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τ Binds and Organizes *Escherichia coli* Replication Proteins through Distinct Domains

DOMAIN III, SHARED BY γ AND τ, BINDS $\delta\delta'$ AND $\chi\psi$*

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The DnaX complex of the DNA polymerase holoenzyme assembles the β_2 processivity factor onto the primed template enabling highly processive replication. The key ATPases within this complex are τ and γ, alternative frameshift products of the *dna*X gene. Of the five domains of τ, I–III are shared with γ *In vivo*, γ binds the auxiliary subunits $\delta\delta'$ and $\chi\psi$ (Glover, B. P., and McHenry, C. S. (2000) *J. Biol. Chem.* 275, 3017–3020). To localize $\delta\delta'$ and $\chi\psi$ binding domains within γ domains I–III, we measured the binding of purified biotin-tagged DnaX proteins lacking specific domains to $\delta\delta'$ and $\chi\psi$ by surface plasmon resonance. Fusion proteins containing either DnaX domains I–III or domains III–V bound $\delta\delta'$ and $\chi\psi$ subunits. A DnaX protein only containing domains I and II did not bind $\delta\delta'$ or $\chi\psi$. The binding affinity of $\chi\psi$ for DnaX domains I–III and domains III–V was the same as that of $\chi\psi$ for full-length τ, indicating that domain III contained all structural elements required for $\chi\psi$ binding. Domain III of τ also contained $\delta\delta'$ binding sites, although the interaction between $\delta\delta'$ and domains III–V of τ was 10-fold weaker than the interaction between $\delta\delta'$ and full length τ. The presence of both δ and $\chi\psi$ strengthened the δ'-C(0)τ interaction by at least 15-fold. Domain III was the only domain common to all of τ fusion proteins whose interaction with δ' was enhanced in the presence of δ and $\chi\psi$. Thus, domain III of the DnaX proteins not only contains the $\delta\delta'$ and $\chi\psi$ binding sites but also contains the elements required for the positive cooperative assembly of the DnaX complex.

Processive and efficient replication of genomic DNA in both prokaryotes and eukaryotes is facilitated by three conserved functional components: a DNA polymerase, a sliding clamp processivity factor and a multiple-subunit complex that loads the processivity factor onto a primed-template. In *Escherichia coli*, the DNA polymerase III holoenzyme is responsible for duplication of the genome in a rapid and processive manner. The holoenzyme contains ten different types of subunits and is composed of two DNA polymerase III cores ($\alpha\epsilon\theta$): two β_2 sliding clamp processivity factors and the clamp-loading DnaX complex. The DnaX complex contains the DnaX proteins plus auxiliary subunits δ, δ', χ, and ψ. The distributive DNA polymerase III core becomes highly processive when the DnaX complex assembles the β processivity factor around DNA in an ATP-dependent process (for reviews see Refs. 1–3).

The *dna*X gene produces two distinct proteins, τ and γ, which have differential interactions with replication proteins in the cell (4, 5). Results presented in the first two reports in this series demonstrate that it is the C-terminal portion of τ, absent in the γ protein, that allows the full-length DnaX gene product to interact with both the DnaB helicase and the DNA polymerase III core. These τ-mediated interactions impart rapid fork movement and coordinated leading and lagging strand synthesis, respectively (6–10). The focus of this investigation is the protein sequence common to both τ and γ.

Functional homomeric DnaX complexes (τ complex, $\tau_3\delta_1\delta'_1\chi_1\psi_1$, and γ complex, $\gamma_3\delta_1\delta'_1\chi_1\psi_1$) can be assembled *in vitro* (11, 12). Thus, the N-terminal 430 residues common to both τ and γ have the minimal protein sequence necessary not only to bind the auxiliary subunits δ, δ', χ, and ψ but also to load the β processivity factor onto a primed template in an ATP-dependent manner. Within the DnaX complex, δ' and ψ bind directly to γ; δ binds δ', and χ binds ψ (13, 14). δ and δ' form a 1:1 complex and function with DnaX to load β onto primed templates in an ATP-dependent manner (10, 15). The χ and ψ subunits also form a 1:1 complex and increase the affinity of DnaX for δ and δ' so that a functional DnaX complex can be assembled at physiological subunit concentrations (16). The χ subunit also interacts with SSB, consistent with the finding that χ-ψ-DnaX bridges strengthen the interactions between the holoenzyme and the SSB-coated lagging strand at the replication fork (17, 18). In the preceding studies, five structural domains were assigned to the τ protein. The focus of this report is to determine which structural domain(s) within the portion of DnaX common to both γ and τ (domains I–III) are responsible for binding the auxiliary subunits. To this end, the relative binding of $\delta\delta'$ and $\chi\psi$ to a series of truncated DnaX proteins lacking specific structural domains was measured using surface plasmon resonance.

EXPERIMENTAL PROCEDURES

Strains—E. coli strains DH5α and HB101 were used for initial molecular cloning procedures and plasmid propagation. *E. coli* strain BL21(λ DE3) was used for protein expression.

Materials and Buffers—CM5 sensor chips (research grade), P-20 surfactant, *N*-hydroxysuccinimide, 1-ethyl-3-[(3-dimethylamino)propyl]-carbodiimide, and ethanolamine hydrochloride were obtained from BIAcore Inc. Ni^{2+}-NTA[1] resin, the QIAquick Gel extraction kit, QIAquick PCR purification kit, and the plasmid preparation kit were purchased from Qiagen. SDS-polyacrylamide gel electrophoresis protein standards were from Life Technologies, Inc. *d*-Biotin was obtained from Sigma. Coomassie Plus protein assay reagent and Immunopure streptavidin were from Pierce. Buffer L, Buffer W, HBS buffer, and HKGM

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[1] The abbreviations used are: NTA, nitrilotriacetic acid; SSB, single-stranded DNA-binding protein; PCR, polymerase chain reaction; RU, resonance unit.

TABLE I
Oligonucleotides used for construction of truncated τ fusion proteins

Oligonucleotide number	Use	Sequence[a]
N-221p1	N-Δ221τ	AACTGCAGGACCAGGCGATTGCCAGCGG
N-221p2	N-Δ221τ	TGCAGACATACTGCGTTGTCGCTCTCC
C-261p1	C-Δ261τ	GCGAGATGCCTTAAGTC
C-261p2	C-Δ261τ	GGACTAGTTGCCGTTGG
C-422p1	C-Δ422τ	TTAAGTCTGACCA
C-422p2	C-Δ422τ	CTAGTGGTCAGAC

[a] The underlined sequences are complementary to the *dnaX* gene template.

buffer were prepared as previously described (19).

Construction of the Fusion Plasmids—Plasmid P_{A1}-N-Δ221τ encodes the fusion protein N-Δ221τ. The starting material for construction of plasmid P_{A1}-N-Δ221τ was plasmid P_{A1}-N-Δ1τ, which encodes the τ protein with the initiating methionine replaced by an N-terminal fusion peptide. PCR primer N-221p1 contained a *Pst*I sequence at the noncomplementary 5′-region followed by a complementary sequence extending from codons 222 to 228 of *dnaX* (Table I). Primer N-221p2 was complimentary to a region located 102 bases downstream of the *Nhe*I site within *dnaX*. The resultant PCR fragment was digested with *Pst*I and *Nhe*I and ligated into the linearized pP_{A1}-N-Δ1τ to generate plasmid P_{A1}-N-Δ221τ.

Plasmid P_{A1}-C-Δ261τ encodes the truncated fusion protein C-Δ261τ. To construct the plasmid P_{A1}-C-Δ261τ, a partial *dnaX* gene sequence encoding the C-terminal 261 amino acids of τ was deleted from the previously constructed plasmid P_{A1}-C(0)τ, which encodes the C-terminal tagged full-length τ protein (19). PCR primer C-Δ261P1 was complementary to a region of *dnaX* located 430 bases upstream of the internal *Rsr*II site. Primer C-Δ261P2 was complementary to the *dnaX* from codons 380 to 382 followed by a noncomplementary *Spe*I cloning site (Table I). After digestion with *Rsr*II and *Spe*I, the resultant PCR fragment was ligated into the linearized pP_{A1}-C(0)τ to generate pP_{A1}-C-Δ261τ.

Plasmid pET11-C-Δ422τ, which lacked the sequence encoding the C-terminal 422 amino acids of τ, was constructed as follows. pET11-C(0)τ was digested with *Afl*II and *Spe*I to delete a *dnaX* sequence encoding from residue 218 to the 3′ end (residue 643) of τ. The annealed oligonucleotides C-422p1 and C-422p2 containing the sequence encoding τ amino acid residues 218–221 were ligated into the linearized pET11-C(0)τ vector to generate pET11-C-Δ422τ (Table I).

Cell Growth and Induction—Plasmids P_{A1}-C-Δ261τ, P_{A1}-N-Δ221τ, and pET11-C-Δ422τ were transformed into *E. coli* strain BL21 (λ DE3) for expression of proteins C-Δ261τ, N-Δ221τ, and C-Δ422τ, respectively. The transformed BL21 (λ DE3) cells were grown at 37 °C in 2 liters of F medium (20) containing 100 μg/ml ampicillin. Cells were induced, biotin-treated, and harvested as previously described (19).

Protein Purification—C(0)τ, N-Δ1τ, and the other holoenzyme subunits were expressed and purified as previously described (19). Induced BL21 cells containing the expression plasmids introduced in this study were lysed in the presence of lysozyme (2.5 mg/g of cells), 5 mM EDTA, 5 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The expressed proteins C-Δ261τ, N-Δ221τ, and C-Δ422τ were precipitated from their corresponding lysate supernatants by addition of 0.226, 0.258, and 0.361 g of ammonium sulfate to each milliliter of the lysates, respectively. The fusion proteins were purified using Ni^{2+}-NTA affinity chromatography as previously described for P_{A1}-N-Δ1τ (19), except that the bound C-Δ261τ was eluted stepwise in Buffer W containing 150 mM imidazole. For purification of C-Δ422τ, the imidazole concentration was 2 mM instead of 1 mM in the binding buffer and 15 mM instead of 23 mM in the washing buffer; the bound proteins were eluted stepwise as for C-Δ261τ. For purification of N-Δ221τ, the imidazole concentration was 15 mM in the washing buffer; the bound N-Δ221τ was eluted with 10 column volumes of 15–100 mM imidazole gradient in buffer W. The imidazole concentration in the peak fraction of N-Δ221τ was ~55 mM.

Surface Plasmon Resonance—A BIAcore™ instrument was used for protein-protein binding analyses. CM5 research grade sensor chips were used for all experiments. Streptavidin was coupled to the sensor chip surface by the *N*-hydroxysuccinimide/1-ethyl-3-[(3-dimethylamino)propyl]-carbodiimide coupling (19). Biotin-tagged τ fusion proteins (700–1600 RU) were then captured onto sensor chips via streptavidin-biotin interaction. τ-χψ binding studies were conducted in HKGM buffer at a flow rate of 25 μl/min at 20 °C. τ-δ′ and τ-δδ′ binding studies were performed in HKGM buffer containing 2% glycerol and 2 mM dithiothreitol at a flow rate of 10 μl/min at 20 °C. In these studies, δ and δ′ were preincubated for 10 min at room temperature before injecting over the τ derivatized sensor chips. Kinetic parameters were determined using the BIAevaluation 2.1 software unless indicated otherwise.

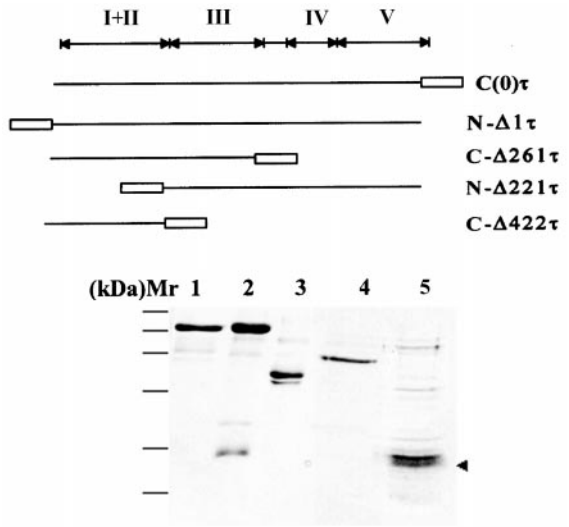

FIG. 1. **Purified truncated τ-fusion proteins.** The *upper panel* shows the truncated biotinylated fusion proteins of τ used in BIAcore analysis. C-Δ261τ contains domains I–III, N-Δ221τ contains domains III–V, and C-Δ422τ contains domains I and II. The *rectangular box* represents the biotinylated fusion peptide. The *lower panel* is the Coomassie Blue-stained 12% SDS-polyacrylamide gel of 1.5 μg of each purified protein after Ni^{2+}-NTA purification. *Lane 1*, C(0)τ; *lane 2*, N-Δ1τ; *lane 3*, C-Δ261τ; *lane 4*, N-Δ221τ; *lane 5*, C-Δ422τ, with the *arrow* indicating the C-Δ422τ protein (two bands are clustered together with the lower band is the C-Δ422τ protein which is ~30% of the total protein of the lane; N-Δ1τ was shown in the first paper of this series).

SDS-Polyacrylamide Gel Electrophoresis—Proteins were separated by electrophoresis at constant current (20 mA) on 12.5% SDS-polyacrylamide mini-gels. The gels were stained and destained as previously described (19).

DNA Polymerization Assays—Activities of τ fusion proteins were measured by their requirement for reconstitution of holoenzyme activity as previously described (19).

RESULTS

Expression and Purification of the Truncated DnaX Fusion Proteins—We constructed three plasmids, each encoding specific structural domains of τ under control of an inducible promoter. Plasmid P_{A1}-N-Δ221τ encoded protein N-Δ221τ (domains III–V), plasmid P_{A1}-C-Δ261τ encoded protein C-Δ261τ (domains I–III), and plasmid pET11-C-Δ422τ encoded protein C-Δ422τ (domains I-II) (Fig. 1). Each of these proteins contained a hexahistidine sequence to facilitate purification using Ni^{2+}-NTA metal affinity chromatography and a short biotinylation sequence to enable immobilization on streptavidin-coated BIAcore sensor chips. The biotin tag also enabled detection of fusion proteins using biotin blots (21). The expression levels of C-Δ261τ, N-Δ221τ, and C-Δ422τ were ~3%, 2, and 0.5% of the total cell protein, respectively. After one round of Ni^{2+}-NTA chromatography, N-Δ221τ and C-Δ261τ preparations at greater than 80% of purity were obtained; the purity of C-Δ422τ was ~30%, as determined by densitometric scanning of the SDS-polyacrylamide gels of the eluted proteins fractions (Fig. 1). Biotin blots verified that these fusion proteins were the only biotinylated proteins in the eluted fractions (results not shown). The biotinylated proteins are presumably the only proteins captured onto the streptavidin sensor chips. This assumption was verified for a similarly purified protein (see footnote 2 in Ref. 19). The activities of C-Δ261τ through its purification were measured in holoenzyme reconstitution assays. Purified C-Δ261τ had a specific activity of 5.3×10^6 units/

TABLE II
Purification of τ deletion fusion proteins

Protein		Fraction	Total protein	Activity	Specific activity[a]
			mg	*units (10^6)*	*units/mg (10^3)*
C-Δ261τ	I	Lysate	700	200	290
	II	Ammonium sulfate	120	160	1000
	III	Ni^{2+}-NTA	16	85	5300
N-Δ221τ	I	Lysate	1060	No activity	
	II	Ammonium sulfate	230		0
	III	Ni^{2+}-NTA	43		0
C-Δ422τ	I	Lysate	710	No activity	
	II	Ammonium sulfate	314		
	III	Ni^{2+}-NTA	2		

[a] For comparison, the specific activity of C(0)τ of the Ni^{2+}-NTA purified fractions is 5.7×10^6 units/mg.

mg, which is comparable with that of C(0)τ (Table II). As expected, N-Δ221τ was inactive in this same assay because its ATPase motif (domains I and II) was deleted. C-Δ422τ (domains I and II) was also inactive in this assay, suggesting that domains I–III represent the minimum protein required to assemble the β processivity factor onto the DNA template.

DnaX Domain III Interacts with χψ—The abilities of the truncated τ fusion proteins C-Δ261τ, N-Δ221τ, and C-Δ422τ to interact with χψ were measured by using BIAcore methodology. Because the χψ binding region is located within the N-terminal 430 amino acids of τ, a fusion peptide tag at the remote C terminus of τ would be unlikely to interfere with χψ binding. For this reason, C(0)τ was used as a positive control for examination of χψ binding to the truncated τ proteins. Biotin-tagged C(0)τ was immobilized onto a streptavidin sensor chip, and various concentrations of χψ (25–300 nM) were passed over immobilized C(0)τ so that binding of free χψ to immobilized C(0)τ could be measured. Representative binding curves (Fig. 2*A*) indicate that χψ bound rapidly to C(0)τ with an association rate of 2.4×10^5 M^{-1} s^{-1}. A dissociation rate of 2.5×10^{-3} s^{-1} (Table III) was obtained; this rate was measured after saturating C(0)τ with χψ to minimize reassociation. The resulting K_d[2] (10 ± 1 nM) was ~5-fold higher than the K_d reported for the native τ-χψ interaction. The observed difference between the K_d values could be due to differences in experimental conditions between the two studies. For example, the present study used biotin-streptavidin rather than amine coupling and employed a 5-fold higher flow rate than did the other study.

The interactions between χψ and the truncated fusion proteins N-Δ1τ, N-Δ221τ, C-Δ422τ, and C-Δ261τ were examined under the same experimental conditions as used for the χψ and C(0)τ interaction. N-Δ1τ, N-Δ221τ, and C-Δ261τ bound χψ, but C-Δ422τ did not (Fig. 2*B*), indicating that domain III of τ, the only domain shared by all of the fusion proteins shown to bind χψ, is required for χψ binding. Similar binding stoichiometries were obtained for the interactions of χψ-N-Δ1τ and χψ-C-Δ261τ compared with that of the χψ-C(0)τ interaction (Table III). The K_d for the interaction between χψ-N-Δ221τ was within 2.5-fold of that measured for the χψ-C(0)τ interaction. These variations are likely within range of accuracy of affinity measurements using a BIAcore and indicate that deletion of domains I and II or deletion of domains IV and V as well as the presence of the tag at the corresponding deletion end of the τ proteins did not decrease the affinity of the τ-χψ interaction. Thus, domain III of τ appears to be fully responsible for χψ binding.

No Observable Binding of δ′ to Individual Domains of the DnaX Protein—The interactions between δ′ and the full-length τ proteins with either an N- or C-terminal tag were characterized in binding studies utilizing the BIAcore instrumentation.

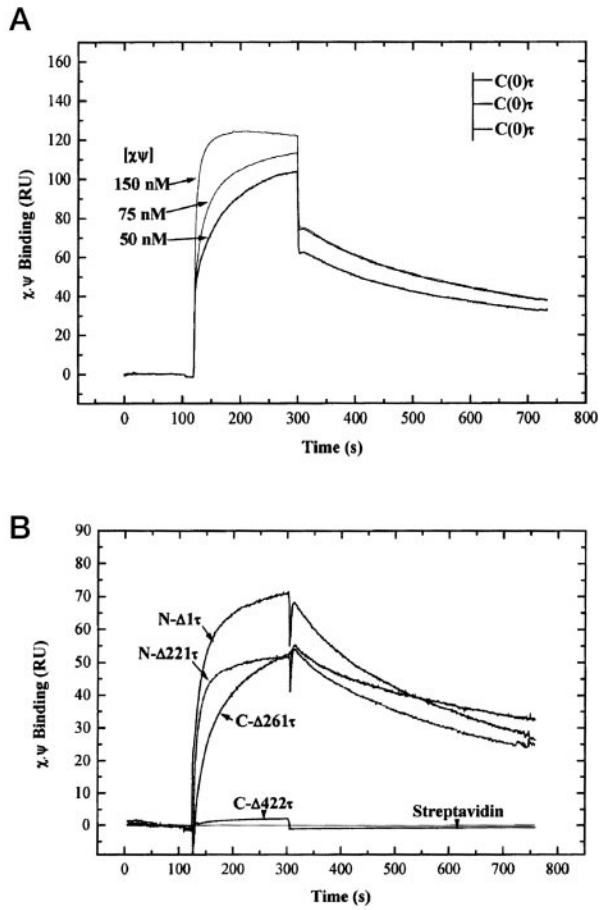

FIG. 2. **The interactions of χψ with immobilized C(0)τ, N-Δ1τ, C-Δ261τ, N-Δ221τ, and C-Δ422τ proteins.** Streptavidin was chemically immobilized to a CM5 sensor chip as described under "Experimental Procedures." Biotinylated τ proteins were attached to sensor chips via biotin-streptavidin interaction. The binding analyses of χψ with truncated τ proteins were conducted in HKGM buffer at a flow rate of 25 μl/min. *A*, Sensorgrams of C(0)τ-χψ binding. C(0)τ (1520 RU) was attached to a sensor chip. Varying concentrations of χψ (50, 75, and 150 nM) were injected over the C(0)τ-immobilized sensor chip for 3 min each. *B*, domain III of τ contains the χψ binding site. The N-Δ1τ (930 RU), C-Δ261τ (710 RU), N-Δ221τ (650 RU), and C-Δ422τ (540 RU) proteins, respectively, were captured onto sensor chips. Solution of χψ (150 nM) was injected over each protein-immobilized sensor chip for 3 min. Control injections, over streptavidin-immobilized sensor chip, were subtracted from the *curves* shown.

TABLE III
Interactions of χψ with truncated and full-length τ fusion proteins: kinetic and equilibrium constants

Results are the averages of two separate experiments, with the error expressed as the range of two data sets.

Immobilized proteins		Dissociation rate constant (k_{off})	Association rate constant (k_{on})	Dissociation constant (K_D)	Stoichiometry ratio of χψ/τ[a]
Name	Domains				
		s^{-1}	$M^{-1}s^{-1}$	*nM*	
C(0)τ	I–V	2.5×10^{-3}	2.4×10^5	10 ± 1	0.66
N-Δ1τ	I–V	2.3×10^{-3}	2.5×10^5	9 ± 1	0.64
C-Δ261τ	I–III	1.5×10^{-3}	1.4×10^5	11 ± 1	0.72
N-Δ221τ	III–V	2.2×10^{-3}	5.3×10^5	4 ± 0.2	0.73
C-Δ422τ	I–II		No detectable interaction		

[a] The stoichiometry of χψ/τ was determined by the following equation: stoichiometry = χψ response units/τ response units × τ Mr/χψ Mr. The tetrameric molecular weights of τ fusion proteins were used.

[2] K_d values were obtained by dividing the measured dissociation rate constant by the association rate constant of a given interaction. In most cases, the K_d values determined in this study were not true equilibrium K_d values but were relative values used to compare the relative affinities of τ-derivatives for the same analytes.

δ′ samples were injected over the immobilized C(0)τ, and the binding curves are shown in Fig. 3*A*. The interaction between δ′ and N-Δ1τ is characterized by weak binding similar to that observed for the δ′-C(0)τ interaction (Table IV). Although measurable, the weak binding observed is close to the limit of BIAcore detection.

Next, the truncated DnaX proteins N-Δ221τ (domains III–V), C-Δ422τ (domains I-II), and C-Δ261τ (domains I–III) were captured onto streptavidin sensor chips so that the binding of δ′ to these proteins could be measured. The K_d observed for the δ′subunit-C-Δ261τ interaction was similar to that of the C(0)τ-δ′ interaction (Fig. 3*B* and Table IV). These observations confirm that the δ′ binding region of τ is entirely within its N-terminal 382 amino acid residues (domains I–III). However, δ′ did not bind to N-Δ221τ or C-Δ422τ at concentrations of δ′ between 0.5–5 μM (Fig. 3*B*). Although we observed no interactions between δ′ and either domains I and II (C-Δ422τ) or domain III (N-Δ221τ), a lower limit for these K_d values could be estimated by comparing N-Δ221τ -δ′ interaction with the C(0)τ-δ′ interaction. When 5 μM of δ′ was injected over the N-Δ221τ-derivatized sensor chip, no binding was observed. However, significant binding was obtained when 0.5 μM of δ′, a 10-fold less concentration, was injected over the C(0)τ derivatized sensor chip (Fig. 3); compatible amounts of C(0)τ and N-Δ221τ were on their respective derivatized sensor chips (see legend of Fig. 3 for details). Thus, if there is an interaction between N-Δ221τ and δ′, the binding affinity is at least 10-fold weaker than that of the C(0)τ-δ′ interaction (500 nM).

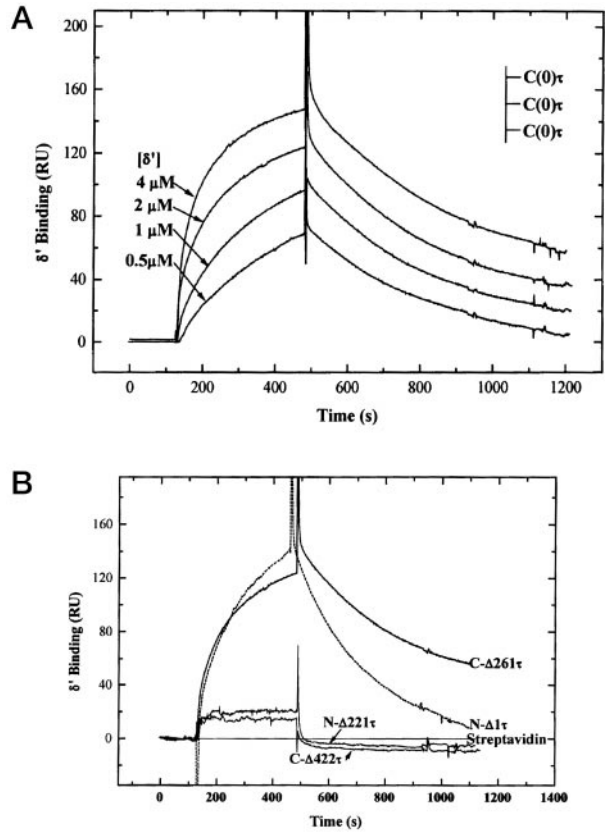

FIG. 3. **Interactions of δ′ with immobilized C(0)τ, N-Δ1τ, C-Δ261τ, N-Δ221τ, and C-Δ422τ proteins.** Streptavidin was chemically immobilized to a CM5 sensor chip as described under "Experimental Procedures." Biotinylated τ proteins were attached to sensor chips via biotin-streptavidin interaction. Binding analyses were conducted in HKGM buffer containing 2% glycerol and 2 mM dithiothreitol at a flow rate of 10 μl/min. *A*, sensorgrams of C(0)τ-δ′ binding. C(0)τ (1500 RU) was attached to a sensor chip. Varying concentrations of the δ′ subunit (0.5, 1, 2, and 4 μM) were injected over the C(0)τ-immobilized sensor chip for 6 min each. *B*, δ′ binds N-Δ1τ and C-Δ261τ but not N-Δ221τ or C-Δ422τ. The N-Δ1τ (2300 RU), C-Δ261τ (1600 RU), N-Δ221τ (1470 RU), and C-Δ422τ (730 RU) proteins, respectively, were captured onto individual sensor chips. For 6 min each, solutions containing 4 μM δ′ subunit were injected over the N-Δ1τ and C-Δ261τ-immobilized sensor chips; solutions containing 5 μM δ′ subunit were injected over the C-Δ422τ and N-Δ221τ sensor chips. Values from control injections obtained via use of an streptavidin-immobilized sensor chip were subtracted from each *curve* shown.

Domain III of τ Contains the δδ′ Binding Site and the Sequence Required for the δ Cooperativity—The δ subunit has a positive cooperative effect on the τ-δ′ interaction (22). Because interactions between δ′ and domain III or domains I and II of DnaX may have been too weak to be detectable by our methodology, we re-examined binding using δδ′ instead of δ′. This enabled us to evaluate whether the cooperative effects of δ strengthen the binding of δ′ to the various DnaX domain constructs to detectable levels. In the following studies, the concentrations of δ′ in all of the δδ′ samples tested were greater than the K_d of δ-δ′ interaction,[3] and the concentrations of δ were 5–10 fold higher than those of δ′. Thus, nearly all the δ′ in these experiments was bound to δ to form δδ′.

When injected over immobilized C(0)τ (Fig. 4*A*), δδ′ bound C(0)τ with an association rate of 4×10^3 M^{-1} s^{-1}. The dissociation of δ and δ′ from the immobilized C(0)τ was complicated because two separate dissociation events were occurring simultaneously. One process was the dissociation of δδ′ from the immobilized C(0)τ, and the other was the dissociation of δ from δ′ still bound to immobilized C (0). Thus, the total dissociation rate measured in these studies was actually reflective of contributions from these two different processes. From the association and dissociation rates, the calculated K_d was 115 nM (Table V).

Samples of δδ′ at the same concentrations used for analysis of the C(0)τ-δδ′ interaction were then passed over the N-Δ221τ and C-Δ422τ derivatized sensor chips. No binding of C-Δ422τ to δδ′ was observed, but N-Δ221τ bound δδ′ at each concentration of δδ′ that was tested (Fig. 4*B*). These results indicate that

[3] M. Song, H. G. Dallmann, P. Pham, R. Schaaper, and C. S. McHenry, manuscript in preparation.

TABLE IV
Interactions of δ′ with truncated and full-length τ fusion proteins: kinetic and equilibrium constants
Results are the averages of two separate experiments, with the error expressed as the range of two data sets.

Immobilized proteins		Dissociation rate constant (k_{off})	Association rate constant (k_{on})	Dissociation constant (K_D)	Stoichiometry ratio of δ′/τ[a]
Name	Domain				
		s^{-1}	$M^{-1}s^{-1}$	μM	
C(0)τ	I–V	1.7×10^{-3}	3.6×10^3	0.47 ± 0.03	0.77
N-Δ1τ	I–V	3.5×10^{-3}	3.4×10^3	0.98 ± 0.04	0.63
C-Δ261τ	I–III	1.9×10^{-3}	3.4×10^3	0.56 ± 0.03	0.6
N-Δ221τ	III–V		No detectable interaction		
C-Δ422τ	I–II		No detectable interaction		

[a] The stoichiometry of δ′/τ was determined by the following equation: stoichiometry = δ′ response units/τ response units × τ M_r/δ′ M_r. The tetrameric molecular weights of τ fusion proteins were used.

N-Δ221τ contains the δδ′ binding site. The K_d of δδ′-N-Δ221τ interaction was 1.5 μM, which is only 10-fold weaker than that of the δδ′-C(0)τ interaction.

10 μM of the δ subunit alone was injected over the immobilized C(0)τ, C-Δ261τ, and N-Δ221τ. No interaction was observed (data not shown), consistent with the report that there is no interaction between τ and δ (13). In contrast, the interactions between C(0)τ-δ′ and C-Δ261τ-δ′ could be easily detected under the same experimental conditions. Thus, the ability to detect the N-Δ221τ- δδ′ interaction resulted from the positive cooperative effect of δ on the τ-δ′ interaction; the deletion of domains I and II of τ did not abolish this cooperativity. Thus, domain III not only binds χψ and δδ′ but also contains the elements required for the positive cooperative effect of δ on the δ′-DnaX interaction. This is also evidenced by similar decreases in the K_d values of C-Δ261τ-δ′ and N-Δ1τ-δ′ interactions in the presence of δ (Table V). An alternative explanation is that δ can weakly interact with DnaX; even though the interaction is too weak to be observed by itself the interaction could lead to an increase in binding of δ′ to DnaX because of the additivity of the binding energies.

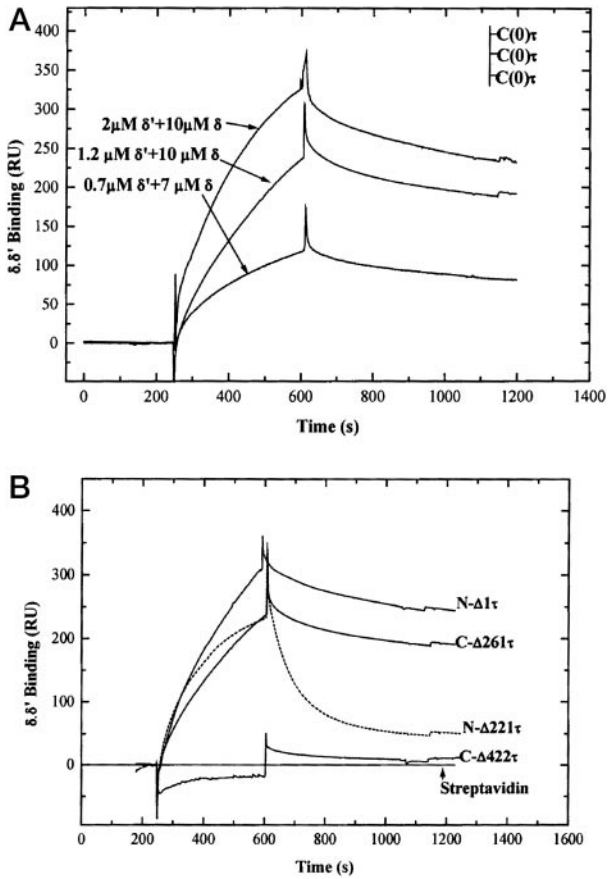

FIG. 4. **Interactions of δδ′ with immobilized C(0)τ, N-Δ1τ, C-Δ261τ, N-Δ221τ, and C-Δ422τ proteins.** Streptavidin was chemically immobilized to a CM5 sensor chip as described under "Experimental Procedures." Binding experiments were conducted in HKGM buffer containing 2% glycerol and 2 mM dithiothreitol at a flow rate of 10 μl/min. *A*, sensorgrams of C(0)τ-δδ′ binding. C(0)τ (1550 RU) was attached to a sensor chip. δ and δ′ samples were mixed at three different concentrations (0.7 μM δ′ + 7 μM δ, 1.2 μM δ′ + 10 μM δ, and 2 μM δ′ + 10 μM δ), incubated for 10 min at room temperature, and injected over a C(0)τ sensor chip for 6 min. *B*, domain III of τ contains the δδ′ binding site. The N-Δ1τ (2300 RU), C-Δ261τ (1600 RU), N-Δ221τ (1470 RU), and C-Δ422τ (720 RU) proteins, respectively, were captured onto the streptavidin-bearing sensor chips. A mixture of 1.2 μM δ′ + 10 μM δ was preincubated and injected over N-Δ1τ, N-Δ221τ, and C-Δ261τ-immobilized sensor chip for 6 min each. A mixture of 10 μM δ′ + 20 μM δ was preincubated and injected over a C-Δ422τ immobilized sensor chip. Control values, obtained by passing injections over a sensor chip containing immobilized streptavidin only, were subtracted from each curve.

Domain III of DnaX Is Sufficient for χψ to Strengthen the δδ′-C(0)τ Interaction—The observation that χψ increases both τ-δδ′ and γ-δδ′ binding affinity indicated that the sequence required for the positive cooperative effect of χψ is localized in the γ portion of DnaX (16). To identify the domain(s) responsible for this cooperativity, C(0)τ and N-Δ221τ were captured onto streptavidin sensor chips, and their relative affinities for δδ′ in the presence of χψ were examined. A solution of χψ (1 μM) was passed over the immobilized C(0)τ until no further binding of χψ was detectable. Samples of δδ′ containing χψ were then injected over the χψ-saturated C(0)τ, and binding was observed. Dissociation was then carried out in the presence of the same buffer containing χψ. This ensured that the RU decrease observed during the dissociation phase was only due to dissociation of δδ′. The C(0)τδδ′χψ complex formed faster and dissociated slower than did the C(0)τδδ′ complex (Fig. 5*A*). In the presence of χψ, the K_d was ~28 nM (Table VI), 4-fold less than that of the the C(0)τ-δδ′ interaction in the absence of χψ.

Under similar experimental conditions, δδ′ samples containing χψ were passed over the N-Δ221τ-derivatized sensor chip. The presence of χψ resulted in a 5-fold reduction in the K_d of N-Δ221τ-δδ′ interaction (Fig. 5*B* and Table VI). This result indicates that the absence of domains I and II did not eliminate the cooperative effect of χψ on the N-Δ221τ-δδ′ interaction. Thus, domain III of τ contains the sequence required for χψ-mediated augmentation of the DnaX-δδ′ interaction.

DISCUSSION

The DnaX complex in *E. coli* functions to assemble the β processivity factor onto the primed template for processive DNA replication. Within the complex, δ′ and ψ bind τ/γ with δ′ bridging the τ/γ-δ interaction and ψ bridging the τ/γ-χ interaction (13). The presence of δ and χψ strengthens the DnaX-δ′ interaction (16, 23). In this study, we identified the δ′ and χψ binding domain of the DnaX proteins by measuring the interactions of these two subunits with truncated τ proteins lacking specific domains. Our results indicate that domain III (amino

TABLE V

Interactions of δ′ with truncated and full-length τ fusion proteins in the presence of δ subunit: kinetic and equilibrium constants

Results are the averages of two separate experiments, with the error expressed as the range of two data sets.

Immobilized proteins		Dissociation rate constant (k_{off})	Association rate constant (k_{on})	Dissociation constant (K_D)	Stoichiometry ratio of δδ′/τ[a]
Name	Domain				
		s^{-1}	$M^{-1}s^{-1}$	nM	
C(0)τ	I–V	4.7×10^{-4}	4.08×10^{3}	114 ± 25	0.72
N-Δ1τ	I–V	4.6×10^{-4}	3.5×10^{3}	130 ± 15	0.63
C-Δ261τ	I–III	5.1×10^{-4}	3.5×10^{3}	150 ± 10	0.58
N-Δ221τ	III–V	8×10^{-3}	5.4×10^{3}	1500 ± 100	0.78
C-Δ422τ	I–II	No detectable interaction			

[a] The stoichiometry of δδ′/τ was determined by the following equation: stoichiometry = δδ′ response units/τ response units × τ M_r/δδ′ M_r. The tetrameric molecular weights of τ fusion proteins were used.

acid residues 222–382) shared by γ and τ binds both χψ and δδ′. Domain III also contains the elements required for the positive cooperative assembly of the DnaX complex.

Among the truncated τ proteins that bound the χψ subunit, C(0)τ (domains I-V), N-Δ1τ (domains I-V), N-Δ221τ (domains III–V), and C-Δ261τ (domains I–III) showed similar affinities for χψ, indicating that deletion of domains I and II or domains IV and V of τ did not decrease the strength of the τ-χψ interaction. Therefore, domain III appears to be responsible for τ-χψ binding.

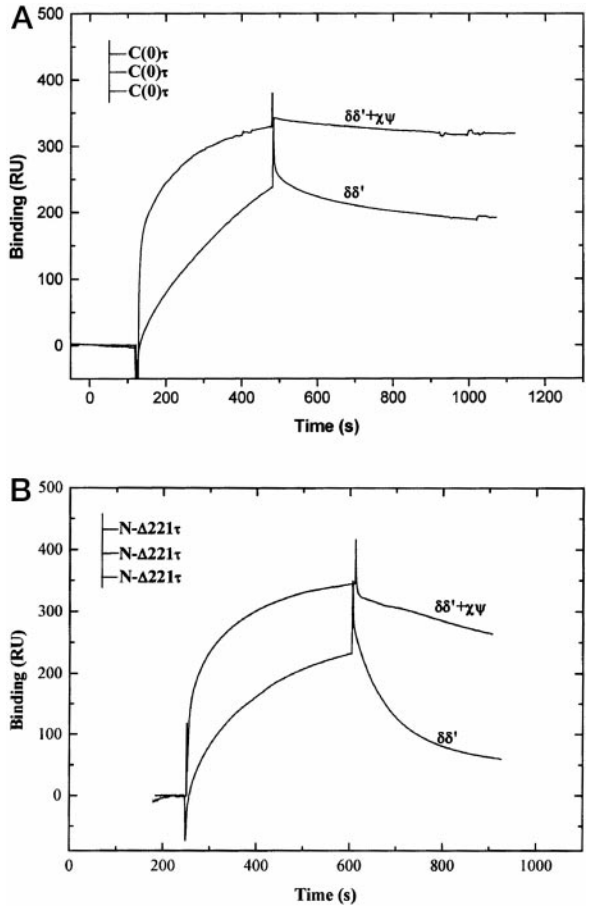

FIG. 5. **Positive cooperative assembly of the DnaX complex on BIAcore sensor chips.** C(0)τ and N-Δ221τ were immobilized to streptavidin sensor chips as described under "Experimental Procedures." The binding study was conducted in HKGM buffer containing 2% glycerol, 2 mM dithiothreitol, and 1 μM χψ at 20 °C. δ′ and δ diluted in the above binding buffer were preincubated for 10 min at room temperature before injection. The injection of the δ′δ samples over the immobilized C(0)τ and N-Δ221τ took 6 min at 10 μl/min. *A*, sensorgram overlays of δ′ (1.2 μM) + δ (10 μM) and δ′ (1.2 μM) + δ (10 μM) + χψ (1 μM) injected over immobilized C(0)τ are shown. *B*, sensorgram overlays of δ′ (1.2 μM) + δ (10 μM) and δ′ (1.2 μM) + δ (10 μM) + χψ (1 μM) injected over immobilized N-Δ221τ. The control injections of the above samples over the streptavidin-derivatized sensor chip were conducted and subtracted from the *curves* shown.

In studies directed toward mapping the δ′ binding domain, δ′ was observed to bind C-Δ261τ (domains I–III) and full-length τ very weakly, near the limit of detection for the BIAcore. However, binding of δ′ to N-Δ221τ (domains III-IV) or C-Δ422τ (domains I-II) could not be detected directly. Instead, we evaluated these interactions by measuring the binding of δδ′ to DnaX derivatives. This exploited the increased affinity of δ′ for DnaX in the presence of δ. In the presence of both δ and δ′, N-Δ221τ was observed to bind δδ′, but C-Δ422τ did not, indicating that domain III of τ contained the δδ′ binding site. The K_d of the N-Δ221τ-δδ′ interaction was 10-fold greater than those of the C(0)τ-δδ′ and C-Δ261τ-δδ′ interactions (Table V). N-Δ221τ interacted less strongly with δδ′ than did C(0)τ and C-Δ261τ, perhaps because the N-terminal peptide tag of N-Δ221τ slightly interfered with the binding of the fusion protein to δδ′. Alternatively, deletion of domains I and II may have perturbed the structure of domain III.

δ′ shares a high sequence similarity to the N-terminal domains I–III of DnaX (24, 25). Both of the DnaX proteins, τ and γ, are tetramers (τ_4, γ_4) when free in solution (12, 23). The DnaX complex ($\tau_2\gamma_1\delta'_1\delta_1\chi_1\psi_1$) contains a total of four copies of homologous subunits (τ, γ, and δ′) (12). It seems reasonable to assume that one DnaX protomer of the tetrameric DnaX proteins is replaced by the homologous δ′ subunit during the formation of the DnaX complex. It is likely that the DnaX proteins bind each other or to the homolog δ′ via similar mechanisms; the same portions of τ probably mediate its binding to other τ subunits and to γ. We have shown that domain III of DnaX is involved in δδ′ binding, likely through the DnaX-δ′ interaction in the presence of δ. Thus, domain III is also likely involved in the τ-τ and τ-γ interactions. That is, the sequences responsible for the tetramerization of DnaX are probably localized in domain III.

The presence of δ decreases the K_d of the C(0)τ-δ′ interaction by approximately 3-fold, as indicated by a comparison of the K_d values of the C(0)τ-δ′ and C(0)τ-δδ′ interactions. The presence of δ also strengthens the C-Δ261τ-δ′ interaction 3-fold. The cooperative effect of δ on the N-Δ221τ-δ′ interaction could not be calculated directly because the interaction of N-Δ221τ, and δ′ in the absence of δ was too weak to be detected. However, the lower boundary of the N-Δ221τ-δ′ interaction K_d was estimated to be 5 μM, based upon the comparison of the concentrations of δ′ and the τ-derivatives used in examination of the C(0)τ-δ′ and N-Δ221τ-δ′ interactions. The effects of δ on the N-Δ221τ-δ′ interaction can be estimated using the lower boundary K_d for the N-Δ221τ-δ′ interaction and the K_d for the N-Δ221τ-δ′δ interaction (Table IV). Using these values, we calculated that δ augments the N-Δ221τ-δ′ interaction ~3-fold, the same degree of enhancement for the interactions between δ′ and τ proteins containing domains I–III. Therefore, domain III appears to contain all sequences required for the full cooperative effect of δδ′ on their interaction(s) with DnaX.

Previous studies indicate that the presence of χψ strengthens both τ-δδ′ and γ-δδ′ interactions (16). The cooperative effect of χψ on the DnaX-δδ′ was examined using BIAcore technology.

TABLE VI

Interactions of δ′ with C(0)τ and N-Δ221τ in the presence of δδ′ + χψ subunits: kinetic and equilibrium constants

Results are the averages of two separate experiments, with the error expressed as the range of two data sets.

DnaX protein		Analytes	Dissociation rate constant (k_{off})	Association rate constant (k_{on})	Dissociation constant (K_D)
Name	Domain				
			$M^{-1}s^{-1}$	s^{-1}	nM
C(0)τ	I–V	χ · ψ + δ · δ′	2.2×10^{-4}	7.9×10^{3}	28 ± 20
		δ · δ′	4.7×10^{-4}	4.08×10^{3}	115 ± 30
N-Δ221τ	III–V	χ · ψ + δ · δ′	2.3×10^{-3}	7.8×10^{3}	295 ± 35
		δ · δ′	8×10^{-3}	5.3×10^{3}	1500 ± 100

The C(0)τ-δδ′ interaction is strengthened approximately 4-fold (Table VI) in the presence of χψ. The absence of domains I and II did not eliminate the cooperative effect of χψ on domain III-δδ′ binding. Rather, χψ decreased the K_d of the N-Δ221τ-δδ′ interaction by 5-fold (Table VI). These results indicate that domain III alone is sufficient for the positive cooperativity of χψ on the τ-δδ′ interaction. The presence of both δ and χψ strengthens the C (0)-δ interaction at least 15-fold, indicating a cooperative assembly of the DnaX complex.

Domain III of DnaX not only contains the δ′ and χψ binding sites but also the sequences required for cooperative assembly of the DnaX complex. This structural arrangement suggests that the cooperativity is a result of an allosteric effect. That is, upon interactions between χψ and the DnaX proteins, the DnaX proteins adopt a conformation with higher affinity for δ′; upon the interaction between δ and δ′, the δ′ subunit adopts a conformation with higher affinity for domain III of DnaX proteins. This allosteric effect is crucial for the efficient assembly of the DnaX complex *in vivo*. The interaction between γ and δδ′ is weak with a K_d of about 100 nM, which is greater that the 28 nM concentration of each component of the DnaX complexes in the cell (26, 27). The affinity between γ and χψ is ~10 nM, and the γ-χψ subassembly can readily form in the cell. Because of the binding of χψ, γ adopts a higher affinity for δδ′ with a K_d of ~28 nM. Thus, the γ-χψ complex efficiently recruits the δδ′ to form γδδ′χψ complex at physiological subunit concentrations.

The DnaX complex functions to load the β processivity factor onto primed template in an ATP-dependent manner. Based on their studies of the crystal structure of the highly homologous δ′ subunit considered in light of structural features of several ATPases, Guenther and colleagues (25) proposed that the N-terminal three domains of γ would also adapt a C-shaped conformation. They also contended that this C-shaped region is likely to open and close in response to ATP binding and hydrolysis by the γ subunit. Experimental results also support these hypotheses. In the absence of ATP or ATP analogs, the DnaX complex does not bind β, presumably because the β binding partner, δ, is buried in the complex. In contrast, DnaX-β interactions occur in the presence of ATP or ATP analogs, indicating that conformational changes occur as ATP binds to γ such that δ subunit becomes exposed, enabling interaction with β (28). Our results show that the δδ′-binding portion of DnaX lies within τ domain III and suggest that this domain may be involved in mediating the ATP effects on the DnaX complex and β interaction. In the absence of ATP, the C-like arrangement of domains I–III of γ is closed, and the auxiliary subunit δ, which is bridged to domain III through δ′, is entrapped within the DnaX complex and not freely accessible to the processivity factor β. In contrast, ATP binding to γ at the interface of domains I and II causes the "C" to open such that domain III of γ, and hence the bridged δδ′ subunits are relatively exposed; δ′ is then free to interact with β. Thus, domain III serves as a "transducer" of the ATP binding signal.

Results from our studies shed light on some of the functional differences between the structurally similar subunits, τ and γ. γ is comprised of domains I–III. Domain III of γ binds the auxiliary subunit δδ′, and χψ functions as the processivity factor (β_2) assembly apparatus. τ contains the same N-terminal three domains, as does γ plus two additional C-terminal domains (domains IV and V). Domain V binds α (polymerase) to form the dimeric polymerase enabling the simultaneous synthesis of the leading and lagging strands. Domain IV interacts with the DnaB helicase to coordinate the replicase and the primosome activities at the replication fork. Domain III is thought to be involved in linking τ to the γ processivity assembly apparatus. The auxiliary subunit χ binds SSB to form a tether between DnaX complex and the SSB-coated lagging strand (18). τ interacts with both the processivity assembly apparatus (via γ) and the polymerase (via α). This direct processivity assembly/polymerase link bridged by DnaX strengthens the interactions between the holoenzyme and the SSB-coated lagging strand at the replication fork. Interactions mediated by domains III–V of τ enable this subunit to serve as a central organizer. The τ subunit effectively couples the processivity assembly process, SSB binding, DnaB helicase activities, and the dimeric replicase into one replicative complex at the replication fork.

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