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A Homology Model of HIV-1 Integrase and Analysis of Mutations Designed to Test the Model

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Abstract

Although there are structures of the different domains of human immunodeficiency virus type 1 (HIV-1) integrase (IN), there is no structure of the entire protein. The recently determined crystal structures of the prototype foamy virus (PFV) IN tetramer, in complexes with viral DNA, led to the generation of models of full-length HIV-1 IN. These models were generated, in part, by superimposing the structures of the domains of HIV-1 IN onto the structure of full-length PFV IN. We developed a model for HIV-1 IN—based solely on its sequence alignment with PFV IN—that differs in several ways from the previous models. Specifically, in our model, the junction between the catalytic core domain and C-terminal domain adopts a helix-loop-helix motif that is similar to the corresponding segment of PFV IN and differs from the crystal structures of these two HIV-1 IN domains. The alignment of residues in the C-terminal domain also differs from the previous models. Our model can be used to explain the phenotype of previously published HIV-1 IN mutants. We made additional mutants, and the behavior of these new mutants provides additional support for the model.

Keywords

HIV; integration; dimerization; infectivity; modeling

Introduction

Retroviral integrase (IN) inserts the linear double-stranded viral DNA (vDNA, the product of reverse transcription) into the chromosomal DNA of a newly infected cell. Integration is a two-step reaction. First, IN dimers bind each of the two ends of the vDNA and remove conserved GT dinucleotides from each of the 3' ends to expose the 3'-hydroxyl groups of the conserved CA dinucleotides that make up the new termini (referred to as 3'-processing, or 3'-P).^{1–5} In the second step, IN catalyzes a nucleophilic attack by the free 3'-hydroxyl groups at the ends of the vDNA on the phosphodiester backbone of the host DNA (hDNA).

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Supplementary Data

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This step is referred to as strand transfer, or ST. The two vDNA ends are inserted, by an IN tetramer, on opposite sides of the major groove of the hDNA target. Different retroviral INs insert the vDNA with different preferential spacing on the hDNA (four to six base pairs). In the case of human immunodeficiency virus type 1 (HIV-1) IN, five base pairs separate the integration sites.^{6–10} The spacing of the inserted vDNA ends means that, after the hDNA is repaired, the integrated HIV vDNA is flanked by a five-nucleotide repeat.^{9,11}

Structural, biochemical, and mutagenesis experiments showed that retroviral INs consist of three distinct domains. The N-terminal domain (NTD, residues 1–49 of HIV-1 IN) forms an HHCC zinc binding motif and contributes to multimerization.¹² The catalytic core domain (CCD, residues 50–212) contains a highly conserved triad of acidic residues (D64, D116, and E152) that binds divalent metal ions, with Mg²⁺ being the biologically relevant metal cofactor.^{13,14} This domain carries out both the 3'-P and ST reactions and contributes to both vDNA and hDNA binding and multimerization. The C-terminal domain (CTD, residues 213–288) is primarily involved in hDNA binding.¹⁵ All three domains contribute to the formation of the IN tetramer (which is a dimer of dimers) that carries out the concerted integration of the two vDNA ends. Structures have been determined for the isolated domains HIV-1 IN and for two-domain fragments; however, the structure and orientations of the linkers that connect the domains differ in the various structures.^{13,14,16–20}

The recent crystal structures of the prototype foamy virus (PFV) IN revealed the proper arrangement of the three domains of IN, the arrangement of the four IN subunits in the functional tetramer, and the modes of vDNA and hDNA binding.^{21–24} The structure is supported by biochemical data showing that a tetramer of IN bound to two vDNA ends is required for concerted integration.²⁵ It has been proposed that one IN dimer binds at each of the vDNA ends, which then assemble into a tetramer formed as a dimer of dimers.⁵ The arrangement of the intasome in the crystal structures suggests that, for each dimer, the inner (catalytic) IN that binds to one of the vDNA ends forms a head-to-head dimer with an outer (non-catalytic) subunit. The two inner subunits then form a head-to-tail dimer to complete the tetramer (Fig. S1). The distance between the active sites of the inner subunits defines the spacing of the integration sites where the ends of the vDNA are inserted into hDNA. Given that both dimerization steps are critical for the concerted integration reaction that inserts vDNA into hDNA, the interactions at the interfaces between the two inner INs and between each of the inner and outer INs of the tetramer are important.

We recently described calculations for the binding of IN inhibitors based on a homology model of HIV-1 IN.²⁶ Others have generated HIV-1 models that were developed based, at least in part, on published crystal structures of incomplete fragments of HIV-1 IN.^{27,28} Our model was derived from, and conforms to, the structure of the full-length PFV IN structure. It includes a helix–loop–helix motif in the C-terminal portion of the CCD that is not present in the available HIV-1 IN fragment structures, nor is this feature described in either of the published HIV-1 IN models. Replacing the partially resolved outer subunit of the PFV IN tetramer with the CCD structure from our model, including the helix–loop–helix motif at the CCD–CTD junction, predicts that the $\alpha 8$ helices from the two subunits could be involved in an intermolecular interaction that would contribute to the stability of this dimer interface. The presence of this motif in our model results in a different alignment of the CTD

compared to the alignment in the previous models of HIV-1 IN. We generated and tested mutations at this proposed CCD dimer interface. The behavior of these IN mutants supports the proposal, based on our model, that this portion of IN forms interacting helices. We also identified residues in the CTD of IN that our model predicts would be involved in hDNA binding. Mutating these residues in an HIV-1 vector leads to the generation of virions that are capable of synthesizing full-length vDNA but have little to no infectivity, consistent with a defect in integration.

Results

Description of the model of HIV-1 IN

The model was generated by aligning the amino acid sequences of several retroviral INs.^{17,23,29,30} The PFV IN structure 3L2T (superseded by 3OYA) was used as a template to build a homology model using the HIV-1 IN sequence in MOE2009.10. Secondary-structure predictions were not used in the sequence alignment because there is poor sequence conservation among SH3-like folds that constitute the CTDs of retroviral INs.³¹ A complete model was generated for the inner protein subunit; then the magnesium ions, vDNA, and inhibitor coordinates were copied directly from the PFV structure into the model, and the resulting structure was energy minimized. When the model of the full-length HIV-1 IN is overlaid on an inner subunit of the PFV IN tetramer, the overall arrangement of the proteins is quite similar. The zinc finger in the NTD is conserved, as are the RNaseH fold of the CCD and the SH3-like fold of the CTD (Fig. 1). These conserved folds are also described in a previous HIV-1 IN model.²⁷ However, our HIV-1 IN model differs from the previous models in the C-terminal portion of the CCD, the CTD, and the linker connecting these regions.

HIV-1 IN consists of 288 amino acids, while PFV IN consists of 395. A substantial portion of that difference lies in the N-terminal extension domain (NED) of PFV IN. However, after accounting for the sequences in the NED, PFV IN is still more than 50 amino acids longer than HIV-1 IN. When we aligned the sequences of HIV-1 IN and PFV IN, the PFV IN sequence included a C-terminal tail that did not align with any of the C-termini of the lentivirus INs we analyzed (HIV-1, SIV_{CPZ}, SIV_{AGM}, or SIV_{SM}). This C-terminal tail is unresolved in crystal structures of PFV IN and, because of the apparent absence of an analogous sequence in lentiviral INs, this tail was not considered in our modeling.

The arrangement of the CTD in our model is different from those seen in any structural studies involving either the isolated CTD of HIV-1 IN or the CCD–CTD fragment (Fig. 2). Two early NMR studies solved the structure of a recombinant peptide corresponding to residues 220–270 of HIV-1 IN.¹⁸ This peptide was identified as the minimal DNA binding domain based on deletion studies.³² The CTD in our model is structurally similar to, but does not match the sequence alignment of, these isolated peptides. The alignment shown in Fig. 2 was scored according to an experimentally determined amino acid similarity matrix.³³ It shows the C-terminal tail of HIV-1 IN (residues 271–288), which is included in our model. A CCD–CTD crystal structure [Protein Data Bank (PDB) ID: 1EX4] comprising residues 52–288 of HIV-1 IN is also shown.¹⁷ Residues 271–288 are not resolved in this structure, but examination of the crystal contacts in the 1EX4 structure shows that the CTD participates in numerous intermolecular interactions. The fold observed in this crystal

structure may have been influenced by this crystal packing. The CTD is a sequence nonspecific DNA binding domain that would be expected to have a positively charged surface that, in its proper configuration, may not have been compatible with the growth of the crystals. The requirement for the protein to interact with itself to form crystals and the absence of DNA in the 1EX4 structure may have favored a refolded configuration that differs from its structure in an intact intasome (Fig. S2). Thus, the constraints imposed by crystal packing may have induced the refolding of the CTD in the two-domain structure.

The 1EX4 CCD–CTD crystal structure of HIV-1 IN includes an extended helix (termed the $\alpha 6$ helix) that is composed of residues 195–220; there is a kink in the helix at T210. In our model, this region forms three distinct helices connected by short loops. The first helix (hereafter referred to as $\alpha 6$) includes residues S195–I200 and superimposes with $\alpha 6$ of the 1EX4 crystal structure. The second helix ($\alpha 7$) in the model includes residues T206–T210; this helix ends in the model where the kink was observed in the two-domain HIV-1 IN crystal structure. In the model, the final helix ($\alpha 8$) spans residues Q214–R224. In the PFV IN crystal structures, the sequence that corresponds to the $\alpha 7$ helix in the HIV-1 IN model forms a long loop, but does not have the single helical turn seen in the HIV-1 IN model. Otherwise, the architecture of this region in our HIV-1 IN model closely resembles the corresponding region of the PFV IN crystal structure (Fig. 3). In addition, the arrangement of residues 50–194 in our model is consistent with recent biochemical assays³⁴ and dimer interactions reported from the CCD structures,^{13,14} particularly near the dimer interface. Importantly, the surface of the CCD shown to bind LEDGF remains exposed in our model and the model is consistent with the previous PFV co-crystal structures.³⁵ Given that the most significant difference between our model and previous HIV-1 IN models and crystal structures of the CCD is in the region that includes the $\alpha 7$ and $\alpha 8$ helices. We present, in later sections, mutational analysis of two residues in the $\alpha 8$ helix that the model predicts are instrumental in dimerization.

Other parts of the structure are affected by the presence of this helix-loop-helix motif. Using the sequence alignment in the 1EX4 crystal structure, the CCD–CTD linker cannot be positioned as it is in the PFV intasome. The 1EX4 CTD is composed of five β -strands: $\beta 1$ = residues 223–228, $\beta 2$ = residues 235–245, $\beta 3$ = residues 248–252, $\beta 4$ = residues 256–260, and $\beta 5$ = residues 265–268. Our model incorporates a similar CTD fold; however, the residues that form the $\beta 1$ and $\beta 2$ strands in the 1EX4 crystal structure form the unstructured CCD–CTD linker in our model. The C-terminal tail (residues 271–288), which is not present in the 1EX4 crystal structure, forms the $\beta 4$ and $\beta 5$ strands of our model; these sequences were not included in the published HIV-1 IN models. Thus, in our model, the CTD is arranged such that $\beta 1$ = residues 242–247, $\beta 2$ = residues 250–260, $\beta 3$ = residues 265–270, $\beta 4$ = residues 273–278, and $\beta 5$ = residues 282–285 (Fig. 4). Deletion analysis showed that residues 280–288 can be removed with only marginal decreases in infectivity.³⁶ In our model, these residues pack against the NTD–CCD linker, suggesting that, in the deletion mutant, this NTD–CCD linker could occupy the space normally occupied by the CTD $\beta 5$ strand (Fig. S3). The model predicts that R269 and K273 are involved in hDNA binding and are located in the $\beta 3$ – $\beta 4$ loop. Truncations of the C-terminus that remove portions of the $\beta 4$ strand may destabilize the fold of the CTD in a manner that cannot be overcome by interactions with the NTD–CCD linker.

The predicted interactions of the vDNA and hDNA with our model of HIV-1 IN are consistent with the previously published DNA cross-linking data (Fig. 5).^{17,27,37–43} C56 interacts with the non-reactive strand of the *cis* vDNA (the vDNA bound to the inner subunit on which C56 resides).^{37–39} P30 lies close to the reactive strand of the *trans* vDNA (the vDNA whose 3' end is bound by the other inner IN subunit).²⁷ Residues critical for recognizing the vDNA ends (G149, S153, and K156) lie in the groove between the reactive and non-reactive strands of the *cis* vDNA.^{37,39,41} The region near the helix-loop-helix motif (including residues G189, G193, K211, K215, and R228) interacts with *cis* vDNA several base pairs from the reactive 3' end.²⁷ Among the specific contacts predicted in our model, the C^α of G193 is positioned 4.0 Å from the C2' ribose atom of the thymidine, which is eight nucleotides from the 3' terminal adenosine of the *cis* reactive vDNA strand;²⁷ this is near enough for van der Waals interactions. Also, the side chain of K215 comes within 4.3 Å of the phosphate group of the C16 nucleotide of the *cis* non-transferred strand.^{17,37} CTD residues of one inner subunit are involved in interactions with *cis* vDNA (K266),^{37,38,42} *trans* vDNA (the end of the vDNA that is processed by the other inner subunit of the intasome) (V250),^{37,38} or hDNA (G247, A248, and R269).^{37, 38,40} The side chain eNH3 of K266 is 2.8 Å from the O1' atom of the A1 nucleotide of the non-transferred *cis* strand.^{37,38,42} R269 has been implicated in hDNA binding,³⁷ and, in our model, the positively charged side chain of R269 extends within 2.9 Å of the phosphate group two nucleotides from the ST site in the hDNA. There is good agreement between the data for the interactions of CTD in the model and the published biochemical data, which supports the structure of this domain proposed in the model.

Mutations in the CTD of IN restrict infectivity

The alignment of the CTD and the inclusion of the C-terminus of HIV-1 IN in our model place the positively charged side chains of R269 and K273 of the two inner INs of the IN tetramer into close proximity with phosphate groups spanning the minor grooves of the hDNA. The R269A mutant has been reported to delay infection and to restrict infectivity measured 24 h post-infection; K273A virions showed infectivity similar to wild type (WT) in the same assays.⁴⁴ Interestingly, this previous report characterized R269A as a Class I mutation; the presence of the mutation in the virus specifically inhibited replication at the integration step. We have analyzed the R269A/K273A double mutant and extended the analysis of the K273A single mutant.

In our model, the positively charged side chains normally present at 269 and 273 in each of the inner subunits of the IN tetramer are close to the negatively charged phosphate groups on the hDNA backbone. Based on the model, the R269A/K273A double mutant would eliminate four ionic interactions with the hDNA. For this reason, we predicted that this double mutant would be impaired for hDNA binding (Fig. 6).

Consistent with the model, the R269A and K273A mutations reduced the infectivity of the virus (Table 1). R269A caused a roughly 7-fold reduction, similar to a previous report,⁴⁴ while K273A reduced infectivity by almost half. Neither of the single mutations completely abolishes infectivity; if both residues are involved in the interaction with hDNA, the remaining residue may be sufficient to permit some interaction with the hDNA that could

account for the residual infectivity. In agreement with this prediction, the infectivity of the double mutant is reduced to levels comparable to the negative controls.

Because IN mutants may impact viral replication at a stage prior to integration,⁴⁵ we determined the levels of late reverse transcription products (following the second strand transfer step, hereafter referred to as vDNA) and 2-LTR circle DNA by qPCR. The R269A/K273A double mutant does produce significantly less full-length vDNA than WT (Table 2). Moreover, the ratio of 2-LTR circles to total vDNA is much higher than WT; this is indicative of reduced integration efficiency. While we cannot rule out the possibility that these residues have some other key function needed between the completion of reverse transcription and integration, the data are consistent with a mechanism by which these mutations weaken hDNA binding.

The near-WT activity of an IN lacking nine C-terminal residues³⁶ can be explained by the proximity of the NTD–CCD linker to the C-terminal residues in our model. It appears that this linker could contribute sufficient H-bonds to maintain the fold of the CTD even in the absence of the C-terminal residues (Fig. S3). Further truncations that show a marked reduction in infectivity extend into the $\beta 4$ strand. R269 is located in the $\beta 3$ – $\beta 4$ loop and K273 is the first residue of the $\beta 4$ strand. In contrast to the minimal effect of removing the last nine residues, removing a portion of the $\beta 4$ strand may compromise the integrity of the DNA binding surface. It is also noteworthy that a separate study identified a deletion mutant of HIV-1 IN consisting of residues 1–269 that still supported viral replication, while viruses carrying deletion mutants that terminated at residues 270, 271, or 272, exhibited Class I mutant phenotypes.⁴⁶ C-terminal IN deletion mutants that had fewer than 269 residues all showed Class II mutant phenotypes. These data are consistent with a key role for the proposed $\beta 3$ – $\beta 4$ loop.

Reduced infectivity of Q214/Q221 double mutants

Superimposing the CCD and portions of the flanking linkers (residues 50–230) of our HIV-1 IN model onto one of the partially resolved outer subunits of the PFV IN tetramer places the $\alpha 8$ helix in close proximity of its counterpart in the inner subunit (Fig. 7). In the model, the glutamine residues at 214 and 221 are positioned on the interacting face of the helix, and their side chains are oriented so that intermolecular H-bonding could occur. Mutations were made to determine the impact of changing the amino acids at these two positions to alanine, cysteine, and complementary charged residues. As predicted by the model, the Q214A/Q221A double mutant reduced the infectivity to ~6% of the WT virus, approximately a 17-fold reduction (Table 3). The Q214A/Q221A mutant also produced slightly more vDNA than a WT vector and the 2-LTR circle-to-vDNA ratio was similar to that in WT (Table 2).

Based on the model, the Q214C/Q221C double mutant should be able to form disulfide-linked dimers. Western blots performed on virions show a greater percentage of IN in a band that migrates where a dimer would be expected; these dimer bands were not present when the samples were treated with a reducing agent prior to loading on the gel (Fig. 8a). The p66 and p51 subunits of RT, the unprocessed Gag (p55), and the processed CA (p24) are clearly distinguished on the same blot, indicating that protease is processing Gag-Pol normally in these mutants. The GeneTools software package (Syngene) was used to quantify the

intensity of the IN monomer and dimer bands. It is noteworthy that neither the Q214C nor the Q221C single mutant showed an increase from the dimer percentages seen in WT, while the Q214C/Q221C showed roughly a 3-fold increase (Fig. 8b). This result is consistent with a direct interaction between these residues. The Q214C/Q221C double mutant was not infectious (Table 3). It also showed a significant reduction in vDNA synthesis (~50-fold) while 2-LTR circles were detected at levels similar to WT virions (Table 2). The reduction of vDNA synthesis suggests that the double mutant, like many other IN mutants, affects reverse transcription.⁴⁷ The increase in the fraction of vDNA forming 2-LTR circles suggests that the double mutant is also impaired in integration. One, or both, of these defects may be the result of the cross-linking of IN.

Surprisingly, the introduction of a single alanine or cysteine mutation (either Q214A, Q214C, Q221A, or Q221C) resulted in infectious viral particles (Table 3). In the Q214A and Q214C mutants, K215 may still be capable of forming an intermolecular H-bond with the side chain of Q221 in the other subunit in the head-to-head dimer. Similarly, these interactions may be formed between Q214 and N222 in the Q221A and Q221C mutants. The double mutant would shift both interactions a full residue along the helical axes. This shift could perturb other interactions at the dimer interface and/or increase the strain required to maintain the H-bonds between the helices, thus making the effects of the double mutant more energetically unfavorable. The low, but consistently detectable, specific infectivity of the double alanine mutant may result from the replacement of the relatively strong intermolecular hydrogen bonding in the WT with a weaker hydrophobic interaction in the Q214A/Q221A double mutant.

Specific activities of cysteine mutants

We expressed and purified the recombinant forms of WT IN, both single cysteine mutant INs, and the double mutant IN (Fig. S4). The 3'-P and ST activities of these enzymes were determined as previously described.⁴⁸ Overall, the single mutants and the double mutants affected the catalytic activities of recombinant IN; ST was affected to a greater extent than 3'-P (the Q214C mutant had 3'-P and ST activities of approximately 60% and 20% of WT IN, respectively; the 3'-P and ST activities of Q221C were approximately 20% and 15% of WT IN, respectively; the activities of the double mutant were approximately 40% and 15% of WT IN, respectively). This may be due to the fact that 3'-P only requires IN to interact appropriately with vDNA, while ST requires that IN interact appropriately with both vDNA and hDNA. Treatment with DTT showed modest (~2.5-fold or less) effects on 3'-P and had relatively little impact on ST for WT IN and all mutants. Thus, the compensation from neighboring amino acids, as described in the previous section, does not appear to be sufficient to maintain the full enzymatic activities of the recombinant proteins. However, the *in vitro* assays measure the overall efficiency of integration, whereas an infection requires only two IN catalyzed integration reactions. It is possible that enough active dimers are present in pre-integration complexes in infected cells to complete the two ST reactions. Also, because the *in vitro* assay involves purified IN, these assays may lack a cellular or viral factor that acts as chaperone, or otherwise enhances the activity of IN.

Rescuing infectivity with complementary charged residues

The results with both the alanine and cysteine mutants support a cooperative, interactive relationship between residues 214 and 221. If this interaction is important, introducing charged residues at these positions could generate either attractive or repulsive forces that would affect infectivity. Substituting residues 214 and 221 with either two glutamates or two lysines would create a repulsive force from the similar electrical charges.

As expected from the model, viruses that carried the Q214E/Q221E and Q214K/Q221K double mutants had infectivities that were not significantly different from the negative controls (Table 3). The Q214E/Q221E mutant synthesized vDNA at levels about 3-fold of less than WT with a corresponding increase in the 2-LTR circle-to-vDNA ratio (Table 2). The Q214K/Q221K mutant produced vDNA at levels similar to WT and showed a modest increase in the 2-LTR circle-to-vDNA ratio. Thus, disrupting the interaction between residues at IN positions 214 and 221 severely impacts infectivity with relatively minor effects on vDNA synthesis and 2-LTR circle formation.

If electrostatic repulsion is a significant factor in reducing the infectivity of the double-glutamate and double-lysine mutants, mutants with complementary charged residues should restore infectivity. The Q214K/Q221E mutant had minimal infectivity; however, the amount of vDNA synthesized by the Q214K/Q221E mutant was not significantly different from the D110E RT negative control. This low level of total vDNA shows that there is a severe restriction in the reverse transcription of the vDNA and presents a possible explanation for the loss of infectivity of this mutant. However, 2-LTR circle DNA was consistently detected, suggesting that a minimal amount of reverse transcription products are being made and that these products apparently cannot be integrated. We propose that this defect in integration could be related to the formation of a salt bridge between a glutamate at position 221 and an arginine at position 224 on the same polypeptide. This would at least partially bury the negative charge of the glutamate at position 221, leaving the lysine at position 214 without a suitable partner on the opposing helix.

However, infectivity was restored with a Q214E/Q221K mutant, whose infectivity was nearly 80% of WT (Table 3). Moreover, this mutant showed a modest 3-fold increase in vDNA synthesized and the 2-LTR circle-to-vDNA ratio showed a correspondingly modest increase compared to WT. Thus, the infectivity was rescued by the introduction of complementary charges at IN position 214 and 221.

Virion core morphology

IN mutants have been defined as either Class I or Class II.⁴⁹ Class I mutants directly impact the enzymatic reactions of IN, while Class II mutants reduce viral infectivity at a stage other than integration. Several Class II mutants have been shown to yield virions with abnormal cores.⁴⁷ To further characterize the mutants described above, we imaged transfected cells by electron microscopy to determine whether the capsid cores of the virions they produced were properly formed (Fig. 9). Core morphologies varied within each sample. Therefore, cores of mature virions were qualitatively categorized as to whether they fit the aberrant Class II phenotype. The percentage of virions in each sample that fit this phenotype and the

total number of mature virions are indicated in Fig. 9. The cores of mutants that synthesized near-WT levels of vDNA resembled those of WT virions. However, all mutants affected in vDNA synthesis (<25% of WT level, including R269A/K273A, Q214C/Q221C, and Q214K/Q221E) had a larger proportion of virions that had an electron-dense mass near the viral membrane, thus fitting the class II phenotype, suggesting that these IN mutations have an impact on viral replication at a stage earlier than integration.

Discussion

The biochemical behavior of HIV-1 IN has precluded detailed structural studies of the full-length protein. While structures of one- and two-domain fragments of HIV-1 IN have been determined, the recent PFV intasome crystal structures provide the best insight into the architecture of the HIV-1 intasome. We used these PFV intasome structures to develop a homology model of HIV-1 IN that has several pronounced differences from the HIV-1 IN fragment structures. The behavior of the IN mutants presented here supports the proposed arrangement of the CTD, with the charged side chains of R269 and K273 interacting with phosphate groups on the hDNA backbone near the ST sites. Also, the proposed intermolecular interactions of the $\alpha 8$ helix are consistent with infectivity data for mutants harboring alanine, cysteine, and charged residues at positions 214 and 221. Together, these data suggest that the proposed model represents a significant advance in our understanding of the structural features of the HIV-1 intasome. It also provides targets for designing inhibitors of IN dimerization and hDNA binding and structural information for drug-resistant mutations.

Materials and Methods

Molecular modeling

All modeling was performed using MOE2009.10 (Chemical Computing Group, Montreal, Quebec). The methods used to generate the homology model of the full-length chain of HIV-1 IN have been described previously.²⁶ This full-length HIV-1 model chain was then superimposed on the outer subunit of the PFV IN crystal structure 3OY9.²¹ The superposition used the C $^{\alpha}$ atoms of residues spanning the DDE motif of each protein (residues 62–152 for HIV-1 and residues 128–221 for PFV). Figures were generated using this superposition with no energy minimization. Additionally, the hDNA structure from the PFV IN target capture complex (PDB ID: 3OS1²⁴) was positioned in our model and energy minimized to an RMS gradient of 0.01 using the AMBER99 force field and Born solvation.

Site-directed mutagenesis

Mutants were generated using QuikChange II mutagenesis kits (Agilent Technologies, Inc.) with primers designed according to the manufacturer's protocol (Integrated DNA Technologies, Inc.). The pSK-RT plasmid was used as a template, and the region between the SalI and SpeI sites was cloned into the appropriate vector plasmid (see the following sections).

Western blots

Pseudotyped virions were produced by transfecting 293T cells with both pNLN_goMIVR-E-HSA and pHCMV-g plasmids.⁵⁰ Supernatants were clarified by low-speed centrifugation (3000g), and then virions were pelleted by ultracentrifugation (35,000g) on a cushion of 20% sucrose in 1 x TNE buffer. Pellets were resuspended in 20 µL of 1 x RIPA buffer and stored at -20 °C. Viral p24 was quantified by ELISA (PerkinElmer, Waltham, MA). Samples were diluted with RIPA to 5 ng/µL and then mixed with an equal volume of loading buffer diluted to 2x with either water or TCEP Bond-Breaker Solution (Thermo Scientific). Samples were heated to 95 °C for 5 min and cooled, and 40 ng of p24 per well was loaded onto a 4–12% 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol gradient gel. Blots were visualized using the SuperSignal West Pico or Femto Substrates (Thermo Scientific) and a G:Box imaging system (Syngene, Cambridge, England).

Infectivity assays

Virions were generated as previously described,⁵¹ with the pNLN_goMIVR⁺ Env.LuC reporter vector used in place of pNLN_goMIVR-E-HSA. Viral p24 was quantified by ELISA, and samples were diluted to equal concentrations of p24 with media. On the day prior to infection, 4×10^3 cells/well were plated on 96-well plates (100 µL from a 4×10^4 cells/mL stock) and incubated at 37 °C overnight. Each well then received 100 µL of virus from transfection supernatants (WT, mutant, or mock) and plates were returned to the incubator for 48 h, at which time luciferase activity was measured using Steady-Lite Plus kits (PerkinElmer) and a microplate reader. Supernatant p24 was determined by ELISA (PerkinElmer), and luciferase activity was normalized for the amount of p24 in each transfection supernatant (see Fig. S5). Luciferase activity of WT virions was set to 100%, and infectivity of the mutants is reported as a percentage of WT activity. Values in figures and tables are the mean ($\pm$ standard deviation) of six infections from at least two independent experiments (at least 12 total infections).

qPCR assays

vDNA amounts were determined using primer and probe sets specific for late reverse transcription products (second strand transfer DNA) and the 2-LTR circle junction at 24 h post-infection. The methods and reagents used have been described previously.⁵⁰ Values from at least two independent infections were normalized such that the WT copy number per microgram of p24 was set to 100%. Reported values in Table 2 are the means of at least two independent infections with standard deviations in parentheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used:

HIV-1	human immunodeficiency virus type 1
IN	integrase
PFV	prototype foamy virus
CCD	catalytic core domain
vDNA	viral DNA
3'-P	3'-processing
hDNA	host DNA
ST	strand transfer
NTD	N-terminal domain
CTD	C-terminal domain
NED	N-terminal extension domain
PDB	Protein Data Bank
WT	wild type

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Fig. 1.

Comparison of full-length PFV IN and our full-length HIV-1 IN model. An inner subunit of the PFV intasome (PDB ID: 3OYA) is shown in gray ribbon and the full-length HIV-1 IN model is shown in blue ribbon. The CCD is at the top of each structure. A flexible loop in this domain, marked in these structures by an asterisk, is not usually resolved in high-resolution structural analysis of the domains of HIV-1 IN. Residues Q214 through Q221 are shown as a yellow ribbon in the HIV-1 IN model and indicate the proposed $\alpha 8$ helix that is discussed in the text. The CTD is located immediately below the CCD with residues R269 through K273 shown as a pink ribbon in the HIV-1 model. This region is also discussed in the text. The NTD is immediately below the CTD. The N-terminal extension domain found in PFV IN is not present in its HIV-1 counterpart.

Fig. 2.

Structure-based sequence alignment of retroviral CTDs. Five retroviral IN CTDs were superimposed based on their secondary structure. (a) Sequences of several retroviral IN CTD structures were compared, based on secondary structure, to the CTD of PFV IN. Amino acid identity (green boxes), similarity (blue boxes), and dissimilarity (pink boxes) are indicated according to a previously reported similarity scoring matrix.³³ (b) The CTD from our model (green) is superimposed on the CTD of the PFV IN CTD (PDB ID: 3OYA, shown in gray). Residues P238 through D288 from our model are shown, as are residues V318 through Q375 of the PFV IN structure. Mutations at PFV residue R362 have been shown to influence hDNA binding; this residue corresponds to K273 in our model. (c) The PFV CTD is shown as in (b), with the CTD from the HIV-1 CCD-CTD crystal structure (PDB ID: 1EX4) shown in cyan. Residues K219 through D270 of the 1EX4 structure are shown, and they adopt a fold similar to that seen in (b) for residues P238 through D288. However, in this HIV-1 IN

domain structure, residue K273 is not resolved and cannot be located near residue R362 of PFV IN.

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Fig. 3.

Positions of CCDs and CTDs in the PFV and HIV-1 IN crystal structures and the HIV-1 IN model. The PFV full-length IN crystal structure (PDB ID: 3OYA, gray), HIV-1 CCD-CTD crystal structure (PDB ID: 1EX4, orange, with residues 214–221 shown in green and residues 269–270 shown in cyan), and our model (blue, with residues 214–221 and 269–273 colored as in Fig. 1) include structurally similar CCDs, but the CTD of 1EX4 is in a different location relative to the CCD than it is in the PFV IN structure or the HIV-1 IN model. The CCD flexible loop is marked by an asterisk; this element is not resolved in the 1EX4 structure.

Fig. 4.

Arrangement of β -strands in the CTD. The linker helix and CTD of the 1EX4 crystal structure (left) differ from our model (right). In our model, the sequence corresponding to β 1 of the crystal structure (blue) is present near the C-terminal end of the α 8 helix. As a result, the sequences corresponding to β 2 (cyan), β 3 (green), β 4 (orange), and β 5 (red) are shifted toward the N-terminus of the structure. The space occupied by β 4 and β 5 strands in the 1EX4 crystal structure is occupied by the C-terminal tail of our model (residues 271 –288, shown in magenta).

Fig. 5.

Positions of residues previously implicated in DNA binding. A surface representation of one inner subunit (white) is shown with both vDNA mimics and hDNA presented as ribbons. The non-reactive and reactive strands of the *cis* DNA end are shown as red and pink ribbons, respectively. The non-reactive and reactive strands of the *trans* DNA end are shown as blue and cyan ribbons, respectively. The hDNA strand targeted by the *cis* DNA end is shown as a light green ribbon, and the strand targeted by the *trans* DNA end is shown as a dark green ribbon. Two wider views of IN with these DNAs are shown at the top, rotated approximately

180° with respect to one another. The surfaces of previously identified DNA-interacting residues are colored red (NTD residues), green (CCD residues), or blue (CTD residues). Each of the lower panels shows an isolated view of the specific residues.

Fig. 6.

Residues in the CTD contributing to hDNA binding. The HIV-1 IN model suggests that R269 and K273 interact with phosphates on the hDNA. The positions of the side chains of residues R269 and K273 from each of the inner INs (colored with gray and cyan carbon atoms and blue nitrogen atoms) are shown relative to the modeled hDNA. The phosphorus atoms of the phosphate groups at the two ST sites, where the vDNA will be inserted, are colored yellow. The R269 side chain is positioned 4.9 Å from the phosphate group two nucleotides 5' of each ST site. The side chain of K273 is 2.3 Å from the phosphate group two nucleotides away from the ST site on the complementary DNA strand. These contacts are in the minor groove on either side of the segment of the major groove where concerted integration occurs.

Fig. 7. Potential dimer contacts in the HIV-1 IN model. The full-length model was superimposed on the partially resolved outer subunit of the crystal structure. The coordinates of that superposed full-length subunit were then added to those of the inner subunit to generate a model of the dimer interface. No energy minimizations were done on this structure. The $\alpha 8$ helix lies very close to its counterpart in the other subunit. Residues Q214 (blue ribbon) and Q221 (red ribbon) are positioned such that intermolecular H-bonds could be present and would contribute to the stability of the head-to-head dimer. Due to van der Waals clashes, only the C β atoms of the Q214 and Q221 side chains are shown.

Fig. 8.

Dimerization of the Q214C and Q221C mutants of HIV-1 IN in virions. (a) Western blot analysis of Q214C, Q221C, and the Q214C/Q221C double mutant is shown. The same blot was probed with primary antibodies for IN, RT, and CA; this analysis was performed twice and a representative blot is shown. The upper bands in the IN panel show the disulfide-linked IN dimers, which are barely visible in lanes in which the sample was treated with TCEP. (b) Monomer and dimer band intensities were quantified for the non-reduced lanes. Total band intensity in each lane was normalized to 100%, and the percentage of that intensity arising from the dimer band is shown. Values represent the mean of two independent blots, with error bars indicating the standard deviation.

Fig. 9.

Electron microscopy of virions. The top panels show representative electron microscopy images of WT virions and the mutants assayed by qPCR, as listed in Table 2. A significant fraction of the mutant viruses that were able to synthesize full-length vDNA within 4-fold of WT (Q214A/Q221A, Q214E/Q221E, Q214K/Q221K, and Q214E/Q221K) contained conical cores that look similar to those in WT virions. In contrast, a much larger fraction of the mutant viruses that were unable to synthesize DNA showed core morphologies characteristic of Class II IN mutants. The graph illustrates the percentage of mature virions that showed the Class II phenotype. Columns are marked as follows: 1, WT; 2, R269A/K273A; 3, Q214A/Q221A; 4, Q214C/Q221C; 5, Q214E/Q221E; 6, Q214K/Q221K; 7, Q214E/Q221K; 8, Q214K/Q221E. The numbers of mature virions counted are indicated under each column.

Table 1.

Infectivity of alanine mutations at positions 269 and 273

IN	Relative (% WT)
WT	100 (8.5)
R269A	13.9 (1.0)
K273A	51.8 (14.4)
R269A/K273A	1.6 (2.1)
MOCK	<1 (—)

Each of the single mutations reduced the infectivity of the virus; the double mutant is severely restricted. Reported values represent the means from three independent infections with standard deviations in parentheses.

Table 2.

Relative vDNA synthesis and 2-LTR circle formation

IN	vDNA (% WT)	2-LTR circles (% WT)
WT	100 (0)	100 (0)
R269A/K273A	1.7 (1.4)	1090 (120)
Q214A/Q221A	290 (20)	130 (29)
Q214C/Q221C	2.1 (3.0)	130 (26)
Q214E/Q221E	32 (10)	32 (6)
Q214K/Q221K	120 (20)	150 (24)
Q214E/Q221K	280 (120)	900 (70)
Q214K/Q221E	0.1 (0.1)	84 (20)
D110E RT	0.4 (0.0)	0 (0)
Mock	0(0)	0 (0)

qPCR analysis was performed using primer and probe sets for both a late reverse transcription product and the 2-LTR circle junction. D110E denotes an RT active-site mutation that is used as an indicator of residual plasmid from the transfection. Mock infections were performed with supernatants from producer cells transfected with plasmid encoding the VSV-g surface protein but not the viral clone. Reported values represent the means from three independent infections with standard deviations in parentheses. For reference, the mean raw values with vectors harboring WT IN were approximately 55,600 copies of vDNA and 14 2-LTR circles per microgram of p24.

Table 3.

Effects of IN mutations at amino acid positions 214 and 221 on viral infectivity

IN		Relative infectivity (% WT)
214	221	
Q	Q	100 (3.3)
A	Q	90.5 (4.5)
Q	A	93.9 (5.4)
A	A	5.9 (1.6)
C	Q	61.7 (12.5)
Q	C	108 (5.5)
C	C	<1 (ND)
E	E	<1 (ND)
K	K	<1 (ND)
E	K	79.1 (14.6)
K	E	<1 (ND)
Mock		<1 (ND)

Infectivity of WT virions (glutamines at positions 214 and 221) was normalized to 100%. The relative infectivities represent luciferase activity in cells 48 h after infection with reporter vectors. Values are the means from three independent infections with standard deviations in parentheses.