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The N-terminal domain of DnaT, a primosomal DNA replication protein, is crucial for PriB binding and self-trimerization

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ABSTRACT

DnaT and PriB are replication restart primosomal proteins required for re-initiating chromosomal DNA replication in bacteria. Although the interaction of DnaT with PriB has been proposed, which region of DnaT is involved in PriB binding and self-trimerization remains unknown. In this study, we identified the N-terminal domain in DnaT (aa 1–83) that is important in PriB binding and self-trimerization but not in single-stranded DNA (ssDNA) binding. DnaT and the deletion mutant DnaT42-179 protein can bind to PriB according to native polyacrylamide gel electrophoresis, Western blot analysis, and pull-down assay, whereas DnaT84-179 cannot bind to PriB. In contrast to DnaT, DnaT26-179, and DnaT42-179 proteins, which form distinct complexes with ssDNA of different lengths, DnaT84-179 forms only a single complex with ssDNA. Analysis of DnaT84-179 protein by gel filtration chromatography showed a stable monomer in solution rather than a trimer, such as DnaT, DnaT26-179, and DnaT42-179 proteins. These results constitute a pioneering study of the domain definition of DnaT. Further research can directly focus on determining how DnaT binds to the PriA–PriB–DNA tricomplex in replication restart by the hand-off mechanism.

1. Introduction

Genetic integrity should be maintained from generation to generation for bacteria to survive [1]. When DNA is damaged, the DNA replication machinery may be arrested anywhere in the chromosome [2]. Collapsed DNA replication forks must be reactivated by the replication restart primosome by the origin-independent reloading of the polymerase to duplicate genomes [2,3]. After DNA repair, the replication restart primosome [4–6], a formidable enzymatic machine, travels along the lagging strand template, unwinds the duplex DNA, and primes the Okazaki fragments required for the progression of replication forks [7,8]. The replication restart primosome preferentially recognizes three-way branched DNA structures with a leading strand [9–12]. In *Escherichia coli*, the PriA-directed primosome includes six essential proteins, namely, PriA, PriB, DnaB, DnaC, DnaT, and DnaG [5]. PriA, an initiator protein, first binds to the forked DNA. PriB and DnaT are the second and third proteins, respectively, to be assembled in the protein–DNA complex. The association of DnaT with PriA is facilitated by PriB [13]. The binding of DnaT to PriA and PriB dissociates PriB from single-stranded DNA (ssDNA) [14]. However, which region of DnaT interacts with PriB remains unclear.

DnaT, formerly known as protein i [15–17], is a key component for the replication of phage ϕX174 [18] and pBR322 plasmid but not for that of R1 plasmid [19]. Genetic analysis of *E. coli* DnaT suggests an essential replication protein for the growth of bacterial cells because the *dnaT822* mutant shows colony size, cell morphology, inability to properly partition nucleoids, UV sensitivity, and basal SOS expression similar to those of *priA2::kan* mutants [20]. DnaT is also required for *E. coli* growth at elevated pressure [21] and for the lytic cycle of Mu growth [22].

DnaT is a homotrimer of approximately 22 kDa subunits [17,23]. The N-terminal region, aa 1–41, is not crucial for the oligomerization of DnaT [23]. However, DnaT also exists in solution as a monomer–trimer equilibrium system [24]. The ssDNA-binding site of DnaT may primarily be located in the C-terminal region (aa 42–179) with a size of 26 ± 2 nt [23]. Yeast two-hybrid analyses revealed that DnaT can interact with PriB alone [25]. Although the role of DnaT in the recruitment of DnaB helicase has been proposed, little is known about the fundamental function of DnaT for the assembly of the replication restart primosome.

Abbreviations: ssDNA, single-stranded DNA; aa, amino acid residues; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; IPTG, isopropyl thiogalactoside; nt, nucleotides; EMSA, electrophoretic mobility shift analysis.

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In this study, we defined a two-domain structure for DnaT and thus identified the involvement of the N-terminal domain (aa 1–83) in PriB binding and of the C-terminal domain (aa 84–179) in ssDNA binding. The lack of the N-terminal domain in DnaT resulted in the failure to trimerize. Further research can focus on determining how PriB and DnaT bind to the PriA–DNA complex in replication restart.

2. Materials and methods

2.1. Construction of expression plasmids of DnaT, DnaT1-83, DnaT1-120, DnaT1-139, DnaT42-179, DnaT84-179, and PriB(dHis)

A fragment containing the coding sequence of DnaT variants was amplified by PCR with the genomic DNA of *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 as template. The PCR products with unique NdeI and XhoI restriction sites were then cloned into the pET21b vector (Novagen, Madison, WI, USA) for protein expression in *E. coli*. The primers used to construct these plasmids are summarized in Supplementary Table 1. These plasmids were verified by DNA sequencing. The expected gene product expressed by these plasmids had a His tag, which is useful for purifying the recombinant protein and Western blot by the anti-His antibody. To distinguish proteins in the Western blot, we also constructed plasmid pET21b-PriB(dHis) to express PriB without a His tag.

2.2. Protein expression and purification

The recombinant DnaT variants were expressed and purified using the protocol described previously for DnaT [23]. Briefly, *E. coli* BL21(DE3) cells were individually transformed with the expression vector and grown to OD_{600} of 0.9 at 37 °C in Luria–Bertani medium containing 250 μg/mL ampicillin with rapid shaking. Overexpression of the expression plasmids was induced by incubating with 1 mM isopropyl thiogalactoside for 3 h at 37 °C. The cells overexpressing the protein were chilled on ice, harvested by centrifugation, resuspended in Buffer A (20 mM Tris–HCl, 5 mM imidazole, and 0.5 M NaCl, pH 7.9) and disrupted by sonication with ice cooling. The protein purified from the soluble supernatant by Ni^{2+}-affinity chromatography (HiTrap HP; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was eluted with Buffer B (20 mM Tris–HCl, 250 mM imidazole, and 0.5 M NaCl, pH 7.9) and dialyzed against a dialysis buffer (20 mM Tris–HCl and 100 mM NaCl, pH 8.0; Buffer C). PriB(dHis) was expressed using the same protocol, but was purified in different way. The soluble supernatant (20 mM Tris–HCl and 100 mM NaCl, pH 5.9; Buffer D) containing PriB(dHis) was applied to the SP column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). PriB(dHis) was eluted with a linear NaCl gradient from 0.1 to 1 M with Buffer D using the AKTA–FPLC system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Purity of these proteins remained greater than 97% as determined by Coomassie-stained SDS–PAGE (Mini-PROTEAN Tetra System; Bio-Rad, CA, USA).

2.3. Gel-filtration chromatography

Gel-filtration chromatography was carried out by the AKTA–FPLC system. Briefly, purified protein (25 mg/mL) in Buffer C was applied to a Superdex 200 HR 10/30 column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with the same buffer. The column was operated at a flow rate of 0.5 mL/min, and 0.5-mL fractions were collected. The proteins were detected by measuring the absorbance at 280 nm. The column was calibrated with proteins of known molecular weight: γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). The K_{av} values for the standard proteins and the DnaT variant were calculated from the equation: $K_{av} = (V_e - V_o)/(V_c - V_o)$, where V_o is column void volume, V_e is elution volume, and V_c is geometric column volume.

2.4. Electrophoretic mobility shift assay (EMSA)

EMSA for DnaT84-179 was conducted according to the protocol previously described for DnaT [23]. ssDNA oligonucleotides of various lengths were radiolabeled with [γ^{32}P]ATP (6000 Ci/mmol; PerkinElmer Life Sciences, Waltham, MA) and T4 polynucleotide kinase (Promega, Madison, WI, USA). DnaT84-179 (0, 1.3, 2.5, 5.0, and 10.0 μM) was individually incubated at 25 °C for 30 min with 1.7 nM DNA substrates (dT10–70) at a total volume of 10 μL in 20 mM Tris–HCl (pH 8.0) and 100 mM NaCl. Aliquots (5 μL) were removed from each reaction solution and were added to 2 μL of gel-loading solution (0.25% bromophenol blue and 40% sucrose). The resulting samples were resolved on a native 8% polyacrylamide gel at 4 °C in TBE buffer (89 mM Tris borate and 1 mM EDTA) at 100 V for 60 min to 90 min and were visualized by autoradiography. Complexed and free DNA bands were scanned and quantified.

2.5. Native PAGE for analyzing protein–protein interaction

DnaT, DnaT42-179, or DnaT84-179 (0–60 μM) was incubated at 25 °C for 20 min with PriB(dHis) (60 μM) at a total volume of 100 μL in Buffer C. Aliquots (10 μL) were removed from each reaction solution and were added to 3 μL of gel-loading solution (0.25% bromophenol blue and 40% sucrose). The resulting samples were resolved on a native 9% polyacrylamide gel at 4 °C in TBE buffer (89 mM Tris borate and 1 mM EDTA) at 150 V for 2 h and were visualized by Coomassie staining.

2.6. Western blot analysis

After native PAGE, the proteins were transferred onto a nitrocellulose membrane (Whattman). After overnight blocking with 1% (w/v) bovine serum albumin, the membrane was sequentially incubated with a mouse anti-6xHis-tag IgG1 monoclonal antibody conjugated to horseradish peroxidase (1:1000) (Serotec). Between these successive 2 h incubations, the membrane was washed with PBS-0.1% Tween 20. A chemiluminescent HRP substrate (Millipore) was used for detection.

2.7. Pull-down assay

DnaT, DnaT42-179, or DnaT84-179 (20 μM) was incubated at 25 °C for 20 min with PriB(dHis) (20 μM) in Buffer C. Nickel-NTA agarose beads (GE Healthsciences) were added to each sample, incubated for 2 min, and centrifuged for 1 min at 6000*g*. The beads were washed several times with Buffer C. Proteins were eluted with Buffer B and visualized by Coomassie stained SDS–PAGE.

2.8. ssDNA-binding ability

The ssDNA binding ability ($[Protein]_{50}$; $K_{d,app}$) for the protein was estimated from the protein concentration that binds 50% of the input ssDNA. Each $[Protein]_{50}$ is calculated as the average of at least three measurements ± SD.

3. Results and discussion

3.1. Purifying DnaT, DnaT1-83, DnaT1-120, DnaT1-139, DnaT42-179, DnaT84-179, and PriB(dHis)

The *K. pneumoniae* proteins were hetero-overexpressed in *E. coli* and were then purified from the soluble supernatant by Ni^{2+}-affinity chromatography and the SP column (Fig. 1A). However, DnaT1-83, DnaT1-120, and DnaT1-139 were all expressed as protein pellets, suggesting that the C-terminal region was important for the solubility of DnaT.

3.2. DnaT84-179 is a monomer

DnaT is a trimeric protein whose N-terminal region (aa 1–41) is unimportant for oligomerization [23]. To determine whether the length of DnaT affects its oligomerization state, we analyzed DnaT84-179 by gel filtration chromatography and found a single peak with an elution volume of 98.08 mL (Fig. 1B). Assuming that DnaT84-179 has a shape and partial specific volume similar to those of standard proteins, the native molecular mass of DnaT84-179 was estimated to be 10816 Da, calculated from a standard linear regression equation, $K_{av} = -0.2921\ (\log Mw) + 1.8984$ (Fig. 1C). The native molecular mass of DnaT84-179 is approximately equal to that of a DnaT84-179 monomer (~11 kDa). Thus, the deletion of the N-terminal 1–83 aa region significantly changed the oligomeric state of DnaT (i.e., from a trimer to a monomer) in contrast to that of DnaT42-179, which remains a trimer [23]. The N-terminal aa 43–83 region was critical for DnaT self-trimerization. The properties of the deletion mutant of DnaT are summarized in Table 1.

3.3. DnaT84-179 bound to ssDNA

In contrast to DnaT, which forms a trimer, DnaT84-179 was a monomeric protein. To confirm whether small protein DnaT84-179 can still bind to ssDNA, we assessed the binding of DnaT84-179 to dT10, dT20, dT30, dT40, dT50, dT60, and dT70 (Fig. 2) at different protein concentrations by EMSA. EMSA, a popular and well-established approach in studies of molecular biology, detects distinct protein–DNA complexes [26]. No significant band shift was observed when DnaT84-179 was incubated with dT10 (Fig. 2A), indicating that DnaT84-179 cannot form a stable complex with dT10 during electrophoresis. In contrast to dT10, long dT homopolymers bound to DnaT84-179 and formed a single complex (Fig. 2B–G). DnaT84-179 can bind to dT20, but DnaT, DnaT26-179, and DnaT42-179 cannot [23]. Surprisingly, in contrast to DnaT, DnaT26-179, and DnaT42-179, which form distinct complexes with ssDNA of varying lengths [23], DnaT84-179 complexed with ssDNA formed a single complex. Because only one complex of DnaT84-179 molecules bound per ssDNA was visible when the length of the dT homopolymers was increased to 70 nt, these interactions were more highly cooperative than those of DnaT, DnaT26-179, and DnaT42-179. No other distinctive complex or intermediate form was observed. Thus, small monomeric protein DnaT84-179 can still bind to ssDNA with unexpected positive cooperativity.

Table 1
The properties of the deletion mutant of DnaT.

	Oliomerization	PriB binding	ssDNA binding
DnaT	Trimer	+	+
DnaT42-179	Trimer	+	+
DnaT84-179	Monomer	–	+
DnaT1-83	Expressed as protein pellets		
DnaT1-120	Expressed as protein pellets		
DnaT1-139	Expressed as protein pellets		

To compare the ssDNA-binding abilities of DnaT, DnaT26-179, DnaT42-179, and DnaT84-179, the midpoint value for the input ssDNA binding of DnaT84-179, calculated from the titration curves of EMSA and referred to as $[Protein]_{50}$, was quantified (Table 2). $[DnaT84\text{-}179]_{50}$ values ranged from 2.7 μM to 5.2 μM, similar to those of DnaT and DnaT26-179 but different from those of DnaT42-179. The reason for such discrepancy remains unknown. Thus, the deletion of the N-terminal 1–83 aa region in DnaT changed the oligomerization state and DNA binding behavior (complex number) but did not decrease ssDNA binding ability (Table 2).

3.4. Native PAGE analysis for identifying regions of interaction of DnaT with PriB

Yeast two-hybrid analyses show the interaction of DnaT with PriB in vivo [25]. To assess such interaction in vitro, native PAGE analysis using purified proteins was conducted together with Western blot. DnaT, DnaT42-179, and DnaT84-179 have a His tag and therefore can be detected by the anti-His antibody. Native PAGE analysis expects that the interaction of a protein with another forms a protein complex(es), whose electrophoretic mobility differs from that of the uncomplexed form(s) of the protein. DnaT and DnaT42-179 (Fig. 3A and B), but not DnaT84-179 (Fig. 3C), incubated with PriB(dHis) caused a significant band shift, indicating different mobilities because of the addition of PriB(dHis). Thus, DnaT can use its N-terminal region (aa 1–83) to interact with PriB in the absence of DNA. Although we wanted to investigate several deletion mutants in the C-terminal region, namely, DnaT1-83,

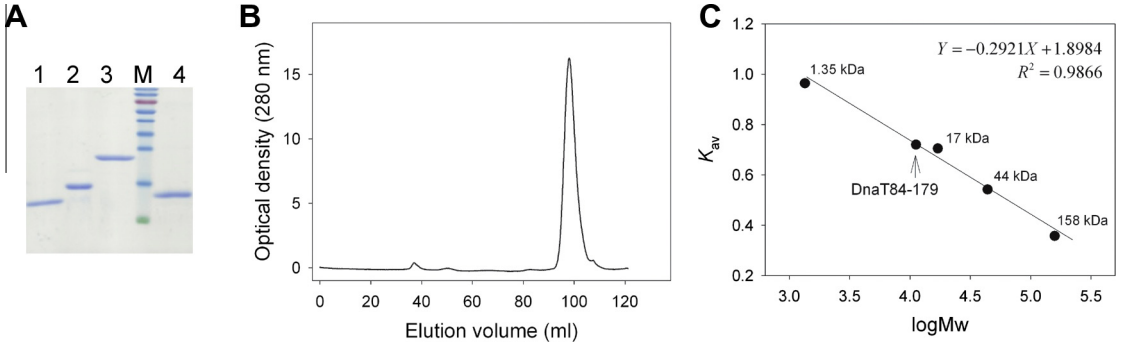

Fig. 1. (A) Protein purity. Coomassie Blue-stained SDS–PAGE (14%) of the purified DnaT84-179 (lane 1), DnaT42-179 (lane 2), DnaT (lane 3), PriB(dHis) (lane 4), and molecular mass standards (M) are shown. (B) Gel-filtration chromatographic analysis of the purified DnaT84-179 protein. The column (Superdex 200 HR 10/30) was calibrated with proteins of known molecular masses: γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). The single peak shows the eluted DnaT84-179 protein. (C) A standard linear regression curve was generated by plotting the log of the molecular mass of the calibration proteins against their K_{av} values.

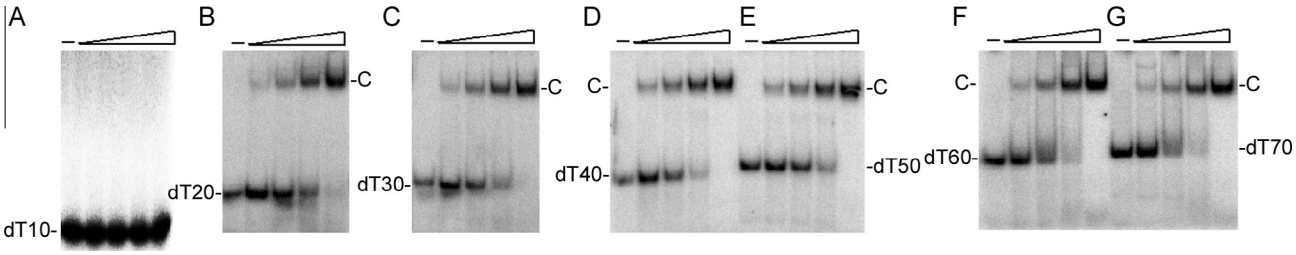

Fig. 2. Binding of DnaT84-179 to (A) dT10, (B) dT20, (C) dT30, (D) dT40, (E) dT50, (F) dT60, and (G) dT70. The reaction solutions contain 1.7 nM of the oligonucleotide and the purified DnaT84-179 protein (0–10 μM).

Table 2
ssDNA binding properties of DnaT84-179 as analyzed by EMSA.

DNA	$[Protein]_{50}$	N
dT10	ND	0
dT20	5.2 ± 0.4	1
dT30	3.1 ± 0.5	1
dT40	2.9 ± 0.2	1
dT50	2.9 ± 0.4	1
dT60	2.7 ± 0.3	1
dT70	2.7 ± 0.4	1

Errors are standard deviations determined by three independent titration experiments.
N, complex number.

DnaT1-120, and DnaT1-139, they were expressed as protein pellets, which cannot be characterized.

3.5. Pull-down assay

To verify direct interaction of DnaT with PriB, we performed pull-down experiments using DnaT variants and PriB(dHis). If there is physical interaction between DnaT and PriB(dHis), untagged PriB(dHis) will be eluted with His-tagged DnaT in this assay. As shown in Fig. 3D–F, DnaT and DnaT42-179, but not DnaT84-179, eluted with PriB(dHis). PriB(dHis) was found in washing solution when DnaT84-179 was used in this experiment (Fig. 3F). Taken together, DnaT and DnaT42-179 can bind to PriB according to native polyacrylamide gel electrophoresis, Western blot analysis, and pull-down assay, whereas DnaT84-179 cannot bind to PriB. In addition, these interactions did not require addition of DNA. These results constitute a pioneering study of the sites of interaction of DnaT with PriB.

3.6. Functional domains of DnaT

Previously we have modeled the DnaT structure by $(PS)^2$ [23]. The C-terminal region of DnaT contains highly conserved positively charged or aromatic residues for ssDNA interaction [23]. In the current study, we found that DnaT84-179, although a monomer, can bind to ssDNA with similar affinity to that of DnaT. The N-terminal region of DnaT was assumed to be insignificant in primosome assembly because this region is variable [23]. However, we found that the N-terminal domain (aa 1–83), especially region aa 43–83, of DnaT, is essential for PriB binding (Table 1). Many bacteria have no recognizable homolog of DnaT and other loading factors, such as PriB, PriC, and DnaC. These Gram-positive bacteria still employ replication restart [27]. We proposed that DnaT serves as an accessory protein for regulating the translocase or helicase activity of replicative DnaB helicase. Thus, several regions that are important for protein–protein interaction and conformational change in DnaT, even those that are important for the interaction of PriB or other primosomal proteins, are not necessarily conserved.

3.7. Domain mapping of DnaT: insight into hand-off mechanism

A hand-off mechanism for primosome assembly [14] has been proposed whereby (i) PriA recognizes and binds to a replication fork, (ii) PriB joins to PriA to form a PriA–PriB–DNA ternary complex, and (iii) DnaT participates in this nucleocomplex to form a triprotein complex, in which the recruitment of DnaT results in the

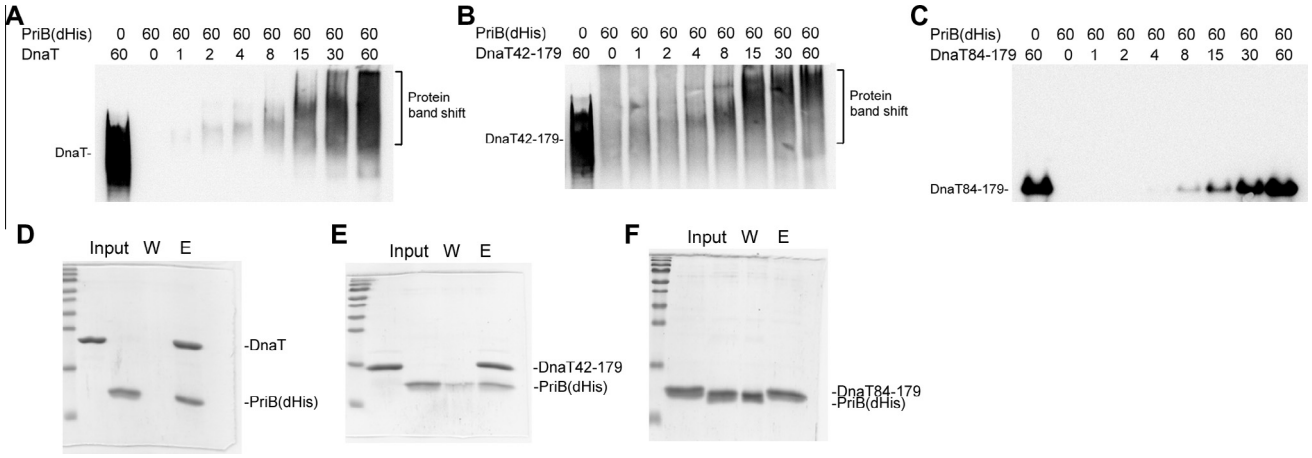

Fig. 3. (A–C) Native PAGE with Western blot analysis. (A) DnaT, (B) DnaT42-179, and (C) DnaT84-179 (0–60 μM) was incubated with PriB(dHis) (60 μM). The reaction solution was analyzed by native PAGE with Western blot. (D–F) Pull-down assay. (D) DnaT, (E) DnaT42-179, and (F) DnaT84-179 was incubated with PriB(dHis) and then the pull-down experiments were performed. W, washing solution; E, elution solution.

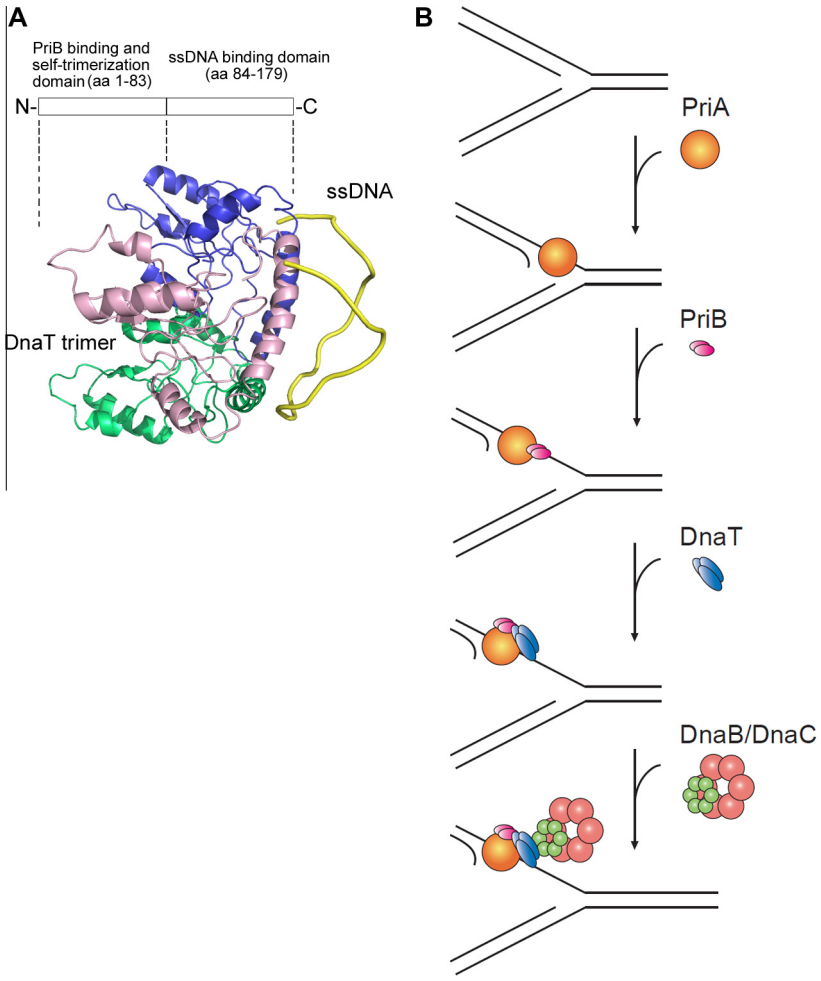

Fig. 4. (A) Domain definition of DnaT. This trimeric DnaT structure (aa 14–165) with ssDNA (25 mer; shown in gold) modeled by $(PS)^2$ was adapted from [23]. (B) On the basis of results in this study, the hand-off mechanism for primosome assembly is updated such that (i) PriA recognizes and binds to a replication fork, (ii) PriB joins to PriA to form a PriA–PriB–DNA ternary complex, and (iii) DnaT interacts with PriA, ssDNA (via the C-terminal domain), and PriB (via the N-terminal domain) and the DnaB/C complex is loaded. This modified model explains the mechanism as to how DnaT simultaneously binds to ssDNA and its partner protein, PriB.

release of ssDNA by PriB and then loads the DnaB/C complex. Very recently, we have identified DnaT as a kind of ssDNA binding protein and then modified the hand-off mechanism for primosome assembly [23]. In the current study, we identified that the N-terminal domain of DnaT is responsible for PriB binding and that its C-terminal domain is involved in ssDNA binding (Fig. 4A). Thus, the hand-off mechanism for primosome assembly is updated (Fig. 4B) such that (i) PriA recognizes and binds to a replication fork, (ii) PriB joins to PriA to form a PriA–PriB–DNA ternary complex, and (iii) DnaT interacts with PriA, ssDNA (via the C-terminal domain), and PriB (via the N-terminal domain) and the DnaB/C complex is loaded. This modified model explains the mechanism as to how DnaT binds to ssDNA and its partner protein, PriB. To further elucidate the function and structure of DnaT, our laboratory is obtaining several crystals of DnaT84-179 in complex with ssDNA for crystallographic analysis. A large number of complex structures of DnaT will enhance our understanding of the mechanism for primosome assembly.

In this study, we constructed and analyzed several deletion mutants of DnaT, in which DnaT84-179 can bind to ssDNA but cannot interact with PriB and cannot form an oligomer. On the basis of this biophysical characterization and extensive evidence from other literature, we mapped the domain functions of DnaT. The N-terminal domain (aa 1–83), especially aa 43–83, is important in PriB binding and self-trimerization, and the C-terminal domain (aa 84–179) is crucial for ssDNA binding. Further research can directly focus on determining how DnaT binds to the PriA–PriB–DNA tricomplex in replication restart by the hand-off mechanism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.069.

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