JOURNAL OF BACTERIOLOGY, Oct. 2008, p. 6918–6921 Vol. 190, No. 20
0021-9193/08/$08.00+0 doi:10.1128/JB.00612-08

Role of the N-Terminal Domain of φC31 Integrase in *attB-attP* Synapsis▽

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Received 2 May 2008/Accepted 1 August 2008

φC31 integrase is a serine recombinase containing an N-terminal domain (NTD) that provides catalytic activity and a large C-terminal domain that controls which pair of DNA substrates is able to synapse. We show here that substitutions in amino acid V129 in the NTD can lead to defects in synapsis and DNA cleavage, indicating that the NTD also has an important role in synapsis.

The integrase (Int) of the *Streptomyces* phage, φC31, is a member of the large serine recombinases, a family of proteins that includes phage Ints and transposases (11). φC31 Int has two domains, the N-terminal domain (NTD), which provides the catalytic activity, and the C-terminal domain (CTD), required for the control of integration versus excision (9, 13). All of the phage-encoded large serine recombinases that have been studied in detail are unidirectional in the absence of accessory factors (2, 7, 13). The DNA substrates (*attB*, *attP*, *attL*, and *attR*) for the phage-encoded serine Ints are small (<50 bp) compared to those used by other directional recombinases (2, 4, 7, 14). For integration, Int dimers bind to *attP* and *attB*, and dimer-dimer interactions are thought to bring the substrates together to form a tetrameric synaptic complex (5, 6, 14). Synapsis is prevented when Int is bound to *attL* and *attR*, and this underpins Int unidirectionality in the absence of accessory factors (12, 14). Mutations in the CTD cause hyperactivity, i.e., Int is able to mediate both *attB* × *attP* and *attL* × *attR* synapsis and recombination. Since these mutations lie in a putative coiled-coil motif, it is possible that the CTD has a direct role in protein-protein interactions in synapsis. In the resolvase/invertase group of serine recombinases, genetic, biochemical, and structural information has shown that synapsis occurs via the NTDs. There is considerable sequence divergence between the NTDs of the large serine recombinases and those of the resolvase/invertases; for instance, the NTDs of γδ and φC31 Int are only 20% identical, as shown by a CLUSTAL W alignment (Fig. 1). Since formation of the synapse is central to understanding how Int controls directionality, we sought to determine whether the φC31 NTD is involved in synapsis of Int bound to *attP* and *attB*.

Despite the sequence divergence, the output from a structure prediction program JPRED (3) for φC31 Int NTD resembled the structure of the NTD of γδ and Tn*3* resolvase, with the conservation of important structural elements. In the resolvases, substitutions at residues 100 to 103 can lead to activated resolvases, which no longer require accessory sites for recombination (1). Whereas wild-type (wt) resolvase is dimeric in solution, activated mutants of resolvase are tetrameric through dimer-dimer interactions that generate the synaptic interface (10). The tertiary structures of wt resolvase dimers and the tetrameric synaptic intermediate are very different, particularly in the positioning of the long E helix (8, 15). Since residues 100 to 103 are at the heart of the synaptic interface and are located where a flexible linker joins the long E helix, substitutions here are thought to facilitate the conformational changes associated with the dimer-to-tetramer transition (8). A mutant φC31 Int, IntV129A, was isolated previously from a library of defective Int mutants (9). Since IntV129A aligned with M103 from resolvase, we were interested to discover the basis for its poor activity. IntV129A was overexpressed, purified, and assayed for in vitro recombination activity. IntV129A was very defective in *attP* × *attB* recombination and had no detectable *attL* × *attR* activity (Fig. 2 and data not shown). To assay synapsis, a doubly substituted Int was generated, IntS12A,V129A. The S12A mutation inactivates the serine nucleophile and permits accumulation of synaptic complexes, which can be detected in vitro using DNA supershifts in nondenaturing gels (12). The amount of free *attB* was reduced at the same rate in reactions with IntS12A and IntS12A,V129A, indicating that binding to *attB* was not affected by the V129A substitution (Fig. 3A). IntS12A, however, accumulated synaptic complexes at a faster rate and to a greater level (sixfold more synapse present after 60 min) than IntS12A,V129A (Fig. 3A).

Since IntV129A is a conservative mutation, site-directed mutagenesis was used to investigate how other amino acid substitutions at V129 influenced Int recombination. Five substitutions were introduced at position V129 (V129G, V129L, V129M, V129F, and V129E). The mutant proteins were purified and tested for in vitro recombination activity with *attB* and *attP* (Fig. 2). Int V129G and Int V129E were inactive in vitro. Gel filtration analysis of the purified mutant Int showed that all V129 mutants were dimeric in solution, indicating that monomer-monomer interactions were not affected by the substitutions at V129 (data not shown).

Since formation of the synaptic complex was inhibited by V129A (Fig. 3A), synapsis by IntV129G, IntV129L, IntV129M, and IntV129F was assayed. The S12A mutation was introduced into each *int* allele and, with the exception of IntS12A,V129E, the proteins purified. Like IntS12A,V129A, IntS12A,V129F was defective in synapsis. IntS12A,V129L, IntS12A,V129M,

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▽ Published ahead of print on 8 August 2008.

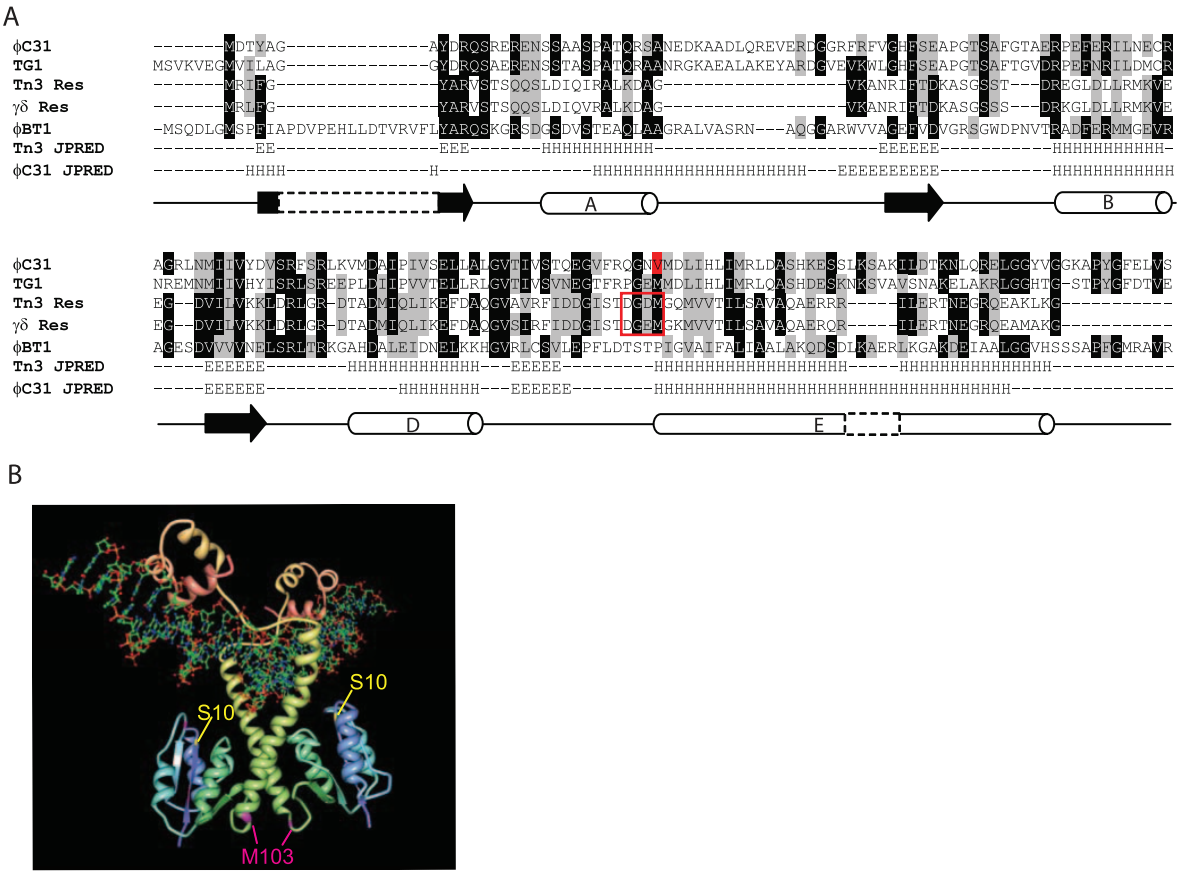

FIG. 1. Sequence alignment and predicted structural similarities between the NTDs of several serine recombinases. (A) CLUSTAL W alignment of γδ resolvase, Tn*3* resolvase, φC31 Int, and the Ints from two φC31-related *Streptomyces* phages, TG1 and φBT1. Gray background shading represents conserved amino acids, and black shading indicates that >50% of the sequences have an identical residue at that position. The position of V129 in φC31 Int is indicated by a red highlight, and amino acids 100 to 103 in the resolvases are boxed. JPRED secondary structure predictions are displayed below the alignment for Tn*3* resolvase and for φC31 Int with a diagram depiction of the γδ secondary structure below (3) (H); cylinders represent α-helix (E), and black arrows represent the β-sheet. (B) Structure of γδ dimer bound to DNA (1GDT; 15). The position of γδ M103, which aligns with φC31 IntV129, is indicated in pink. The catalytic serine, S10, is highlighted in yellow. The image was created with PDB Protein Workshop.

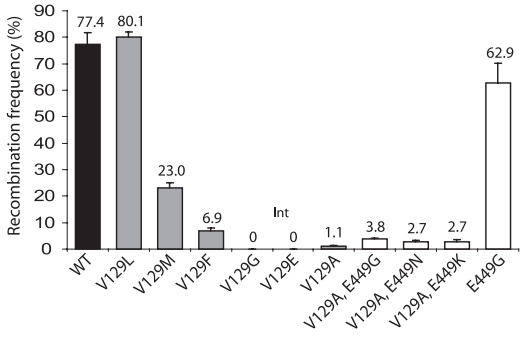

FIG. 2. In vitro activities of purified Ints containing different substitutions at V129. Quantitative assay of recombination activity in vitro of wt Int (■) versus Int mutants with single substitutions (▨) and double substitutions (□). Ints were purified and assayed as described previously (9, 12). Ints (733 nM) were incubated (2 h, 30°C in R buffer) with the reporter plasmid, pRT508 (0.15 nM) encoding *attP* and *attB* flanking *lacZ*. Deletion of the *lacZ* marker in pRT508 by *attP* × *attB* recombination was assayed by introduction of the whole reaction mixture into DH5α and scoring the fraction of white colonies after selection for transformants on plates containing carbenicillin, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and IPTG (isopropyl-β-D-thiogalactopyranoside). The data are the averages and standard errors for two replicates.

and IntS12A,V129G were as efficient as IntS12A in the synapsis of *attB* and *attP* (Fig. 3B and data not shown). Since IntV129G was completely defective in the *attP* × *attB* recombination but able to synapse as well as wt Int, we tested whether V129G might be defective in the step that follows synapsis, i.e., DNA cleavage.

Concomitant with DNA cleavage by Int a phospho-serine bond is made, most likely between the 5′ end of the DNA and amino acid S12. This cleaved intermediate can be readily detected when Int is provided with linear substrates (one of which is radiolabeled) and, after incubation is treated with a protease (12). IntV129G showed a noticeable reduction in the amount of cleaved substrate compared to wt Int and a corresponding increase in the amount of synaptic complex (Fig. 3C). Thus, recombination-defective mutations at V129 can be affected in synapsis and activation of DNA cleavage.

In the crystal structures of γδ resolvase, the flexing of the linker region adjacent to M103 is an obvious feature of the transition from the dimeric to the tetrameric structures, a conformational change that is centered on the movement and packing of four E-helices (8). It seems likely that V129 has a similar function in Int as M103 has in the resolvases, playing a part in both synapsis

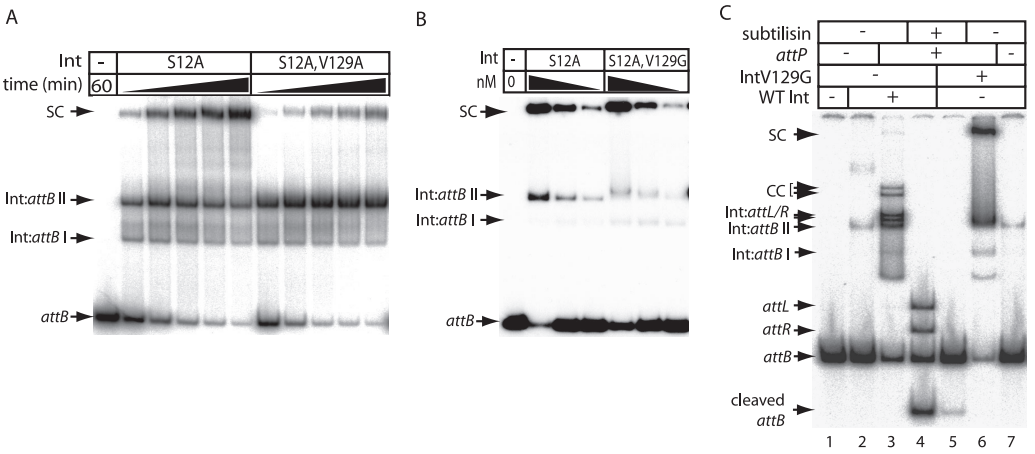

FIG. 3. Effects of the V129 substitutions on DNA synapsis and cleavage. (A) The catalytically inactive mutants IntS12A and IntS12A,V129A were used to assay the formation of synaptic complexes. Reactions contained radiolabeled *attB* (1.5 nM), unlabeled *attP* (14 nM), and Int (733 nM) in binding buffer (12) and were incubated (30°C) for 10, 20, 40, 60, or 120 min. Reactions were analyzed by polyacrylamide gel electrophoresis (5% polyacrylamide, 1× Tris-borate-EDTA). The complexes observed were as described previously (12) and are Int bound to *attB* (Int:*attB* complexes I and II, which are most likely Int monomers and dimers, respectively) or synaptic complexes (SC). (B) A V129G mutation did not adversely affect the ability of IntS12A to synapse. Three concentrations (92, 46, or 23 nM) of IntS12A or IntS12A,V129G were incubated (30°C, 2 h) with radiolabeled *attB* and unlabeled *attP* as described in panel A. (C) The mutation V129G inhibits DNA cleavage. wt Int (733 nM; lanes 5 to 7) or IntV129G (733 nM; lanes 2 to 4) was incubated with radiolabeled *attB* with or without unlabeled *attP* as described in panel A. *attB*-bound complexes are indicated (Int:*attB* I and II). Recombination intermediates were characterized previously (12) and are indicated as SC (for synaptic complexes) and CC (for covalent complexes). Free recombination products, *attL* and *attR*, or products bound to Int, Int:*attL*, and Int:*attR* are also indicated. Subtilisin was added to lanes 4 and 5 to release the DNA from bound Int. Free *attB* is shown in lane 1.

and in the conformation changes that are thought to occur postsynapsis to activate DNA cleavage. It is noteworthy that cleavage defects of this kind have yet to be reported for any mutations within the same region of the resolvases.

Mutations in the CTD of Int partly rescue the unstable synapsis of V129A. Previous work has demonstrated that a single mutation within the Int CTD at position E449 enable the recombinase to undertake a variety of novel recombination reactions (9). The basis for this extended recombinational repertoire is thought to be the ability of the mutant Int to stabilize a synapse between *att* sites that would not normally form a synaptic complex (9). Since V129 appeared to be involved in synapsis, the question arose as to whether this residue interacts independently of, or in concert with, the Int CTD during Int synapsis and recombination. To test this, plasmids encoding IntV129A and IntV129G were used as substrates for site-directed mutagenesis to introduce mutations at E449. The double mutants were tested in an in vivo recombination assay in which deletion of *lacZ* by *attP* × *attB* recombination resulted in a white colony color on selective media (9). IntV129A,E449G

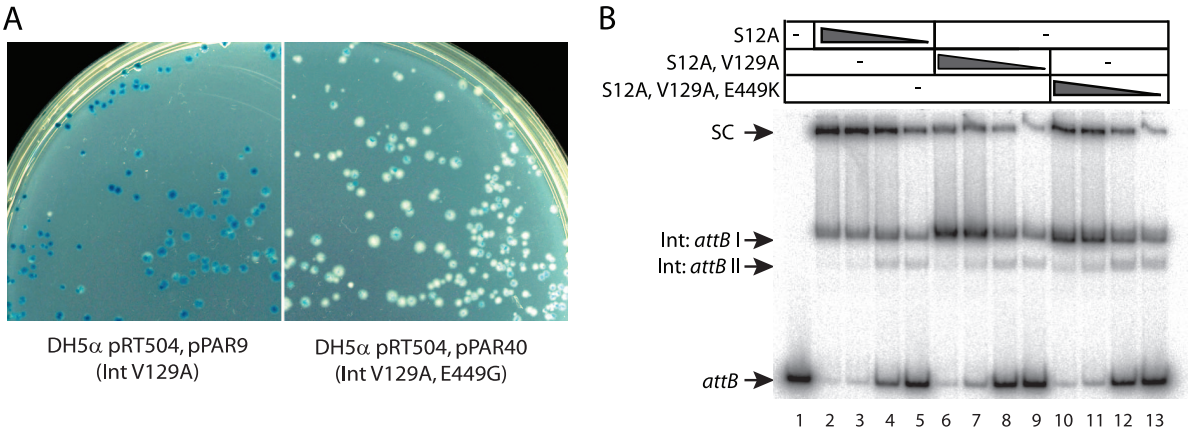

FIG. 4. Partial rescue of recombination activity and synapse formation in V129A by hyperactive mutations at E449. (A) In vivo recombination by IntV129A versus IntV129A,E449G. Almost every colony containing pPAR40 (encoding IntV129A,E449G) is mainly white, with some blue sectors, indicating loss of the *lacZ* gene in pRT504 after *attP* × *attB* recombination (9). This is compared to largely blue colonies obtained when pPAR9 (encoding IntV129A) is present with pRT504. (B) Synapsis by IntS12A (lanes 2 to 5), IntS12A,V129A (lanes 6 to 9), and IntS12A,V129A, E449K (lanes 10 to 13). Radiolabeled *attB* was incubated with unlabeled *attP* and various concentrations of Int—246 nM (lanes 2, 6, and 10), 123 nM (lanes 3, 7, and 11), 61 nM (lanes 4, 8, and 12), or 31 nM (lanes 5, 9, and 13)—as described in the legend to Fig. 3. Quantitation of the signal intensities in the synaptic complexes formed with 246 nM Int revealed that over two independent experiments the mean percentage of counts in the IntS12A,V129A,E449K synapse was 39.6% ± 0.03% (standard error) compared to 19.7% ± 0.23% with IntS12A,V129A.

showed a restoration of *attP* × *attB* recombination in vivo, indicated by extensive white sectoring in the transformants compared to largely blue colonies in transformants expressing IntV129A (Fig. 4A). Plasmids encoding double mutants that combined IntV129A with E449K, E449H, E449F, E449N, or E449R all gave similar sectored white and blue colonies (data not shown). In contrast, plasmids encoding either IntV129G, E449K or IntV129G,E449N gave only blue colonies, indistinguishable from those obtained in the presence of the single mutant IntV129G (data not shown). IntV129A,E449G, IntV129A, E449K, and IntV129A,E449N were purified and used in in vitro recombination assays and compared to wt Int and IntV129A. A small but consistent increase in the frequency of *attP* × *attB* recombination was observed when V129A was combined with E449G, E449N, or E449K (Fig. 2). The modest rescue of activity was investigated further through the construction of an S12A derivative of IntV129A,E449K in order to test the ability of this triple mutant to synapse *attB* and *attP*. Under the same conditions IntS12A,V129A,E449K was able to trap, reproducibly, twice the amount of *attB* probe into synaptic complexes compared to the double mutant IntS12A,V129A (Fig. 4B). The substitution E449K was, therefore, partially able to rescue the synapse defect caused by the mutation V129A, which may have resulted in the small increase in in vivo *attP* × *attB* recombination activity (Fig. 2). It is not known to what extent IntV129A could be affected in DNA cleavage in addition to the synapse defect. Since E449K appeared to be unable to rescue the DNA cleavage defect in IntV129G, introduction of the E449K mutation into IntV129A would not be expected to enhance DNA cleavage, and this could help to explain the low recombination activity in the IntV129A mutants, particularly IntV129A,E449K. None of the double mutants containing substitutions in both V129 and E449 were able to recombine *attL* × *attR* (data not shown).

The data presented here indicate that the NTD of the large serine recombinase ϕC31 Int has a role in synapsis and activation of recombination. We propose that, like resolvase, Int has a flexible region around V129 that is crucial for the communication of conformation changes and activation of catalysis (8). Due to the fact that recombination is regulated by the formation of a stable synapse, we and others have proposed that Int bound to *attP* and *attB* adopts specific conformers that allow synapsis (5, 12, 14). It seems likely that protein conformations in the CTD, adopted through binding to different *att* sites, influence the stability of the synaptic interface in the N-terminal domain and the activation of DNA cleavage.

This study was supported by the Biotechnology and Biological Science Research Council of the United Kingdom (grant reference BB/D007836/1) and by a University of Aberdeen studentship to P.A.R.

We are grateful to The Medical Illustration Unit, University of Aberdeen, for photography.

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