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Amino-terminal domain interactions of λ integrase on arm-type DNA

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ABSTRACT

In contrast to the other tyrosine recombinase family members, integrase protein (Int) of bacteriophage λ has an additional amino-terminal domain that binds to "arm-type" DNA sequences distant from those involved in strand exchange. The homomeric interaction between neighboring amino-terminal domains of Int is contributed by R30-D71 salt-bridge in a non-equivalent manner on Holliday-junction intermediates. In this report, R30 and D71 residues were investigated in regard to Int's cooperative binding to "arm-type" DNA and the attenuating function of "arm-type" DNA. The results suggest the electrostatic interaction between residues 30 and 71 is dependent on "arm-type" DNA and contributes the "selective" inhibition of catalytic activity of λ Int by "arm-type" DNA.

Viral genome of *Escherichia coli* phage λ gets into and out of its host chromosome by site-specific DNA recombination. The integrase protein (Int) catalyzes this reaction at specific DNA loci; *att*P/*att*B for 'integrative' and *att*L/*att*R for 'excisive' recombination. Int belongs to the large λ Int family of recombinases, which use transient covalent phosphor-tyrosine intermediates to exchange DNA strands for DNA recombination [1–3] (Fig. 1).

Int is a heterobivalent DNA-binding protein, which can simultaneously bind two distinct, separate, 'arm-type' and 'core-type' DNA sequences. This 'Int-bridge' is manifested by accessory DNA-binding proteins, IHF, Xis, and Fis that bind to specific DNA sequences between arm-type and core-type DNA. Int, accessory DNA-binding proteins, arm and core type DNA comprise higher order recombinogenic complex by which Int molecules are precisely positioned to execute recombination reaction [4–6].

Int is composed of three main functional domains; amino-terminal (residues 1–74), core-binding (residues 75–170) and catalytic domains (residues 171–356). The CB (core-binding) and catalytic domains comprise carboxy-terminal domain of Int and bind to the core-type DNA site. This carboxy-terminal domain has a RHR triad (Arg212-His308-Arg311), which activates the scissile phosphate of sugar-phosphate backbone in DNA strands, and tyrosine nucleophile (Tyr342) to execute DNA strand cleavage and ligation [7–9]. Previously, the salt-bridge interactions between carboxy-terminal domains of Int were investigated and the functional importance of those interactions were reported in the activation of Int protomers and proper DNA ligation via correct purine–pyrimidine base pairing [7,10,17].

The amino-terminal domain of Int binds specifically to arm-type DNA site. This domain interacts homomerically with the amino-terminal domain of neighboring Int and heteromerically with the Xis accessory protein [11,12]. The twofold symmetric interactions between residues R30 and D71 from adjacent amino-terminal domains bias the resolution of Holliday-junction DNA intermediate [13]. It was suggested that the arm-type DNA may play a critical role in this interaction [13].

The results reported here provide evidence that the homomeric interaction of the amino-terminal domains of Int is dependent on the Int-arm-type DNA binding. Thus, the R30-D71 salt-bridge and the Int-arm-type DNA interaction contribute to the regulation of the catalytic activity of λ Int.

Materials and methods

Oligonucleotides and protein preparation. The wild-type, R30A, D71A, R30D, D71R, R30D;D71R mutant Int proteins were prepared from expression plasmids as previously described method [13].

HPLC purified P′1,2 arm-type DNA and COC' core-type DNA were designed as previously described [14,15] and prepared by Operon Technologies (Alameda, CA). The top strands of the P'1,2 arm-type DNA and COC′ core-type DNA were 5′ end-labeled with γ-P^{32}-ATP (NEN) using T4 polynucleotide kinase and precipitated in 0.3 M Na-acetate and 95% ethanol.

Cleavage assay. The indicated amounts of wild-type and mutant Int proteins were added to 100 ml assay mixtures (10 mM Tris–HCl (pH 8.00), 55 mM NaCl, 5 mM DTT, 0.5 mg/ml of bovine serum albumin (BSA) and 20 nM ^{32}P-labeled COC' core-type DNA. 20 nM P'1,2 arm-type DNA was added for the reactions with arm-type DNA. The reactions were incubated for 30 min at 19 °C. The samples were mixed with 4 ml of Ficoll (final concentration, 2%)

Abbreviations: Int, integrase; HJ, Holliday-junction

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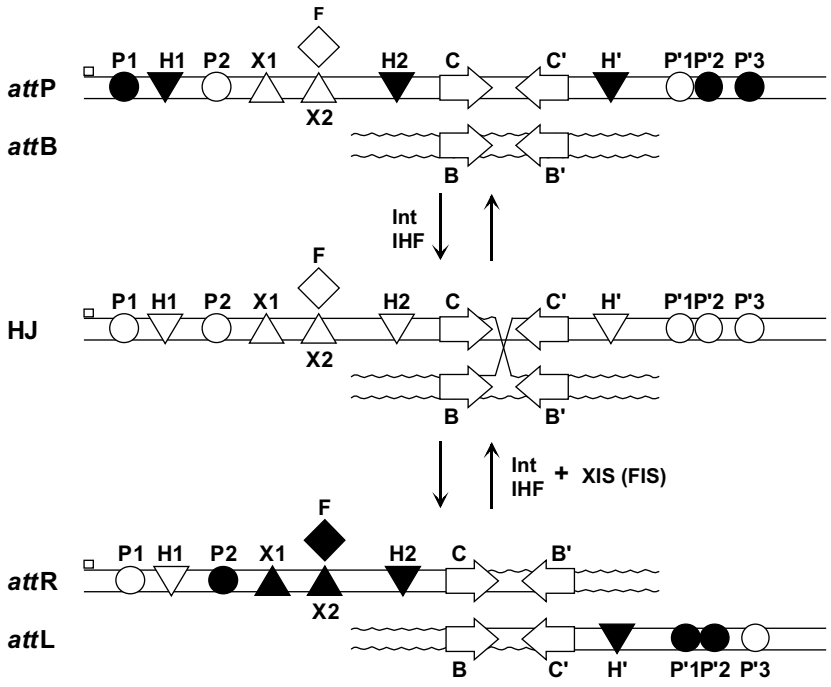

Fig. 1. Diagram of the Int-dependent recombination reaction. Integrative recombination between the phage *att*P and bacterial *att*B sites requires the phageencoded Int protein and the host-encoded IHF to generate *att*L and *att*R prophage sites. Excisive recombination between *att*L and *att*R sites additionally requires the phage-encoded Xis and the host-encoded Fis proteins. Int has four core-type binding sites (C, C′, B, and B′) that flank the 7 bp overlap region as inverted repeats (inverted open arrows). Int cleavage, strand swapping, and ligation at the left boundary of the overlap region generates a four-way DNA junction (Holliday junction recombination intermediate, middle panel), which is resolved to recombinant products by the same sequence of events at the right boundary of the overlap region. There are five arm-type Int binding sites (circles): two single sites in the P arm (P1 and P2) and three adjacent sites in the P' arm (P′1, P′2, and P′3). There are six binding sites for the three DNA-binding accessory proteins: IHF (H1, H2, and H′, inverted triangles), Xis (X1 and X2, upright triangles), and Fis (F, diamond), which overlaps with the X2 site. Protein-binding sites in the arms of *att*P, occupied during integrative recombination, and in the arms of *att*L and *att*R, occupied during excisive recombination, are shown as filled symbols.

loading solution containing SDS and the resulting products were separated by electrophoresis on a 0.4% SDS/7% polyacrylamide gel at 200 V for 30 min. The radio-labeled bands were detected by autoradiography and quantified by a Fuji BAS 2500 phosphorimager system.

Gel retardation assay. Binding of Int to double-stranded DNA fragments was carried out in 10 mM Tris–HCl (pH 8.0), 55 mM NaCl, 5 mM DTT, 0.5 mg/ml of BSA and 10 nM radio-labeled DNA for 30 min at 19 °C. The samples were mixed with loading solution without SDS and separated by electrophoresis (200 V for 30 min) through native, non-denaturing 6% (w/v) polyacrylamide gels. Dried gel was visualized autoradiography and phosphorimaging by Fuji BAS 2500 phosphorimager system.

Results and discussions

R30 and D71 residues of int and the Int-arm-type DNA complex formation

To see whether R30 and D71 are involved in homomeric interaction of Int on arm-type DNA site, two alanine mutants, R30A and D71A, and wild-type Int were tested in regards to arm-type DNA binding. The P′1,2 arm-type DNA has two adjacent binding-sites for the amino-terminal domain of Int, thus allowing the formation of nucleoprotein complex of 2 Int's and a P′1,2 arm-type DNA. Both alanine mutants, R30A, and D71A, were significantly depressed in the formation of 2-Int-P′1,2 nucleoprotein complex compared to the wild-type Int (Fig. 2). This strongly suggests both residues are involved in the 2 Int nucleoprotein complex formation with arm-type DNA.

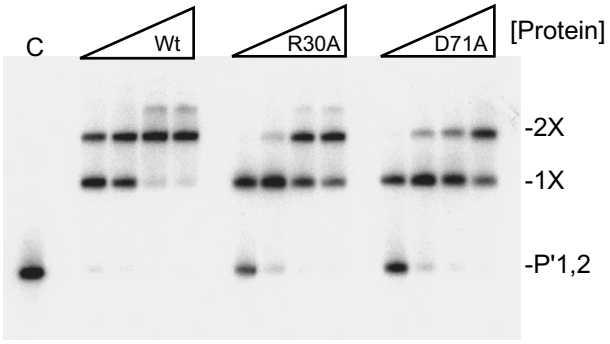

Fig. 2. P′1,2 arm-type DNA binding for Int and Ala substitution mutants. A ^{32}P-labeled P′1,2 arm-type site was incubated with the indicated amounts (nM) on wild-type of Ala mutants and electrophoresed on a native 6% polyacrylamide gel to separate the singly-bound (1×) and doubly-bound (2×) protein–DNA complexes.

R30-D71 interaction of int on arm-type DNA

In the previous study, allele-specific suppression showed the R30-D71 interaction during HJ resolution; the single mutants, R30D and D71R were depressed in HJ resolution while the R30D;D71R double mutant was fully competent in HJ resolution [13]. The depression of single mutants is due to the inability of electrostatic interaction between R30 and D71 residues by charge reversal amino-acid substitution. This inability is rescued by simultaneously reversed charges on two amino-acid residues (R30 to D30, D71 to R71), which allows the reformation of salt-bridge based on the oppositely charged residues. The same logic was em-

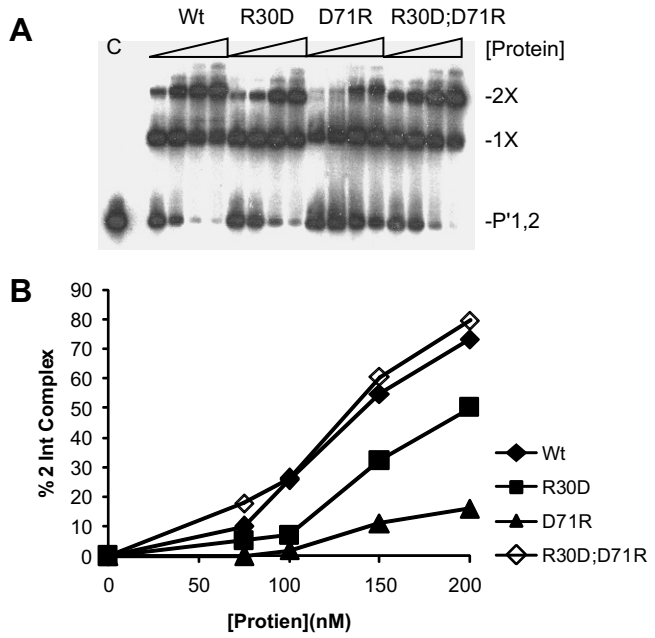

Fig. 3. P′1,2 arm-type DNA binding for charge reversal substitution mutants and wild-type Ints. (A) PAGE profiles of P′1,2 arm-type att site binding assays for the indicated proteins were carried out as described in Fig. 2 and in Materials and methods. (B) Quantification of the gel profiles in (A).

ployed in this study regarding Int's binding to the P′1,2 arm-type DNA. If R30-D71 interaction does exist on the arm-type DNA, any depression of 2 Int-P′1,2 arm-type DNA complex by a single mutant would be recovered by a double mutant.

Both single charge reversal mutants were depressed for the 2-Int complex formation on the P′1,2 DNA substrate in comparison to the wild-type Int (Fig. 3). This result supports the prediction that the charges of residues 30 and 71 are involved in the homomeric interaction of Int on the arm-type DNA. The R30D;D71R double mutant was fully functional in 2-Int complex formation indicating the restoration of an ion pair between residues 30 and 71.

R30-D71 Interaction in the tuning functions of arm-type DNA

The arm-type *att* site binding of Int provides asymmetrical constraints on the recombinogenic complex in conjunction with the twofold symmetric interactions between R30 and D71 of neighboring N-domains of Int [16]. The experimental evidences presented here suggest that the arm-type DNA attenuate one Int protomer from the two protomers bound to it. In this selective inhibition, R30-D71 interaction seems to play a critical role.

In this study, suicide substrates with a single-strand nick at position 3/4 of the seven base-pair overlap region were utilized in order to trap and visualize Int-dependent core-type DNA cleavages (Fig. 4A).

The catalytic activity of Int for the cleavage of core-type DNA is inhibited by the presence of arm-type DNA. In the presence of arm-type DNA, the cleavage percentage was inhibited significantly. It looked as if approximately half of Int protomers were attenuated by arm-type DNA (Fig. 4B).

To look at the role of R30-D71 salt-bridge in the attenuating function of the arm-type DNA, single charge reversal mutants, R30D and D71R, as well as double mutant, R30D;D71R were tested for cleavage reaction in the presence and the absence of arm-type DNA.

In the case of the R30D mutant, the arm-type DNA inhibition of catalytic activity was remarkably higher. Less than 15% of substrate was cleaved in the presence of arm-type DNA indicating a greater inhibition of arm-type DNA than in the wild-type Int (Fig. 4C). The arm-type DNA inhibited the catalytic activities of every Int protomer bound to it.

However, the arm-type effect was different for the other single mutant, D71R. There's no difference in the cleavage activity of the D71R mutant in the presence and the absence of arm-type DNA (Fig. 4D). The attenuation of arm-type DNA does not function for this single mutant Int. Thus, two Int protomers bound to arm-type DNA are fully active regardless of the existence of arm-type DNA without proper electrostatic interaction between residues 30 and 71.

The two opposing effects of each single mutation were compensated for by double charge reversal mutations. The R30D;D71R double mutant responded to the arm-type DNA in the same manner as wild-type Int did (Fig. 4E). Only one Int protomer on the

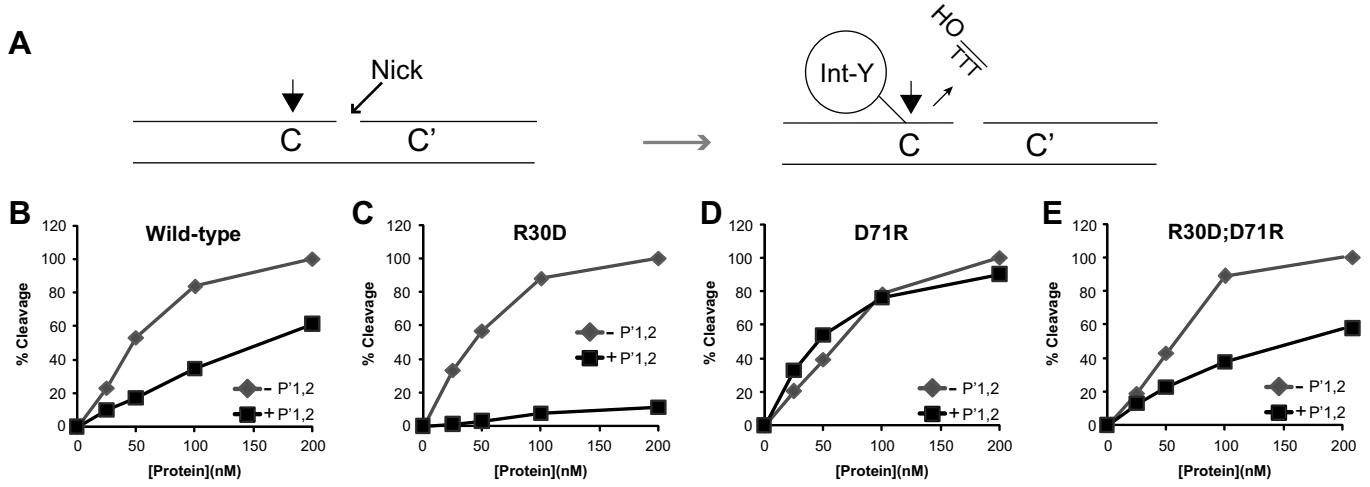

Fig. 4. Diagram of suicide core-type DNA substrate and cleavages of core-type DNA by charge reversal mutant and wild-type Ints in the absence and the presence of P′1,2 arm-type DNA. (A) A suicide *att* site substrate contains either a hydroxyl or a phosphate at the 5′ side of the nick at position 3/4 of the overlap region. When the suicide substrate is cleaved by Int at the TS cleavage site (down vertical arrow) it releases a three base oligonucleotide and thus traps the covalently bound Int cleavage intermediate. (B) Cleavage activity of wild-type Int. (C) Cleavage activity of R30D single mutant Int. (D) Cleavage activity of D71R single mutant Int. (E) Cleavage activity of R30D;D71 double mutant. Cleavage assays were carried out as described in Materials and methods.

arm-type DNA was attenuated. The restoration of the salt-bridge between residues 30 and 71 may contribute to the recovery of "selective" inhibition of catalytic activity of λ Int by arm-type DNA.

This report proposes a new theory on the role of arm-type DNA, which selectively inhibits the catalytic activity of λ Int. As seen Fig. 4B, arm-type DNA decreases Int's catalytic activity for the cleavage of core-type *att* site by half without arm-type DNA. This result may be interpreted to indicate that the arm-binding selectively inhibits a single Int out of two Int protomers on the same arm-type DNA molecule. This attenuation function is closely related to the electrostatic interaction between residues 30 and 71. Loss of this salt-bridge by single charge reversals, R30D and D71R, resulted in opposing interactions with arm-type DNA; greater inhibition and no inhibition, respectively. This may be interpreted to indicate that the inhibition of catalytic activities of both Int protomers on the single arm-type DNA and no inhibition of any Int protomers on the arm-type DNA molecule. The restoration of the electrostatic interaction between residues 30 and 71 by double charge reversals R30D;D71R resulted in the same level of attenuation function of arm-type DNA as for the wild-type Int. This suggests the "selective" inhibition of catalytic activity of Int by the arm-type DNA may be directly related with the amino-terminal domain interactions of Int.

The selective inhibition of the arm-type DNA with R30-D71 salt-bridge may be the core mechanism of the recombination reaction that requires highly coordinated activation of Int protomers.

These results have significant implications for understanding the mechanism of the allosteric regulation of the heterobivalent DNA-binding λ integrase and possibly of other DNA-binding multimeric enzymes.

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