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Integration Requires a Specific Interaction of the Donor DNA Terminal 5′-Cytosine with Glutamine 148 of the HIV-1 Integrase Flexible Loop*

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Integration is essential for retroviral replication and gene therapy using retroviral vectors. Human immunodeficiency virus, type 1 (HIV-1), integrase specifically recognizes the terminal sequences of each long terminal repeat (LTR) and cleaves the 3′-end terminal dinucleotide 5′-GT. The exposed 3′-hydroxyl is then positioned for nucleophilic attack and subsequent strand transfer into another DNA duplex (target or chromosomal DNA). We report that both the terminal cytosine at the protruding 5′-end of the long terminal repeats (5′-C) and the integrase residue Gln-148 are critical for strand transfer. Proximity of the 5′-C and Gln-148 was demonstrated by disulfide cross-linking. Cross-linking is inhibited by the inhibitor 5CITEP 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2-tetrazol-5-yl)-propene. We propose that strand transfer requires a conformational change of the integrase-viral (donor) DNA complex with formation of an H-bond between the N-3 of the 5′-C and the amine group of Gln-148. These findings have implications for the molecular mechanisms coupling 3′-processing and strand transfer as well as for the molecular pharmacology of integrase inhibitors.

Integration of the HIV-1 DNA into a host chromosome is required to complete viral infection. Integration is also required for retroviral gene transfer. HIV-1 integrase (integrase) catalyzes integration in two steps (1–3). First, an endonucleolytic cleavage reaction releases a terminal 5′-GT dinucleotide from the end of each viral long terminal repeat (LTR) in a reaction called 3′-processing (3′-P). Subsequently, the viral and host DNAs are joined by insertion of each exposed viral DNA 3′-hydroxyl end into a host chromosome (strand transfer, ST). The integrase-DNA complex during ST consists of several integrase monomers, the two ends of the viral DNA, and the target DNA. Although the arrangement of the integrase dimers has been elucidated by x-ray diffraction of the structures of various integrase domains (4–10), determination of the structure of an integrase-DNA co-crystal has remained elusive.

HIV-1 integrase binds to the tips (‘att’ sites) of each viral LTR. The roles of several bases at the extremities of the viral DNA have been examined. The release of the 5′-GT dinucleotide during 3′-P leaves a complementary 5′-AC overhang (see Fig. 1A for the sequence of the terminal 21 bp of the HIV-1 US LTR). The 5′-AC overhang is required for ST (11) and has been proposed to stabilize the viral DNA end in the proper position for ST (11, 12). Yet the roles of these individual nucleotides, as well as their functional association with an integrase amino acid during ST have not been established.

A flexible loop consisting of amino acids 140–149 resides over the integrase active site. The conformational flexibility of this loop is suggested to be important for the catalytic step following DNA binding (4). Amino acids Gln-148 and Tyr-143 have been identified through photocross-linking as possible contacts for the 5′-C and Gln-148 through disulfide cross-linking, and propose that ST requires the formation of a hydrogen bond between the cytosine N-3 and the glutamine amine group. These results directly relate to the mechanism and structure of the ST reaction as well as to the mechanism of action of integrase inhibitors that block 3′-P and ST.

**EXPERIMENTAL PROCEDURES**

Oligonucleotide Synthesis—Oligonucleotides, except those used for disulfide cross-linking, were commercially synthesized by (Integrated DNA Technologies, Coralville, IA). O-4-Triazolyl-dU-CE phosphoramidite was purchased from Glen Research (Sterling, VA). Oligonucleotides containing convertible nucleosides were synthesized on an Applied Biosystems 392 DNA synthesizer. The two carbon tether (cystamine) was added post-synthetically using the convertible nucleoside approach (15), reduced with excess dithiothreitol, and purified using a NAP-5 column. Incubation of the DNA with a 10-fold molar excess of 5′,5′-dithiobis(2-nitrobenzoic acid) (Sigma) using pH 8.5 phosphate buffer and subsequent ethanol precipitation provided the activated DNA (16). The activated DNA was annealed with the complementary strand before cross-linking experiments were performed.

Mutagenesis—Integrase mutants were created using the Stratagene QuickChange site-directed mutagenesis kit (La Jolla, CA), according to the manufacturer’s recommendations. Primers containing mutations were as follows: for C56S, 5′-CTGAGATGACACGTTAGCCAGGA-3′;
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for C6SS, 5′-GGCCAGCTAGTCTCTACACATTAG-3′; for C28OS, 5′-GGCCAGGTGATGATAGTGTGGCAAGTAG-3′; for Q148N, 5′-CCCCAAAGTAACGGGGTAATAG-3′; for Q148C, 5′-CCCCAAAAGTGCGGGGTAATAG-3′. A complementary primer was used for each mutant. The presence of desired mutations and the integrity of the remainder of the integrase sequence were verified by DNA sequencing.

Integrase Purification—Recombinant wild-type or mutant integrase was purified from Escherichia coli as described (17) with the addition of 10% glycerol to all buffers. Reducing agents were omitted from the elution and dialysis steps of proteins used in disulfide cross-linking reactions.

Integrase Reactions—Integrase reactions contained 400 nM integrase, 20 nM DNA, 7.5 mM MgCl₂ or MnCl₂, 7.5 mM NaCl, 14 mM 2-mercaptoethanol, and 7.5 mM MgCl₂. Schiff base DNA (20 nM) was quenched by addition of an equal volume of gel loading dye (formamide containing 0.25% bromphenol blue and xylene cyanol). Products were separated on 20% polyacrylamide denaturing sequencing gels. Dried gels were visualized using a 445 SI PhosphorImager (Amersham Biosciences). Densitometry analyses were performed using ImageQuant software from Amersham Biosciences.

Disulfide Cross-linking—Integrase (10 μM) was incubated with DNA duplexes (10 μM) containing tethered thioles in the presence of 20 mM Tris, pH 7.4, 7.5 mM MgCl₂, and 10% glycerol for 20 min (unless otherwise noted) at 37 °C. Reactions were capped by the addition of 20 mM methyl methanethiosulfonate. Nonreducing gel loading buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol) was added, and samples were heated at 95 °C prior to loading onto 16% polyacrylamide gels (Invitrogen). Gels were stained with Microwave Blue according to the manufacturer’s recommendations (Protiga, Gaithersburg, MD).

Alternatively, 500 nM integrase was incubated with 5CITEP in the presence of 20 mM Tris, pH 7.4, 7.5 mM MgCl₂, and 10% glycerol for 20 min. DNA (20 nM) containing a 5′-32P label on one strand and a thiol-modified cytosine on the other strand was added, and reactions were capped with methyl methanethiosulfonate at 0.016, 0.33, 0.66, 1, 2, 5, 7.5, 10, 15, and 20 min or at 1 min (see Fig. 6). Following capping, nonreducing gel loading buffer was added, and samples were directly loaded on 16% polyacrylamide gels.

Schiff Base Cross-linking—Inhibition of DNA binding by 5CITEP was examined by using 5′-32P-labeled oligonucleotide containing an abasic site (X) substitution for adenine of the conserved 5′-CA, 5′-GTGTTGGGATACCACTCTCAGAGGTGATGATAGTGTGGCAAGTAG-3′. An abasic dot. 21 ab is the full-length lower strand with an abasic adenosine as the 5′-nucleotide. 20 is the lower strand without the entire 5′-terminal adenine. 20 ab is the lower strand without the 5′-terminal adenine and the neighboring cytosine base. 19 is the lower strand without the two 5′-terminal nucleotides. Note that removal of the 5′-C, as in substrates 20 ab and 19, selectively blocked ST without changing 3′-P.

Strand transfer efficiency for each substrate is indicated below each lane in A and C.

RESULTS

The 5′-C Is Required for ST—Integrase 3′-P and ST activities can be measured by using a duplex DNA derived from the terminal 21 bp of the HIV-1 U5 LTR (Fig. 1A). The base pair on the 3′-side of the integrase 3′-P site, which normally is guanine/cytosine, was replaced by each of the other 15 possible base pair combinations at the X/Y position (Fig. 1B) to examine the importance of the base pair sequence for the 3′-P and ST reactions. By using a full-length substrate, the 3′-P reaction must
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Table 1

| Species | Loop residues | Base |
|---------|---------------|------|
| HIV-1   | 140GIPYNPGQGVE152 | C    |
| SIV     | 140GIPYNPGQGVE152 | C    |
| FIV     | 142GIPYNPGQGVLVE154 | C    |
| ASV     | 145GIPYNPGQGANVE157 | A    |
| RSV     | 145GIPYNPGQGANVE157 | A    |

Glutamine 148 of Integrase Promotes ST—We next looked for a potential integrase hydrogen bond donor residue. An alignment of the retroviral flexible loop residues through the adjacent catalytic glutamate residue and the DNA base corresponding to the 5’-C are shown in Table 1. Of the potential hydrogen bond donating residues, only Asn-144 and Glu-148 are conserved and required for viral replication (19, 20). We chose those residues as potential hydrogen bond donors, as well as Tyr-143 because of published results indicating this amino acid cross-linked the 5’-A of the LTR tip (13). Additionally, residues Thr-122 and Lys-127 were selected from modeling studies of integrase and viral DNA.3 Mutating Thr-122 (to Ala or Cys) or Tyr-143 (to Ser, Phe, or Ile) had no effect on ST. Mutating Asn-144 (to Lys, Ala, or Cys) had a minimal effect on ST. Mutation of Lys-127 (to Ala or Cys) blocked 3’-P and ST (data not shown). Therefore, positions 122, 127, 144, and 143 were not investigated further.

Fig. 4A shows the close proximity of Gln-148 (blue) to the three acidic, catalytic amino acids (red) (from the crystal structure 1BI4).

3 R. Karki and M. Nicklaus, personal communication.
encompassing the flexible loop (21)). We created the mutants Q148N and Q148A to examine the efficiency of ST when the amino acid 148 side chain is shortened by one methylene group or changed to a methyl group (Fig. 4B). Q148N completed 3′-P at a level similar to wild-type integrase but had a 40% reduction in ST activity (Fig. 4B and data not shown). Q148A had a slight decrease in 3′-P and lacked ST. Other Gln-148 mutants (Glu and Lys) were also deficient for ST (data not shown). The 5′-C requirement was tested with the Q148N mutant, and the results were similar to wild-type integrase (see Fig. 1) for both 3′-P and ST, except for the globally lower ST efficiency (data not shown).

Direct Cross-linking of Integrase Gln-148 to the 5′-C—Because changes to either the 5′-C and Gln-148 diminished ST, and both the 5′-C and Gln-148 are plausibly partners for hydrogen bonding, we tested their potential direct interaction using disulfide cross-linking. Disulfide cross-linking between proteins and DNA has been accomplished for several protein-DNA complexes, including HIV-1 reverse transcriptase and DNA repair proteins (for review see Ref. 22). Additionally, the integrase mutant E246C has been cross-linked previously with viral DNA substrate at the seventh base (adenine) from the 5′-end of the lower DNA strand by using a similar method (23).

Three surface cysteine residues of integrase were mutated to serine to eliminate background cross-linking. Therefore, in cross-linking experiments the control protein had the mutations C56S/C66S/C280S ("SSS"). Sequential mutations indicated Cys-56 was responsible for background cross-linking, in contrast to a previous study where Cys-65 and Cys-280 provided background cross-linking to the 5′-adenine of the lower DNA strand (23). The mutation Q148C was added to the triple serine mutant integrase to place a reactive sulfur at residue 148 ("Q148C/SSS").

The activity of these mutants is shown in Fig. 4B. The triple serine (SSS) retains wild-type activity for both 3′-P and ST. The Q148C/SSS mutant exhibits no ST and altered 3′-P specificity. 3′-P was reduced at the canonical cleavage site (CA↓ GT-3′ generating the 19-mer) and enhanced at the adjacent site CAG↓ T-3′, resulting in the formation of a 20-mer product and a similar level of total 3′-P. Altered 3′-P has also been reported for a Q148L mutant of HIV-2 integrase with enhanced generation of circular dinucleotide products (24). Thus, it is possible that Gln-148 helps position the 3′-P reagents, allowing dinucleotide release, and the role of Gln-148 in ST becomes important only once the 5′-C is unpaired. Activity of the Q148C/SSS mutant was tested with precleaved DNA in the presence of manganese. These conditions permitted a partial "rescue" (19%) of ST. Presumably cysteine can interact with manganese, and not magnesium, because of the ability of manganese to bind cysteine (25).

The cross-linking components and reaction are shown schematically in Fig. 5A. DNA was synthesized with an alkanethiol tether at the 5′-C position. The tether was activated by 5,5′-dithiothreitol(2-nitrobenzoic acid) to yield the substrate shown before reaction with integrase in Fig. 5A.

The DNA substrates used to examine position-specific cross-linking are shown in Fig. 5B. Cross-linking of the Q148C/SSS integrase to the thiol-modified 5′-C-containing DNA (DNA X-1) was observed as the band migrating between the monomer and dimer species of integrase (highlighted by horizontal arrow in Fig. 5C). Cross-linking efficiency was between 30 and 50% relative to the monomer concentration. The cross-link band was not observed when unmodified DNA was used (Fig. 5, C and D). As expected, dithiothreitol reduced the level of protein-DNA cross-link, and no cross-link was observed with the SSS integrase lacking Q148C (Fig. 5C). Thus, the core domain surface residue Cys-130 does not cross-link with the thiol-containing DNA. Cross-linking was not observed when the reactive thiol group was placed distal from the 5′-end of the duplex (DNA X-2), which rules out nonspecific cross-linking (Fig. 5D). Finally, cross-linking was observed in the presence of manganese and magnesium, precleaved or full-length DNA (Fig. 5, E and F) in agreement with the previous observation of DNA binding in the absence and presence of metal (26, 27). The recently reported flexibility of the viral DNA end may allow cross-linking of nonprocessed substrate (28). Together, these experiments indicate that the integrase residue 148 is in the vicinity of the cytosine of the 5′-overhang and that catalysis is not required for DNA cross-linking.

Cross-linking Interference by Integrase Inhibitors—Diketo acid derivatives have emerged recently as specific ST inhibitors with antiviral activity (29, 30) and are reviewed in Refs. 3 and 31. Interaction between 5CITEP and Gln-148 was observed crystallographically (25). Inhibition of cross-linking by the integrase inhibitor 5CITEP was examined at concentrations of integrase and DNA used in standard catalytic exper-
iments in the presence of magnesium. The DNA was radioactively labeled to permit visualization of integrase-DNA complexes. A time course experiment indicated cross-linking was complete in less than 2 min (Fig. 6, A and B). Addition of 5CITEP decreased the rate of cross-link formation. A 1-min cross-linking time was chosen to examine the inhibition of cross-linking at different 5CITEP concentrations (Fig. 6 C). The IC50 of cross-link inhibition was 29 μM. 5CITEP had no effect on overall binding of integrase to DNA, as measured by Schiff base assay (Fig. 6, D and E), suggesting inhibition of disulfide cross-linking reflects the 5CITEP-binding site.

**DISCUSSION**

The present study demonstrates that efficient ST requires both the presence of a 5'-C at the end of the donor (viral) DNA and Gln-148 in the flexible loop of HIV-1 integrase. The 5'-C is conserved in the LTR sequences of HIV-1, HIV-2, simian immunodeficiency virus, and feline immunodeficiency virus (Table 1). In contrast, avian sarcoma virus and Rous sarcoma virus LTRs contain an adenine at that position, where the nitrogen at the first ring position could contribute to a similar interaction in an appropriately structured active site. Gln-148 is conserved among retroviral integrases, including HIV-1, simian immunodeficiency virus, avian sarcoma virus, Rous sarcoma virus, and feline immunodeficiency virus (32). Gln-148 resides in a flexible loop disordered in most integrase crystal structures or crystallized in a conformation that is unsuitable for catalysis (4–9). It has been suggested that the loop becomes ordered upon DNA binding and acts to stabilize the 5'-end of the viral DNA (5, 13).

Mutations that reduce the flexibility of the loop containing Gln-148 impair catalysis without affecting DNA binding (4). A conformational change following 3'-P has been observed (23). This flexibility might
allow Gln-148 and the 5'-C to interact following the 3'-P reaction, allowing efficient ST. This structural change could involve movement of the flexible loop, rotation of the 5'-C base out of the DNA duplex, or both, as illustrated in Fig. 7. In our model, release of the 5'-GT dinucleotide provides the rotational freedom to the lower strand 5'-C, leaving the area near the catalytic 3'-hydroxyl available for subsequent ST. The 5'-AC overhang is then anchored away from the catalytic 3'-hydroxyl by a hydrogen bond between the Gln-148 side chain amine and the cytosine N-3 group. Note that integrase is active as a multimer, and our model does not confine the active site performing catalysis and the Gln-148 facilitating ST to the same subunit.

A precedent for a similar glutamine-cytosine N-3 hydrogen-bonding interaction exists for human deoxycytidine kinase (33). Deoxycytidine kinase efficiently phosphorylates deoxycytidine but also deoxynucleosine and deoxyadenosine by interaction with Gln-97. The bound base dictates rotation of the glutamine side chain into an appropriate hydrogen-bonding position. Discrimination of deoxycytidine by deoxycytidine kinase is achieved through H-bonding of the cytosine N-3 group. Note that integrase is active as a multimer, and our " flexible loop, rotation of the 5'-C following 3'-P reaction, allowing Gln-148 and the 5'-C cross-linking by 5CITEP (42), and docking studies suggest the participation of Gln-148 in the drug-binding pocket for several other integrase inhibitors (35, 43, 44). A drug-resistant mutation Q148K developed after exposure to S-1360, a diketo acid integrase inhibitor in clinical trial, and this mutant exhibited poor viral replication lending support to the important role of Gln-148 (20). Diketo acids have been proposed to bind the integrase-DNA complex and block ST by interacting with the 5'-AC overhang (45). We observed that cross-link between the LTR 5'-C and residue 148 of integrase was inhibited by 5CITEP at an IC50 of 29 µM (Fig. 6, C and D). 5CITEP inhibits 3'-P and ST at IC50 values of 35 and 0.65 µM, respectively (45). Inhibition of Q148C/5'-C cross-linking by 5CITEP suggests the binding site for this inhibitor overlaps the region of the Gln-148/5'-C interaction. This is supported by the lack of inhibition of Schiff base formation between integrase and an abasic site substitution for the conserved adenine at the 3'-P site (Fig. 6, D and E). The cross-linking approach could be used to scan the molecular contacts between integrase and its DNA substrates, as well as inhibitor-binding sites. Stabilization of integrase-DNA complexes may also enable co-crystal structure determination.

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