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Reversion of a Human Immunodeficiency Virus Type 1 Integrase Mutant at a Second Site Restores Enzyme Function and Virus Infectivity

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The integration of a DNA copy of the retroviral RNA genome into the host cell genome is essential for viral replication. The virion-associated integrase protein, encoded by the 3′ end of the viral *pol* gene, is required for integration. Stable virus-producing T-cell lines were established for replication-defective human immunodeficiency virus type 1 carrying single amino acid substitutions at conserved residues in the catalytic domain of integrase. Phenotypically reverted virus was detected 12 weeks after transfection with the integrase mutant carrying the P-109→S mutation (P109S). Unlike the defective P109S virus, the revertant virus (designated P109S^R) grew in CD4$^+$ SupT1 cells. In addition to the Ser substitution at Pro-109, P109S^R had a second substitution of Ala for Thr at position 125 in integrase. Site-directed mutagenesis was used to show that the P109S T125A genotype was responsible for the P109S^R replication phenotype. The T125A substitution also rescued the in vitro enzyme activities of recombinant P109S integrase protein. P109S integrase did not display detectable 3′ processing or DNA strand transfer activity, although 5 to 10% of wild-type disintegration activity was detected. P109S T125A integrase displayed nearly wild-type levels of 3′ processing, DNA strand transfer, and disintegration activities, confirming that T125A is a second-site intragenic suppressor of P109S. P109S integrase ran as a large aggregate on a size exclusion column, whereas wild-type integrase ran as a monomer and P109S T125A integrase ran as a mixed population. Pro-109 and Thr-125 are not immediately adjacent in the crystal structure of the integrase catalytic domain. We suggest that the T125A substitution restores integrase function by stabilizing a structural alteration(s) induced by the P109S mutation.

Integration of a double-stranded DNA copy of the viral RNA into the host cell genome is a crucial step in a productive retroviral infection (35). Genetic studies have shown that two regions of the viral genome are required for integration: the long terminal repeat (LTR) ends of linear viral DNA (10, 47, 49) and the 3′ region of the *pol* gene, which encodes the integrase protein (14, 50, 52, 57). Integrase cleaves the LTRs at conserved CA dinucleotides within U3 and U5 (3′ processing reaction) (2, 54) and subsequently joins the recessed viral ends to the 5′ phosphates of a staggered double-strand cut in chromosomal target DNA (strand transfer reaction) (2, 25). Both 3′ processing and DNA strand transfer activities are carried out in vitro by purified integrase proteins using duplex oligonucleotide substrates that mimic the ends of the viral LTR (4, 11, 34, 36, 58, 63, 68). In addition, purified integrases can catalyze an apparent reversal of the DNA strand transfer reaction, a process termed disintegration (9).

Comparison of a number of retroviral integrases, retrotransposases, and bacterial transposases identified two highly conserved amino acid sequence motifs, HHCC and D,D(35)E (32, 37, 39), important for polynucleotidyl transferase activity. Human immunodeficiency virus type 1 (HIV-1) integrase proteins lacking the amino-terminal HHCC motif are defective for 3′ processing and DNA strand transfer activities but display appreciable levels of disintegration activity (5, 66, 67). The most highly conserved region of retroviral integrases is the central part of the proteins containing the D,D(35)E motif in which the invariable Asp residues are located at positions 64 and 116, and the Glu residue is at position 152 (39). HIV-1 proteins carrying certain single amino acid substitutions of the Asp and Glu residues are defective for 3′ processing, DNA strand transfer, and disintegration activities as assessed by conventional oligonucleotide assays (20, 42, 64). Strand transfer activities of HHCC deletion mutant proteins and D,D(35)E point mutant proteins, however, can be detected by PCR (26).

The crystal structures of the catalytically active core domains of HIV-1 and avian sarcoma virus integrases have revealed a five-stranded β sheet flanked by helical regions (3, 17). The folding topology is conserved in other polynucleotidyl transferases, including bacteriophage MuA transposase (53), RNase H, and the Holliday junction-resolving enzyme RuvC (17). The active-site region in the HIV-1 structure is identified by the positions of the conserved aspartate residues of the D,D(35)E sequence motif. Active-site aspartates are located at similar positions in all members of the polynucleotidyl transferase superfamily. The active-site residues bind and coordinate divalent metal ions for catalysis (17).

In addition to analyses of recombinant integrase function in in vitro biochemical assays, amino acid substitutions of conserved residues in the catalytic domain have also been assessed for their effects on HIV-1 replication in tissue culture cells (1, 6, 22, 40, 41, 44, 59, 61, 69). The results indicate the occurrence of defects in integration, reverse transcription, and virion particle assembly, suggesting that integrase mutations can interfere with a variety of steps in the early and late stages of the viral replication cycle.

To further investigate the integration process in HIV-1-infected cells, we have established T-cell lines chronically producing replication-defective viruses containing single amino

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acid substitutions in the catalytic domain of integrase (59, 61). In this report we describe the isolation and characterization of a phenotypically reverted virus which appeared spontaneously in the supernatant of cells stably producing the P-109→S (P109S) integrase mutant. We previously described that the P109S virus was replication defective as a consequence of a block in integration (61). The P109S substitution had no discernible effects on viral protein synthesis and processing, virion morphology, and viral DNA synthesis. This amino acid substitution was shown by others to abolish DNA binding (15) and to reduce the 3′ processing and DNA strand transfer activities (16) of recombinant HIV-1 integrase in in vitro biochemical assays. The P109S revertant virus replicated normally in $CD4^+$ human SupT1 T cells. The reversion event was identified as a second-site suppressor mutation that restored in vitro enzyme activity and in vivo viral infectivity to the P109S integrase-defective mutant.

MATERIALS AND METHODS

Cell culture, transfection, and infection. Human SupT1 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum. Cells (5×10^6) were cotransfected with 5 μg of integrase-defective HIV-1 proviral DNA and 0.5 μg of pBS-Neo (kindly provided by Klaus Uberla, University of Erlangen-Nurnberg, Erlangen, Germany) by electroporation. G418 (0.8 mg/ml) was added to the cells 48 h after transfection, and a G418-resistant population was selected 3 weeks later. Singly pBS-Neo-transfected cells were cleared from the culture via syncytium formation with doubly transfected cells, resulting in a G418-resistant culture in which most, if not all, cells were stably expressing the mutant HIV-1 provirus.

COS-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells (2×10^6) were seeded in 10-cm-diameter plates 24 h before transfection. Cells were transfected with 10 μg of proviral DNA by using DEAE-dextran (55). Virus production was evaluated by measurement of reverse transcriptase (RT) activity 48 h posttransfection as previously described (61). SupT1 cells (10^6) were infected with cell-free virus equivalent to 10^5 cpm of RT activity. Virus production was monitored by measuring the culture supernatant RT activity every third day.

DNA analysis. Total DNA was prepared from stably transfected and freshly infected SupT1 cells by standard techniques (55). DNAs were subjected to PCR with primers P1 (nucleotides 4137 to 4157 of HXBc2 HIV-1 [46]) and Art5 (nucleotides 5174 to 5151), which were designed to amplify a 1,037-bp fragment encompassing the entire integrase-coding region. PCR mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.15 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.6 μg of each primer, 1 μg of total DNA, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) in a volume of 100 μl. Reaction mixtures were kept at 94°C for 4 min and then subjected to 35 cycles of amplification. Each cycle consisted of 1 min of denaturation (94°C), 2 min of annealing (55°C), and 3 min of extension (72°C). The PCR products were purified following agarose gel electrophoresis and cloned into TA3 pCR II vector DNA under conditions specified by the manufacturer (Invitrogen, San Diego, Calif.). At least four clones from each PCR were sequenced by the chain termination method (56).

Construction of mutant HIV-1. Overlapping PCR was used to introduce site-specific mutations in the 3′ region of the *pol* gene in pHXB-SV, which contains infectious HXBc2 proviral DNA and a simian virus 40 origin of replication for episomal propagation in COS-1 cells (13). The T125A mutation was incorporated into the 1.8-kb *Pfl*MI restriction site fragment (bases 3486 to 5297), essentially as previously described (22, 29). Wild-type and P109S plasmids were used as templates to generate half fragments, each containing a *Pfl*MI site near one end and the T125A mutation near the other end. Full-length fragments were generated in a second PCR with the overlapping halves as the template and the two *Pfl*MI restriction site primers. The reaction mixtures contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.2 mM each dNTP, 0.5 μg of DNA template, 0.6 μg of each primer, and 2 U of Vent DNA polymerase (New England Biolabs, Beverly, Mass.). The reaction mixtures were subjected to 15 cycles of denaturation at 94°C (1 min), annealing at 58°C (1.5 min), and extension at 72°C (2.5 min). The full-length fragments were digested with *Pfl*MI and then ligated with *Pfl*MI-digested pHXB-SV DNA. The presence of the mutations was confirmed by DNA sequencing.

Expression and purification of recombinant integrase. The mutant integrase-coding regions were amplified by PCR as previously described (5) with the different proviral plasmids as templates. One primer (5′-ATACATATGTTTTT AGATGGA-3′) contained an *Nde*I restriction site (underlined) and an ATG codon for initiation of translation in *Escherichia coli*, and the other primer (5′-TGTGGATCCTAATCCTCATCC-3′) contained a *Bam*HI restriction site (underlined) and a TAG termination codon. The amplified products were cloned into the T7 expression vector pET-15b (Novagen, Madison, Wis.), which results in expression of integrase fused to an amino-terminal affinity tag with six adjacent histidine residues. The His tag facilitates purification by metal-chelating affinity chromatography (62). The PCR-amplified regions of the plasmids were analyzed by DNA sequencing.

Plasmids were transformed into *E. coli* BL21(DE3) (60), and cells were grown at 37°C in Terrific broth (21) containing 100 μg of ampicillin per ml until the optical density at 600 nm was 0.8 to 1. Protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM, and the culture was grown for an additional 3 h. The cells were harvested and resuspended in 35 ml of ice-cold 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethansulfonic acid (HEPES) (pH 7.6)–0.1 mM EDTA, frozen in liquid nitrogen, and stored at −80°C. The remainder of the preparation was done at 4°C or on ice. The cells were thawed overnight and resuspended in 35 ml of lysis buffer (20 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 2 mM β-mercaptoethanol [β-ME], 0.1 mM EDTA, 5 mM imidazole, 0.2 mg of lysozyme per ml). After 30 min, the cells were subjected to six cycles of sonication for 25 s at 100 W, allowing 4 min for cooling between each cycle. The lysate was centrifuged at 40,000 × *g* for 35 min, and the supernatant (fraction I) was saved. The pellet was resuspended by homogenization in 35 ml of buffer A (20 mM Tris-HCl [pH 8.0], 1.5 M NaCl, 2 mM β-ME, 0.1 mM EDTA, 5 mM imidazole). The suspension was centrifuged at 40,000 × *g* for 35 min, and the supernatant (fraction II) was saved. The pellet was resuspended by homogenization in 25 ml of buffer B (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 6 M guanidine-HCl, 2 mM β-ME, 0.1 mM EDTA, 5 mM imidazole). The suspension was stirred slowly for 30 min and centrifuged at 40,000 × *g* for 35 min, and the supernatant (fraction III) was saved. Integrase-containing fractions were identified following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

(i) Purification under native conditions. A 2-ml Chelating Sepharose Fast Flow (Pharmacia Biotech, Piscataway, N.J.) column was precharged with 50 mM $NiSO_4 \cdot 6H_2O$ as previously described (12) and then equilibrated with buffer A. Fraction II was loaded, and the column was washed with approximately 500 ml of buffer A and then with about 500 ml of buffer A containing 60 mM imidazole. The protein was eluted with a linear gradient of 60 mM to 1 M imidazole in buffer A. Integrase-containing fractions were pooled, and EDTA was added to a final concentration of 5 mM. The protein was dialyzed against 20 mM HEPES (pH 7.6)–1 M NaCl–2 mM EDTA–2 mM β-ME–0.3 M imidazole–10% (wt/vol) glycerol. The His tag was removed from the amino terminus of integrase by treatment with human thrombin (Sigma, St. Louis, Mo.). Four vector-derived amino acids, Gly-Ser-His-Met, remain after cleavage. Reaction mixtures (30 National Institutes of Health units per mg of fusion protein) were incubated at 26°C for 40 min, the concentration of thrombin was doubled, and the incubation was continued for another 40 min. The extent of cleavage was monitored by SDS-PAGE. Thrombin was removed by adsorption to a Benzamidine Sepharose 6B (Pharmacia Biotech) column. Cleaved protein was dialyzed against 20 mM HEPES (pH 7.6)–1 mM EDTA–1 M NaCl–10% glycerol–1 mM dithiothreitol (DTT) and centrifuged at 19,000 × *g* for 10 min. The supernatant, which contained soluble integrase, was frozen in liquid nitrogen and stored at −80°C.

Wild-type integrase from the NL4-3 strain of HIV-1 was expressed and purified as previously described (12, 58). The D116N (19, 20) and 50-288 (5, 19) mutant integrase proteins were purified under native and denaturing conditions, respectively, as previously described.

(ii) Purification under denaturing conditions. Fraction III was loaded over a precharged 2-ml Ni^{2+} chelating column equilibrated with buffer B. The column was washed with approximately 500 ml of buffer B and then with about 500 ml of buffer B containing 20 mM imidazole. Protein was eluted with a linear gradient of 20 to 600 mM imidazole in buffer B. Integrase-containing fractions were pooled, and EDTA was added to a final concentration of 5 mM. The pooled fractions were dialyzed against 6 M guanidine-HCl–20 mM HEPES (pH 7.6)–2 mM EDTA–2 mM β-ME. The protein concentration was adjusted to 1 mg/ml in dialysis buffer, and then the mixture was diluted with an equal volume of buffer containing 1 M NaCl, 20 mM HEPES (pH 7.6), 2 mM EDTA, and 2 mM β-ME. The protein was sequentially dialyzed against three buffers which were progressively less denaturing: (i) 20 mM HEPES (pH 7.6)–2 M urea–0.5 M NaCl–5 mM EDTA–10 mM β-ME–10 mM [(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), (ii) 20 mM HEPES (pH 7.6)–1 M NaCl–1 mM EDTA–1 mM DTT–10 mM CHAPS–10% glycerol, and (iii) 20 mM HEPES (pH 7.6)–0.5 M NaCl–1 mM EDTA–1 mM DTT–10 mM CHAPS–10% glycerol (buffer C). The dialysate was centrifuged at 19,000 × *g* for 10 min, and the supernatant containing soluble refolded integrase was treated with thrombin as described above. The flowthrough from the Benzamidine Sepharose column was dialyzed against buffer C and then centrifuged at 19,000 × *g* for 10 min. The P109S T125A and P109S proteins, which were recovered at 0.4 and 0.25 mg/ml, respectively, were frozen in liquid nitrogen and stored at −80°C.

Integrase assays. The DNA substrate for assaying 3′ processing activity corresponded to the terminal 30 bp of the U5 end of HIV-1 LTR. Substrate DNA was prepared by labeling the 5′ end of a single-stranded oligonucleotide (AE144) with ^{32}P and then annealing the complementary single strand (AE143), as previously described (21). The preprocessed substrate for assaying strand transfer activity was prepared by labeling AE155 (5′-TTTTAGTCAGTGTGGAAAAT CTCTAGCA-3′) at the 5′ end and annealing AE143. The substrate for measuring disintegration activity, the Y oligomer, was prepared by labeling AE157 and annealing AE146, AE117, and AE156, as previously described (20). Labeled

substrates were separated from unincorporated nucleotides as previously described (21).

Integrase reaction mixtures (16 μl) contained 25 mM morpholinepropanesulfonic acid (MOPS) (pH 7.2), 0.1 mg of bovine serum albumin per ml, 10 mM β-ME, 10% glycerol, 25 nM labeled DNA substrate, 0.7 mM CHAPS, 63 mM NaCl, 7.5 mM $MnCl_2$, and the indicated integrase protein at either 0.25 or 0.5 μM. The in vitro complementation reactions were identical except for the concentrations of CHAPS (1.4 mM), NaCl (36 mM), and integrase (0.4 μM). Mutant proteins (7.7 μM) were premixed in protein storage buffer and then added to reaction mixtures such that the total concentration of integrase was 0.4 μM.

Reactions were terminated after 60 min at 37°C, and the products were analyzed by electrophoresis on 15% polyacrylamide–urea gels as previously described (12). The results were visualized by autoradiography and quantitated by densitometry (IS-1000 Digital Imaging System; Alpha Innotech Corp., San Leandro, Calif.).

Gel filtration chromatography. Proteins were dialyzed against buffer C and centrifuged at 19,000 × *g* for 10 min prior to size exclusion chromatography. Estimates of molecular mass were based on retention times with a 24-ml Superdex 200 HR 10/30 column (Pharmacia Biotech) and a Pharmacia fast protein liquid chromatograph at 4°C. The column was calibrated at 0.5 ml/min in buffer containing 20 mM HEPES (pH 7.6), 0.5 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM CHAPS, and 10% glycerol with molecular mass standards (Bio-Rad, Hercules, Calif.). Integrase proteins (0.25 mg/ml) were injected (200 μl) and analyzed under the same conditions.

RESULTS

Isolation of a revertant virus. Stable expression of integrase-defective viruses was achieved in SupT1 cells by cotransfecting integrase mutant proviruses and a selectable marker. The cells were passaged repeatedly, and the culture medium was collected and tested for the presence of replication-competent virus by infecting fresh SupT1 cells and measuring the RT activity of the infected-cell supernatants. Following the initial release of virus displaying the expected replication-defective phenotype, infectious virus appeared after 12 weeks in the supernatant of cells stably transfected with the P109S integrase mutant. The revertant virus, which we refer to as $P109S^R$, exhibited a 7-day delay in peak virus production compared with wild-type HIV-1 (Fig. 1A). $P109S^R$ was analyzed to identify the genetic basis for the reversion.

Identification of a second-site suppressor mutation. Total DNA extracted from the G418-resistant cell line, as well as from SupT1 cells freshly infected with $P109S^R$, was analyzed by DNA sequencing, as described in Materials and Methods. Eight of eight sequenced clones contained the original P109S mutation in the 3′ end of the *pol* gene. A second, single A-to-G base change was identified in each clone at position 4601 (codon ACG to GCG), resulting in a predicted substitution of Ala for Thr at position 125 in the integrase protein (T125A). No other mutations were identified in the integrase-coding regions of these eight clones.

To directly test if this second mutation was responsible for the revertant phenotype, the T125A change was introduced into wild-type and P109S proviral DNAs by site-directed mutagenesis. The plasmids were transfected into COS-1 cells, and the replication potentials of the COS-1 cell supernatants were assessed by infecting SupT1 cells. Parallel cultures of SupT1 cells were infected with equivalent RT counts per minute of wild-type and mutant viruses. The T125A substitution had no discernible effect on HIV-1 replication, as the growth curve of this virus was indistinguishable from that of the wild type (Fig. 1B). The parental P109S virus showed no sign of replication over the 21-day observation period (Fig. 1B) (61). The virus containing both the P109S and T125A changes was able to replicate in SupT1 cells, showing a 3-day delay in appearance of peak RT activity compared with the wild type (Fig. 1B).

These data show that the T125A amino acid substitution restores infectivity to the P109S integrase mutant virus. The T125A mutant virus grew like the wild type, indicating that the suppressor mutation alone does not affect HIV-1 replication. To further investigate the loss of integration activity in the P109S mutant and the mechanism of second-site reversion, the in vitro enzymatic activities of purified integrase proteins containing the P109S, T125A, and P109S and T125A amino acid substitutions were compared with those of the wild-type protein.

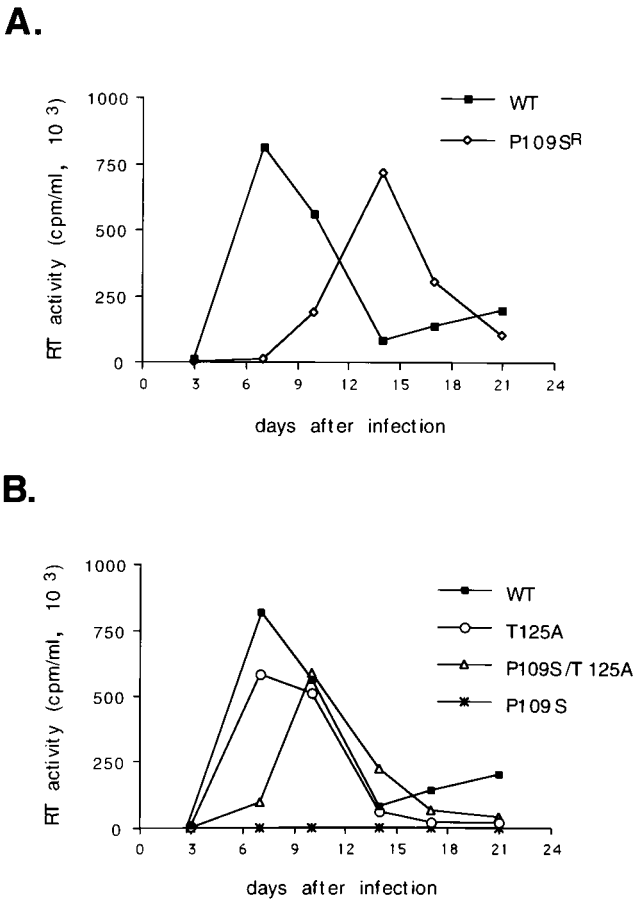

FIG. 1. Replication of wild-type (WT) and integrase mutant viruses in SupT1 cells. (A) Cells were infected with the $P109S^R$ virus recovered from the T-cell line stably transfected with P109S proviral DNA and with the supernatant of COS-1 cells transfected with WT proviral DNA. The culture medium was changed and assayed for RT activity every 3 days. (B) Cells were infected with the supernatants of COS-1 cells transfected with the indicated proviral plasmid DNAs. The growth curve for wild-type HIV-1 was from the experiment with the results shown in panel A. The cultures were analyzed as described for panel A.

Biochemical activities of the mutant integrase proteins. The T125A integrase protein was purified by nickel affinity chromatography after extraction of lysed *E. coli* cells with high salt concentrations. Wild-type (NL4-3) integrase was purified under native conditions as previously described (12, 58). P109S and P109S T125A integrase proteins were purified by nickel affinity chromatography under denaturing conditions and were subsequently refolded in the presence of detergent. No differences have been detected in the activities of wild-type and mutant HIV-1 integrase proteins purified by using the alternative approaches (5, 12, 26, 27). The 3′ processing, DNA strand transfer, and disintegration activities of the proteins were assayed under identical reaction conditions.

T125A integrase displayed Mn^{2+}-dependent 3′ processing, strand transfer, and disintegration activities (Fig. 2, lanes 8 and 9) indistinguishable from those of wild-type integrase (Fig. 2,

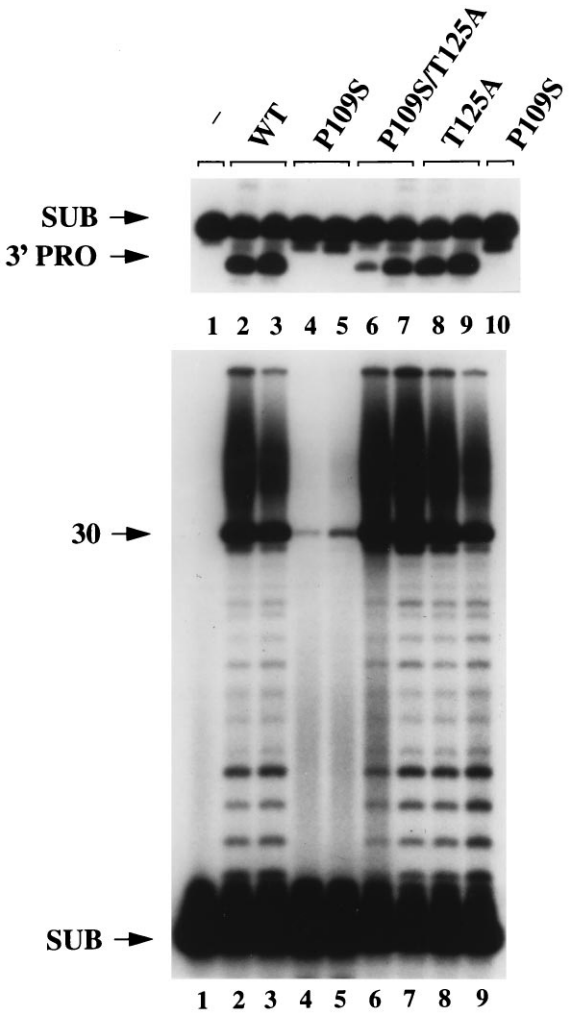

FIG. 2. 3′ processing and disintegration activities of wild-type (WT) and mutant integrase proteins. Each protein was assayed for 3′ processing (top) and disintegration (bottom) activities at two different concentrations: 0.25 μM (lanes 2, 4, 6, and 8) and 0.5 μM (lanes 3, 5, 7, and 9). The 3′ processing activity of P109S integrase was also assayed at 1 μM (upper panel, lane 10). Integrase was omitted from the reactions in lane 1. The other lanes contained reaction mixtures with the indicated mutant proteins. SUB, substrate DNAs; 3′PRO, 3′ processing product; 30, disintegration product.

lanes 2 and 3). The 3′ processing and DNA strand transfer activities of the P109S integrase mutant were undetectable (Fig. 2, upper panel, lanes 4 and 5), even when 1 μM protein was tested (Fig. 2, upper panel, lane 10). However, this mutant did display 5 to 10% of wild-type disintegration activity (Fig. 2, lower panel, lanes 4 and 5). The T125A second-site substitution partially restored activities to the P109S mutant protein (Fig. 2, lanes 6 and 7): at 0.5 μM, the P109S T125A integrase showed 3′ processing (Fig. 2, upper panel, lane 7) and disintegration (Fig. 2, lower panel, lane 7) activities comparable to those of the wild type (Fig. 2, lane 3). The DNA strand transfer activity of the P109S T125A mutant, assayed at 0.5 μM protein with the precleaved U5 substrate, was also similar to the level of wild-type integrase activity (data not shown).

We have shown that the T125A amino acid substitution is a second-site intragenic suppressor of P109S as assayed by HIV-1 replication and the in vitro biochemical activities of recombinant integrase protein. It is generally believed that multimerization of integrase subunits is an essential step in the formation of an enzymatically active complex with the DNA substrate (8, 19, 65). We therefore wondered if the P109S substitution might affect integrase multimerization. Wild-type integrase exists in a concentration-dependent equilibrium between monomeric, dimeric, and tetrameric species under conditions of high ionic strength (27, 30, 63, 66). We analyzed wild-type, P109S, T125A, and P109S T125A integrases by size exclusion chromatography to assess the multimeric states of the proteins.

Size exclusion chromatography. Wild-type and T125A integrase proteins migrated predominantly as single species on a gel filtration column. The retention times of these proteins compared with those of molecular mass standards were consistent with monomeric integrase (Fig. 3). The T125A protein also showed evidence of dimeric and higher-order aggregates. P109S integrase also ran predominantly as a single species, but in this case the protein migrated as a large aggregate at the void volume of the column, showing only a trace of monomer (Fig. 3). P109S T125A integrase eluted as a mixture of the

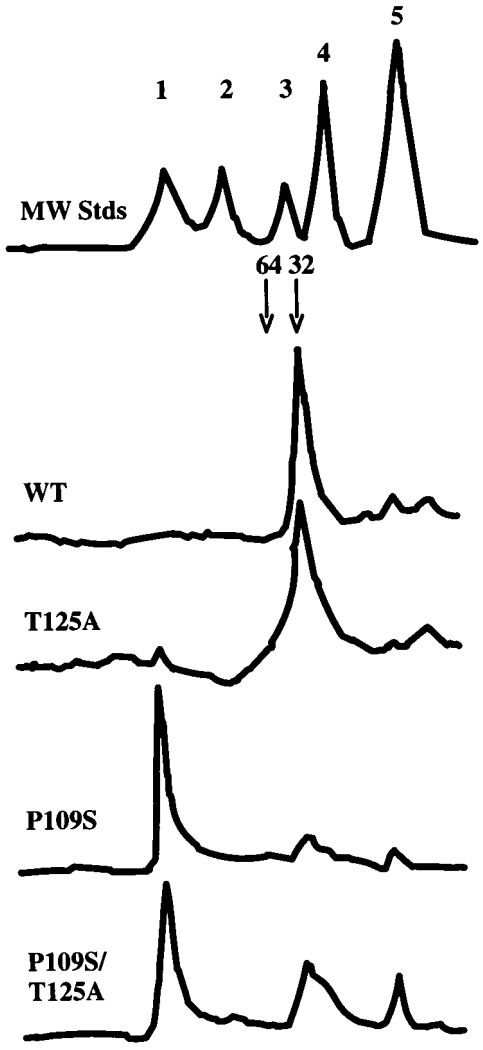

FIG. 3. Size exclusion profiles of wild-type (WT), T125A, P109S, and P109S T125A integrase proteins. The chromatogram of the molecular mass standards (MW Stds) is also shown (1,670 kDa; 2, 158 kDa; 3, 44 kDa; 4, 17 kDa; 5, 1.35 kDa). The predicted migration positions of monomeric and dimeric integrase are marked 32 and 64, respectively, by arrows.

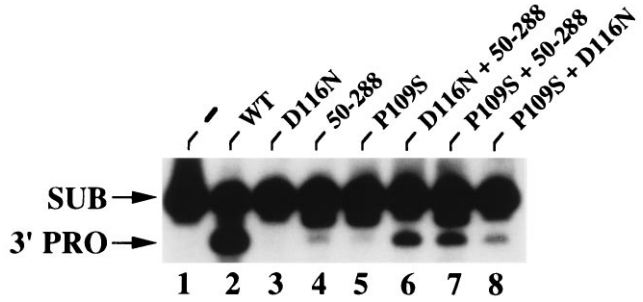

FIG. 4. In vitro complementation with P109S integrase. Integrase was omitted from the reaction in lane 1. The reaction in lane 2 contained wild-type (WT) integrase, and those in lanes 3 through 8 contained the indicated mutant proteins. The low level of 3′ processing activity detected in lanes 4 and 5 is due to contaminating *E. coli* exonuclease activity (23), not integrase. Other labeling is the same as in Fig. 2.

two forms, containing similar levels of aggregates and monomers (Fig. 3). These results show that the P109S substitution can affect the multimeric state of recombinant integrase and that the T125A second-site mutation is capable of correcting, at least partially, the alteration induced by the P109S mutation. Similar results were detected following digestion with V8 protease. Both the wild-type and T125A proteins yielded the expected protease-resistant catalytic and carboxyl-terminal fragments (20). The P109S mutant, in contrast, displayed an aberrant pattern, most likely because the protein aggregates prohibited access to some of the protease sites (data not shown). The cleavage pattern of the P109S T125A protein was more similar to the pattern of wild-type integrase than it was to that of the P109S protein (data not shown).

These results show that the P109S amino acid substitution induces the aggregation of recombinant HIV-1 integrase. The P109S protein was purified under denaturing conditions and was subsequently refolded. The P109S T125A protein was also refolded and contained both aggregated and monomeric integrase (Fig. 3). We were concerned that the P109S T125A aggregates might be inactive, accounting for the lower specific 3′ processing and DNA strand transfer activities of this protein compared with the T125A and wild-type integrases (Fig. 2). To test this, the P109S T125A monomers were isolated from the aggregated material by gel filtration chromatography. The specific 3′ processing and DNA strand transfer activities of the purified monomers were identical to those of the starting P109S T125A aggregate-monomer mixture (data not shown). If the P109S T125A aggregates were inactive, we would have expected the purified monomers to display a higher specific activity than the aggregate-monomer mixture. We conclude that the aggregated forms of P109S T125A are most likely active. The P109S mutant protein was next analyzed in in vitro complementation assays to further investigate the defective nature of this protein.

In vitro complementation. We and others have previously shown that mixtures of certain defective integrase proteins display 10 to 50% of wild-type 3′ processing and DNA strand transfer activities in vitro (19, 65). For example, the 50-288 integrase deletion mutant protein, which lacks the amino-terminal HHCC domain, does not display detectable 3′ processing (Fig. 4, lane 4) or DNA strand transfer activity (5) on its own. Full-length integrase containing the single amino acid substitution of Asn for active-site residue Asp-116 (D116N) does not display detectable 3′ processing (Fig. 4, lane 3) or DNA strand transfer or disintegration activity (20). An equimolar mixture of these two proteins, however, displays approximately 10 to 20% of wild-type 3′ processing (Fig. 4, lane 6) and DNA strand transfer (19) activities. In this reaction mixture, both 3′ processing and DNA strand transfer are catalyzed by the intact active site of the 50-288 protomer, while the essential amino-terminal domain function(s) is supplied in *trans* by the D116N protomer. P109S integrase was next tested in place of the D116N protein. Remarkably, this reaction mixture yielded levels of 3′ processing (Fig. 4, compare lane 7 with lane 6) and DNA strand transfer (data not shown) activities similar to those of the mixture containing the 50-288 and D116N proteins. An equimolar mixture of the P109S and D116N mutant proteins also displayed detectable 3′ processing and DNA strand transfer activities, although these levels were reduced approximately fivefold compared with those of the mixture of the P109S and 50-288 proteins (Fig. 4, compare lane 8 with lane 7).

DISCUSSION

A number of conserved residues in the catalytic domain of HIV-1 integrase have been targeted by site-directed mutagenesis. Substitution of the active-site residue Asp-64, Asp-116, or Glu-152 abolishes HIV-1 replication in human macrophages (24, 69) and T-cell lines (1, 6, 22, 40, 41, 44, 59, 61, 69) and can abolish the 3′ processing, DNA strand transfer, and disintegration activities of recombinant integrase protein in vitro (20, 42, 64). Substitution of less well conserved residues in the catalytic domain of integrase can also affect HIV-1 replication (30, 41, 59, 61, 69) and in vitro enzyme activities (16, 39, 42, 64). However, in these cases recombinant proteins display some polynucleotidyl transferase activity as assessed by using conventional oligonucleotide assays.

A common defect of integrase active-site mutant proteins is likely improper divalent metal ion coordination. For example, recombinant integrase proteins containing conservative charge-to-charge substitutions support partial activities under conditions in which conservative charge-to-neutral substitutions destroy activity (20). Defects caused by changing less well conserved residues in the catalytic domain are less likely to occur through a common mechanism. Indeed, such mutations have been shown to affect reverse transcription (41, 59) and particle assembly (30) in addition to integration (41, 61, 69) in vivo. To further investigate the mechanistic bases for these pleiotropic defects in HIV-1-infected cells, we established T-cell lines chronically producing replication-defective viruses for several of our previously characterized integrase mutants (59, 61).

A second amino acid change in integrase, T125A, restores replication to the defective P109S mutant virus. In this study, we analyzed a T-cell line chronically producing the P109S integrase mutant virus, which is replication defective because of a block in integration (61). We identified and characterized a phenotypically reverted virus ($P109S^R$) which appeared while passaging the stably transfected T-cell line. An intragenic second-site amino acid substitution, T125A, that restored both viral infectivity and recombinant enzyme function to the P109S integrase-defective mutant was identified. The biological mechanism(s) underlying the emergence of the revertant virus is unclear. Assuming that the appearance of the second-site mutation occurred during reverse transcription, we suspect that the original virus bearing the P109S substitution is able to integrate viral DNA to a minimum extent. This level of replication must be below the detection limit of the exogenous RT (Fig. 1B) and p24 antigen capture (61) assays.

Virus recovered following transfection with molecularly cloned P109S T125A proviral DNA showed a replication pro-

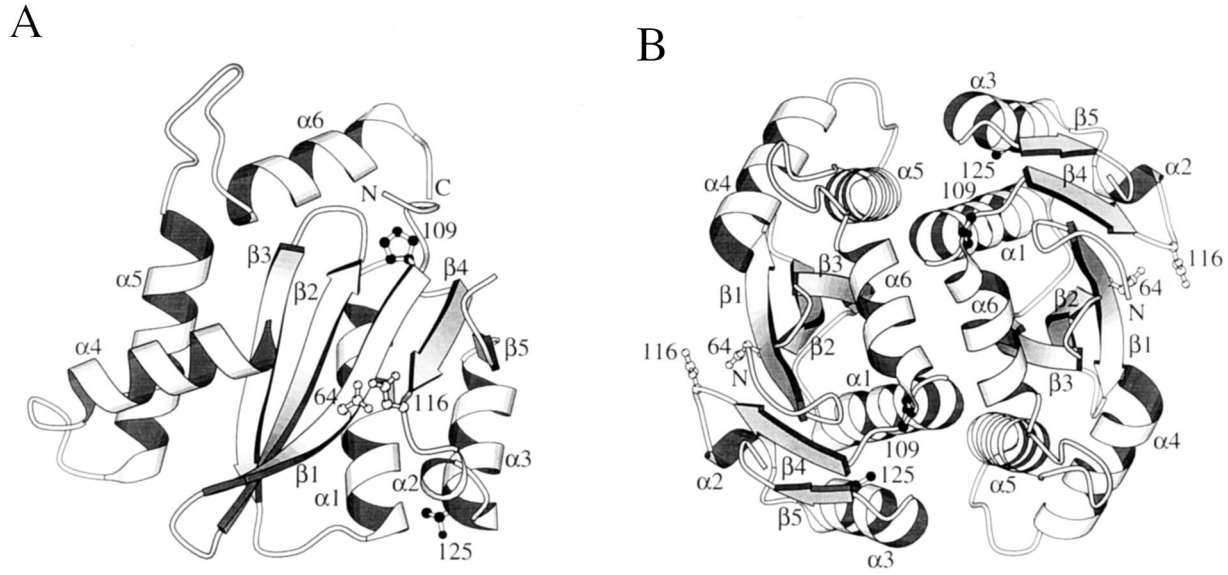

FIG. 5. Locations of Pro-109 and Thr-125 in the crystal structure of the catalytic domain of NL4-3 integrase. The structures were drawn with the program MOLSCRIPT v1.1 (38). (A) Structure of the integrase monomer. Pro-109 and Thr-125 are shown as filled-in ball-and-stick models, and active-site residues Asp-64 and Asp-116 are shown as empty ball-and-stick models. Secondary structural elements are labeled as in reference 17. The amino and carboxyl termini are labeled N and C, respectively. (B) Dimer in the crystal, showing parts of α1, α3, α5, α6, and β3, which form the dimer interface (17). In this view, the carboxyl terminus lies over Pro-109, and Thr-125 is partially hidden in back of α3. Other labeling is as in panel A.

file similar to that of the P109S^R virus (Fig. 1). The 4-day delay in peak RT activities in cells infected with P109S^R is most likely due to the presence of the parental P109S mutant in this viral stock. This mixture would effectively reduce the number of infectious particles when equal RT counts per minute for the P109S^R and P109S T125A viruses are compared. Consistent with this interpretation, virus harvested from the peak of P109S^R replication (day 14 in Fig. 1A) showed the same replication kinetics as the P109S T125A virus (data not shown).

The T125A substitution restores function to recombinant P109S integrase. The T125A amino acid substitution also rescued the in vitro biochemical activities of recombinant HIV-1 integrase protein. P109S integrase was defective for 3′ processing and DNA strand transfer activities but displayed about 5 to 10% of wild-type disintegration activity. The P109S T125A integrase displayed wild-type disintegration and nearly wild-type levels of 3′ processing and DNA strand transfer activities (Fig. 2).

The in vitro activities of the recombinant integrase proteins correlated well with the replication profiles of the different viruses. In both cases, P109S was defective, T125A was equivalent to the wild type, and P109S T125A displayed nearly wild-type activities; the P109S T125A virus showed a 3-day delay in peak virus production compared with the wild type (Fig. 1B), and P109S T125A recombinant integrase displayed approximately 50% of wild-type 3′ processing and DNA strand transfer activities (Fig. 2). These results establish that T125A is an intragenic second-site suppressor of P109S in the context of both HIV-1 replication and the in vitro biochemical activities of recombinant integrase protein. To our knowledge, this is the first example of an intragenic second-site suppressor of a retroviral integrase mutation.

How does T125A restore function to a defective P109S integrase? Pro-109 and Thr-125 are not in close proximity to each other in the crystal structure of the catalytic domain of HIV-1 integrase (Fig. 5A). Thus, it is not immediately obvious how the T125A substitution might rescue the P109S defect(s). Since Pro-109 is in the turn between α1 and β4, it is tempting to speculate that the Ser substitution at position 109 may extend α1 and, in doing so, alter the positions of β4 and active-site residue Asp-116 (Fig. 5A). In this model the T125A substitution would act intramolecularly to restore the integrity of the active site. Analysis of the crystal structure in this detail, however, is complicated by three amino acid differences in the catalytic domains of the NL4-3 and HXBc2 strains of integrase (7, 46). The core domain of NL4-3 integrase was crystallized (17), whereas HXBc2 viruses and mutant integrase proteins were studied in this work. Two of the strain differences lie between Pro-109 and Thr-125: the crystal contains Val-113 and Thr-124 where HXBc2 integrase contains Il3-113 and Ala-124.

Both the catalytic (8, 19, 33, 45, 65) and carboxyl-terminal (30) domains of HIV-1 integrase are important for multimerization, and multimerization is required for 3′ processing and DNA strand transfer activities in vitro (19, 65). Pro-109 is located near the crystallographic dimer interface, and Thr-125 is part of α3, which also forms part of this interface (Fig. 5B) (17). Since the crystal structure of the avian sarcoma virus integrase catalytic domain shows a similar dimer (3), it seems likely that the dimer in the HIV-1 structure is important for 3′ processing and DNA strand transfer activities. We therefore wondered if the primary effect of the P109S substitution might be the disruption of the dimer interface and, hence, integrase activity. In this model T125A would restore function by stabilizing the dimer interface. P109S, P109S T125A, T125A, and wild-type integrases were analyzed by gel filtration chromatography to assess the multimeric states of the proteins.

The results of these analyses showed that P109S integrase was aggregated. Aggregation may be related to the loss of activity, but the P109S T125A protein, which displayed appreciable levels of 3′ processing and DNA strand transfer activities, contained both aggregates and monomers (Fig. 3). Certain substitutions of other residues in the HIV-1 catalytic domain, including Ser-81 (42, 64), Cys-65, Phe-121, and Ser-123 (64), have previously been shown to lower the solubility of

recombinant integrase. Both S81A and S81R recombinant proteins display 3′ processing and DNA strand transfer activities, showing that marginally soluble integrase proteins can be active (42, 64). Purified P109S T125A monomers displayed the same specific 3′ processing and DNA strand transfer activities as the monomer-aggregate mixture, implying that the P109S T125A aggregates are also active. We speculate that aggregation per se is not the reason for the lack of P109S integrase activity.

Crystal structures of P109S and P109S T125A integrase proteins would help decipher the mechanism of second-site reversion. These changes would have to be introduced into the catalytic domain (amino acid residues 50 to 212) of integrase along with the F185K amino acid substitution, which was necessary for crystallization (17, 31). We feel that the tendencies for both the P109S and P109S T125A full-length HXBc2 proteins to aggregate would make the crystallization of the 50-212/F185K core domains extremely difficult, if not impossible.

We therefore probed the P109S integrase defect by using an in vitro transcomplementation assay (19, 65). Premixing P109S integrase with the amino-terminal deletion mutant 50-288 yielded 10 to 20% of wild-type levels of 3′ processing and DNA strand transfer activities (Fig. 4). How might P109S integrase, which behaved as a large aggregate on a sizing column, form functional heteromultimers with the 50-288 protein? In vitro, 3′ processing and DNA strand transfer activities are catalyzed by large insoluble aggregates of integrase and DNA (63). It is therefore not unreasonable that an aggregated protein like the P109S integrase would form functional mixed multimers in vitro. The carboxyl-terminal domain, which is a dimer in solution (18, 43), also contributes to the multimerization of HIV-1 integrase (30). P109S and wild-type integrases displayed equal metal-independent DNA binding to the 30-bp U5 substrate as detected by UV cross-linking (data not shown). The carboxyl-terminal domain of P109S integrase is most likely intact, as this region is responsible for the metal-independent binding of HIV-1 integrase to DNA (23, 28, 51, 67, 70). It is therefore likely that the carboxyl-terminal domains of the 50-288 and P109S protomers help form and/or stabilize the functional heteromultimers in the in vitro complementation reaction mixture. The reaction mixture containing the D116N and P109S mutant proteins also displayed detectable 3′ processing and DNA strand transfer activities (Fig. 4), although these levels were only $\leq$5% of the levels of wild-type activities. Thus, the P109S protomer can donate the active site for 3′ processing and DNA strand transfer activities under these reaction conditions.

At present we are unable to form a definitive model as to how the T125A second-site amino acid substitution restores function to the defective P109S integrase. We suggest that the T125A substitution stabilizes a structural alteration(s) induced by the P109S mutation. Both intermolecular (48) and intramolecular (71) models of second-site reversion have been proposed for other systems in which the participating amino acid residues are not in close proximity to each other. It is important to note that the amino- and carboxyl-terminal domains, which are necessary for the 3′ processing and DNA strand transfer activities of recombinant HIV-1 integrase, are absent in the crystal structure. The T125A second-site substitution may correct the P109S defect primarily through intramolecular contacts in the integrase monomer or through intermolecular contacts involving the catalytic, amino-, and/or carboxyl-terminal domains. Characterization of additional integrase-defective viruses, together with potentially rescuing second-site suppressor mutations, is expected to reveal more information about the structure and function of HIV-1 integrase.

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