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NOTES

Tn5 Synaptic Complex Formation: Role of Transposase Residue W450[▽]

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A series of Tn5 transposases (Tnp's) with mutations at the conserved amino acid position W450, which was structurally predicted to be important for synapsis, have been generated and characterized. This study demonstrates that W450 is involved in hydrophobic (and possibly aromatic) contacts within the Tnp monomer that negatively regulate synaptic complex formation.

Tn5 transposase (Tnp) is a member of the retroviral integrase superfamily of proteins. This superfamily includes other Tnp's, as well as proteins such as human immunodeficiency virus type 1 integrase and the RAG-1 recombinase of V(D)J recombination. The members of this superfamily share many mechanistic details, as well as structurally conserved active sites (4, 6, 12). In order to catalyze DNA movements, these proteins adopt highly ordered protein-DNA synaptic complexes. Based on structural studies with Tn5 Tnp, it is known that these complexes involve large numbers of protein-protein and protein-DNA contacts (3) and generally involve the rearrangement of the proteins. The catalytic steps of Tn5 transposition are depicted in Fig. 1.

Tn5 transposition is a tightly regulated process, as excessive transposition activity would likely lead to instability of the host cell genome. Previous studies led to a model in which Tn5 Tnp monomers manifest an intramolecular interaction between the N terminus, containing end sequence (ES) DNA binding residues, and the C terminus, containing the protein-protein dimerization domain, that inhibits synaptic complex formation (9, 15). Tn5 Tnp is monomeric in solution and binds to ES DNA substrates only as a dimer (5, 9, 15). Tn5 inhibitor Inh (lacking 55 N-terminal residues) exists as a dimer in solution, suggesting that the N terminus of free Tnp blocks dimerization (9). When the C-terminal dimerization domain is removed from Tnp (in a C-terminal truncation mutant protein, Δ369), Tnp is unable to form dimers or synaptic complexes (14, 16, 17). However, Δ369 is able to bind to ES DNA as a monomer (17). Thus, the removal of the C terminus allows ES DNA binding. It is likely that C-terminal deletions of Mu and IS911 transposases also enhance ES DNA binding (1, 7).

In this study, we investigated the role of amino acid W450 in transposition and demonstrated that this residue is inhibitory for synapsis and may contribute to the N terminus-C terminus inhibitory interaction. W450 is conserved across IS50/Tn5 transposase-related proteins (11). W450 is located at the beginning of the C-terminal α-helix (3) that is spatially dislocated by the hyperactive L372P mutation (10, 15). W450 is also located adjacent to a previously identified, modestly hyperactive mutation, E451Q (8). Mutations at position 450 were introduced via site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). All control and mutant Tnp constructs in this study contained three hyperactive mutations, E54K (19), M56A, and L372P (15), unless otherwise specified. All Tnp mutant forms were overexpressed and purified as previously described (2).

In vivo analysis of W450 substitutions. The in vivo activities of the proteins with W450 substitutions were analyzed utilizing a qualitative, colorimetric papillation assay performed as previously described (19). In this assay, transposition levels were estimated by using the total number of blue papillae and mutant Tnp's were compared to the control Tnp.

Compared to the control, the W450C, W450S, and W450R mutant forms demonstrated a hyperpapillation phenotype after ~48 h incubation (Fig. 2A). It is important that these mutant proteins not only displayed a net increase in total papillae but also formed papillae faster than the control Tnp (they exhibited blue papillae ~8 h earlier than the control) (data not shown). When the tryptophan group was replaced with another hydrophobic, aromatic functional group through a W450F mutation, the proteins formed papillae at the same relative rate as the control and arrived at the same relative amount of total papillae formed after ~48 h. These results indicate that the hydrophobic (and possibly aromatic) properties of residue W450 are deleterious for transposition in vivo.

The effect of the W450C mutation was also investigated in relation to the mutation that is likely to be involved in a spatial distortion of the C-terminal α-helix and that may compromise the N terminus-C terminus inhibitory interaction, L372P (10, 15). W450C mutant Tnp (lacking L372P) was shown to be hyperactive versus a control Tnp lacking both the W450C and L372P mutations (Fig. 2B). This result indicates that the mu-
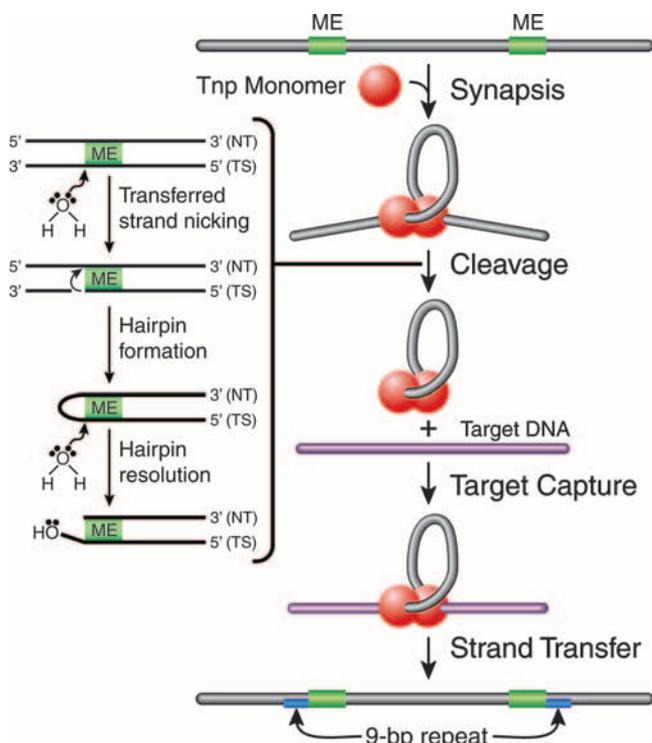
FIG. 1. Tn5 transposition mechanism. Transposition is initiated by Tnp binding to the transposon-specific ESs and the formation of a highly ordered nucleoprotein complex (synaptic complex) through a process called synapsis. The synaptic complex contains two protomers of Tnp, which exist as a dimer, and two ESs. Catalytic cleavage occurs when an activated water molecule coordinated by Mg^{2+} nicks the transferred DNA strand (TS) on both sides of the transposon, through a nucleophilic attack, forming a 3′-hydroxyl group. The free 3′-hydroxyl group acts as a nucleophile and cleaves the nontransferred DNA strand (NT), forming a hairpin. A second activated water molecule resolves the hairpin, resulting in a double-stranded DNA cleavage product. The postcleavage synaptic complex is now free to bind to target DNA through target capture. The 3′-hydroxyl group of the transposon end attacks the phosphodiester backbone of target DNA during strand transfer. A 9-bp duplication in the target results, due to the staggered strand transfer reactions followed by DNA repair by host enzymes.
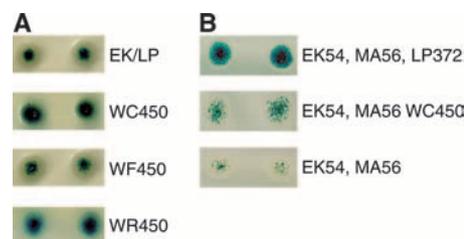

FIG. 2. In vivo (papillation) transposition assay. Transposition levels were estimated by using the total number of blue papillae, and mutant Tnp's were compared to control Tnp to qualitatively assess transposase activity levels. (A) The W450F mutant Tnp (WF450) showed the same relative transposition activity as the control, indicating that W450 is involved in hydrophobic (and possibly aromatic) contacts. The W450C (WC450), W450S, and W450R (WR450) mutant Tnp's led to an ~6-fold increase in transposition activity and proceeded to catalyze transposition (form blue papillae) ~8 h before the control (EK/LP) (data not shown). (B) Transposition activity levels of the W450C mutant Tnp (lacking L372P) and the control were compared. The W450C mutant Tnp was shown to be less active in transposition than control Tnp, but the mutation at position 450 still led to a general increase in transposition activity compared to that of Tnp containing the E54K (EK54) and M56A (MA56) mutations. This finding indicates that the increase in transposition activity conferred by mutations at position 450 is not dependent on the presence of the L372P (LP372) mutation.

tations at position 450 are independent of (and likely synergistic with) the L372P mutation and that mutations at both sites are likely to be involved in overcoming the inhibitory conformation.

The primary mechanistic step of transposition affected by W450 mutations is synaptic complex formation. This study was conducted with the belief that position W450 is involved in inhibitory contact(s) with the N terminus of the Tnp. Therefore, it stood to reason that mutations at this position would have large effects on synaptic complex formation. This assumption was assayed by measuring the effects of the mutant Tnp's on paired-end complex (PEC) formation. The PEC is an experimental analog of the synaptic complex. Tnp was incubated with fluorescently labeled 60-bp oligonucleotides containing the hyperactive 19-bp mosaic-end (ME) ES, and PECs were fractionated from free DNA by means of native polyacrylamide gel electrophoresis (PAGE) as previously described (13).

Tnp forms with substitutions that replaced the tryptophan

group that were shown to be hyperactive for in vivo transposition led to dramatic increases in synaptic complex formation in vitro. The hyperactive W450C, W450S, and W450R mutant proteins formed 81, 70, and 76% PECs (Fig. 3, lanes W450C, W450S, and W450R), respectively, which represented increases in complex formation of 1.8-, 1.5-, and 1.8-fold, respectively, compared to that by the control, and none of the mutant Tnp's displayed any ability to bind to DNA in a monomeric fashion. Consistent with in vivo transposition data, the replacement of the tryptophan group with a phenyl group in the W450F mutant Tnp led to control-like activity for PEC formation (Fig. 3, lanes W450F).

The results of the PEC formation assay indicate that the native amino acid W450 is deleterious for synaptic complex formation. Furthermore, these results indicate that this tryptophan is likely to be involved in hydrophobic (and possibly aromatic) contacts which are most likely responsible for the inhibition of synaptic complex formation.

The W450 Tnp mutant forms were further investigated in vitro to determine if the mutations had additional effects on the remaining mechanistic steps of transposition: cleavage and strand transfer (current experimental techniques do not allow for the independent analysis of target capture and strand transfer). The cleavage assay was performed by incubating mutant Tnp's with fluorescently labeled 60-bp oligonucleotides containing the 19-bp ME to form PECs and then adding 10 mM Mg^{2+} to induce cleavage of the transposon from donor backbone (dbb) DNA as previously described (13). The cleavage reaction gives rise to two products: the single-end-break product (SEB) that represents the cleavage of one dbb-ES junction and the double-end-break product (DEB) that represents the cleavage of both dbb-ES junctions. The three protein-DNA complexes were separated by fractionation of the PECs from
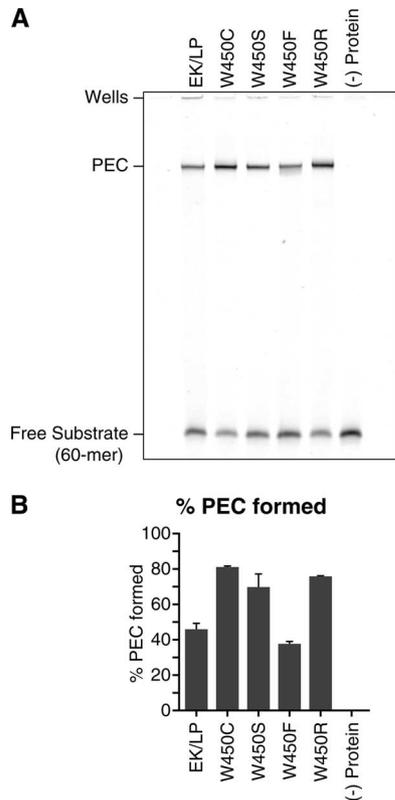
FIG. 3. PEC formation assay. (A) PECs were formed by incubating Tnp with double-stranded, fluorescently labeled DNA oligomers (60-mers) for 1 h at 37°C as previously described, with the addition of 100 μg of tRNA/ml to the reaction buffer (13). Products were visualized via 6% native PAGE. These experiments were repeated three times, and the gel shown is representative of all of these experiments. EK/LP, control Tnp; (−) protein, no protein. (B) PEC formation was quantified by comparing the amount of labeled DNA in the PEC to the total amount of labeled DNA. These experiments were repeated three times, and the results shown are representative of all of these experiments. PEC formation was enhanced for W450C, W450S, and W450R mutant Tnp's, which formed 81, 70, and 76% PECs, respectively, in contrast to the control Tnp, which formed 46% PECs. These differences represent increases of 1.8-, 1.5-, and 1.8-fold, respectively. The W450F mutant Tnp formed 38% PECs, which represents an 18% reduction compared to the control Tnp. Increases in PEC formation were most likely due to the removal of the hydrophobic (and possibly aromatic) contacts between amino acid W450 and its binding partners, presumably on the N-terminal portion of the Tnp monomer. In this model, the removal of these contacts shifts the conformation equilibrium of the Tnp monomer away from the inhibitory conformation.

free DNA by means of native PAGE as previously described (13).

With the exception of that by the W450R mutant Tnp, cleavage was largely unaffected by mutations at position W450. At each mechanistic step of cleavage, W450C, W450F, and W450S mutant Tnp's proceeded at rates that were comparable with that of the control Tnp, and the mutant forms proceeded to the same end point in the reactions (Fig. 4). Surprisingly, the W450R mutant Tnp led to a general increase in cleavage at each mechanistic step of cleavage (Fig. 4). It is possible that larger structural changes were introduced into the PEC by substituting the large, charged guanidine group for the wild-type tryptophan group. These structural changes may in turn
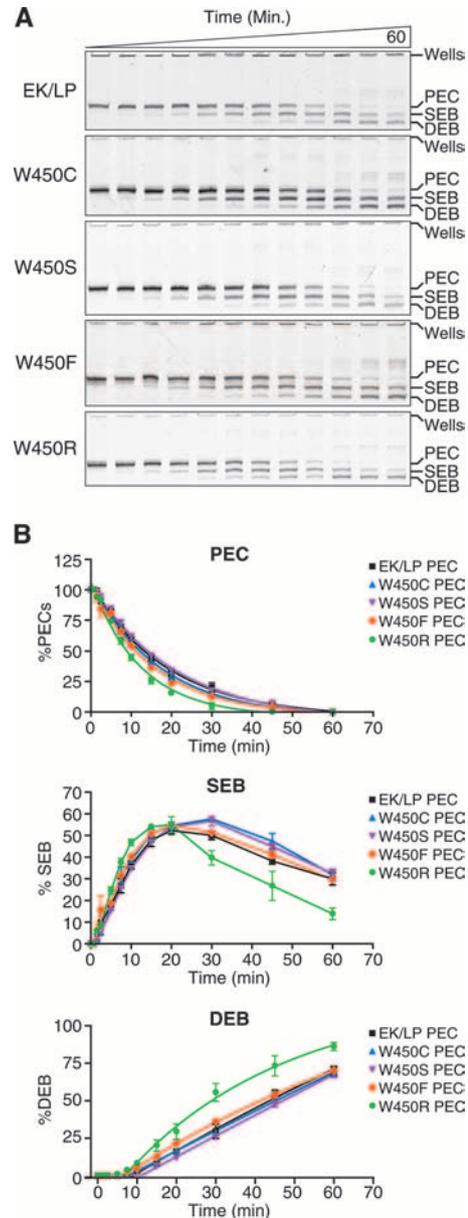

FIG. 4. Cleavage assay. (A) Cleavage activity was assessed by forming PECs with fluorescently labeled DNA oligomers (60-mer) at 37°C for 1 h as previously described, with the addition of 100 μg of tRNA/ml to the reaction buffer (13). After complex formation, Mg^{2+} was added to a final concentration of 10 mM to catalyze the cleavage mechanism. Cleavage results in two products: the SEB and the DEB. Cleavage products, along with unreacted PEC, were visualized via 6% native PAGE. The time course proceeded for 1 h. These experiments were repeated three times, and the gel shown is representative of all of these experiments. EK/LP, control Tnp. (B) The percentage of labeled oligonucleotide in each complex (the PEC, SEB, or DEB) relative to the total amount of the substrate contained in the complex was determined. With the exception of the W450R mutant Tnp, the Tnp's with mutations at position W450 behaved similarly to the control Tnp. The mutant Tnp forms had approximately the same rates and total yields of PEC cleavage, SEB formation and destruction, and DEB formation. The W450R mutant Tnp led to an increase in the rate of cleavage over that of the control, forming SEB complexes and converting SEB complexes into DEB complexes faster than the control. This increased rate of cleavage was repeatable over all three separate experiments. The data indicate that position W450 does not play a large role in the cleavage mechanism but that the substitution of a large charged amino acid leads to hyperactivity, possibly through steric interference with the W450 binding partners.

have led to a Tnp conformation that was more reactive for cleavage. Except for the W450R result, these results indicate that residue W450 does not play a large role in cleavage.

The strand transfer capabilities of the mutant Tnp's were assayed by preforming PECs with fluorescently labeled 40-bp oligonucleotides containing the ME as previously described (13). Following PEC formation, the reaction mixtures were incubated at 20°C for 30 min. Strand transfer was induced with the addition of 10 mM Mg^{2+} and 15 nM supercoiled pUC19 DNA (to serve as target DNA) as previously described (13). Strand transfer leads to two products: an open-circle single-end strand transfer product and a linear double-end strand transfer product. These products were separated from unreacted substrate DNA via electrophoresis on a 2% agarose gel.

Tnp's with mutations at residue W450 have strand transfer activities very similar to that of the control Tnp. The three mutant Tnp's that have been shown to be hyperactive for in vivo transposition and synapsis (the W450C, W450R, and W450S mutant forms) were slightly hyperactive for total strand transfer, with each showing an increase of ~10% compared to that by the control Tnp (data not shown). The W450F mutant Tnp showed a slight deficiency in strand transfer (~10% decrease in total strand transfer activity) compared to that by the control (data not shown). These slight changes in strand transfer activity led us to conclude that residue W450 does not play a significant role in either target capture or strand transfer.

Model for W450 activity in Tn5 transposition. It has been shown previously that W450 is inhibitory for transposition in vivo, and this inhibition seems to act at the level of synaptic complex formation that is critical in controlling the initiation of transposition. Position W450 is likely to be involved in hydrophobic (possibly aromatic) interactions, as replacement with phenylalanine leads to wild-type activity levels. These results are in agreement with the prediction that W450 is involved in the N terminus-C terminus inhibitory interaction. It is believed that by removing the tryptophan functional group, the C-terminal α-helix is better able to move away from the N terminus. In this model, W450 serves as a tether that keeps the helix in proximity to the N terminus.

One explanation for the effect of residue W450 on transposition posits that the W450 contacts other, presumably N-terminal, residues in monomeric Tn5 Tnp. It is difficult to predict the residues that W450 would contact prior to DNA binding, as no X-ray crystal structure of the full-length, monomeric, DNA-free form of the Tnp exists. However, one candidate region is located near residue 40; thus, we introduced mutations into position 40 and nearby residues and examined the resulting mutant proteins for effects on Tn5 transposition. All of these mutant transposases were defective in transposition. This result is not surprising given that ES binding contacts are located in the same region (3, 18). Therefore, the results of these

analyses were uninformative about the various N-terminal residues potentially playing a role in the N terminus-C terminus inhibitory interactions because the negative effects of the loss of ES binding would be epistatic for any possible beneficial effects on the Tnp conformation.

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