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Structure-Based Mutational Analysis of the C-Terminal DNA-Binding Domain of Human Immunodeficiency Virus Type 1 Integrase: Critical Residues for Protein Oligomerization and DNA Binding

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The C-terminal domain of human immunodeficiency virus type 1 (HIV-1) integrase (IN) is a dimer that binds to DNA in a nonspecific manner. The structure of the minimal region required for DNA binding ($IN_{220–270}$) has been solved by nuclear magnetic resonance spectroscopy. The overall fold of the C-terminal domain of HIV-1 IN is similar to those of Src homology region 3 domains. Based on the structure of $IN_{220–270}$, we studied the role of 15 amino acid residues potentially involved in DNA binding and oligomerization by mutational analysis. We found that two amino acid residues, arginine 262 and leucine 234, contribute to DNA binding in the context of $IN_{220–270}$, as indicated by protein-DNA UV cross-link analysis. We also analyzed mutant proteins representing portions of the full-length IN protein. Amino acid substitution of residues located in the hydrophobic dimer interface, such as L241A and L242A, results in the loss of oligomerization of IN; consequently, the levels of 3′ processing, DNA strand transfer, and intramolecular disintegration are strongly reduced. These results suggest that dimerization of the C-terminal domain of IN is important for correct multimerization of IN.

Retroviral DNA integration is mediated by the viral integrase (IN) protein. This essential step in the retroviral life cycle can be subdivided into two steps: (i) in the cytoplasm of the infected cell IN cleaves two nucleotides from the 3′ viral DNA ends (3′ processing), and (ii) in the nucleus IN couples the 3′-recessed DNA ends to the host DNA (DNA strand transfer). The unpaired, 5′-overhanging dinucleotides of the viral DNA are removed from the integration intermediate, and the single-stranded gaps are repaired, most likely by cellular repair enzymes (reviewed in references 25, 36, and 65).

Both IN-mediated reactions, 3′ processing and DNA strand transfer, can be carried out in vitro with synthetic DNA oligonucleotide substrates which mimic the viral DNA ends, divalent metal ions, and purified recombinant IN protein. By use of these assays, the *cis* and *trans* requirements of retroviral DNA integration have been determined in great detail (for recent reviews, see references 2, 36, and 62). Concerning DNA requirements, it has been demonstrated that terminal nucleotides of the viral U5 and U3 DNA ends are important for IN activity and that catalysis is enhanced in the presence of frayed DNA ends (9, 54, 56, 64). Furthermore, it has been shown that human immunodeficiency virus type 1 (HIV-1) IN protein contains three functional domains (10, 61). The N-terminal domain harbors a conserved HHCC motif, and by virtue of these conserved histidine and cysteine residues it is able to bind to zinc (8, 10, 72). Zinc induces proper folding of the N terminus and promotes tetramerization of IN, which leads to enhanced catalytic activity (43, 72). The central, catalytic domain contains the three active-site residues, D64, D116, and E152 (DDE motif), which together form the catalytic triad of IN. Amino acid substitution of one of the three active-site residues abolishes the catalytic activity of the protein (15, 22, 39, 58). The DDE motif is highly conserved among retroviruses, retrotransposons, and some transposable elements (14, 24, 32, 39). Recently, it was shown that two lysine residues (K156 and K159) which are located in close proximity to the DDE motif are involved in viral DNA binding (30). In contrast to the other two IN domains, the C-terminal, DNA-binding domain does not show sequence homology with any known protein motifs, based on the primary amino acid sequence. It has been shown that the C terminus of IN binds to DNA in a nonspecific fashion (23, 37, 51, 61, 67). The minimal region required for DNA binding comprises residues 220 to 270 and is hereafter termed $IN_{220–270}$ (51). DNA binding of $IN_{220–270}$ occurs in an ion-independent fashion; by mutational analysis it has been shown that lysine 264 is involved in nonspecific DNA binding (51).

Furthermore, it became apparent that all three structures of isolated HIV IN domains are dimeric (16, 18, 19, 44). This finding is supported by the observation that the catalytic core and C-terminal domains of IN show self-association properties (1, 29). The active oligomeric organization of IN within a ternary complex with DNA and metal ions is not clear at present, and attempts to determine the three-dimensional structure of IN have been hampered by the poor solubility of the protein. However, precise domain definition and "brute-force" mutagenesis approaches (10, 31, 51, 61) have led to determination of the structures of all three IN domains by either X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy. The N-terminal domains of HIV-1 IN and HIV type 2 IN consist of a three-α-helix bundle stabilized by zinc binding of the HHCC motif (11, 19). The overall structure is similar to those of helix-turn-helix domains of DNA-binding proteins, such as the Trp repressor (42), the paired

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domain (70), and the Tc3 transposase (60). The structures of the catalytic domains of HIV-1 and avian sarcoma virus (ASV) IN have been solved by crystallography (5, 6, 16); for ASV IN, divalent cation binding to active-site residues has been reported (7). The structure of the catalytic core domain of IN shows similarities to those of other, functionally related polynucleotidyl transferases (reviewed in references 26, 52, and 71). The C-terminal, DNA-binding domain of HIV-1 IN is dimeric in solution, and the monomer of $IN_{220–270}$ consists of five β strands which form two antiparallel β sheets, as demonstrated by NMR spectroscopy (18, 44). The overall fold of $IN_{220–270}$ is surprisingly similar to those of Src homology region 3 (SH3) domains. SH3 domains are small (approximately 60 amino acids), monomeric modules which are involved in protein-protein interactions in signal transduction pathways (13, 50). Intermolecular interactions of SH3 domains (55, 69) and ligand binding to peptide substrates are well defined, based on structural information (reviewed in references 40 and 45).

Here, we focus on the functional activities of the C-terminal, DNA-binding domain of HIV-1 IN, which are DNA binding and dimerization. Based on the structure of $IN_{220–270}$ (18), we analyzed the role of several amino acid residues of this domain both in DNA binding of the isolated domain ($IN_{220–270}$) and in functional activities and oligomerization of the full-length protein. We found two amino acid residues to be involved in DNA binding. Additionally, we found that amino acid substitution in the hydrophobic dimer interface of the C-terminal domain of IN resulted in the loss of oligomerization of the full-length protein and in decreased functional activity. These experiments indicated that self-association of the C-terminal domain of IN is important for functional oligomerization.

MATERIALS AND METHODS

DNA techniques. An 890-bp *Nde*I fragment derived from plasmid pRP274 (64), which contains HIV-1 IN, was ligated to *Nde*I sites of expression vector pET15b (Novagen); the resulting plasmid, pRP1012, was used as a template for site-directed mutagenesis. Point mutations were introduced by an overlapping PCR approach (28, 56a). Full-length IN was amplified with the following primers: T7F, 5′-CGAAATTAATACGACTCACTATAGG-3′, and T7R, 5′-GCTAGTTATTGCTCAGCGGTGGATCCATC-3′. The resulting PCR fragments were gel purified, digested with *Nde*I, and subsequently cloned into expression vector pET15b. In order to clone the mutants in the context of $IN_{220–270}$, we used a protocol described previously (51).

Protein purification. Wild-type and mutant proteins were expressed in *Escherichia coli* BL21(DE3)/pLysS upon induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside.

Full-length IN and mutant derivatives were purified by affinity chromatography. The following protein purification steps were performed at 4°C. Cells were lysed in low-salt buffer containing 25 mM HEPES (pH 7.5), 0.1 mM EDTA, and 1 mM β-mercaptoethanol (β-me), sonicated, and then centrifuged at 15,000 × *g* for 45 min (SS34 rotor; Beckman). The pellet was resuspended in high-salt buffer (1 M NaCl, 25 mM HEPES [pH 7.5], 0.1 mM EDTA, 1 mM β-me) and then subjected to Dounce homogenization 10 times. The IN-containing extract was cleared by centrifugation (15,000 × *g* for 30 min), and imidazole (pH 8.0) was added to a final concentration of 5 mM. IN was bound batchwise to Ni-nitrilotriacetic acid beads (Qiagen) as described by the manufacturer. The column material was transferred to another column (Pharmacia; 9.5 by 1 cm) and washed once each with four column volumes of high-salt buffer containing 5 and 25 mM imidazole (pH 8.8), respectively, and 0.2% Tween 20. IN was eluted from the Ni affinity column with high-salt buffer supplemented with 200 mM imidazole (pH 8.0), and IN-containing fractions were dialyzed against high-salt buffer containing 10% glycerol and frozen at −80°C.

$IN_{220–270}$ and mutant derivatives were purified as reported earlier (51).

IN activity assays. For the 3′-processing and DNA strand transfer reactions, we used 1 μM HIV-1 IN or mutant proteins and DNA oligonucleotide substrates (28 bp) which mimic the HIV-1 U5 DNA termini, and reactions were carried out as described previously (61). Reaction products were separated on a denaturing 12% polyacrylamide gel and quantitated by phosphoimager analysis (FujiBas).

Inter- and intramolecular disintegrations were carried out as described previously with disintegration substrate IV5 (72). Reaction products were separated on a denaturing 20% polyacrylamide gel and quantitated by phosphoimager analysis.

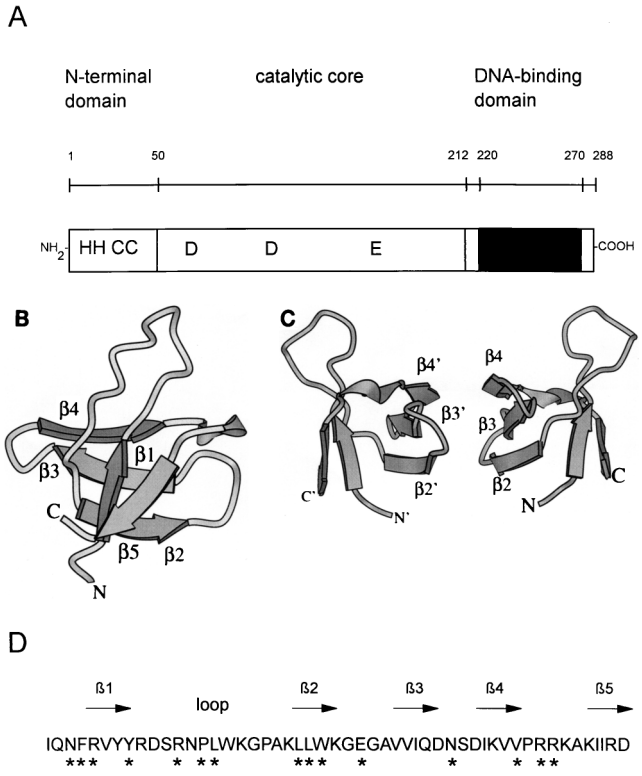

FIG. 1. Domain structure of HIV-1 IN. (A) The three functional domains of HIV-1 IN are indicated: N-terminal domain (residues 1 to 50), catalytic core region (50 to 212), and C-terminal, nonspecific DNA-binding domain (220 to 270; black box). Conserved histidine and cysteine residues in the N-terminal region (HHCC) and the catalytic triad (DDE) within the catalytic core are indicated. (B and C) Ribbon diagrams of HIV-1 $IN_{220–270}$ monomer (B) and $IN_{220–270}$ dimer (C) (18). Both figures were generated by use of the program MOLSCRIPT (38). (D) Amino acid sequence of $IN_{220–270}$. Secondary structural elements (β strands 1 to 5) are indicated above the sequence, as determined by data in panels B and C. Amino acids targeted by mutagenesis are marked by asterisks.

Protein-DNA UV cross-link analysis. Reaction mixtures for UV cross-link analysis contained a ^{32}P-labeled DNA substrate similar to that used in the 3′-processing reaction (10 nM) and 0.42 μM $IN_{220–270}$ in 25 mM HEPES (pH 7.5)–50 mM NaCl–0.5% Tween 20–1% glycerol–0.1 mM β-me in a final volume of 20 μl. Samples were preincubated for 15 min on ice prior to UV exposure and then irradiated on a Chromato-vue transilluminator at 254 nm on ice (50 Hz, 0.6 A; Ultraviolet Products Inc.). Reactions were stopped by the addition of 20 μl of protein loading dye, and the samples were boiled for 10 min and subsequently loaded on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel. Protein-DNA UV cross-link products were visualized by autoradiography and quantitated by phosphoimager analysis.

Gel filtration chromatography. All HIV-1 IN proteins were centrifuged for 30 min at 14,000 rpm prior to chromatography in order to remove IN aggregates. Size exclusion chromatography was performed on a fast protein liquid chromatography system at 4°C. The gel filtration columns were calibrated with molecular mass markers (see the legend to Fig. 4). Protein elution was monitored at A_{280}.

Typically, 100-μl samples of 30 μM full-length HIV-1 IN protein in high-salt buffer containing 10% glycerol were applied to a Superdex 200 HR 10/30 column (Pharmacia) at a flow rate of 0.25 ml/min.

RESULTS

Strategy. HIV-1 IN contains three functional domains, one of them being the C-terminal, nonspecific DNA-binding domain (Fig. 1). The three-dimensional structure of $IN_{220–270}$ has been solved by NMR spectroscopy (18, 44), and the structure revealed that $IN_{220–270}$ consists of five β strands which form two antiparallel β sheets (Fig. 1B). The homodimer of $IN_{220–270}$ is shown in Fig. 1C. Based on this structure (18), we

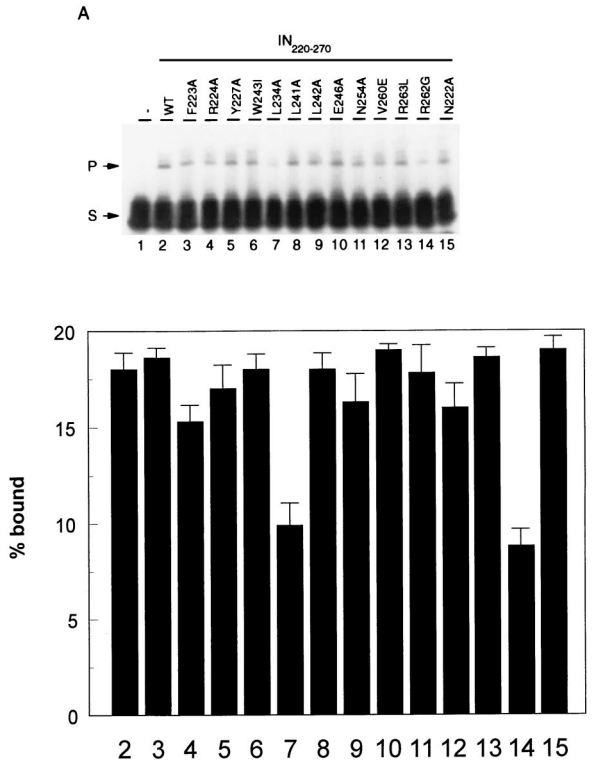

FIG. 2. DNA-binding activities of various mutants analyzed by protein-DNA UV cross-linking. (A) Amino acid substitutions in the context of the C-terminal, DNA-binding domain of HIV-1 IN ($IN_{220–270}$) are shown at the top of the panel. Lanes: 1, protein was omitted from the reaction; 2, wild-type $IN_{220–270}$ (WT); 3 to 15, various mutant proteins. Reactions were carried out as described in Materials and Methods and analyzed by SDS–15% polyacrylamide gel electrophoresis. S, DNA substrate; P, covalent protein-DNA UV cross-link product. (B) Quantitation of protein-DNA UV cross-link products relative to the DNA substrate (% bound). Lanes are as in panel A. Error bars show the deviation among three experiments.

introduced several amino acid substitutions, mainly to alanine (Fig. 1D). The mutant proteins, in the context of either $IN_{220–270}$ or the full-length protein, were analyzed for (i) in vitro activity, (ii) DNA binding, and (iii) oligomerization.

DNA-binding activity of $IN_{220–270}$. In order to study the effects of amino acid substitutions on DNA-binding ability, we introduced several mutations in the context of polypeptide $IN_{220–270}$ by site-directed mutagenesis. All mutant constructs were expressed in *E. coli*, and proteins were subsequently purified by means of three chromatographic steps as described earlier (see Materials and Methods and reference 51). Protein preparations were more than 95% homogeneous, as judged by Coomassie blue staining. The chromatographic behavior of the mutant proteins was found to be similar to that of wild-type $IN_{220–270}$, so we assume that the overall folding is similar. DNA-binding activity was subsequently tested by protein-DNA UV cross-linking. A radiolabeled oligonucleotide was incubated with mutant $IN_{220–270}$ and UV irradiated, and the products were analyzed by denaturing SDS-polyacrylamide gel electrophoresis (Fig. 2A).

In this DNA-binding assay, wild-type $IN_{220–270}$ bound to approximately 18% of the radiolabeled DNA substrate (Fig. 2, lanes 2). Most of the $IN_{220–270}$ mutant proteins had binding efficiencies similar to that of the wild-type polypeptide (Fig. 2B). Two mutants, however, had reduced DNA-binding activity. (i) In R262G, arginine 262 is located in a small, basic helical turn between β strands 4 and 5 (Fig. 1B). Amino acid substitution of arginine 262 with glycine decreased the DNA-binding activity of $IN_{220–270}$ by about 50% (Fig. 2A, lane 14). (ii) In L234A, leucine 234 is part of a long loop region which connects β strands 1 and 2. Compared to wild-type $IN_{220–270}$, L234A has reduced DNA-binding activity (Fig. 2A, lane 7). The results obtained when leucine 234 was substituted with alanine suggested that L234 interacts with DNA by hydrophobic interactions, whereas R262 may bind to DNA by electrostatic interactions.

3′ Processing and DNA strand transfer. Site-directed mutations were also introduced into full-length HIV-1 IN. The IN gene which harbored the desired mutation was cloned into expression vector pET15b, expressed in *E. coli*, and purified by affinity chromatography. Most of the mutant proteins did not display a remarkable increase in solubility compared to wild-type IN (data not shown).

We tested the in vitro activities of the mutant proteins by using DNA oligonucleotide substrates which represent the viral U5 termini. Reaction products were separated on denaturing polyacrylamide gels and quantitated (see Materials and Methods). The results are summarized in Table 1. Many mutant proteins displayed 3′-processing and DNA strand transfer activities similar to those of wild-type IN. Some other mutant proteins, however, showed reduced IN activities.

(i) R231A. Based on the structure of the DNA-binding domain of IN, it has been proposed that R231 is involved in DNA binding, since arginine 231 is largely solvent exposed (44). Unfortunately, mutant protein $IN_{220–270}$ R231A was found to be expressed at low levels and therefore was not tested for DNA binding. However, mutant protein R231A in the context of full-length IN had reduced (50%) 3′-processing and DNA strand transfer activities compared to wild-type activity (Table 1).

(ii) R262G. Due to the reduced DNA-binding activity of R262G (see above), mutant protein R262G had reduced (53%) 3′-processing activity compared to wild-type activity.

TABLE 1. In vitro activities of HIV-1 mutant proteins

Mutant	Location[a]	% Wild-type activity[b]			
		3′ Processing	Strand transfer	Disintegration	
				Intramolecular	Intermolecular
N222A		++	++	++	++
F223A	β1	++	++	++	++
R224A	β1	++	++	++	++
Y227A	Loop	++	++	++	++
R231A	Loop	++[c]	+	+	+
P233A	Loop	++	++	++	++
L234A	Loop	++	++	++	++
L241A	β2	−	−	−	++
L242A	β2	−	−	+	++
W243A	β2	++	++	++	++
E246A		++	++	++	++
N254A		++	++	++	++
V260E	β4	−	−	−	++
R262G		++[d]	++	+	++
R263L		++	++	++	++

[a] For example, β1 is β strand 1.
[b] ++, 50 to 100%; +, 10 to 50%; −, 0 to 10%. See also Fig. 3 and reference 56.
[c] Value was 51%.
[d] Value was 53%.

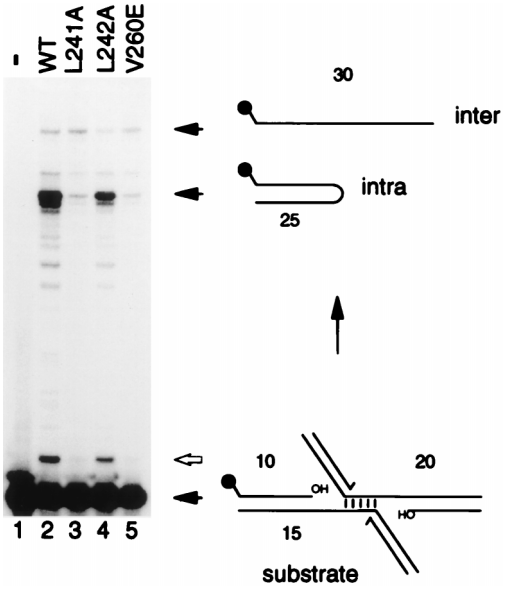

FIG. 3. Inter- and intramolecular disintegration activities of mutant proteins. The substrate of the disintegration reaction resembled an integration intermediate with viral U5 and U3 sequences and 5-bp complementary bases at the site of integration (for details, see reference 72). Intramolecular disintegration resulted in a hairpin loop which migrated at a position of 25 bp (intra) on a denaturing 24% polyacrylamide gel. Intermolecular disintegration resulted in the formation of a 30-bp product (inter). The open arrow indicates reintegration products characterized earlier (61). The circles mark the 5′ ^{32}P label of the reaction substrate and products. Lanes: 1, protein was omitted from the reaction; 2 to 5, disintegration activities of wild-type, L241A, L242A, and V260E proteins, respectively.

(iii) L234A. The other mutant protein with reduced DNA-binding activity, L234A, also had reduced 3′-processing activity (73% wild-type activity).

(iv) V260E. Mutant protein V260E was generated based on the observation that this mutant does not interact with IN in the yeast two-hybrid assay (35). Here we show that mutant protein V260E had greatly reduced 3′-processing and DNA strand transfer activities (8 and 11% wild-type activity, respectively).

(v) L241A and L242A. Leucine 241 and leucine 242 are located in β strand 2, which forms, together with β strands 3 and 4, an antiparallel β sheet. This three-stranded antiparallel β sheet forms the hydrophobic dimer interface of $IN_{220–270}$ (18) (Fig. 1C). Both mutant proteins had reduced 3′-processing activities (8 and 12% wild-type activity, respectively).

Disintegration. We analyzed whether the full-length IN mutant proteins were also able to catalyze the disintegration reaction (12). One might expect that amino acid residues in the C-terminal, DNA-binding domain are not directly involved in phosphoryl transfer itself and therefore that it is likely that mutations in the C terminus will not affect disintegration activity. Indeed, all mutants were able to perform the disintegration reaction (Table 1). Next, we analyzed whether the mutant proteins affected the ratio of inter- and intramolecular disintegration products (46, 56). The substrate of the disintegration reaction resembled the integration intermediate after DNA strand transfer and before the repair of single-stranded gaps (a schematic presentation is shown in Fig. 3). Full-length IN can catalyze the nucleophilic attack of the 3′-hydroxyl either at the phosphate group on the adjacent strand (intermolecular disintegration), which corresponds to the conventional disintegration reaction (12) or, alternatively, at a similar position but on the bottom strand, which leads to the formation of an intramolecular disintegration product (for more details, see the legend to Fig. 3 and reference 56).

Most of the mutant proteins showed ratios of inter- and intramolecular disintegration products similar to that of wild-type HIV-1 IN (Table 1). Mutants with different patterns are shown in Fig. 3. The predominant product of wild-type IN is the intramolecular disintegration product (Fig. 3, lane 2). For mutant L242A (Fig. 3, lane 4), both reaction products were reduced compared to the results for wild-type IN, but the ratio between inter- and intramolecular disintegration products was comparable. In contrast, mutants L241A and V260E (Fig. 3, lanes 3 and 5, respectively) showed strongly reduced intramolecular disintegration, whereas intermolecular disintegration was present at approximately wild-type levels.

The dimeric catalytic core of IN ($IN_{50–212}$) is able to carry out the conventional disintegration reaction (comparable to intermolecular disintegration) but not intramolecular disintegration. It is likely that intramolecular disintegration requires all three domains in a higher-order complex of IN (46, 56). We found that mutants L241A, L242A, and V260E had a phenotype similar to that described for a C-terminal deletion of IN (46). Therefore, we speculate that the functional oligomerization of IN might be partially disturbed by amino acid substitutions in residues L241, L242, and V260.

Oligomerization of IN. In order to test the hypothesis that point mutants L241A, L242A, and V260E were impaired for oligomerization of full-length IN, we investigated the oligomeric state of these mutants by size exclusion chromatography. Previously, it was shown that IN exists in an equilibrium among monomers, dimers, and tetramers and that the oligomerization of IN is concentration dependent (29, 57). For gel filtration chromatography, we chose protein concentrations at which IN exists in an equilibrium between dimers and tetramers (see Materials and Methods).

Wild-type and mutant IN proteins were loaded on a Superdex 200 gel filtration column, and the absorbance was monitored by UV. Wild-type IN eluted at positions which corresponded to IN dimers and tetramers relative to molecular mass standards (Fig. 4C). Eluted protein samples (wild-type IN) were analyzed on a denaturing polyacrylamide gel at various retention times (Fig. 4D). Mutant protein V260E was mainly misfolded (data not shown). Next, we analyzed the elution profiles of L241A and L242A. For mutant protein L242A the IN dimer-tetramer ratio was shifted to the dimer position (Fig. 4B) compared to that for wild-type IN (Fig. 4C). More strikingly, mutant protein L241A eluted mainly at the position of dimeric IN and less at the position of tetrameric IN (Fig. 4A). We observed that this effect was concentration dependent. High concentrations of L241A increased the amount of tetramer but also gave rise to aggregation of IN (data not shown).

Taken together, mutations L241A and L242A in the C-terminal, DNA-binding domain of IN resulted in a loss of oligomerization ability in the context of full-length IN, especially for L241A. This result correlated with the in vitro activities of the L241 and L242 mutant proteins (described above). We conclude that point mutation L241A disturbs hydrophobic interactions in the C terminus of IN and thereby tetramerization of the protein which, in turn, explains the loss of functional activities, such as 3′ processing, DNA strand transfer, and intramolecular disintegration.

DISCUSSION

DNA binding. In vivo, IN is able to discriminate between specific viral DNA and nonspecific target DNA. In vitro, how-

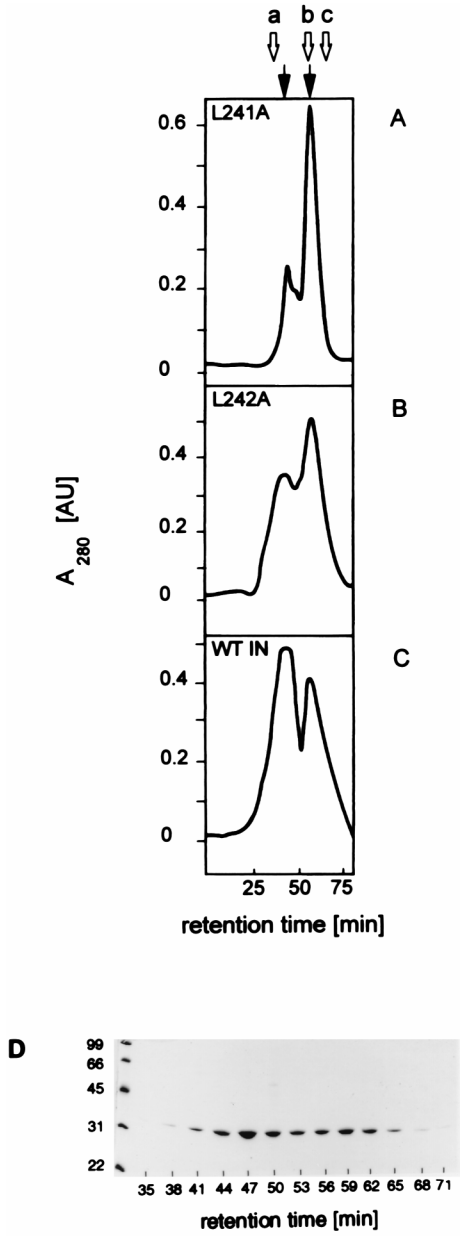

FIG. 4. Size exclusion chromatography of HIV-1 proteins. Elution profiles of wild-type (C) and mutant proteins L241A and L242A (A and B, respectively). The retention time in minutes is indicated on the *x* axis, and the A_{280} in arbitrary units (AU) is indicated on the *y* axis. Molecular mass markers for size exclusion chromatography are indicated by open arrows above panel A: a, aldolase, 158 kDa, retention time, 40 min; b, bovine serum albumin, 67 kDa, retention time, 54 min; c, chymotrypsin, 25 kDa, retention time, 66 min. The retention time of the IN tetramer is 47 min, and that of the IN dimer is 59 min (filled arrows). (D) IN samples analyzed by SDS–12.5% PAGE at various retention times. Molecular mass standards are indicated on the left in kilodaltons.

ever, binding of IN to either specific viral DNA or nonspecific target DNA occurs with similar efficiencies (41, 48, 53, 57, 66). Furthermore, it has been shown that metal ion-dependent IN-DNA assembly is required for specific in vitro activities (20, 63). Therefore, DNA-binding specificity may be determined by the nucleoprotein structure of the preintegration complex.

Two of the three functional domains of IN possess DNA-binding activity. The isolated N-terminal domain appears not to bind to DNA (48, 61, 66), although the overall fold is similar to those of helix-turn-helix domains which are involved in DNA binding (for a discussion, see references 11 and 19). The central, catalytic domain binds in an ion-dependent fashion to the disintegration substrate (23), and specific photo-cross-linking experiments indicated that lysine 156 and lysine 159 are involved in viral DNA binding (30). The third IN domain, the C-terminal domain, binds to DNA in a nonspecific manner (23, 37, 51, 61, 67). DNA binding occurs in an ion-dependent fashion, and the minimal region required for DNA interaction is located at amino acid residues 220–270 ($IN_{220–270}$) (51).

Residues within HIV-2 $IN_{220–270}$ that are important for nonspecific DNA binding reside in a stretch of basic amino acids (R262, R263, and K264), and substitution of lysine 264 by glutamate especially results in a strong reduction of DNA binding and IN activities (51). In the present study, we examined the role of several residues in DNA binding by site-directed mutagenesis, based on the dimeric structure of HIV-1 $IN_{220–270}$ (18). Amino acid residues were changed mainly to nonpolar amino acids, and the resulting effects of the mutant proteins on DNA binding was obviously biased by the choice of the substituted residue. Previously, it was speculated that R231 is involved in DNA binding by virtue of the dimeric saddle-shaped groove of $IN_{220–270}$ (44). We found that R231A showed reduced functional activity in the context of the full-length protein, but we could not distinguish whether this reduction was due to a loss of DNA binding or intra- or intermolecular protein interactions. We found, however, two $IN_{220–270}$ mutants which showed reduced DNA-binding activity. One had a mutation of arginine 262 to glycine (R262G). This amino acid residue lies in a small helical turn between β strands 4 and 5 (Fig. 1B), and this mutant protein displayed DNA-binding and 3′-processing activities similar to those of HIV type 2 IN R262D (51). A similar basic RRK motif has been identified in domain III of MuA transposase; moreover, it has been shown that this 26-amino-acid polypeptide has nonspecific DNA-binding and nucleolytic activities (68). Unlike domain III of MuA transposase, HIV-1 $IN_{220–270}$ does not exhibit this nucleolytic activity (data not shown). The other mutant which showed reduced DNA-binding properties had a mutation of leucine 234 to alanine (L234A). L234 is located in a long loop between β strands 1 and 2 (Fig. 1B). The types of DNA interaction of R262 and L234 might be different. Reduction of DNA binding due to substitution of leucine by alanine at position 234 suggests that L234 interacts with DNA via hydrophobic interactions, whereas R262 and K264 may interact with DNA by electrostatic interactions.

Comparison of the HIV-1 $IN_{220–270}$ fold with SH3 folds. The overall fold of HIV-1 $IN_{220–270}$ is similar to those of SH3 domains (44, 51). Other DNA-binding proteins which have an SH3-like fold are the Sso7d and Sac7d proteins of the archaebacteria *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*, respectively (3, 4, 17). The function of these small, basic, histone-like proteins is not known. Sso7d and Sac7d are monomeric, and NMR experiments suggest that the three-stranded β sheet of Sso7d interacts with the DNA major groove (3).

SH3 domains are involved in signal transduction pathways in eukaryotes, and the molecular bases for both intramolecular interactions of Src-tyrosine kinases (55, 69) and ligand interactions with proline-rich peptides have been determined by X-ray crystallography and NMR spectroscopy (reviewed in references 40 and 45). SH3 domains are usually monomeric, and the ligands are proline-rich peptide sequences which harbor a so-called PxxP consensus motif. For the c-Abl and Fyn SH3 domains, the peptide ligands bind mainly by hydrophobic interactions (49). Only two residues of the SH3 domains, asparagine 58 (in c-Abl SH3) and tryptophan 41, form hydrogen bonds

with their ligands; these residues also show van der Waals interactions (49). Interestingly, asparagine 58 in c-Abl SH3 is located between β strands 4 and 5 (distal loop), a position similar to that of the RRK motif of HIV-1 IN (R262, R263, and K264; see above). The second amino acid in $IN_{220–270}$ which appeared to be involved in DNA binding, leucine 234, is located in a long loop between β strands 1 and 2. The homologous region of the SH3 domains is the so-called RT loop, which also interacts specifically with the peptide ligands.

Whether the ligand interface of SH3 domains and the dimeric C-terminal, DNA-binding domain of IN are the same is not clear at present, since no structural information is available for $IN_{220–270}$ in complex with DNA. As indicated in a previous study (51) and the present study, some protein-ligand interactions might be similar, as in the cases of R262, K264, and L234. On the other hand, tryptophan 41 in the c-Abl SH3 domain is clearly involved in peptide binding, whereas valine 249 at a similar position in $IN_{220–270}$ is part of the three-stranded antiparallel β sheet which forms the hydrophobic dimer interface of $IN_{220–270}$. Further elucidation of the protein-DNA interface of $IN_{220–270}$ will require more biochemical and structural data.

Taken together, these data indicate that a common fold, such as the SH3 fold, can serve different biological functions, DNA binding in the case of the C-terminal domain of HIV-1 IN, Sso7d, and Sac7d and protein-protein interactions in the case of SH3 domains of tyrosine kinases.

Multimerization of IN and functional consequences. So far, it is apparent that all three isolated domains of IN have self-association properties. Size exclusion experiments indicated that $IN_{1–55}$ is dimeric (19), and an NMR analysis revealed that helix 3 of HIV-1 $IN_{1–55}$ is involved in dimerization (11). The central, catalytic domains of HIV-1 IN and ASV IN are also dimeric, as indicated by both biophysical analysis and X-ray crystallography (1, 6, 16, 27). The C-terminal, DNA-binding domain of IN also harbors a multimerization interface (1, 29). The solution structure of $IN_{220–270}$ revealed that the dimer interface is formed by the three-stranded antiparallel β sheet (Fig. 1C) (18, 44). The dimer interface is predominantly formed by hydrophobic interactions which involve β strands 2, 3, and 4. Protein-mixing experiments with unlabeled and ^{13}C- and ^{15}N-labeled $IN_{220–270}$ unambiguously identified hydrophobic residues L241, L242, W243, and I257 as being involved in intersubunit contacts (18).

In this study, we examined whether the dimer interface within the C-terminal domain of IN is functionally important for IN activity. We analyzed the functional activities and oligomeric state of several point mutants. Mutant protein V260E was mainly misfolded, as indicated by size exclusion chromatography, and consequently was not active in functional IN assays (Table 1 and Fig. 3). Mutation V260E in the context of $IN_{220–270}$, however, resulted in DNA binding at wild-type levels. This result is consistent with previous results showing that mutant protein V260E does not display intermolecular interactions, whereas the C terminus of IN is dispensable for IN-IN interactions, based on a transcriptional reporter assay in yeast (34, 35). We speculate that amino acid substitution of valine 260 by glutamate may disturb intermolecular interactions with other IN domains or the folding pathway of IN in general.

Previously, it was shown that wild-type IN exists in an equilibrium between dimers and tetramers (29). We found that two dimer interface mutant proteins, L241 and L242, are impaired in the tetramerization of IN (Fig. 4). Mutant protein L242A has an intermediate effect in size exclusion chromatography. The equilibrium between dimers and tetramers is modestly shifted, compared to that in wild-type IN. Amino acid substitution L241, in contrast, results in a more severe phenotype. Tetramerization of this mutant protein is largely impaired (Fig. 4). Functional activities of L241A and L242A reflect the above observations. Whereas L242A is able to perform the intramolecular disintegration reaction, this IN activity in L241A is highly reduced. In view of this result, mutation L241A results in a phenotype similar to that caused by a C-terminal deletion in IN (46). In summary, we found that amino acid substitution of leucine by alanine at position 241 within the dimer interface of the C-terminal domain of IN disturbs the tetramerization of IN and thereby strongly reduces the 3′-processing and strand transfer activities of IN. These results indicate that the dimer interface of the C-terminal domain of IN is important for the functional multimerization of IN.

What is the active oligomeric state of IN? Complementation studies (21, 59), biophysical experiments (1, 29, 33), and genetic approaches (34) have demonstrated that IN is a multimer (reviewed in references 2 and 62). The oligomeric state of IN and the precise stoichiometry within the ternary complex with DNA and metal ions are, however, not clear at present. Several lines of evidence suggest that at least a tetramer of IN is the active multimeric organization: (i) zinc stimulates the tetramerization of IN and thereby increases activity (43, 72), (ii) disturbance of the dimer interface of the C-terminal domain results in a reduction of tetramer formation and subsequently in decreased IN activity (this study), and (iii) the functionally related MuA transposase is active as a tetramer in both 3′ processing and DNA strand transfer (reviewed in reference 72 and references therein). Taking into account the facts that the stereochemical course of DNA cleavage and DNA strand transfer of MuA proceeds via a mechanism similar to that in IN (recently reviewed in reference 47) and, in addition, that both catalytic domains share a fold common to polynucleotidyl transferases, we believe that it is likely that IN and MuA have similar multimeric organizations.

Formally, however, we cannot exclude the possibility that the multimeric organization of IN is higher than tetrameric (e.g., octameric). In order to dissect subsequent steps of IN action, such as intra- and intermolecular protein interactions, metal ion binding, and DNA binding, more high-resolution structures of IN in a ternary complex with metal ions and DNA are required. Meanwhile, biochemical studies, such as those reported here, may complement the presently known structures of IN in order to determine the ligand binding site and to model an oligomeric complex of IN.

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