Complementation between HIV integrase proteins mutated in different domains

Dik C. van Gent, Cornelis Vink, Antoinette A.M. Oude Groeneger and Ronald H.A. Plasterk

Division of Molecular Biology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Communicated by P. Borst

HIV integrase (IN) cleaves two nucleotides off the 3' end of viral DNA and integrates viral DNA into target DNA. Previously, three functional domains in the HIV IN protein have been identified: (i) the central catalytic domain, (ii) the C-terminal DNA binding domain, and (iii) the N-terminal region, which is also necessary for activity. We have now investigated whether IN proteins mutated in different domains can complement each other. Mutant D116I does not contain an intact active site, but does bind DNA, whereas the C-terminal deletion mutant CΔ73 does not bind DNA, but does have an intact active site. Neither mutant protein mediates site-specific cleavage or integration. However, a mixture of both proteins is active, suggesting that IN functions as an oligomer, and that two subunits can have different functions; one subunit binds the (viral) DNA and another subunit provides the active site. We found three classes of mutants, corresponding to the three domains mentioned above. Mutants from different classes, but not from the same class, can complement each other. However, complementation is most efficient when the N- and C-termini are present on the same molecule.

Key words: complementation/human immunodeficiency virus/integration/protein–protein interactions/transposition

Introduction

Integration of viral DNA into the human chromosome is an essential step in HIV replication (Stevenson et al., 1990; LaFemina et al., 1992). Integration is catalysed by the viral integrase (IN) protein. After reverse transcription of the viral genomic RNA, IN removes two nucleotides from the 3' ends of the linear viral cDNA (donor cut) (Katzman et al., 1989; Roth et al., 1989; Sherman and Fyfe, 1990; Vora et al., 1990). Donor cut is a one-step reaction: IN makes a specific phosphodiester bond accessible for nucleophilic attack (Engelman et al., 1991; Vink et al., 1991). The natural nucleophile is probably water, resulting in the release of a dinucleotide with a 5' phosphate and a 3' hydroxyl group. However, other nucleophiles, such as glycerol or the alcoholic amino acid serine (Vink et al., 1991), or the 3' hydroxyl group of the viral DNA (Engelman et al., 1991) can also be used.

The newly formed 3' hydroxyl ends of the viral DNA are coupled to phosphate groups in the target DNA (strand transfer) (Craigie et al., 1990; Katz et al., 1990; Bushman and Craigie, 1991). Strand transfer is a one-step reaction: a phosphodiester bond in the target DNA is broken and a new one (between viral DNA and target DNA) is made in one concerted reaction (Engelman et al., 1991). The viral 3' hydroxyl ends are coupled to phosphate groups in opposite strands of the target DNA, a few base pairs apart. Subsequently, the gaps are filled in, resulting in an integrated provirus flanked by a duplication of a short stretch of the target sequence (reviewed in Varmus and Brown, 1989).

Mutational analyses of HIV-1 (Drelich et al., 1992; Engelman and Craigie, 1992; Kulkosky et al., 1992; Vink et al., 1993) and HIV-2 IN (van Gent et al., 1992) have identified three distinct regions in the protein.

(i) The N-terminus is required for donor cut and strand transfer (Bushman et al., 1993; Vincent et al., 1993; Vink et al., 1993). This region contains the zinc-finger-like motif His-Xaa2-His-Xaa20-30-Cys-Xaa2-Cys, which has been shown to bind Zn²⁺ (Burke et al., 1992; Bushman et al., 1993). Mutation of one of the His or Cys residues results in reduction of IN activity, especially donor cut (Engelman and Craigie, 1992; LaFemina et al., 1992; van Gent et al., 1992). The function of this region is not clear yet.

(ii) The central region, approximately between amino acids 60 and 160, is the most conserved part of retroviral integrases (Fayet et al., 1990; Khan et al., 1991). Mutational analysis identified three potential active-site residues in this region. Asp64, Asp116 and Glu152; mutation of each one of these residues leads to inactivation of IN (Drelich et al., 1992; Engelman and Craigie, 1992; Kulkosky et al., 1992; van Gent et al., 1992). These amino acids are conserved among all retroviral integrases and also in transposases of certain bacterial insertion sequences (Fayet et al., 1990; Khan et al., 1991). They will be referred to as active-site residues. In general both donor cut and strand transfer activity are equally affected by mutations in IN, indicating that both activities are probably carried out by one active site.

(iii) The C-terminus is the least conserved part of retroviral integrases. It contains a DNA binding domain (Khan et al., 1991; Mumm and Grandgenett, 1991; Woerner et al., 1992), located between amino acids 200 and 270 of the 288 amino acid HIV-1 IN protein (Vink et al., 1993). HIV IN has only slightly more affinity for specific DNA (LaFemina et al., 1991; van Gent et al., 1991).

Here we report that mixing of different classes of inactive IN mutants restores activity. One subunit can provide the C-terminal DNA binding domain (and preferably also the N-terminus), and the other one the active site. This suggests that IN is active as a dimer (or oligomer), in which the different subunits can have different functions.

Results

Complementation between active-site mutants and C-terminal deletion mutants

We expressed and purified the HIV-2 IN active-site mutants D64V, D116I and E152L, and a C-terminal deletion mutant
of HIV-2 IN containing amino acids 1–220 (CA73). These mutants do not mediate site-specific cleavage or integration of viral DNA (Figure 1A and B). In contrast to the active-site mutants, the deletion mutant contains an intact active site, which is inferred from the ability of the deletion mutant to perform the reversal of the integration reaction, called disintegration (Chow et al., 1992; Figure 1C). The level of disintegration by the deletion mutant, however, is reduced compared with wild-type (~6% of the wild-type level). The C-terminus of the HIV-1 IN has been found to contain a DNA binding domain (Woerner et al., 1992; Vink et al., 1993). In agreement with this finding for HIV-1, the HIV-2 IN mutant CA73 does not bind DNA, whereas the active-site mutants do (data not shown).

Several recombination proteins act as dimers or oligomers in site-specific recombination reactions. Kinetic analysis of Rous sarcoma virus IN indicates that IN acts as an oligomer in cleavage and integration (Jones et al., 1992). We studied whether different subunits of IN might play different roles in the reaction. To investigate whether an active-site mutant could rescue a DNA binding mutant, we mixed the active-site mutant D116I and the C-terminal deletion mutant CA73, and assayed the mixture for site-specific cleavage and integration. Both mutant proteins alone are inactive; the mixture, however, mediates both reactions (Figure 2A and B). We found that a mixture of approximately equimolar

Fig. 1. Activities of mutant HIV-2 IN proteins. (A) Cleavage, (B) integration and (C) disintegration reactions using immobilized proteins. The nomenclature is as follows: the first letter signifies the amino acid in wild-type HIV-2 IN, then the position of the residue is given and the amino acid into which this residue is changed. C-terminal deletion mutants are named CA followed by the number of amino acids that have been deleted from the C-terminus, and N-terminal deletion mutants are indicated by NΔ followed by the number of amino acids that have been deleted from the N-terminus. G, glycerol product; C, cyclic dinucleotide; D, linear dinucleotide; IP, integration products; WT, wild-type HIV-2 IN; DNase, DNase I degradation ladder of the oligonucleotide used. The positions of the 28-mer cleavage substrate, the 26-mer integration substrate, the 13-mer disintegration substrate, and the 29-mer disintegration product are indicated on the left. A schematic representation of the substrates is drawn below the autoradiographs; the position of the radiolabel is depicted by an open circle.

Fig. 2. Complementation between the HIV-2 IN mutants CA73 and D116I. Cleavage (A) and integration (B) reactions were carried out with CA73 (lane 1), D116I (lane 7) and mixtures of these mutants (lanes 2–6). The proteins were mixed in molar ratios of 9:1, 3:1, 1:1, 1:3 and 1:9 respectively in lanes 2–6. The abbreviations were as in Figure 1. Figures 1, 2 and 3 are the same exposure of different parts of the same gel.
amounts of the two mutants yielded the highest activity (Figure 2A and B). Since an active-site mutant acts together with the deletion mutant, we conclude that HIV IN can act as a dimer or oligomer in both cleavage and integration. One subunit probably binds the (viral) DNA and another subunit provides the active site for hydrolysis of a specific phosphodiester bond in the viral DNA, or integration of viral DNA into target DNA (see Discussion).

Previously, two other active-site residues were identified: Asp64 and Glu152 (Drellich et al., 1992; Engelman and Craigie, 1992; Kulkosky et al., 1992; van Gent et al., 1992). The active-site mutants D64V and E152L were also tested for their ability to complement the C-terminal deletion mutant CA73 and each other. As shown in Figure 3, they indeed complement mutant CA73 (although mutant E152L somewhat less than the other two active-site mutants). They do not complement mutant D116f nor each other, showing that they are members of the same complementation group.

We investigated which part of the C-terminus is required for complementation using a series of fusion proteins of maltose binding protein and (mutant) HIV-1 IN (Vink et al., 1993). As shown in Figure 4, the wild-type fusion protein mediates site-specific cleavage and integration, whereas the active-site mutant MD116f and the C-terminal deletion mutants do not. However, mixtures of the active-site mutant and the C-terminal deletion mutants, were active. Complementation was very efficient with the short deletions MCA51 and MCA66, weak with the larger deletion mutants MCA81 and MCA94, and only detectable after prolonged exposure with the largest deletion mutants MCA105 and MCA117 (Figure 4A and B and Table I). The gradual decrease in the level of complementation parallels the gradual loss of disintegration activity of these mutants (Figure 4C and Table I).

All experiments described above have been performed with IN preparations that were immobilized (after mixing). These preparations contain a lower non-specific nuclease background, and remain active for a longer period of time, resulting in a much higher level of cleavage and integration (van Gent et al., 1992). We performed some of the reactions also with soluble proteins and found that the results were similar to what was found for immobilized proteins (Table I).

To investigate the specificity of the interactions between HIV IN monomers, we asked whether HIV-1 and HIV-2 IN mutants could complement each other. We found that the HIV-2 deletion mutant CA73 could complement the HIV-1 IN active-site mutant MD116f in both site-specific cleavage and integration (data not shown). The level of

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Fig. 3. Complementation between HIV-2 IN active-site mutants and the C-terminal deletion mutant CA73. Cleavage (A) and integration (B) reactions with mixtures of mutant HIV-2 IN proteins. The abbreviations were as in Figure 1. Figures 1, 2 and 3 are the same exposure of different parts of the same gel.

Fig. 4. Complementation reactions with C-terminal deletion mutants and an active-site mutant of fusion proteins of maltose binding protein and HIV-1 IN. (A) Cleavage, (B) integration and (C) disintegration reactions. The abbreviations and nomenclature are as in Figure 1, the 'M' is added to signify that a mutant is a fusion protein of maltose binding protein and HIV-1 IN. WT, fusion protein of maltose binding protein and wild-type HIV-1 IN. The band at the 16-mer position is probably the result of reintegration (integration following disintegration; Vink et al., 1993).
complementation, however, was lower (−5% of wild-type HIV-1 or HIV-2 IN) than between HIV-1 IN mutants MCA66 and MD161 (37% of wild-type level) or between HIV-2 IN mutants CA73 and D161 (30% of wild-type level), suggesting that the interaction between IN subunits is rather specific and that dimerization signals are not completely conserved between HIV-1 and HIV-2.

### N-terminal deletion mutants from a separate complementation group

Previously the N-terminus has been identified as a region in the protein that is required for site-specific cleavage and integration, but not for disintegration (Bushman et al., 1993; Vink et al., 1993). An N-terminal deletion mutant in which the first five amino acids have been deleted (MNΔ5) mediates site-specific cleavage and integration at a reduced level compared with the wild-type protein (Figure 5 and Table I). Larger deletions up to 50 amino acids are inactive in these two reactions, but can still mediate disintegration (Table I). Deletion of 72 amino acids from the N-terminus results in a protein that does not mediate disintegration, presumably because part of the active-site (including residue Asp64) has been deleted.

N-terminal deletion mutants were tested for their ability to complement the active-site mutant MD161. Mixtures of both types of mutants mediate site-specific cleavage and integration (Figure 5 and Table I). Mutant MNΔ72 does not complement mutant MD161, which is in line with the hypothesis that this deletion mutant lacks part of the active site.

In order to find out whether the N-terminal and C-terminal deletion mutants belong to the same complementation group, we also mixed both types of deletion mutant. As shown in Figure 6 and Table I they complement each other, showing that they belong to different complementation groups, and that the N-terminus can be provided in trans to the C-terminal DNA binding region. Complementation between N-terminal and C-terminal deletion mutants is, however, less efficient than between either of the deletion mutants and the active-site mutant. In summary, we identified three complementation groups that correspond to the three domains that were previously characterized.

### The three domains can be provided either in cis or in trans

As shown above, we identified three classes of mutants that can complement each other. In other words, the three domains can be supplied from different molecules. We investigated whether a protein mutated in two domains could complement a protein mutated in the third domain (schematically depicted in Figure 7A). We constructed a set of mutants containing two mutations of different complementation groups and mixed them with a mutant from the third group. When the N- and C-termini are present on one molecule and the active site on another one (Figure 7A, combination 4), complementation is rather efficient (Figure

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**Table I. Complementation between HIV-1 IN mutants**

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*a*These reactions were also performed in solution and yielded similar results; MNΔ50 + MD161, ++ ++; MCAΔ51 + MD161, ++ +; MNΔ50 + MCAΔ51, ++.

*b*These reactions yielded a very low level of integration products, only detectable after prolonged autoradiography.

The levels of activity of the various mutants were determined by scanning of autoradiographs and comparison with the fusion protein of maltose binding protein and wild-type HIV-1 IN. The nomenclature of mutants is as in Figure 4, cl, site-specific cleavage; int, integration; dis, disintegration. The activity is scored as follows: +++ +++, >40% of wild-type activity; ++, 10–40% of wild-type activity; +, 1–10% of wild-type activity; −, <1% of wild-type activity; nd, not determined.
Complementation between HIV IN mutants

7B and C and Table 1), only ~2-fold lower than between an active-site mutant and a mutant containing either the N-terminal or the C-terminal deletion alone (Figure 7A, combinations 1 and 2). Complementation between N- and C-terminal deletion mutants is less efficient than between either of these and an active-site mutant. If the N- and C-termi are present on separate molecules, complementation is more efficient when the active site is present on the same molecule as the C-terminus (Figure 7A, combination 6 and 7B and C) than when it is located on the other molecule (Figure 7A, combination 5 and 7B and C). In conclusion, these experiments show that the N-terminus and the C-terminal DNA binding domain should preferentially be present on the same molecule, and the active site on another one. However, other combinations can also complement each other, suggesting that there is a high degree of flexibility in the interactions between these three domains.

Deletion of the N-terminal 72 amino acids deletes part of the active site. Therefore, one would expect that it belongs to the same class of mutants as the double mutant in which both the N-terminal 50 amino acids have been deleted and the active site has been disrupted by a point mutation (Figure 7A, combination 6). However, this mutant does not complement the C-terminal deletion mutant (Figure 7B and C, lane 16). This indicates that the region between amino acids 50 and 72 is either important for dimerization or that this mutant does not fold correctly. Mixing all three double mutants together did not result in preparations that contain detectable cleavage and integration activity (data not shown). This indicates that functional complementation can only occur between two and not between three IN monomers.

Discussion

Three IN complementation groups

HIV IN contains three regions that are important for activity: (i) the central catalytic domain, contained within amino acids 50–194, (ii) the DNA binding domain, between amino acids 200 and 270, and (iii) the N-terminal region, spanning residues 1–50. IN mutants deleted in the N-terminal region or the C-terminal DNA binding region contain an intact active site, but are no longer able to carry out site-specific cleavage of the viral DNA termini or DNA strand transfer. However, mixing of these deletion mutants with active-site mutants restores both site-specific cleavage and strand transfer activity, showing that these domains can be supplied on different IN molecules. This shows that IN molecules can interact functionally with each other, and suggests that IN functions as oligomer. However, we cannot formally exclude that wild-type HIV IN might be able to act as a monomer. In that case one would have to assume that the IN domains of the same monomer of wild-type IN interact, and that only in our complementation experiments do the domains of different monomers functionally interact. This would require a very high degree of flexibility of the relatively small IN protein, which is why we favour the interpretation that functional interaction between mutant monomers suggests that wild-type IN also acts as oligomer.

Functional complementation was not observed between different active-site IN mutants. These mutants therefore belong to the same complementation group, which is consistent with the hypothesis that Asp64, Asp116 and Glu152 are key residues within the single active site of IN.

The other complementation groups are formed by N-terminal deletion mutants and IN mutants that have deletions of the C-terminal DNA binding region. Similar results were independently obtained by Engelman et al. (see accompanying paper): they identified similar complementation groups, and reach the conclusion that IN contains three more or less independently functioning domains. Minor differences in levels of complementation can be attributed mainly to differences in definition of activity levels and in reaction conditions.

Oligomerization of IN

Mutant IN proteins that contain amino acids 50–220 efficiently complement active-site mutants. Efficient complementation between IN mutants is no longer observed when a single mutant has a deletion of >50 residues from the N-terminus or more than ~70 residues from the C-terminus. This might indicate that the region between amino acids 50 and 220 of HIV IN is important for oligomerization. The signals that govern oligomerization remain to be determined.

Based on the symmetry of the integration reaction, in which the two viral DNA ends are inserted into both strands of the target DNA, it is likely that an even number of IN monomers is involved in the process. Models have been proposed in which either IN dimers (Vink et al., 1991; Vincent et al., 1993) or IN tetramers (Engelman et al., 1991) carry out the concerted integration of one viral DNA molecule. A tetrameric IN complex is consistent with the results presented here: cleavage and integration of one viral DNA end is accomplished by (at least) two IN molecules, suggesting that integration of two DNA termini would require (at least) four IN molecules.

Intra-protein complementation

The results presented here suggest that one IN subunit binds the viral DNA terminus and that another subunit provides
the active site for site-specific cleavage. The only other recombinase for which complementation between different mutant proteins has been described is Flp recombinase from *Saccharomyces cerevisiae* (Chen et al., 1992). Flp contains a catalytic domain with four amino acids that are conserved among the Int family recombinases. The key catalytic residue within one monomer, Tyr343, forms a catalytic site in conjunction with the other three invariable residues of another Flp monomer. This tyrosine residue leads the nucleophile attack on the target phosphodiester bond in the DNA cleavage step of Flp recombination. Tyr343 attacks DNA in *trans*; a DNA site is not cleaved by the Flp monomer which is bound to it, but rather by a second Flp monomer. IN is probably also active in *trans*; the active site can be provided by an IN monomer that is not able to bind DNA. It is not clear whether the active site is provided by an IN molecule that is bound to another DNA site or by an IN molecule that forms a dimer on one viral DNA end. In contrast to Flp, IN active-site mutants cannot complement other active-site mutants.

**Materials and methods**

**DNA techniques**

Standard DNA procedures were carried out as described (Sambrook et al., 1989). Point mutants of HIV-1 and HIV-2 IN were generated by site-directed mutagenesis as described (van Gent et al., 1992). The generation of HIV-1 IN deletion mutants has been described previously (Vink et al., 1993). In the HIV-2 IN deletion mutant CΔ72 three stop-codons were introduced 3' of triplet codon for amino acid Leu220, and subsequently the region encoding amino acids 221–280 was deleted. The double mutant MNΔ50/CΔ51 was generated by exchanging restriction fragments between plasmids encoding proteins MNΔ50 and MCΔ51. For the expression of proteins MNΔ50/D116I and MCΔ51/D116I constructs were generated by site-directed mutagenesis of plasmids encoding MNΔ50 and MCΔ51, respectively.

**Protein purification**

HIV-2 IN was purified and immobilized on Thiopropyl Sepharose as described (van Gent et al., 1992). For the complementation assays the purified mutant proteins were mixed, incubated for 30 min at 0°C, and immobilized on Thiopropyl Sepharose. The total protein concentration was 10 pmol/µl of a 50% slurry of beads. The fusion proteins of HIV-1 IN and maltose binding protein were purified as described (Vink et al., 1993). For the complementation assays the purified proteins were dialysed to 20 mM
Tris–HCl pH 7.5, 1 M NaCl, 1 mM EDTA for 2 h at 4°C, mixed and incubated for 30 min at 0°C, and immobilized on Thiopropyl Sepharose at a total protein concentration of 10 pmol/μl of a 50% slurry of beads. Unless stated otherwise, proteins were mixed in a 1:1 ratio in the complementation reactions.

**Cleavage, integration and disintegration assays**

Oligonucleotide cleavage reactions were done with an HIV-1 U5 substrate labelled at the 3' end as described (Vink et al., 1991). Reaction mixtures contained 20 mM MOPS pH 7.2, 25 mM NaCl, 1 mM (HIV-2) or 3 mM (HIV-1) MnCl₂, 10 mM DTT, 10% (v/v) glycerol, 0.04 μM oligonucleotide substrate and 1 μM immobilized IN, and were incubated for 1 h at 30°C.

Integration reactions were carried out as described (van Gent et al., 1992). Reaction mixtures were incubated for 3 h at 30°C. Disintegration reactions were carried out using the Y-shaped substrate depicted in Figure 1 as described (van Gent et al., 1992). Activities were quantified by densitometry, using an Ultrascan XL Enhanced Laser Densitometer (LKB).

**Acknowledgements**

D.C.v.G. and C.V. contributed equally to the work presented in this paper. We thank Chris Vos, Sean Colloms and Piet Borst for critically reading the manuscript, and Cindy Gerhardt at the Free University of Amsterdam for assistance with densitometry. This work was supported by the Dutch Ministry of Health (RGO), the Netherlands Organization for Scientific Research (NOW), and by a grant from Glaxo Group Research Limited to C.V.

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