ 1%) in these regions was observed: 55.1% (27/49), 74.9% (122/163), and 57.9% (44/76), respectively.

N-terminal domain

In particular, analyzing the N-terminal domain (1-49 amino acids), in addition to the conserved HHCC motif ($H_{12}H_{16}C_{40}C_{43}$), other invariant residues were scattered throughout the sequence either individually or in small stretches of two to four amino acids (F1-L2, G4-I5, A8-Q9, Y15-H16, N18-W19, E35-I36, C43-Q44, K46-A49). The high conservation of the zinc-binding $H_{12}H_{16}C_{40}C_{43}$ motif, involved in the multimerization of the integrase subunits and stabilization folding[25-27], confirms its essential maintenance for the enzyme

function and/or virus replication. The HHCC is involved also in the interaction with LEDGF/p75: the IN_{H12N} mutant heavily alters this viral domain, reducing the affinity for the cellular cofactor[36]. Previous reports showed that HHCC mutants abolished viral infectivity[75,76]. Indeed, changes, such as H12N, H12A, H16A, and C40S, cause replication-defective mutants and a strong diminution of the activities of 3' processing and of strand transfer of this viral protein[23,75,76].

Similarly, the high conservation of residues A33, E35-I36 is consistent with previous reports that showed that catalytic activities (processing and integration) of integrase with mutations such as V32G, A33L, K34A were between 0-10% of wild-type activity[77]. Moreover, Lu, et al.[78] showed that HIV-1_{K34A} is a virus failing to replicate despite encoding catalytically competent integrase, supporting the fact that such mutation was never found *in vivo*.

Catalytic core domain

In the catalytic core domain (50-212 amino acids), the highest integrase conserved domain, the majority of invariant residues (81/122, 66.4%) were scattered throughout the sequence, either individually or in small stretches of amino acids; the remaining 41 invariant amino acids (33.6%) clustered into four large regions of wide conservation, comprising 7-14 consecutive invariant residues (Fig. 1). These invariant regions, containing some functionally important residues, were: I (W61-K71), II (V75-Y83), III (K127-A133) and the longest IV (Q137-V150). The I region contains the first (D64) amino acid of the catalytic triad ($D_{64}D_{116}E_{152}$). The conserved D116 residue was within a short, highly conserved region (H114-G118). Drelich, et al.[79,80] proposed that mutations of conserved residues D64, P109, D116, and E152 adversely affect integrase function *in vitro*, while mutation of a conserved T115 to alanine causes a near complete loss of Mg^{2+}-dependent integration activity. In our dataset, in addition to the catalytic triad, also P109 and T115 residues were highly conserved (0 and 0.45% of variability, respectively).

Interestingly, the KRK motif ($K_{186}R_{187}K_{188}$), important for the integrase:integrase oligomerization[31] at the dimer:dimer interface[32], was not fully conserved. Only residues K186 and R187 were highly conserved (variability ≤ 1%). The residue K188 had a 2.2% variability in B-subtype dataset and 5% variability in the group M dataset[67,68], though the only mutation observed was in both groups the conservative arginine substitution (K188R), which maintains the basic positive charge character. A previous study showed that mutant substitutions K186T and K188T define an integrase with absent infectivity in lymphocytic cell lines C8166 and H9[81], confirming the necessity to maintain the basic positive charge character in the region for stability of the enzyme structure and thus viral infectivity.

Another region within the catalytic core domain, the non-canonical nuclear localization signal (I161-K173)[30], showed in both datasets (B-subtype and group M) an overall high conservation, with a small exception of variability at two residues associated with *in vitro* and/or *in vivo* resistance to different integrase inhibitors (G163 and V165), at residue D167 and at the above-mentioned H171 (Fig. 1). However, the two mutations V165A and R166A that abolish nuclear import, provirus formation, and consequently virus replication[30,76], were absent (the residue R166 was highly conserved), supporting the important role of this region that has been proposed as mediator of pre-integration complex nuclear import; this function is required for productive viral infection of dividing and non-dividing cell targets.

C-terminal domain

The C-terminal domain (213-288 amino acids), involved in the binding with viral and cellular DNA, also contained two large regions of consecutive invariant residues, area V (L241-Q252) and area VI (I257-K264) (Fig. 1). Both regions are almost overlapping with the known conserved regions C (^{235}WKGPAKLLWKGEGAVV^{250}) and N (^{259}VVPRRK^{264}), conserved in all retroviruses and essential for HIV-1 replication[81]. Indeed, the C region (W235-V250) was fully conserved, with the only and partial exception of the residue K240, which was rarely mutated in K240R (1.5% variability in our dataset and 1% variability in Rhee's dataset[67,68]) without changing the basic positive charge character. Other integrase mutations, E246K and G247W, known to be associated with an excessive RNA-splicing phenotype, Gag protein processing, and virus particle production[82], were completely absent. Indeed, these mutations, generated by site-directed mutagenesis because they overlap with one of the four HIV splice site donors (the 5' SS D2), activate the splicing at 3' SS A1, therefore decreasing the accumulation of unspliced mRNA (that encodes messages for Gag and Gag-Pol genes and serves as genome RNA for packaging into virions), reduces the production of virions (approximately 90%) and are lethal for virus infectivity[82]. Besides, the integrase E246K cross-links the adenine located near the end of U5 plus strand of viral *att* site[83].

On the other hand, in the VI conserved area (I257-K264), there are several known important residues such as V260, important for the integrase multimerization[84], and K258, R262, R263, and K264, implicated in DNA binding[40,85]. Cells infected with HIV-1 IN_{V260E} failed to yield a detectable level of virus growth[86]; triple mutant $IN_{R262D/R263V/K264E}$ was not able to bind the DNA substrate[40]. Other residues E246 and K273 implicated in DNA binding[85] were fully conserved.

Finally, the known sequence Q (^{211}KELQKQITK^{219}), previously described as a region with notable concentration of glutamine and basic residues in lentiviruses[81], was very variable in almost all positions in both B-subtype's and M-group's dataset[50,67,68], although overall maintaining the basic positive charge character.

Structural interpretation of the HIV-1 integrase conserved residues and regions

Although full-length HIV-1 integrase has not been crystallized, several NMR and crystal structures for individual- or two-domain integrase fragments have been solved[32,87-89], providing insight into the mechanism of host and viral DNA binding and multimerization of integrase. The HIV-1 integrase 1EX4 PDB model, deposited in the Protein Data Bank (PDB, http://www.rcsb.org/PDB/, with a resolution of 2.80 Å[87]), consisting of two chains, A and B, and reporting the catalytic core domain and C-terminal domain integrase fragments, was used to rationalize our observations also in structural terms, by coloring the integrase residues according to the mutation frequency rate observed in 448 HIV-1 B subtype integrase inhibitor-naive patients (Fig. 2). All the large conserved regions, which are completely confirmed in the HIV-1 M group[67], are localized in the molecular surface, suggesting that large areas of consecutive residues, more than single residues, are involved in protein stability, multimerization, DNA binding, catalytic activity, and LEDGF binding. The secondary structure of these regions showed a preponderance of β-sheets. In particular, regions I (61-71), II (75-83), V (241-252), and VI (257-264) reported β-sheets separated by random coiled loops. Differently, region III (127-133) is a well-defined α-helix.

Within the large conserved region III, there is the residue C130 that resulted crucial for maintaining the tertiary structure of integrase. It has also been implicated in HIV-1 nuclear import through affecting integrase multimerization more than functioning as a nuclear localization signal *per se*[90]. The residue C130 establishes three hydrogen bonds with the contiguous amino acids A133, G134 and I135 (Artese A. personal observation), providing structural explanation of the drastic destabilizing effect onto the integrase catalytic core observed by mutations occurring at these positions. Indeed, it is reported that mutations at C130 residue caused instability of the integrase protein, presumably by de-structuring the catalytic core itself. Viruses with mutations at this position proved (i) deficient for the interaction with LEDGF/p75, (ii) replication defective, (iii) blocking the reverse transcription *in vivo*, and (iv) with an incorrect rearrangement of the integrase C-terminal domain for productive interactions with reverse transcriptase[33,90-92].

The largest conserved region (IV, residues 137-150) contains the known flexible loop G140-G149, whose conformational flexibility is suggested to be important for the catalytic step after the DNA binding[93]. It has been reported that this loop becomes ordered upon DNA binding and stabilizes the 5'-end of the viral DNA[94,95]. Indeed, mutations reducing the flexibility of this region impaired the catalytic activity without affecting the DNA binding[93]. Several important residues associated with *in vitro* and/or *in vivo* resistance to raltegravir and/or elvitegravir (E138, G140, Y143, Q146, S147, Q148) are also located in this large conserved area.

The C-terminal domain, particularly involved in the DNA-binding, has an overall SH3 fold[96]. Within this domain, there are several known important amino acids spanning the conserved area VI (257-264): residue V260, crucial for the integrase multimerization[84], and residues K258, R262, R263, and K264, implicated in the DNA binding[40,85]. In particular, we observed that the conserved residue V260 establishes two hydrogen bonds with another conserved residue V249 (Artese A. personal observation), suggesting an important role of both amino acids for the integrase structure stabilization.

HIV-1 integrase resistance mutations

All primary signature resistance mutations found in patients failing raltegravir (Y143R, Q148H/K/R, N155H)[17,49] or elvitegravir (T66I, E92Q, S147G, Q148H/K/R, N155H)[18] were completely absent or highly infrequent (≤ 0.5%) in integrase inhibitor-naive patients, either infected with HIV-1 B subtype (antiretroviral-naive or -treated[50,68], or non-B subtypes/group N and O[65-67] (Fig. 3). Indeed, the T66I mutation was found only in two HIV-1 B antiretroviral-treated patients[68], while N155H and Q148H were reported in unique samples in the Los Alamos

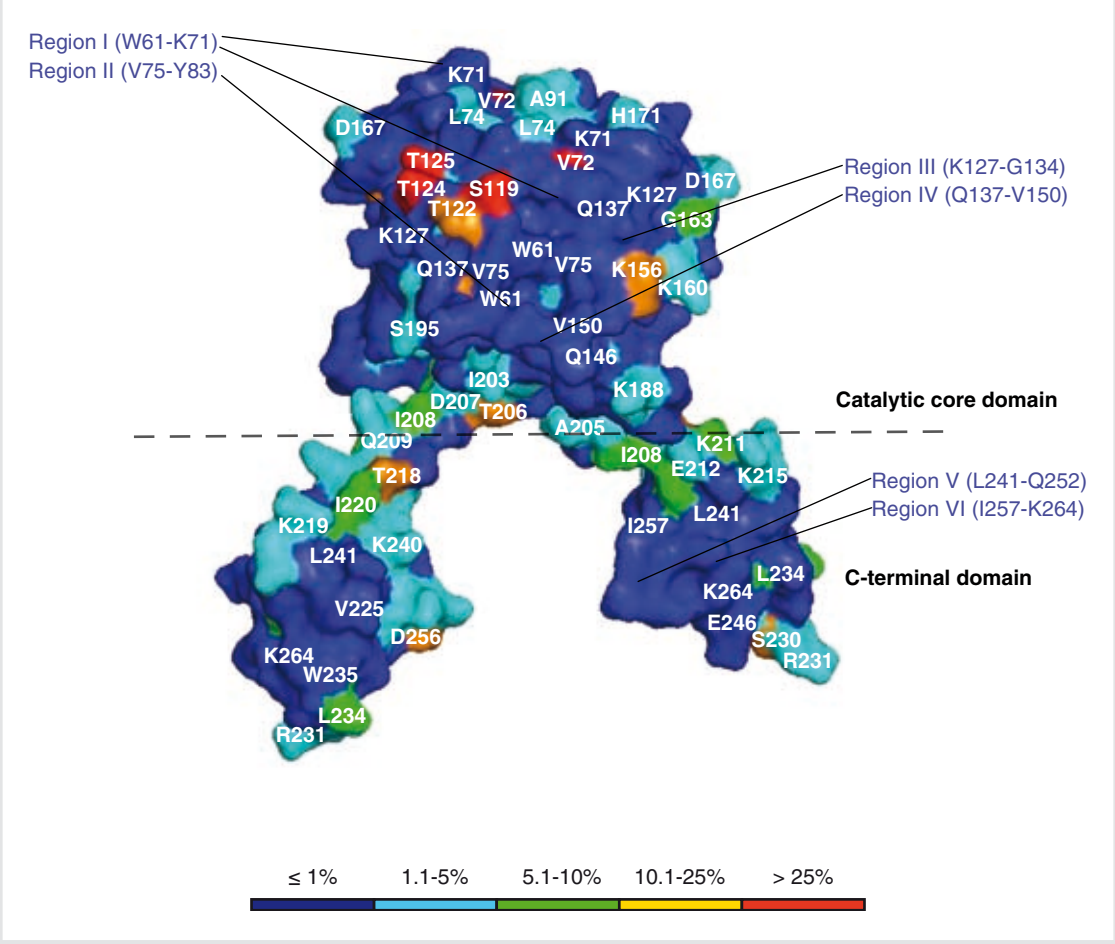

Figure 2. *Molecular surface representation of HIV-1 integrase. The X-ray crystallographic coordinates of HIV-1 integrase deposited in the Protein Data Bank (PDB, http://www.rcsb.org/PDB/) with code 1EX4 with a resolution of 2.80 Å[87] were used to map residues in a three-dimensional representation. Such a structure consists of two chains, A and B, presenting some deletions and mutations in the catalytic core and the C-terminal domains, defined by a dashed black line. In particular, in the diffraction experiment, in both integrase chains the residues F1-V54 and Y271-D288 were missing; amino acids D55 and P142-N144 were not located in chain A, while residues E138-G149 were not located in chain B; such an aspect could explain the asymmetric shape of the integrase homodimer. PyMOL package was employed for visualization and display (DeLano Scientific LLC, USA). The molecular surface representation was defined as solvent accessible surface area according to the "Connolly" method[119] as implemented in PyMOL program.The residues are colored according to the mutation frequency rate obtained in 448 integrase inhibitor-naive patients. All the conserved regions are located on the structure surface and highlighted by labeling the first and the last residue by one-letter amino acid symbols. Also, the superficial amino acids polymorphic are shown.*

Database[50]. Secondary mutations found in patients failing raltegravir- and/or elvitegravir-containing regimens, such as H51Y, T66A/K, E138K, G140S/A/C, K160N, R166S, E170A, S230R, R263K[17,18,49,57-59] or other mutations known to reduce HIV-1 susceptibility *in vitro* to elvitegravir (Q95K, H114Y, F121Y, T125K, Q146P, S153Y)[51,55,64], were also completely absent in all datasets[50,65-68] (Fig. 3). In contrast, some secondary mutations found in patients failing raltegravir- and/or elvitegravir-containing regimens[17,18,49,59], such as L68I/V, E138A, E157Q, G163K/R, and D232N mutations, were rare (frequency < 1%, with the exceptions of G163K/R > 10% prevalence only in subtype F, and E157Q with 3 and 4% prevalence in subtype AG and D, respectively[67]), while L74M, T97A, S119G/R, and V151I were present as natural polymorphisms with a frequency of 1.3-6%; K156N, T206S and S230N were remarkably frequent (≥ 10%)[50,65-68].

The mutation T112I selected *in vitro* under pressure of MK-2048, a potent second integrase generation strand transfer inhibitor with high genetic barrier able to inhibit HIV-1-resistant variants generated with

Raltegravir-associated mutations	Primary: **Y143R**, **Q148H/K/R**, **N155H**
	Secondary: **L74M**, **E92Q/A**, T97A, **E138K**/A, **G140S/A**, **Y143C/H**, V151I, K156N, E157Q, G163K/R, T206S, D232N
	In vitro: **L68V**, S119R/G, **F121Y**, **N155S**, I203M, **S230R/N**
Elvitegravir-associated mutations	Primary: T66I, **E92Q**, **S147G**, **Q148H/K/R**, **N155H**
	Secondary: **H51Y**, **T66K/A**, L68I/V, S119R/G, **E138K**, **G140S/C**, E157Q, **K160N**, **R166S**, **E170A**, **S230R**, D232N
	In vitro: **E92G**, **Q95K**, **H114Y**, **F121Y**, **T125K**, **Q146P**, V151I, **S153Y**, M154I, **N155S**, **R263K**
Mutations associated with other integrase inhibitors	*In vitro*: V72I, **T112I**, **A128T**, **Q146K**, S153A, **N155S**, **K160D**, V165I, V201I, **V249I**, **C280Y**

≤ 1% 1.1-10% > 10%

Figure 3. *Prevalence of mutations in positions associated with* in vivo *and/or* in vitro *resistance to integrase inhibitors. The prevalence of integrase mutations associated with integrase inhibitor resistance by previous* in vitro *or* in vivo *studies[6,12,17,18,46-64] (Stanford HIV Drug Resistance Database, http://hivdb.stanford.edu), in integrase inhibitor-naive patients, either infected with HIV-1 B subtype (antiretroviral-naive or -treated[50,68]), or non-B subtypes/group N and O[65-67] are reported. The amino acid sequence of HIV-1 integrase of clade B consensus is shown as a reference. Primary and secondary mutations found* in vivo *in patients failing raltegravir[6,17,49,56-58,60-63] or elvitegravir[18,59] are shown. Mutations known to be associated by themselves with > 10-fold decrease susceptibility are F121Y, Q148H/R/K, N155H for both raltegravir and elvitegravir[51,52,56,58,59], Y143R only for raltegravir[58], while T66I, E92Q/G, Q146P, S147G and V151I mutations only for elvitegravir[18,48,51,59]. Mutations known to increase resistance in presence of primary mutations are L68V, E138K, G140A/S, V151I, for both raltegravir and elvitegravir[51,52,58,59]; L74M, E92Q, T97A, E138A, Y143H only for raltegravir; H51Y, L68I, Q95K, T125K, Q146P, S153Y, M154I, E157Q, R263K only for elvitegravir[51,52,59,64]. G140S + Q148H reduces raltegravir and elvitegravir susceptibility > 1,000-fold[18,52]. Resistance mutations completely absent (or found only in single isolates among all studies) are shown in black bold; mutations found with ≤ 1% variability are shown in black; mutations with 1.1-10% variability are shown in dark grey bold; mutations with > 10% variability are shown in grey.*

first-generation compounds[54], occurred in integrase inhibitor-naive patients at a frequency of 7%[50,65-68].

Additional mutations associated with *in vitro* resistance to elvitegravir (M154I, that in the co-presence of T66I primary mutation was associated with reduced susceptibility to elvitegravir[52]) or to other integrase inhibitors different than raltegravir or elvitegravir (I72V, V165I, V201I)[50] showed > 10% variability (Fig. 3).

Taken together, all these data consistently show that all primary mutations associated with resistance to integrase inhibitors clinically relevant today are absent or highly infrequent in integrase inhibitor-naive patients.

However, for some secondary integrase inhibitor resistance-associated mutations, differences in prevalence between the distinct studies were observed. For instance, four integrase mutations (I84V, M154I/L, V165I) showed a significant increase of prevalence in HIV-1 B antiretroviral-treated patients compared to antiretroviral-naive patients[68]. Two of them, previously associated with *in vitro* resistance to integrase inhibitors (strand transfer inhibitors as well as DNA binding inhibitors and 3' processing inhibitors)[50,52], M154I and V165I, occurred at 6% frequency in untreated patients, reaching 21.3% ($p < 0.001$) and 13.4% ($p = 0.022$), respectively, in antiretroviral-treated patients. Mutation M154L was absent (0%) in antiretroviral-naive patients, and reached 5.7% in antiretroviral-treated patients ($p = 0.003$). Similarly, the I84V mutation occurred at 1.5% frequency in untreated patients, reaching in antiretroviral-treated patients a frequency of 5.7% ($p = 0.048$)[68]. All these mutations within the Los Alamos Database, that mostly came from antiretroviral-naive patients, were with a frequency similar to what we observed in HIV-1 B subtype antiretroviral-naive patients[50,67].

The mechanisms of this observed difference on the prevalence of some integrase mutations between drug-naive and antiretroviral-treated patient populations need further investigation. It is conceivable that specific drug

pressure induced by protease inhibitors or in particular reverse transcriptase inhibitors may select or induce mutations also in different target regions within the same gene. For instance, very recent observations by us and by other groups indicate that there are some associations between integrase and reverse transcriptase resistance mutations in antiretroviral-failing patients[68,97,98], supporting the hypothesis of a tight physical interaction between the viral integrase and reverse transcriptase, and a potential co-evolution of some of their mutations[68,99]. Further studies are required to elucidate this point with potentially relevant implications in clinical practice.

So far, in patients failing raltegravir-containing regimens, three main different pathways of raltegravir resistance have been generally associated with virologic failure, each involving one signature primary mutation at positions N155 or Q148 or Y143, plus one or more secondary mutations (L74M, E92A/Q, T97A, E138A/K, G140S/A, V151I, G163R/K, D232N) important for viral fitness rescue and/or increasing resistance[17,49,53,54,56-63]. However, recent analyses suggest that in addition to these common resistance profiles, there are other pathways associated with raltegravir resistance *in vivo*, involving E92Q or E157Q or T97A+G163R mutations[57,63].

The existence of distinct integrase resistance profiles is similar to what has been described for other antiretroviral classes, such as nonnucleoside reverse transcriptase inhibitors, nucleoside reverse transcriptase inhibitors, or protease inhibitors. However, it is unknown what the determinants of the evolution toward these different profiles are. The potential role of natural occurring polymorphisms in HIV-1 integrase may have clinical and virologic implications for integrase inhibitors, and in clinical practice has yet to be established.

It is possible that preexisting integrase mutations, both occurring as natural polymorphisms and/or acquired/selected by previous virologic failures with antiviral regimens different from integrase inhibitors, may influence the integrase genetic pathways to develop resistance, and could reduce the "genetic barrier" and thus accelerate treatment failure to integrase inhibitors.

In this context, HIV-1 group and subtype differences may also have an impact on the evolution of resistance to integrase inhibitors, as has been described for protease inhibitors, nucleoside reverse transcriptase inhibitors, and nonnucleoside reverse transcriptase inhibitors[100-111]. Hackett, et al., by analyzing 1,304 sequences from group M, N, and O viruses, have recently reported that some of the mutations associated with resistance to raltegravir and/or elvitegravir, such as L74M, T97A, E157Q, as well as other integrase inhibitor resistance mutations (V165I, V201I, T206S) occurred as natural polymorphisms (≥ 1%) and occurred differently according to different HIV-1 subtype/circulating recombinant form/group[65]. Similarly, Rhee, et al., by analyzing more than 1,500 published integrase sequences of group M, showed that some secondary mutations associated with resistance to raltegravir and/or elvitegravir, such as L74M, T97A, V151I, E157Q, G163K/R, and S230N, occurred differently according to different HIV-1 subtypes/circulating recombinant form[67]. In some cases, the prevalence was > 10% in specific subtypes (T97A only in subtype A; V151I and S230N only in subtype B; G163K/R only in subtype F). The mutation E157Q occurred in about 2-4% of integrase inhibitor-naive patients with subtype B, AG, and D. In addition, the comparison of integrase amino acid sequences between subtype B and CRF02-AG showed that 13 positions (K/R14, V/I31, L/I101, T/V112, T/A124, T/A125, G/N134, I/V135, K/T136, V/I201, T/S206, L/I234, S/G283) differed between the HIV-1 integrase of these two subtypes[112].

The significance of all the polymorphic residues to the current generation of integrase inhibitors is not yet well known. However, a recent study showed that the contribution of integrase polymorphisms to raltegravir and elvitegravir phenotypic susceptibility was minimal not only in subtype B, but also in all non-B subtypes tested[113]. Similarly, preliminary results showed that HIV-1 subtype did not affect the response to raltegravir in phase III clinical trials[17,53]. However, more clinical trials including patients infected with non-B subtype HIV-1 are required to further elucidate the efficacy of raltegravir and elvitegravir on non-B subtypes.

Interestingly in this context, recent studies showed also promising results for the efficacy of integrase inhibitors in HIV-2. Despite a 40% heterogeneity between the HIV-1 and HIV-2 integrase genes, phenotypic susceptibility to raltegravir and elvitegravir in HIV-2 is similar to that of HIV-1[114], and virologic and immunologic response to a HAART regimen containing raltegravir in HIV-2-infected patients experiencing immuno virologic failure to several previous antiretroviral lines has been reported[115]. Very recently, it has been also reported that HIV-1 and HIV-2 share similar integrase inhibitor resistance pathways. Indeed both N155H and Q148KR mutations were observed in HIV-2-infected patients failing a raltegravir-containing regimen[116,117]. It should be noted that HIV-2 is naturally resistant to current nonnucleoside reverse transcriptase inhibitors

and fusion inhibitors[118], and therefore the so far short-term immunologic and virologic efficacy of an integrase inhibitor-containing regimen also in heavily pretreated HIV-2-infected patients is really promising and clinically relevant.

In conclusion, the HIV-1 integrase *in vivo* is an enzyme requiring the full preservation of almost two-thirds of its amino acids in the absence of specific integrase inhibitor pressure. It will be interesting to evaluate in future the minimal degree of conservation of the integrase under the specific pressure of integrase inhibitors. The characterization of the highly conserved residues (involved in protein stability, multimerization, DNA-binding, catalytic activity, LEDGF-binding, and some with still poorly understood function) could help in the rational design of new HIV-1 inhibitors with alternative mechanisms of action and more favorable resistance profiles.

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