Carboxyl-terminal Domain III of the δ′ Subunit of DNA Polymerase III Holoenzyme Binds DnaX and Supports Cooperative DnaX Complex Assembly

Min-Sun Song and Charles S. McHenry‡
From the Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, Colorado 80262

The δ′ subunit of the DNA polymerase-III holoenzyme is a key component of the DnaX complex; it is required for loading the β₂ processivity factor onto a primed template. The x-ray crystal structure of δ′ indicates a three domain C-shaped structure (Guenther, B., Onrust, R., Sali, A., O’Donnell, M., and Kuriyan, J. (1997) Cell 91, 335–345). In this study, we localized the DnaX-binding domain of δ′ to its carboxy-terminal domain III by quantifying protein-protein interactions using a series of δ′ fusion proteins lacking specific domains. The fusion protein corresponding to domain III of δ′ bound to DnaX with an affinity approaching that of full-length δ′. In contrast, a construct bearing δ′ domains I-II did not bind DnaX at detectable levels. The presence of δ and χψ strengthened the interaction of DnaX with full-length δ′ and δ′ domain III. Thus, domain III of δ′ not only contains the DnaX-binding site, but also contains the elements required for positive cooperative assembly of the DnaX complex. A domain III-specific anti-δ′ monoclonal antibody interfered with DnaX complex formation and abolished the replication activity of DNA polymerase III holoenzyme.

In Escherichia coli, chromosomal replication is catalyzed by the DNA polymerase III holoenzyme (holoenzyme),¹ a complex that contains 10 different types of subunits: α, α', γ, β, δ, δ', ε, θ, χ, and θ. Pol III core (αεθ) contains DNA polymerase and 3′–5′ exonuclease proofreading activities. Pol III is tethered to the template via the sliding clamp processivity factor (β₂). The DnaX complex (DnaX₃δ′ψ) assembles β₂ onto the primer template in an ATP-dependent reaction, and plays a central role in organization and communication at the replication fork (1–3). This complex contains a trimer of DnaX protein plus one copy each of the δ, δ', χ, and ψ subunits (1, 4). The DNA gene of E. coli encodes two distinct products known as DnaX: τ, the full-length translation product (70 kDa); and γ, a shorter protein (47.4 kDa) that arises by translational frameshifting (5–8). Both DnaX proteins are contained in the native DnaX complex, which has a subunit stoichiometry of τ₂γδδ'ψψ (4). In vitro, functional homonomeric DnaX complexes (τ-complex, τ₂δδ'ψψ, and γ-complex, γ₂δδ'ψψ) can also be assembled (4, 10).

Within the DnaX complex, δ′ and δ bind directly to γ (11); δ binds δ′ and χ binds ψ (12, 13). δ and δ′ form a 1:1 complex (13, 14) and function with DnaX to load β onto primed templates (13, 15). In the presence of δ, the δ-DnaX interaction is strengthened, resulting in the positive cooperative assembly of the DnaX₃δ′ complex (16). The χ and ψ subunits also form a 1:1 complex (17) and increase the affinity of DnaX for δδ′ (12, 17) so that the DnaX complex can be assembled at physiological subunit concentrations.

The δ′ subunit is a C-shaped molecule comprised of three structural domains determined by x-ray crystallography (9, 18). Sequence alignment comparisons predict that the structure of the γ subunit resembles that of the δ′ subunit (18). The limited proteolysis of DnaX (τγ) and expression of the predicted domains of DnaX further support this prediction (19). This redundancy between subunits within the DnaX complex (τ₂γδδ'ψψ) is echoed in other homologous proteins of the clamp loader family, including T4 phage gp44/62 proteins, eukaryotic replication factor-C (RFC), and archael RFC-like proteins (18, 20–25). Sequence-based alignments suggest that the gene 44 protein of bacteriophage T4 and the subunits of the RFC complex are also likely to have the same general architecture of δ′, particularly within the NH₂-terminal and central domains of δ′ (18). Furthermore, x-ray crystallography studies have shown that the hexamerization domain of N-ethylmaleimide-sensitive fusion protein which is the ATPase of the conserved membrane fusion machinery, and the RuvB family of hexameric DNA helicases which is also a DNA-dependent ATPases, have folds similar to that of δ′ (18, 26, 27).

Understanding the mechanism of clamp loading requires a detailed structure-function study of the prototypical E. coli DnaX complex. Among the DnaX complex subunits, δ′ provides a representative example to achieve insight into the architecture and possible mechanisms of action of the basic components of these molecular machines. We previously used a panel of δ′ fusion proteins to map the δ′-binding domain of δ′ to its COOH-terminal domain (domain III) (14). In the present work, we determined that domain III of δ′ also binds the DnaX protein, and supports cooperative DnaX complex formation of DNA polymerase III holoenzyme.

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† To whom correspondence should be addressed.

¹ The abbreviations used are: holoenzyme, DNA polymerase III holoenzyme; δ′, fusion peptide was tagged at the amino-terminal end of full-length δ′ except that first amino acid (Met, start codon) was omitted to enable fusion of a peptide tag; δ′, carboxy-terminal end fusion peptide tagged full-length δ′; Iₜ, carboxy-terminal end fusion peptide-tagged domain I (amino acid residues 1–168) of δ′; I + Iₜ, carboxy-terminal end fusion peptide-tagged domains I-II (amino acid residues 1–206) of δ′; Iₜ, carboxy-terminal end fusion peptide-tagged domain III (amino acid residues 207–334) of δ′; II + IIIₜ, amino-terminal end fusion peptide-tagged domains II-III (amino acid residues 169–334) of δ′; mAb, monoclonal antibody; DTT, dithiothreitol; RFC, replication factor-C.

**EXPERIMENTAL PROCEDURES**

**Reagents, Materials, and Buffers—**Centricor-10 microconcentrators were obtained from Amicon (Millipore, Bedford, MA). Superose 6 HR 10/10 FPLC gel filtration column and NAP-25 column were from Amersham Bioscience, Inc. (Piscataway, NJ). Ready Gel™ SDS-polyacrylamide gel and Coomassie Blue were from Bio-Rad (Hercules, CA). Biacore CM5 sensor chips (research grade), P-20 surfactant, N-hydroxysuccinimide, 2-N,N-diethylaminopropylcarbodiimide, and ethanolamine hydrochloride were obtained from Biacore Inc. (Piscataway, NJ). SDS sample buffer was 60 mM Tris-HCl (pH 6.8), 10% sucrose, 2% SDS, 0.007% Coomassie Blue, and 60 mM DTT. Buffer A was 20 mM Hepes (pH 7.4), 100 mM NaCl, 5% glycerol, 10 mM MgCl₂, 5 mM Mg(OAc)₂, 1 mM EDTA, 100 mM potassium glutamate, 5 mM DTT, and 200 μM ATP. Buffer B is 50 mM Hepes (pH 7.4), 10% (v/v) glycerol, 100 mM potassium glutamate, 5 mM DTT, 10 mM Mg(OAc)₂, 200 μM ATP, 2% SDS, 0.007% Coomassie Blue, and 60 mM DTT. Buffer A is supplemented with 18 mM -(3-diethylamino-propyl)carbodiimide, and 2% DTT, and 200 μM ATP. HBS buffer, HKGM buffer, and assay reaction mixtures were prepared as previously described (14).

**Proteins, Enzymes, and Antibodies—**ΔIIIB fusion proteins ΔIIIB, ΔIIIB, ΔIIIC, I + ΔIIIB, I + ΔIIIC, and I + ΔIIIC, were generated and purified as described (14). The pol III core (αεθν, the β(29), γ, and τ(10), β-, and δ(-30), and χ(+17) subunits were purified as previously described. Prior to conducting gel filtration-based protein binding studies, the purified ΔIIIB fusion proteins were desalted and exchanged into Buffer A via filtered through NAP-25 prepacked desalting columns (1.5 × 4.9 cm; Amersham Bioscience, Inc.) equilibrated in that buffer. Fusion proteins I + ΔIIIB, I + ΔIIIC, and I + ΔIIIC, were concentrated in a Centricor-10 microconcentrator (molecular mass cut off of 10 kDa), respectively, at 5,000 × g at 4 °C before use in the binding studies. Anti-mouse Fc (a rabbit polyclonal antibody prepared against the Fc fragment of mouse IgG) was obtained from Pierce Inc. (Rockford, IL). Anti-δ monoclonal antibodies were purified and characterized as described (14, 30).

**Gel Filtration-based Binding Studies—**A 24-ml Superose 6 HR 10/10 FPLC gel filtration column was used to study the interaction of ΔIIIB fusion protein with DnaX. Samples containing fusion proteins were run alone and in various combinations with or without δ and χ were incubated in a total volume of 300–500 μl in Buffer A at 15 °C for 30 min, then injected onto the column at 4 °C. In the absence of δ and χ, the following samples were evaluated (gels are shown in Fig. 1): (a) alone (1.5 nmol as τΔ, δ, χ, alone (1.3 nmol); δ, χ, and r(δ′, 1.3 nmol, and r, 1.5 nmol as τΔ, ΔIIIB, r + ΔIIIC, and r + ΔIIIC, 3.0 nmol, and r, 0.77 nmol as τΔ). For the interaction of ΔIIIB fusion proteins and DNAx in the presence of δ and χ, samples contained δ + χ (2.2 nmol each): δ, r(δ′, 0.27 nmol, or 0.86 nmol, and r, 0.77 nmol as τΔ); I + ΔIIIB, r(II + ΔIIIC), 0.77 nmol, and r, 0.77 nmol as τΔ); I + ΔIIIC, 3.0 nmol, and r, 0.77 nmol as τΔ); r + ΔIIIC, 1.0 nmol, and r, 0.77 nmol as τΔ). These gels were used in gel filtration (32, 33). SDS-Polyacrylamide Gel Electrophoresis—Superose 6 gel filtration column fractions were trichloroacetic acid precipitated by adding an equal volume of 30% of trichloroacetic acid followed by incubation for 5 min on ice. 300 μl of each column fraction (total fraction size, 0.4 ml/tube) were subjected to the trichloroacetic acid precipitation and loaded onto a SDS-polyacrylamide gel. Samples were centrifuged at 11,200 × g for 10 min and the resultant pellet was washed with acetone. 25 μl of SDS sample buffer were added per each sample, boiled for 5 min, and loaded onto a SDS-polyacrylamide 8–16% gradient gel. The gel was stained with Coomassie Blue for about 4 h and destained overnight in a solution of 10% methanol and 10% acetic acid.

**Estimation of the K_d for the ΔIIIB-DnaX Interaction by Gel Filtration—**Specific Domain—To estimate the dissociation constants (K_d values) of the interaction between DnaX and ΔIIIB fusion proteins using gel filtration, stained gels of the column fractions were subjected to a laser densitometric scan (Molecular Dynamics, Sunnyvale, CA). The bound and unbound fractions of ΔIIIB to DnaX were quantified using the method of Kim and McHenry (31) except that δ and χ were also included when indicated. The amount of ΔIIIB bound to DnaX was determined by comparing the shift in the detection position of ΔIIIB in the presence and absence of DnaX. Although δ can associate equally well with either of the two DnaX proteins in vitro (16), we recently discovered that it binds to γ, but not τ, within the DnaX complex of the native holoenzyme (11). Many of the experiments presented in this report use the τ subunit; these studies were conducted before we learned of the exclusive δ-γ interaction within the native holoenzyme. However, we have confirmed that the in vitro interactions between δ and either of the two different DnaX proteins are similar (experiments reported in Fig. 3 and other data that are not shown). Thus, these data may be taken as reflective of general DnaX binding properties.

Molar excesses of δ and χ were used in all binding experiments. The interaction of DnaX and ΔIIIB in the absence or presence of molar excesses of δ and χ was modeled as ΔIIIB + DnaX = δΔIIIB-DnaX, where the apparent K_d of ΔIIIB-DnaX interaction was defined as K_d = [δ]/[DnaX]δ/[δΔIIIB-DnaX]X. The molar concentration of δ bound to DnaX was determined from the equation: [δΔIIIB-DnaX]X = (I + δ_{ΔIIIB} + γ)/[δΔIIIB-[δΔIIIB-DnaX]. The total molar concentration of δ load onto the column, γ_{ΔIIIB} is the total intensity from all the δ bands (bound plus unbound), and I_{ΔIIIB} is the total intensity of bands corresponding to δ bound to DnaX from the stained gel. The amount of free DnaX (τ_{ΔIIIB}) and free δ (δ_{ΔIIIB}) were then determined from mass conservation: [DnaX]_{ΔIIIB} = [DnaX]_{ΔIIIB} - [δΔIIIB-DnaX] (DnaX_{ΔIIIB} is the total initial molar concentration of DnaX) and [δ_{ΔIIIB} = [δΔIIIB-DnaX] - [δΔIIIB-DnaX]. Subsaturation amounts of δ relative to DnaX were applied to gel filtration for the K_d quantification; at least two different concentrations of δ were used in these experiments.

**Surface Plasmon Resonance Analysis—**The effect of monoclonal antibodies on the ΔIIIB-DnaX interaction and cooperative DnaX complex assembly were assessed using Biacore technology. To immobilize anti-δ mAb, anti-mouse Fc was chemically coupled to the CM5 sensor chip using an amine coupling reaction of N-hydroxysuccinimide and N-ethyl-N,N′-dihydroxypropylcarbodiimide followed by ethanalamine inactivation as described (14), and then anti-δ mAb was captured by the immobilized anti-mouse Fc in HBS buffer. Unoccupied binding sites were blocked by injecting the nonspecific mouse IgG. Native δ was then injected repeatedly to saturate the immobilized anti-δ mAb-binding sites. In separate experiments, samples containing 2 μg δ, r, and τ were injected over the immobilized δ mAb complex at 20 °C in HBS buffer at a flow rate of 5 μl/min. For a control experiment, each of δ, r, τ, or δ + r + τ, was injected over δ in the absence of mAb, where δ is immobilized directly without using mAb on the sensor chip via amine coupling methods as described (14). K_d values were calculated from the determined kinetic parameters (K_d = k_{diss} / k_{ass}, where k_{diss} is association rate constant and k_{ass} is dissociation rate constant) obtained from binding association and dissociation phases of Biacore sensogram using the BIAevaluation 2.1 software (Biacore).

**Biotin Blot—**After separation by SDS-PAGE, proteins were transferred onto the transfer membranes and developed as described (14).

**RESULTS**

**Interaction of DnaX and Fusion Proteins of ΔIIIB Lacking Specific Domains—**We have reported the construction of a series of plasmids that produce proteins lacking specific domains of δ (14). Either the amino or carboxyl terminus of each of these δ derivatives is fused with a hexahistidine sequence and a short sequence that is biotinylated in vivo to facilitate purification, detection, and immobilization (14). We assessed the ability of δ fusion proteins to interact with DnaX using gel filtration. Different concentrations (0.83, 2.3, and 5.0 μM) of full-length δ tagged at its COOH-terminal end, δ_{C}, were incubated with τ followed by gel filtration, and binding was monitored by evaluating Coomassie-stained SDS-polyacrylamide gels of Superose 6 gel filtration fractions (Fig. 1). The τ subunit by itself eluted earlier (Fig. 1A, fractions 31–33) due to its higher molecular weight (τ_{ΔIIIB}, 280 kDa), and a lower molecular weight δ_{C} (40 kDa). The δ_{C} was bound to DnaX by itself eluted in later fractions (Fig. 1B, fractions 44–45). When δ_{C} was preincubated with τ prior to gel filtration, its elution position was shifted to a higher molecular weight (Fig. 1C, fractions 41–47 to 29–33), indicating an interaction. The densitometric scan of the protein bands in the gel permitted estimation an δ_{C}-τ-K_{δ}, between 1.9 and 3.8 μM αδ indicating a relatively weak interaction (Table 1). The K_d determined by gel filtration was comparable to the value obtained using a Biacore for the binding of native δ and immobilized,
tagged DnaX ($K_D = 0.5–1 \mu M$) (16). Unlike full-length δ’ fusion protein with either an amino- or carboxyl-terminal tag, I_C, II + III_N, or I + II_C at concentrations up to 9–11 μM, did not co-elute with τ in gel filtration (Table I) (Fig. 1). This indicates that the δ’ fusion proteins lacking domain I or domain III did not form a gel filterable complex with DnaX at least a detectable level. However, due to the relatively weak interaction of δ’ with DnaX alone, even a modest decrease in $K_D$ would result in undetectable complex formation under our experimental conditions. To overcome this obstacle to binding determination, we exploited the ability of δ and χ’ to enhance the apparent affinity of δ’ and DnaX.

δ and χ’ Enhance the Interaction of DnaX and the Fusion Proteins of δ’ Lacking Specific Domains—Both δ and χ’ have positive cooperative effects on the δ’-DnaX interaction, strengthening its binding by at least 10–15-fold (12, 16, 17). χ’ alone tightly binds DnaX and strengthens both δ’-DnaX and δδ’-DnaX interactions (12, 16, 17). We previously found that although δ does not interact with DnaX by itself, it binds δ’ and strengthens the δ’-DnaX interaction (16). Since interactions between DnaX and domain III or domains I-II may have been too weak to be detectable by our methodology, we exploited the cooperative contribution of δ and χ’ to the δ’-DnaX interaction. A 3-fold molar excess of both δ and χ’ over DnaX were used for gel filtration-based binding studies. The concentrations of the fusion δ’ proteins employed were 1.5–19-fold greater than the $K_D$ of δ-δ’ interaction ($K_D = 570 \mu M$) (14), and the molar concentrations of χ’ were 600-fold higher than the $K_D$ of DnaX-χ’ interaction ($K_D = 10 \mu M$) (16, 17). The interaction between full-length δ’ fusion protein and τ was first assessed (Fig. 2). In the presence of δ and χ’, full-length δ’ fusion protein bound τ with greater affinity; the concentration of δ’ required for half-maximal levels of complex formation was decreased more than 5-fold (for δ_C, Fig. 2 and Table I; for δ_N, Table I). For quantifying the apparent $K_D$ values, subsaturating amounts of δ’ proteins δ_N or δ_C relative to τ were used for gel filtration (Table I, Fig. 2 and Table I; for δ_N, Table I). The identities of the δ’ fusion proteins were confirmed by biotin blotting (data not shown). Densitometric scans of the stained δ_C- or τ-subunit bands of column fractions permitted estimation of an δ_C-τ apparent $K_D$ between 0.24 and 1.0 μM (Table I). This indicates that δ and χ’ strengthened the full-length δ’ fusion protein-DnaX interaction by 4–10-fold.

**Domain III of δ’ Contains the DnaX-binding Site and the Elements Required for Cooperative DnaX Complex Formation**—Next, we examined the interactions of DnaX to the truncated δ’ proteins lacking specific domains in the presence of δ and χ’. Samples of DnaX, δ, and χ’ at the same concentrations used for analysis of full-length δ’ were used. II + III_N by itself, which contains domains II-III, eluted in fractions 39–40 (data not shown). In the presence of molar excesses of δ and χ’, II + III_N co-eluted with DnaX in early column fractions (Fig. 3A, fractions 29–31 for δ, Fig. 3B, fractions 33–35 for χ’) indicating its interaction with DnaX.

At a concentration of 2 μM DnaX, most of the II + III_N co-eluted with DnaX (Fig. 3, A (τ) or B (γ)), indicating that II + III_N was saturated with DnaX. This also indicates that the majority of II + III_N fractions were functional. Detection of this II + III_N-DnaX interaction is enabled by positive cooperative contributions of δ and χ’, since there was no detectable II + III_N-DnaX interaction in the absence of δ and χ’ (Fig. 1D).

Thus, deletion of domain I did not abolish this cooperativity. This is also evidenced by the decrease in the apparent $K_D$ values by virtue of the presence of δ and χ’, based on the comparison between II + III_N-δδ’ (−5–20-fold) and δ’-γδ’.
interactions (–4–10-fold) (Table I). Deletion of domain I resulted in only a 3–4-fold reduction in the observed affinity values (Table I).

Domains I–II and domain III were then used to further localize the domain boundary for DnaX binding and for cooperative DnaX complex formation. The NH₂-terminal half of δ' containing domains I–II at a concentration up to 9 μM did not bind DnaX even in the presence of δ and ψ (Table I, Fig. 3, C (τ) and D (γ)). However, the COOH-terminal domain III bound to τ and supported the complex formation; the synergistic effect of δ and ψ on the δ′-DnaX interaction was still present (Table I). Deletion of domain III of δ' abolished both DnaX and δ binding (Fig. 3, C and D, Table I). To assess the role of domain II on DnaX binding and the DnaX complex formation, we compared the differences in apparent $K_D$ values between the NH₂-terminal tagged δ′N-τ and II + III-τ interactions; and likewise, between the COOH-terminal tagged δ′C-τ and III-τ interactions, respectively. These pairwise comparisons were performed to be consistent in corresponding values due to the tag orientation of fusion domains (Table I). In the presence of a molar excess of δ and ψ, the difference in apparent $K_D$ values for the δ′C-τ and II + III-τ interactions was 3–4-fold, and that for the δ′C-τ and III-τ interactions was 2–3-fold (Table I). The presence or absence of domain II did not provide a significant difference in the observed binding affinities. This indicates that domain II appeared to have little or no effect on DnaX binding and cooperative complex formation.

As a verification of our quantification of truncated δ' protein, we substituted the molar quantity of δ bound in a complex and recalculated apparent $K_D$ values for the δ' derivative for DnaX. This exploited our knowledge that δ does not bind detectably to DnaX by itself and enters complexes in a 1:1 ratio with δ' (12, 14, 32). The obtained results were similar to those calculated for domains II + III, domain III, and δ' (data not shown).

Since both τ and γ bind δ' similarly (16), we also used γ instead of τ to verify that both bound δ' in the absence of domain I. Again, domains II–III, but not domains I–II, bound γ with a similar magnitude to that of τ (Fig. 3, B and D) (Table I). Overall, domain III was the only domain common to all of δ' fusion proteins whose interaction with DnaX was enhanced in the presence of δ and ψ.

**Domain III-specific MAb s Inhibit Cooperative DnaX Complex Assembly and Holoenzyme Activity**—We have reported the production of a panel of mAbs against native δ' (14, 30). The domain specificity of each anti-δ' mAb was characterized using δ' proteins lacking specific domains in Biacore-based experiments (14). We exploited the availability of this battery of domain-specific anti-δ' mAbs to further probe the domain's function in δ'-DnaX interaction and DnaX complex assembly using Biacore. Anti-δ' mAb was immobilized indirectly via binding to goat anti-mouse IgG cross-linked on the sensor chip. The native δ' subunit was then injected to saturate the binding
FIG. 4. Domain III-specific anti-δ' mAb prevents a cooperative complex formation. δ' was immobilized by a domain-specific anti-δ' mAb on the Biacore sensor chip as described under "Experimental Procedures." A, domain I-specific anti-δ' Mab874B2 was immobilized on the anti-mouse Fc derivatized sensor chip, and 310 response units (RU) δ' was then immobilized by Mab874B2. B, domain III-specific anti-δ' Mab583H9 was immobilized on the anti-mouse Fc derivatized sensor chip, and 450 RU δ' was then immobilized by Mab583H9. Samples containing 2 μM of each τ, δ + τ, or δ + τ + χψ were then injected over the derivatized sensor chip. All binding analyses were carried out in HBS buffer at a flow rate of 5 μl/min at 20°C. Control injections over the anti-mouse Fc derivatized sensor chip were performed and subtracted from the data shown. Significant signal to noise ratio (>0.7–0.9) were observed in the data set shown in B, and the base lines of sensor grams were modestly declined after the subtraction of background signal. Neither anti-δ' Mab583H9 nor Mab874B2 displayed cross-reactivity against τ, δ, and χψ (14).

COOH-terminal Domain III Binds DnaX

In this study, we have identified the DnaX-binding domain of δ' by measuring the binding of DnaX to recombinant proteins corresponding to the δ' subunit lacking specific domains. Furthermore, we found that the interaction of δ's domain III with DnaX is stimulated by inclusion of the other DnaX complex components δ and χψ. We previously found that this same domain of δ' contains the determinants necessary and sufficient for binding the δ subunit (14). The sum of these data indicates that δ' domain III binds both DnaX and δ, and suggests that it bridges these two components within the DnaX complex, and facilitates cooperative DnaX complex assembly. Our findings were surprisingly similar to the previous observation that DnaX also requires domain III for δ' binding, oligomerization, and cooperative DnaX complex assembly (Table III (16, 34)).

Domain III of δ' bound to DnaX nearly as strongly as did full-length δ'. The KD values for the δ'-C and III-C interactions in the presence of molar excesses of δ and χψ differed by only 3–7-fold. This indicates that domain III alone possesses the major DnaX binding determinants of δ'. The presence of δ and χψ strengthened the δ'-C interaction by 4–10-fold, which is a similar level of enhancement previously observed using a native δ' and a full-length DnaX fusion protein (16). Thus, the fusion peptide tag located at the COOH terminus of δ' had no significant effect on the synergistic effect of δ and χψ on δ'-C interaction. Moreover, a comparable level of stimulation of δ and χψ on the interaction of DnaX with the δ' proteins containing domains II-III (5–20-fold) and domains I-III (4–10-fold), was observed. This shows that δ' domains II-III contain the elements required for the full cooperative effect of δ and χψ on DnaX-δ' interaction and that domain I is not a significant participant in cooperativity. Unlike full-length δ', the cooperative effect of δ and χψ on the II + III-C or III-C interaction could not be directly calculated, since the interaction of II + III or III-C with τ in the absence of δ and χψ was too weak to be detected using our methodology. However, the lower boundary limit of the II + III-C interaction KD was presumed to be greater than 11 μM. Relative comparisons of the apparent KD values in the presence or absence of δ and χψ for the interactions between II-C and II + III-C provided comparable values. These results indicate that domain II did not have a significant role in DnaX and δ binding or cooperative complex assembly and that the COOH-terminal domain of δ' is sufficient for the DnaX complex assembly.

We did not observe any detectable binding contribution from δ' domain I. A parallel observation was made for the RFC, a five-subunit protein complex required for eukaryotic DNA replication and repair; Uhlmann et al. (9, 33) found that the NH2-terminal half of all five RFC subunits lack detectable
COOH-terminal Domain III Binds DnaX

**TABLE II**

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% inhibition of holoenzyme replication activity in reconstitution assay

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**TABLE III**

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<td>COOH-terminal half</td>
<td>Full-length</td>
<td>Full-length</td>
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</table>

Results obtained using mAbs further confirm the results from the deletion study of δ'. Domain III-specific Mab853H9 specifically interfered with the δ'-τ interaction. By blocking individual τ binding (but not δ binding), Mab853H9 also interfered with cooperative DnaX complex formation. Furthermore, this mAb abolished the replication activity of holoenzyme. This suggests an essential function of δ' domain III in holoenzyme activity, consistent with the observed pivotal role of δ' domain III for DnaX complex assembly. We observed that the domain III-specific mAbs which block δ'-δ' interaction (Mab629G10 and 1755D9) (14) had little or no significant effect on δ'-τ binding (data not shown). These data suggest that δ' and τ bind distinct sites on δ' domain III, consistent with the ability of δ' to bind DnaX and δ simultaneously. The observed inhibitory effect of the domain I-specific mAb on δ'-τ binding was unexpected. Although the domain I-specific mAb interferes with the δ'-τ interaction, it does not block cooperative complex assembly (Fig. 4A). It should be noted that the domain I-specific Mab874B2 did not interfere with the δ'-δ' interaction (14), and there was no detectable effect of domain I on the δ'-δ' interaction (14). One possibility is that Mab874B2 inhibits formation indirectly by inducing conformational changes. Alternatively, Mab874B2 may sterically interfere with τ domain III interaction via binding to domain I, which is conceivable, given that IgG (150 kDa) and the τ subunit (284 kDa) are both large molecules. If this is the case, then positive allosteric effects of δ' and χψ may overcome the steric hindrance effect of domain I-specific mAb on δ'-τ interaction. Interestingly, this domain I-specific Mab874B2 dramatically inhibited the replication activity of holoenzyme, despite all holoenzyme subunits including δ' and χψ being present in the reconstitution activity assay. These findings are consistent with the observation that δ' constructs lacking domain I or I-II of δ' abolished holoenzyme activity (14). These data suggest that although domain III is responsible for complex formation, domain I is also critical for holoenzyme activity.

Multiple sequence alignment comparisons and limited proteolysis analysis suggest that the structure of the γ subunit resembles that of the δ' subunit (18, 19). A deletion study of DnaX protein showed that DnaX domain III also contains the binding sites of δ' and χψ as well as the sequence for the cooperative assembly of the DnaX complex (16). Domain III of DnaX is also responsible for the homomeric tetramerization of DnaX (34). Within the DnaX complex (τ, χψ, δ'γ), a total of four copies of homologous subunits (δ', τ, and γ) are found (4). It seems that despite their lack of significant sequence similarity, the COOH-terminal regions of homologous proteins of DnaX complex may share tertiary structural elements and mediate conserved functional themes. It is reasonable to assume that δ', binding function for RFC complex assembly. However either NH2-terminal or COOH-terminal deletions of each component of RFC diminished the replication activity of Pol δ, reminiscent of our previous observation that deletion of either domain I or domain III of δ' abolished holoenzyme activity (14). All of these NH2-terminal deletions of RFC subunits contained the conserved sequence element designated as the RFC box-II (18, 26). Thus, δ' may share some structure/function themes with RFC, but it appears to employ some unique molecular features as well.

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COOH-terminal Domain III Binds DnaX

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τ, and γ are linked together by related interactions, forming a core for the addition of the δ and χ-ψ components.

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REFERENCES