THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 50, Issue of December 14, pp. 47185–47194, 2001
Printed in U.S.A.

Interplay of Clamp Loader Subunits in Opening the β Sliding Clamp of *Escherichia coli* DNA Polymerase III Holoenzyme*

Received for publication, July 18, 2001, and in revised form, September 21, 2001
Published, JBC Papers in Press, September 25, 2001, DOI 10.1074/jbc.M106780200

Frank P. Leu‡ and Mike O'Donnell§¶

From the ‡Department of Pharmacology, Joan and Sanford I. Weill Graduate School of Medical Sciences of Cornell University and the §Rockefeller University, Howard Hughes Medical Institute, New York, New York 10021

The *Escherichia coli* β dimer is a ring-shaped protein that encircles DNA and acts as a sliding clamp to tether the replicase, DNA polymerase III holoenzyme, to DNA. The γ complex (γδδ′χψ) clamp loader couples ATP to the opening and closing of β in assembly of the ring onto DNA. These proteins are functionally and structurally conserved in all cells. The eukaryotic equivalents are the replication factor C (RFC) clamp loader and the proliferating cell nuclear antigen (PCNA) clamp. The δ subunit of the *E. coli* γ complex clamp loader is known to bind β and open it by parting one of the dimer interfaces. This study demonstrates that other subunits of γ complex also bind β, although weaker than δ. The γ subunit like δ, affects the opening of β, but with a lower efficiency than δ. The δ′ subunit regulates both γ and δ ring opening activities in a fashion that is modulated by ATP interaction with γ. The implications of these actions for the workings of the *E. coli* clamp loading machinery and for eukaryotic RFC and PCNA are discussed.

Chromosomal replicases are highly processive machines owing to a sliding clamp subunit that encircles and slides on DNA, acting as a mobile tether for the replicase during synthesis (1–4). These circular clamps require a multimeric clamp loader assembly for their opening and closure around DNA in a process that consumes ATP. In *Escherichia coli* the clamp is the β dimer, formed from two crescent-shaped protomers (5), and the β ring is opened and closed by the γ complex clamp loader (γδδ′χψ). Once on DNA, β acts as a mobile tether for the replicase, DNA polymerase III holoenzyme, holding it to DNA for highly processive synthesis (1). In fact, the β subunit can also couple with all the other *E. coli* DNA polymerases (DNA polymerases I, II, IV, and V) (6–9) and with DNA ligase and MutS (10). The eukaryotic system is similar (11). Here, the RFC[1] clamp loader assembles the ring-shaped PCNA clamp onto DNA for processive DNA polymerase action (12, 13). PCNA is also known to interact with several other proteins indicating that, like β, these clamps serve multiple roles in cellular DNA metabolism (14).

This report is part of a continuing study on the mechanism of the *E. coli* γ complex clamp loader. The γ complex consists of five different subunits (γδδ′χψ) (15), three of which (γδδ′) are essential to clamp loading action (16). One copy each of the χ and ψ subunits bind the γδδ′ core but are not essential to clamp loading activity (17). The crystal structure of the γδδ′ complex has recently been solved, and it shows that there are three γ subunits and one each of δ and δ′ in a circular pentameric arrangement (18). A protein in the holoenzyme known as τ is encoded by the same gene as γ (*dnaX*) and therefore is essentially identical to γ except for an extra C-terminal section in τ (19, 20). Fully active clamp loading complexes can be reconstituted and are composed of one each of δ, δ′, and either τ_3 or γ_3, or mixtures of τ and γ (*i.e.* $\tau_1\gamma_2$ and $\tau_2\gamma_1$) (17, 21). The unique C terminus of τ, lacking in γ, binds to the DNA polymerase III core and DnaB, thereby acting to organize the replisome machinery (22, 23).

The γ (τ) subunit of the clamp loader is the only one that binds and hydrolyzes ATP, and thus is the motor of the clamp loader (19). The δ subunit of γ complex forms a strong attachment to β and, in fact, opens or destabilizes one of the β dimer interfaces (25–27). No ATP is required for this (δ does not bind ATP); therefore, the energy for ring opening is derived from protein-protein interaction between δ and β. The recent crystal structure analysis of δ in complex with a monomer of β provides detailed insight into δ action (28). The way in which the δ subunit binds β leads to disruption of one of the dimer interfaces, preventing the ring from closing. Further, monomeric β forms a shallower crescent than each β protomer in the dimer, and thus the β subunit structure would appear strained to bend into a half-circle shape upon partnering with another β protomer to form a closed ring. Hence, upon cracking one dimer interface, the strain is released in the two β halves, allowing them to adopt shallower crescent shapes and resulting in significant widening of the gap at the open interface.

The energy for clamp opening is supplied by the protein-protein interaction between δ and β. However, ATP is required by γ complex. What is the role of ATP if it is not required for clamp opening? Our studies on this subject reveal that the δ subunit is buried within γ complex such that its interaction with β is weak compared with the δ·B β complex (26). Upon ATP binding to the γ subunits, however, the γ complex undergoes a conformational change that exposes δ for interaction with β (26, 29). Only in the presence of ATP and β does the ATP·γ complex·β composite show appreciable affinity for ssDNA (*i.e.* a site for DNA binding becomes exposed) (29, 30). Upon binding to DNA, especially a primed site, ATP is hydrolyzed and the connection between β and γ complex is severed (25). At a primed site, this process results in a closed β ring encircling DNA.

Docking of β onto the δ subunit of the crystal structure of γδδ′ (by replacing δ in the γδδ′ structure with the δ-β structure) suggests that γ may also bind to β (18). Our previous studies utilized gel filtration to detect the relatively strong interaction of δ subunit with β, but failed to detect an interaction of β with

* This work was supported by the Howard Hughes Medical Institute and by National Institutes of Health Grant GM38839. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 212-327-7251.

[1] The abbreviations used are: RFC, replication factor C; PCNA, proliferating cell nuclear antigen; SPR, surface plasmon resonance; ssDNA, single-stranded DNA; DTT, dithiothreitol; ATPγS, adenosine 5′-*O*-(thiotriphosphate); RU, response unit(s).

γ or any other subunit of γ complex (26). Hence, we reexamined γ complex subunits for interaction with β in such a manner that we could detect even very weak interactions.

This report reveals that, in addition to δ, γ and χ also interact with β, and possibly δ′ as well. Further, like δ, the γ subunit can open β, as inferred from its ability to increase its rate of dissociation from circular DNA. However, γ binds β weaker than δ and is ~20-fold less efficient in unloading β from DNA compared with δ ($k\delta_{unloading}$ = 0.42 min^{-1}; $k\gamma_{unloading}$ = 0.016 min^{-1}). The δ′ subunit does not appear to unload the β ring from DNA, but it binds δ and prevents δ from unloading β. δ′ also inhibits the γ unloading activity. Interestingly, the δ′-mediated inhibition of γ, and of γδ, is relieved upon adding ATP. Hence, δ′ is essential for coupling ATP to the action of γ and δ with β, even though ATP binds γ and not δ′. These interactions between γ complex subunits among themselves and β, and their regulation by δ′ and ATP, are discussed in terms of a molecular model of γ complex mechanism.

EXPERIMENTAL PROCEDURES

Proteins and Other Reagents

Radioactive nucleotides were purchased from PerkinElmer Life Sciences. Unlabeled nucleotides were purchased from Amersham Pharmacia Biotech. Bio-Gel A-15m gel was purchased from Bio-Rad. Oligodeoxyribonucleotides were synthesized by Life Technologies, Inc. Singly nicked plasmid DNA was prepared as described (21) using M13 gpII endonuclease and pBluscript plasmid DNA, which is nicked once at the M13 origin by gpII. Proteins were purified as described: α, ε, and γ (31); δ and δ′ (32); χ and ψ (33); θ (34); and SSB (31). The γ complex was reconstituted from pure subunits and purified from unassociated proteins as described in our earlier study (17). The $\beta_{monomer}$ (I272A, L273A) was purified as described (35). ^{32}P-β, a derivative of β containing six C-terminal amino acid residues (NH_2-RRASVP-COOH) that serve as an efficient substrate for cAMP-dependent protein kinase, was labeled using [γ-^{32}P]ATP as described (36, 37). ^{32}P-β used in this study had a specific activity of 150 dpm/fmol. The catalytic subunit of cAMP-dependent protein kinase produced in *E. coli* was a gift from Dr. Susan Taylor (University of California, San Diego, CA).

Buffers

Buffer A is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2 mM DTT, 4% (v/v) glycerol, 1 mM ATP, and 8 mM $MgCl_2$. Buffer B is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 100 μg/ml bovine serum albumin (Sigma), 2 mM DTT, 4% (v/v) glycerol, 8 mM $MgCl_2$. Buffer C is Buffer B, but contains 2 mM $MgCl_2$ and lacks ATP. 6× sample loading dye contains 0.25% bromophenol blue, 15% Ficoll, and 0.25% xylene cyanol. Buffer D contains 8.9 mM Tris, 8.9 mM sodium borate, and 0.2 mM EDTA. Surface plasmon resonance (SPR) buffer contains 10 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20. Replication Buffer contained 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 40 g/ml bovine serum albumin, 5 mM DTT, 1 mM ATP, and 8 mM $MgCl_2$.

Surface Plasmon Resonance

The $\beta_{monomer}$ was immobilized on a carboxymethyldextran matrix-coated sensor chip CM5 using carbodiimide covalent linkage in 10 mM sodium acetate (pH 5.5) to yield a final value of ~7000 response units (RU) of immobilized $\beta_{monomer}$. The mobile phase (SPR buffer) contained 1 μM δ, 1 μM γ, 1 μM χ, or 1 μM δ′ or 1 μM χψ. SPR buffer containing protein was passed over the immobilized β at a flow rate of 6 μl/min for 5 min, after which SPR buffer lacking protein was injected over the chip.

Preparation of β·DNA Complex

The β·DNA complex was prepared as substrate for clamp loading assays as follows. ^{32}P-β (1.5 pmol) was incubated with 1 pmol of γ complex and 1.25 pmol of gpII nicked pBluescript plasmid DNA at 37 °C for 10 min in 50 μl of Buffer A. The reaction was applied to a 5-ml Bio-Gel A-15m gel filtration column (Bio-Rad) equilibrated in Buffer B at room temperature, and fractions of 180 μl were collected. Because of the large size of the DNA, the ^{32}P-β·DNA complex elutes early (usually fractions 11–14) and separates from free ^{32}P-β, γ complex, and ATP (in fractions 21–31). Three peak fractions (usually 11–13) containing ^{32}P-β·DNA (determined by scintillation counting) were pooled for use as substrate in the unloading reactions.

β Clamp Unloading Assays

Proteins were analyzed for ability to unload clamps by mixing 0.4 nM ^{32}P-β·DNA substrate in 25 μl of Buffer B on ice with 25 μl of Buffer C containing δ, δ′, or γ_3 (0.5–3.0 μM, as indicated in the plots or figure legend), and then the reaction was shifted to 37 °C for incubation. Reactions were quenched at various times (5–180 min, as indicated in the plots or figure legend) upon addition of 5 μM $\beta_{monomer}$ (3 μl of 82 μM $\beta_{monomer}$), and then the quenched reaction was immediately placed on ice. We have shown previously that $\beta_{monomer}$ effectively quenches δ-mediated β unloading (35) and we find that it also quenches γ-mediated unloading of β (as discussed under "Results" and shown in Fig. 3). Next, 8 μl of 6× sample loading dye was added to the quenched reactions, followed by analysis in a 1.5% neutral agarose gel, which separates free ^{32}P-β from ^{32}P-β·DNA complex. Electrophoresis was for 1 h (100 V) at room temperature in Buffer D. Gels were then removed, fixed with 20% acetic acid for 10 min, and overlaid with one layer each of DE-81 paper, nitrocellulose membrane, Whatman 3M paper, and several paper towels, and then flattened under a lead brick until ~3 mm thick. The flattened gel was then wrapped in plastic wrap and exposed to a phosphor screen (Amersham Pharmacia Biotech) for ~12 h. Bands corresponding to ^{32}P-β on and off DNA were visualized using a PhosphorImager (Amersham Pharmacia Biotech), and the amount of ^{32}P-β in each band was quantitated using ImageQuant (Amersham Pharmacia Biotech). The fraction of β on DNA at each time point was calculated as the ratio of ^{32}P-β·DNA complex to total β (summation of free ^{32}P-β and ^{32}P-β·DNA). This value was then normalized to 1.0 by dividing by the fraction of β on DNA at time 0 ([β on DNA]$_t$/[β on DNA]$_{t=0}$). Typically the percentage of total β on DNA at time 0 was 70–95%; the variability is likely the result of some spontaneous loss of β from DNA between the initial isolation of the substrate ^{32}P-β·DNA, and the unloading experiment.

Overall, clamp unloading reactions are second order, but because the concentration of catalyst in the reaction (*e.g.* δ or γ) is much higher than the substrate (^{32}P-β·DNA), the reaction becomes pseudo first-order and the β unloading rate ($k_{unloading}$) can be obtained at any particular δ or γ subunit concentration using the first-order equation: ([β·DNA]$_t$/[β on DNA]$_{t=0}$) = $e^{(-k_{unloading})(t)}$, where t = time. Data points from unloading time courses were fit to this equation to obtain the observed $k_{unloading}$ value at a given concentration of protein subunit (*i.e.* γ_3, δ, δ′, or combination thereof). The $k_{unloading}$ values were then plotted *versus* the concentration of the protein subunit used as the catalyst for β unloading and then the best fit to the hyperbolic equation: $k_{unloading}$ = ($k_{unloading(max)}$)([γ_3])/([γ_3] + K_d) was determined to obtain the apparent maximal β unloading rate ($k_{unloading(max)}$) and the apparent K_d value for interaction of the catalyst with the β·DNA complex.

Assays that examine subunit mixtures for ^{32}P-β unloading activity were performed upon mixing 0.4 nM ^{32}P-β·DNA in 25 μl of Buffer B with 25 μl of Buffer C containing some combination of δ (0.2–1.0 μM), γ_3 (0.5–3 μM), and δ′ (0.5–5 μM). Subunit mixtures were preincubated for 10 min on ice before addition to the assay. Specifics are as follows. Assays that examined the effect of γ and/or δ′ on the ability of δ to unload β at a fixed time point (3 min) all contained 0.4 nM ^{32}P-β·DNA in Buffer B (25 μl) to which was added 25 μl of a mixture containing 1 μM δ and either γ (0.5, 1, 2, or 3 μM) or δ′ (0.5, 1, 2, 4, or 5 μM). Time courses of β unloading assays (0, 5, 15, and 30 min) performed using mixtures of either δγ or δδ′ contained 0.2 μM δ that was preincubated (25 μl) with either 1 μM γ_3 or 1 μM δ′ before addition to the ^{32}P-β·DNA substrate. Reactions containing three subunits were performed by preincubating 0.2 μM δ with either 2 μM each γ_3 and δ′ or 3 μM each γ_3 and δ′, in the presence or absence of 1 mM ATP in 25 μl of Buffer B. Reactions were incubated at 37 °C for the indicated times or as described in the legend, and then quenched using $\beta_{monomer}$ and analyzed on an agarose gel as described above. In reactions containing ATP, 1 mM final concentration of ATP was included in the protein pre-incubation reaction.

$\beta_{monomer}$ Inhibition Reactions

DNA Synthesis—In assays examining $\beta_{monomer}$ inhibition of DNA synthesis, reactions contained 420 nM SSB (as tetramer), 1.4 nM M13mp18 ssDNA primed with a DNA 30-mer, and 0.3 μM γ complex in 25 μl of Replication Buffer. Following this, $\beta_{monomer}$ was added to reactions on ice at 0, 0.2, 0.4, 0.8, 1.2, 1.6, or 2.0 μM concentration. Next, a mixture was added yielding final concentrations of 60 μM each of dATP, dGTP, and dCTP; 20 μM [α-^{32}P]dTTP; 4.8 nM core (αεθ); and 8 nM

β. The mixture was shifted to 37 °C for 3 min, and then quenched upon addition of 25 μl of 1% SDS, 40 mM EDTA. Reactions were spotted onto DE81 filters, washed as described (38), and analyzed by liquid scintillation counting.

Clamp Loading—The effect of $\beta_{monomer}$ on β clamp loading by the γ complex was performed in 100 μl of Buffer A containing 3 μM SSB (as tetramer), 10 nM M13mp18 ssDNA primed with a DNA 30-mer, 8 nM γ complex, and 5 μM $\beta_{monomer}$ (when present). This reaction was brought to room temperature for 5 min, ^{32}P-β was added to a final concentration of 10 nM, and the reaction was shifted to 37 °C for 5 min. Reactions were analyzed by gel filtration on a 5-ml Bio-Gel A15m column equilibrated in Buffer A as described above for preparation of the ^{32}P-β clamp unloading substrate.

Clamp Unloading—In assays that examine the effect of $\beta_{monomer}$ on β clamp unloading by γ and δ, the 50-μl reactions contained 0.2 nM ^{32}P-β·DNA, and reactions were initiated by the addition of either 0.5 μM δ or a mixture of 0.5 μM δ and 2 μM $\beta_{monomer}$. Reactions were incubated at 37 °C for 3 min and then were analyzed in a native agarose gel as described above for the clamp unloading assay. In assays that examine the effect of $\beta_{monomer}$ on β clamp unloading by γ, procedures were as described above except that 1 μM γ, or a mixture of 1 μM γ and 5 μM $\beta_{monomer}$, was added and reactions were incubated at 37 °C for 80 min before analysis in a native agarose gel.

RESULTS

Interaction of γ Complex Subunits with β—To increase the possibility of detecting weak γ complex subunit interactions with β, we employed the SPR technique. In the SPR experiment of Fig. 1, we immobilized β on the chip surface. For this study we utilized a β mutant that contains two amino acid replacements, which disrupt the dimer interface resulting in a $\beta_{monomer}$ (27). Use of monomer β provides a stabile base line in SPR, compared with immobilized β dimer, which drifts down over time, probably because of slow dissociation of the protomer that is not directly cross-linked to the chip. In Fig. 1*A*, δ was passed over the immobilized β and the formation of a δ-β complex was indicated by the resulting increase in mass (recorded as RU). Following the protein injection, buffer lacking δ was passed over the chip, resulting in dissociation of δ from β as indicated by loss of the signal. The time courses of mass accumulation, and mass loss provide information from which the rates of association and dissociation of δ-β complex can be calculated. The equilibrium constant calculated from these rates is ~0.03 μM. In Fig. 1*B*, passage of γ over β also demonstrated an interaction between them with an approximate K_d of 0.9 μM (assuming γ as a trimer). γ appears to be a tetramer in solution (39), but it is trimeric when in association with δ and δ' (40). Therefore, for ease in comparing kinetic constants obtained using γ alone, and with δ and δ', we have calculated the concentration of γ as a trimer for consistency throughout this report. If γ is considered a tetramer, the calculated equilibrium constant for the experiment in Fig. 1*B* is ~25% lower.

Fig. 1*C* examines δ' for interaction with β; however, only very slight, or no, interaction was detected. Fig. 1*D* demonstrates that χ interacts with β (~K_d = 1.1 μM). This χ-to-β interaction is not explored further in this report because neither the χ nor ψ subunits are required for clamp loading (41–43). Further, we did not detect a significant effect of χ in the experiments of this report. The ψ subunit is not soluble, which prevented us from analyzing ψ for a ψ-to-β interaction. However, the ψ subunit is soluble as a $\chi\psi$ complex (34, 44). Fig. 1*E* indicates that $\chi\psi$ complex binding to β gives a similar signal as χ alone (~K_d = 1.0 μM), suggesting that χ forms the major contact to β and that ψ may not make a significant contribution to the interaction between $\chi\psi$ and β. However, we cannot rigorously exclude the possibility of a ψ-β interaction from this data.

The SPR analysis in Fig. 1 was performed using immobilized $\beta_{monomer}$ because use of β dimer in SPR analysis is limited by dissociation of β_2 during the experiment. However, it is important to keep in mind that δ interacts with $\beta_{monomer}$ much tighter than β dimer. We do not know at present whether other γ complex subunits bind the $\beta_{monomer}$ and β dimer with different affinities.

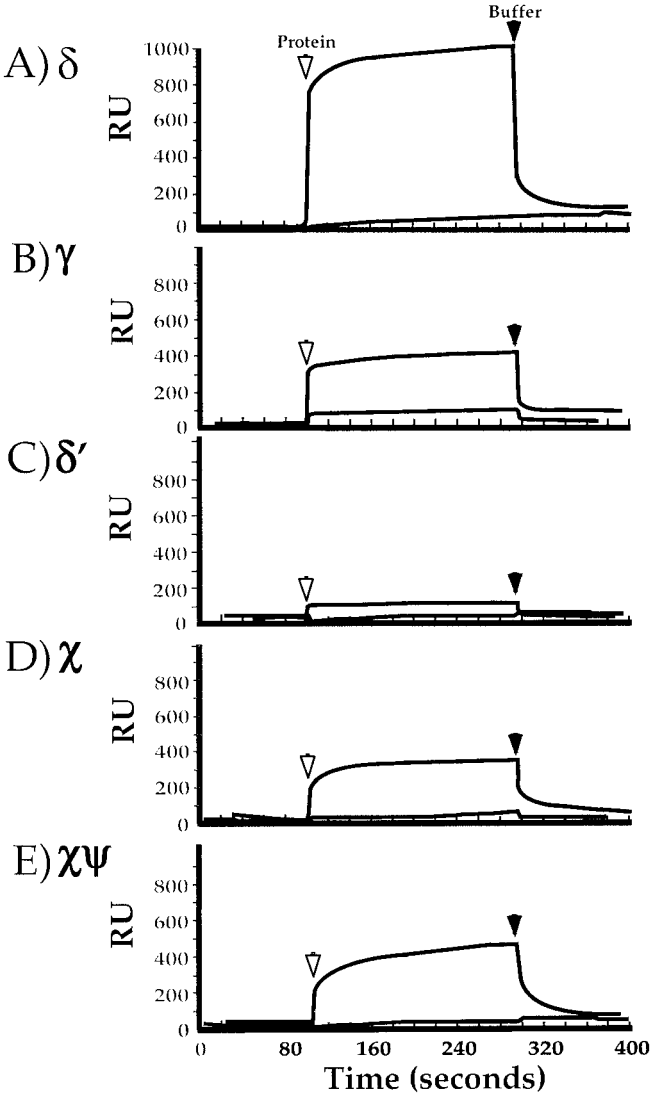

FIG. 1. **Interaction of γ complex subunits with the β clamp.** Interaction between γ complex subunits and immobilized β were examined using SPR. The *open arrow* in each sensorgram indicates the start of an injection of the indicated subunit over the immobilized β. The *solid arrow* indicates the end of the protein injection and the start of an injection of buffer. The following subunits were injected over immobilized β: *panel A*, δ; *panel B*, γ; *panel C*, δ'; *panel D*, χ; *panel E*, $\chi\psi$. Complex formation with immobilized β is indicated by an increase in mass, registered as RU. Each *panel* shows a pair of sensorgrams. In each *panel*, the lower sensorgram shows the result of injecting the indicated γ complex subunit over a sensor chip that lacked immobilized β.

We have reexamined ability to detect these complexes by gel filtration analysis using a Superose 12 column. Although we routinely detect δ-β complex by this method (26, 27), we have not been successful in detecting an interaction between β and either γ, δ', χ, $\chi\psi$, $\gamma\chi\psi$, or $\gamma\delta'\chi\psi$ by gel filtration.[2] As gel filtration is not an equilibrium technique, we presume that these complexes are too weak, and dissociate too fast, to be detected by this method. Inability to detect γ-β, χ-β, and $\chi\psi$-β complex by gel filtration is consistent with the high K_d values

[2] A. Johnson and M. O'Donnell, unpublished results.

for these complexes determined from the SPR experiments of Fig. 1.

γ Catalyzes β Unloading—Our previous studies showed that δ can open the β ring (25, 27, 35). Ring opening by δ was deduced from experiments in which β was first assembled onto circular DNA, then adding δ subunit. Ability of δ to open β is detected by removal of the β ring from the circular DNA. To follow β dissociation from DNA, this assay utilizes a kinase-tagged version of β, which can be radiolabeled using [γ-^{32}P]ATP and protein kinase. The ^{32}P-β is then placed onto circular DNA using γ complex and ATP, followed by gel filtration to isolate the ^{32}P-β·DNA complex from free β and γ complex/ATP. The ^{32}P-β·DNA complex is then used as a substrate to examine γ complex and δ for unloading activity. If ^{32}P-β is unloaded from DNA, the amount of ^{32}P-β unloaded from DNA can be determined by analysis of the reaction in a native agarose gel (or by a second gel filtration column analysis). The ^{32}P-β on DNA comigrates with the DNA substrate in the agarose gel and resolves from the free ^{32}P-β, which migrates faster through the gel. Using this procedure, we showed earlier that ^{32}P-β has a half-life for spontaneous dissociation from DNA of ~120 min at 37 °C, but if the ring is opened by δ or γ complex, the ^{32}P-β is unloaded from DNA much quicker (19). In Fig. 2, this assay was used to examine γ for ability to open β.

First, in Fig. 2*A*, is a control reaction using the δ subunit. In the absence of added δ, β spontaneously dissociates from DNA with a half-life of ~140 min. Addition of δ to the assay results in much more rapid dissociation of ^{32}P-β from the DNA ($t_{1/2}$ = 5 min or less). This experiment was repeated using 0.2–1.0 μM δ, and the autoradiograms of the agarose gels are shown to the *left* of Fig. 2*A*. From the amount of ^{32}P-β on and off the DNA, the ratio of β remaining on DNA can be obtained at each time point and is plotted in the *top right* of Fig. 2*A*. The data points were fit to a model of this kinetic process (see "Experimental Procedures") to obtain the observed rates of β dissociation from DNA at each concentration of δ. A replot of the observed rates *versus* δ subunit concentration (Fig. 2*A*, *bottom right*) yields an apparent maximal $k\delta_{unloading}$ value of 0.42 min^{-1}.

Next, we examined the effect of increasing concentrations of γ on the stability of ^{32}P-β on DNA. Previous studies using this assay did not detect β opening by γ, but only a low concentration of γ was used in that study and the incubation time was limited to 10 min (25). The assay in Fig. 2*B* utilizes several different concentrations of γ and extends the incubation time with ^{32}P-β·DNA for up to 120 min. The results show that, in the presence of 1 μM γ, the dissociation time of ^{32}P-β from DNA is reduced to 75 min and is further decreased to 40 min at the highest concentration of γ tested (3 μM γ). A replot of these observed rates *versus* γ subunit concentration indicate an apparent maximal rate of unloading ($k\gamma_{unloading}$) of 0.023 min^{-1}. Hence, γ can unload β from DNA, but is less efficient compared with δ.

These experiments were performed in the absence of ATP, yet γ is an ATP interactive protein. Does ATP alter the results? We examined the effect of ATP on γ-catalyzed β unloading in Fig. 2*C*, but the results were essentially the same as those observed in the absence of ATP.

In these unloading experiments, time points are removed from a reaction, placed on ice, and quenched from further unloading by adding the $\beta_{monomer}$ mutant, which acts as a competitor and prevents further δ- or γ-mediated unloading of ^{32}P-β_2. SDS can not be used to quench the reaction because it would simply denature β, causing all the ^{32}P-β_2 to be released from DNA. We have shown previously that $\beta_{monomer}$ (β_1) stops δ-mediated clamp unloading (35), and this is demonstrated in Fig. 3*A* for γ as well. In fact, the $\beta_{monomer}$ also

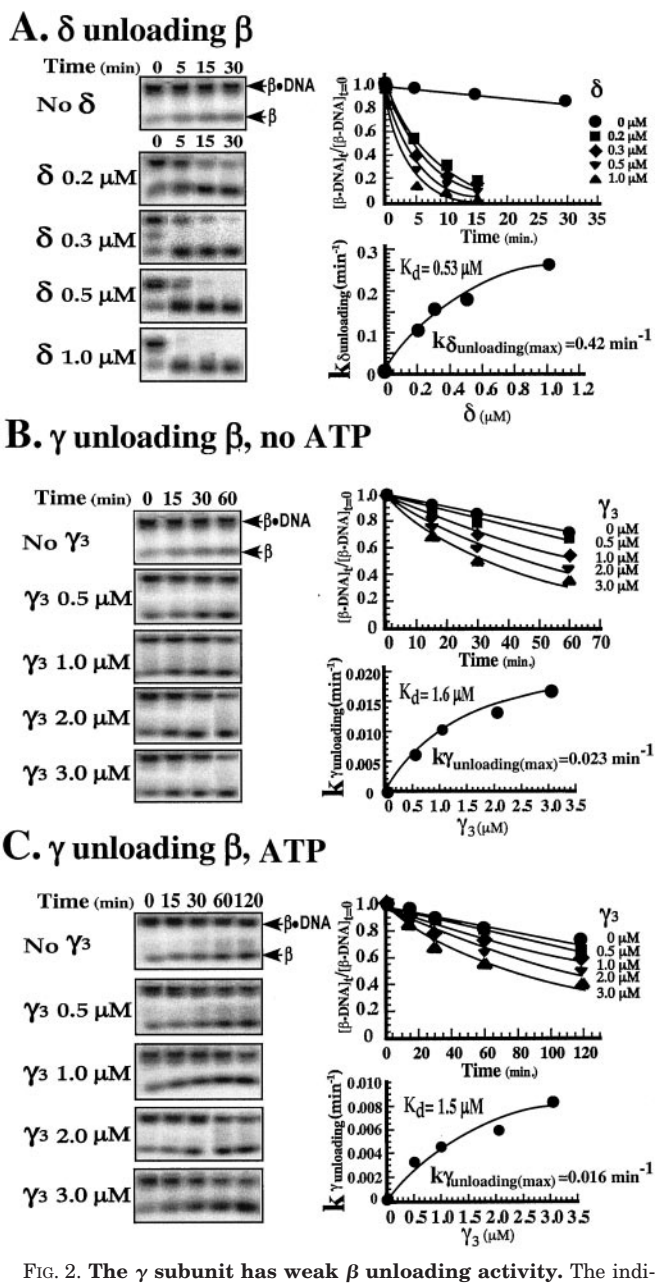

FIG. 2. **The γ subunit has weak β unloading activity.** The indicated amounts of δ (*A*), γ (*B*), or γ + 1 mM ATP (*C*) were incubated with ^{32}P-β·DNA for 5, 15, or 30 min before being stopped and analyzed by native agarose gel electrophoresis. The autoradiograms of the gels are shown to the *left* in each *panel*. The *top band* is ^{32}P-β on DNA; the *bottom band* is free ^{32}P-β. Observed off rates of ^{32}P-β dissociation from DNA were obtained by fitting data to a first order decay process. These rates were as follows. *Panel A*, no δ added, 0.005 min^{-1}; 0.2 μM δ, 0.11 min^{-1}; 0.3 μM δ, 0.16 min^{-1}, 0.5 μM δ, 0.18 min^{-1}; 1 μM δ, 0.27 min^{-1}. *Panel B*, no γ, 0.005 min^{-1}; 0.5 μM γ_3, 0.006 min^{-1}, 1.0 μM γ_3, 0.016 min^{-1} 2 μM γ_3, 0.014 min^{-1}; 3 μM γ_3, 0.016 min^{-1}. *Panel C*, no γ_3 + 1 mM ATP, 0.003 min^{-1}; 0.5 μM γ_3 + 1 mM ATP 0.004 min^{-1}, 1.0 μM γ_3 + 1 mM ATP, 0.010 min^{-1}; 2 μM γ_3 + 1 mM ATP, 0.008 min^{-1}; 3 μM γ_3 + 1 mM ATP, 0.010 min^{-1}. Replots of the observed off rates *versus* subunit concentration are shown at the *bottom right* in each *panel*. The best fit to the data yielded the following maximum unloading rate ($k_{unloading}$) and apparent K_d, respectively: *panel A* for δ, 0.42 min^{-1}, 0.53 μM; *panel B* for γ, 0.023 min^{-1}, 1.6 μM; *panel C* for γ + ATP, 0.016 min^{-1}, 1.5 μM.

inhibits γ complex-mediated clamp loading, as illustrated in Fig. 3*B*. In the Fig. 3*B* experiment, γ complex is used to assemble ^{32}P-β_2 onto DNA, then the reaction is filtered over a Bio-Gel A15m column, which resolves the large ^{32}P-β_2·DNA complex (fractions 12–16) from free ^{32}P-β_2 that is unattached to DNA. The $\beta_{monomer}$ prevents γ complex from loading the

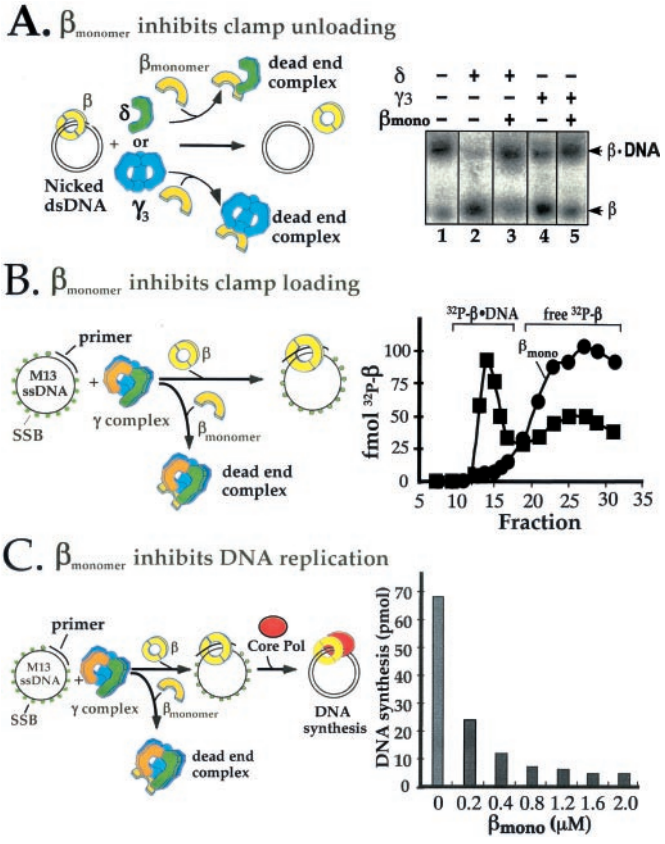

FIG. 3. **$\beta_{monomer}$ inhibits clamp unloading, clamp loading, and replication.** The *schemes* at the *left* illustrate how a dead end complex may form between $\beta_{monomer}$ and δ, γ, or γ complex, which could explain the observed inhibition by $\beta_{monomer}$ of β unloading, β loading, and DNA synthesis. *Panel A*, effect of $\beta_{monomer}$ on β unloading by δ and γ. Reactions contained ^{32}P-β·DNA complex and were incubated 3 min at 37 °C with: *lane 1*, no addition; *lane 2*, 0.5 μM δ; *lane 3*, 0.5 μM δ + 2 μM $\beta_{monomer}$; *lane 4*, 1 μM γ_3; *lane 5*, 1 μM γ_3 + 5 μM $\beta_{monomer}$. The positions of ^{32}P-β·DNA complex (*top band*) and free ^{32}P-β (*bottom band*) are indicated to the *right* of the autoradiogram of the agarose gel. *Panel B*, $\beta_{monomer}$ inhibits clamp loading by γ complex. Reactions contained ^{32}P-β, γ complex, SSB-coated primed M13mp18 ssDNA in the presence (*squares*) or absence (*circles*) of $\beta_{monomer}$. After incubation at 37 °C for 5 min, reactions were analyzed for ^{32}P-β on DNA by gel filtration as described under "Experimental Procedures," which resolves ^{32}P-β·DNA complex (fractions 12–16) from free ^{32}P-β in solution (fractions 20–32). *Panel C*, $\beta_{monomer}$ inhibits DNA synthesis. The γ complex was preincubated with DNA, β, and increasing concentrations of $\beta_{monomer}$, and then core polymerase was added to initiate primer extension around the primed M13mp18 ssDNA template. DNA synthesis was monitored by following the incorporation of radioactive dNTPs.

^{32}P-β_2 onto DNA. We have also tested the $\beta_{monomer}$ for an effect on DNA synthesis using β_2, γ complex, and core polymerase. The results, in Fig. 3*C*, demonstrate that $\beta_{monomer}$ inhibits DNA synthesis. What underlies ability of $\beta_{monomer}$ to inhibit in each of these assays? We have demonstrated previously that δ binds $\beta_{monomer}$ at least 50-fold tighter than the dimer (26). Hence, formation of dead end complexes between $\beta_{monomer}$ and δ (or γ or γ complex) likely underlies the mechanism of inhibition in each of the assays of Fig. 3, as diagrammed in the *schemes* to the *left* of each *panel*.

The finding that $\beta_{monomer}$ is a potent inhibitor of replication *in vitro* begs the question of whether it may do so *in vivo* as well. However, the K_d of the β dimer to monomer equilibrium is below 50 pM (45), and its concentration in the cell is ~500 nM (35). Hence, there should be very little of the monomeric species of β in the cell, especially relative to the amount of dimer.

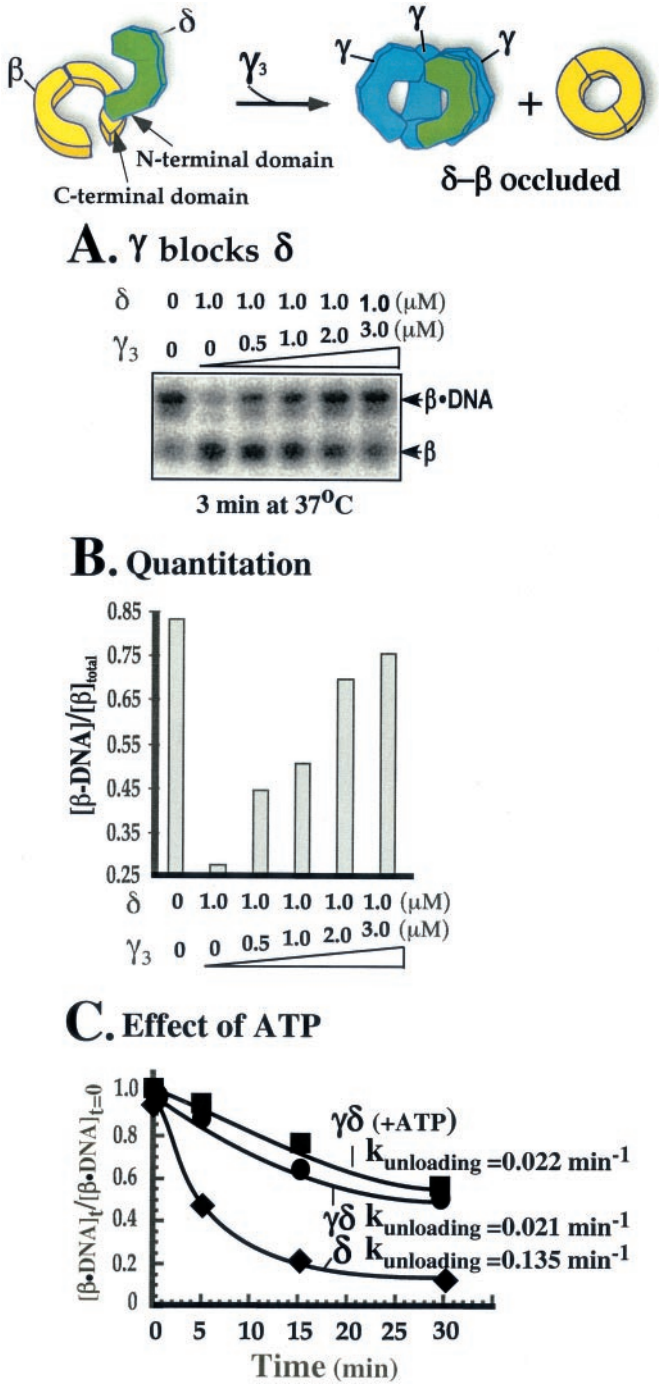

FIG. 4. **γ blocks δ from unloading β clamps from DNA.** The *scheme* at the *top* shows δ bound to an open β ring and suggests that γ interaction with δ may block the δ-β interaction, thereby largely preventing δ from opening β and unloading it from DNA. *Panel A*, 1 μM δ was incubated with the indicated amounts of γ_3 and ^{32}P-β·DNA complex at 37 °C for 3 min, then quenched with $\beta_{monomer}$ and analyzed on a native agarose gel. *Panel B*, the *bar plot* is a quantitation of the autoradiogram shown in *panel A*. *Panel C*, the effect of ATP on ability of γ to inhibit δ in clamp unloading was examined. The control reaction contained 0.2 μM δ, and the best fit to the data (*diamonds*) yields $k_{unloading}$ = 0.135 min^{-1}. In the presence of both 3 μM γ_3 and 0.2 μM δ, the rate was decreased to $k_{unloading}$ = 0.021 min^{-1} in the absence of ATP (*circles*) and 0.022 min^{-1} in the presence of ATP (*squares*).

γ Inhibits δ-Mediated β Unloading—Next, we studied the effect of a mixture of γ and δ on the stability of ^{32}P-β on DNA. In the experiment of Fig. 4 (*A* and *B*), we added 1 μM δ and various concentrations of γ subunit to ^{32}P-β_2·DNA complex. Reactions were then incubated for 3 min at 37 °C before

quenching with $\beta_{monomer}$ and analysis in an agarose gel. If γ and δ act independently, the expected rate of β unloading using the mixture would be approximately the same rate as using δ without γ, because 1 μM δ is much more efficient at unloading β than even 3 μM γ. However, we observed a markedly different result; the presence of γ with δ resulted in a marked decrease in the rate at which δ unloaded β. In Fig. 4*C*, the time course of γ inhibition of δ, using δ at 0.2 μM and γ at 3 μM, yielded a $t_{1/2}$ ~ 35 min for β unloading, ~7 times slower than the rate of β dissociation in the presence of δ by itself.

This apparent dilemma, in which a mix of two unloading proteins results in slower unloading of β from DNA compared with the rate observed using δ subunit separately, may be explained by at least two different mechanisms. In one case, γ interaction with β may be competitive with δ, thereby preventing the more effective δ from even binding β. This seems unlikely, given the higher affinity of δ for β compared with γ for β. Another possibility is that, when γ binds δ, it occludes the sites on γ and δ from interaction with β. This last possibility is illustrated in Fig. 4.

Next, we examined the γ/δ reaction for an effect of ATP. Fig. 4*C* shows a comparison of the γ/δ activity in ^{32}P-β unloading in the presence and absence of ATP. The result shows that ATP has no significant effect on the reaction. We have examined this reaction at 0.2 μM δ and several different concentrations of γ (0.5, 1, 2, and 3 μM) but have not detected a significant difference plus or minus ATP. This result is quite interesting given the fact that γ complex is an efficient β unloader only when ATP is present (25, 35, 45). Hence, it would appear that δ′ (which is not present in the reactions of Fig. 4) must be present for ATP to stimulate clamp unloading, even though γ, and not δ′, is the ATP binding subunit.

δ′ Blocks δ from Unloading β—In Fig. 5 we utilized the β unloading assay to examine δ′ for ability to open β and unload it from DNA, and for ability of δ′ to block δ in β unloading. The experiment in Fig. 5*A* compares the stability of ^{32}P-β on DNA in the presence and absence of 3 μM δ′. The results show that δ′ exerts no apparent effect, positive or negative, on the stability of ^{32}P-β on DNA. This result is consistent with the very low affinity interaction, or no interaction, between δ′ and β in the SPR experiment of Fig. 1.

Does the presence of δ′ influence the activity of δ in unloading β from DNA? The next experiment demonstrates that δ′, like γ, is an inhibitor of δ catalyzed β unloading. Fig. 5*B* shows a time course of ^{32}P-β unloading in the presence of 0.2 μM δ, which yields a half-life for β on DNA of ~5 min. Addition of 3 μM δ′ to this assay greatly reduces the unloading activity of δ subunit. Fig. 5*C* shows the results of titrating δ′ into a β unloading assay in the presence of 1 μM δ. The results show that δ′ inhibits δ-catalyzed unloading of β. Because δ′ does not appear to bind β tightly, but is known to form a tight 1:1 complex with δ (16), the mechanism of inhibition is likely via δ′ forming a complex with δ, preventing interaction of δ with β, as illustrated in the *scheme* of Fig. 5. This interpretation is also consistent with previous studies demonstrating that δ′ and β compete for binding to δ (25). ATP has no effect in β unloading reactions containing δ and/or δ′, consistent with lack of ATP interaction with either δ or δ′ (data not shown).

Effect of δ′ on γ—γ and δ′ are known to interact with one another (17). Does δ′ block γ unloading action? The experiments in Fig. 6 explore the effect of δ′ on γ. In Fig. 6*A* the results of a mixture of 1 μM γ and δ′ demonstrate that δ′ inhibits γ unloading β from DNA. The effect of ATP on these dynamics is examined in Fig. 6*B*. The presence of ATP reverses the inhibitory effect of δ′ on γ activity in unloading β from DNA.

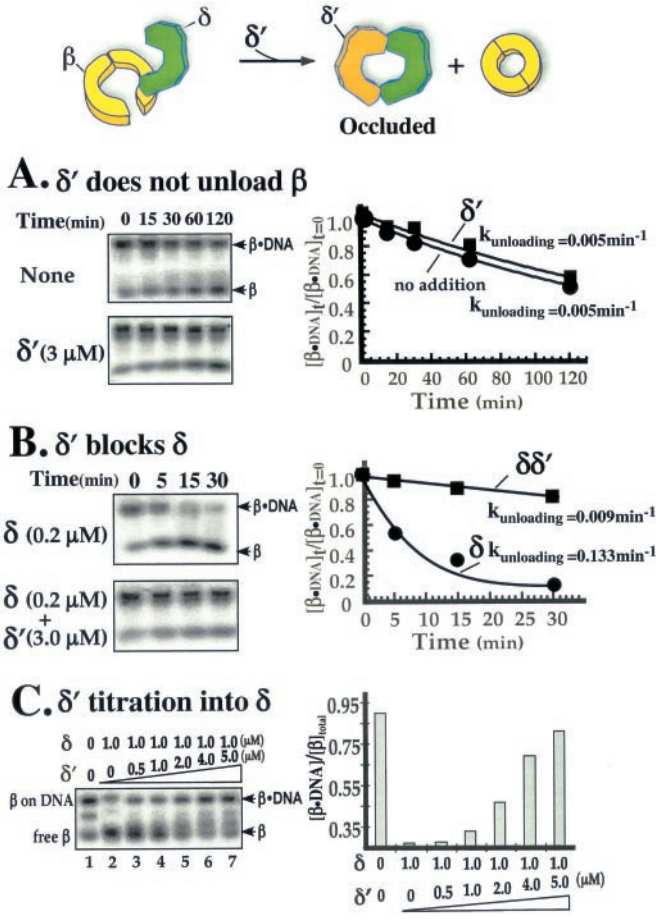

FIG. 5. **δ′ inhibits β unloading by δ.** The *scheme* at the *top* indicates that δ binds and opens β, but δ′ binds to δ, blocking its ability to interact with β, thereby preventing δ from unloading β from DNA. *Panel A*, stability of ^{32}P-β on DNA is compared in the presence and absence of 3 μM δ′. The autoradiograms of the neutral agarose gels are shown to the *left*, and the data are quantitated in the plot to the *right*. Curve fitting yields $k_{unloading}$ values of 0.005 min^{-1} in both the absence (*circles*) and presence (*squares*) of δ′. *Panel B*, the presence of δ′ inhibits β unloading by δ subunit. Unloading reactions contained 0.2 μM δ in the absence or presence of 3 μM δ′. The autoradiograms of the gels are to the *left*, and the quantitation to the *right* yields $k_{unloading}$ values of 0.133 min^{-1} for δ alone (*circles*) and 0.009 min^{-1} for δ plus δ′ (*squares*). *Panel C* shows a titration of δ′ into δ meditated clamp unloading reactions. *Lane 1* in the autoradiogram of the gel, to the *left*, is a reaction lacking added protein that was incubated the same amount of time (3 min at 37 °C) as the rest of the reactions. *Lanes 2–7* are reactions that contain 1 μM δ and the indicated amount of δ′. Results are quantitated in the *bar plot* to the *right*.

Effect of γδ′ on δ Activity—The γ complex has been shown previously to require ATP to unload β from DNA (25, 35, 45). We expect that the same will be true for γδδ′, because these three subunits are the only ones that are required for efficient clamp loading activity (16). This was tested in the experiment of Fig. 7 by adding an equimolar mixture of γ (as trimer) and δ′ to a solution of 0.2 μM δ in a β unloading reaction. The expected outcome is that, in the absence of ATP, these three subunits will be less efficient in unloading β than in the presence of ATP. *Panel A* shows that the γδ′ mixture blocks δ from unloading β in the absence of ATP, the expected result. *Panel B* shows the effect of ATP on ability of γδ′ to block δ-mediated β unloading. The result demonstrates that ATP reverses the blocking action of γδ′ on δ, the predicted result. The three subunits, γ, δ, and δ′, freely assemble into the $\gamma_3\delta\delta'$ pentamer (17). The illustration in the figure depicts ATP binding to γ, resulting in a more open structure at the points of subunit interactions with the β ring.

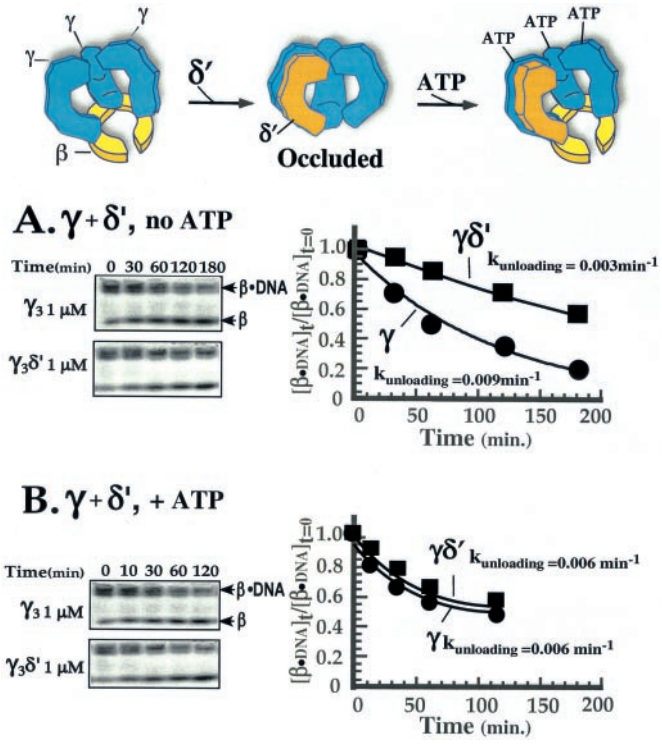

FIG. 6. ***δ′* blocks *γ* from unloading *β*.** The *scheme* at the *top* illustrates a mechanism by which δ′ binds γ, occluding interaction with β, but ATP binding to γ induces a conformation change in γ, allowing it to bind β even while bound to δ′. *Panel A*, the reactions compare the rate of β unloading in the presence of 1 μM γ_3, and either 1 μM δ′ (*squares*) or no δ′ (*circles*). Best fits to the data yield $k_{unloading}$ values of 0.009 min^{-1} (γ only) and 0.003 min^{-1} (γ + δ′). *Panel B*, δ′ does not inhibit γ in β unloading when ATP is present. Reactions comparing rates of β unloading were performed as in the experiments of *panel A* except 1 mM ATP was present. The autoradiograms of the gels are to the *left*, and the quantitation is to the *right*. Best fits to the data yield $k_{unloading}$ values of 0.006 min^{-1} for both γ alone (*circles*) and γ plus δ′ (*squares*).

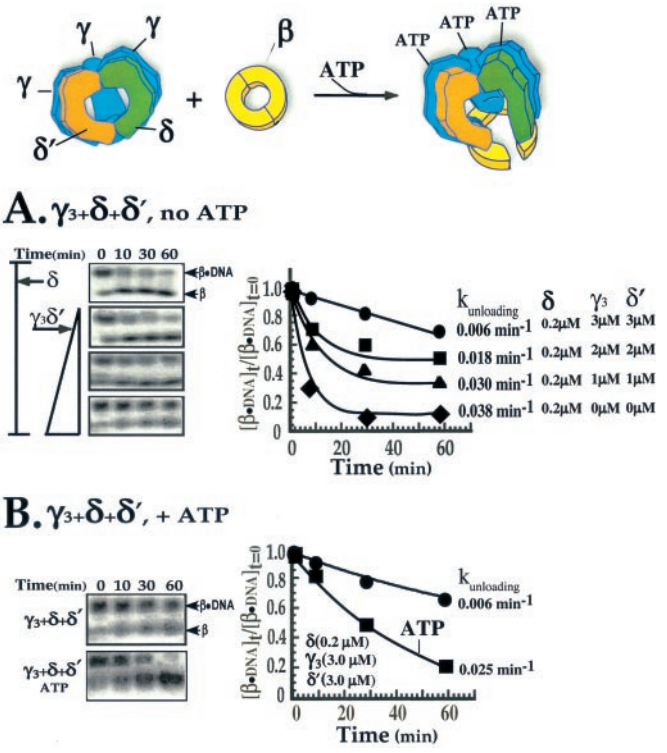

FIG. 7. ***γδ′* blocks δ-mediated unloading of *β*, but not in the presence of ATP.** The *scheme* at the *top* illustrates the subunits of γ complex arranged such that δ′ prevents γδ binding to β, but ATP binding to γ causes a conformational change allowing γδ to bind β. *Panel A*, δ (0.2 μM) was analyzed for the rate of unloading β from DNA in the absence (*diamonds*) or presence of both γ_3 and δ′. The γ and δ′ subunits were at the concentrations indicated in the figure. The autoradiograms of the native gels are to the *left*, and the quantitation is shown in the plots to the *right*. The $k_{unloading}$ values indicated in the plot are derived from the best fit curve (*solid line*) through the data points. *Panel B*, ATP stimulates unloading by a mixture of γδ and δ′. Reactions contained 0.2 μM δ and 3 μM each of γ_3 and δ′ to assemble all of the δ subunit into $\gamma_3\delta_1\delta'_1$ complex. Autoradiograms of the native agarose gels are shown to the *left* for unloading reactions in the absence (*top*) and presence (*bottom*) of 1 mM ATP. The plots to the *right* show the best fit to the data quantitated from the analysis. The resulting $k_{unloading}$ values were 0.006 min^{-1} in the absence of ATP (*circles*) and 0.25 min^{-1} in the presence of ATP (*squares*).

DISCUSSION

Multiple Subunits of the Clamp Loader Contact β—This report demonstrates the existence of multiple contacts between the β clamp and subunits of γ complex. Previous studies identified the δ subunit contact to β (26). This study demonstrates that the γ and χ subunits, and possibly δ′, also bind β, but they bind β weaker than δ binds β.[3] The γ subunit is required, along with δ and δ′, for clamp loading function, but neither χ nor ψ are needed for clamp loading (16). The χ subunit binds to SSB (44, 46) and aids in the switch of a primed site between primase and DNA polymerase III holoenzyme (22). The current study demonstrates a function of the γ-β interaction in ring opening (by observing unloading of β from DNA), and δ′ in modulating γ- and δ-mediated β unloading, but we have not yet observed a function of the χ subunit in assays with β. Therefore, this report focuses on functions of γ, δ, and δ′ with β. The function of the χ-β contact must await future study.

The γ and δ subunits of γ complex are capable of accelerating the dissociation of β from DNA. We presume that the underlying function observed in this assay is the opening of the ring, whereupon the DNA then escapes from the open ring. The δ subunit binds the $\beta_{monomer}$ over 50-fold tighter than the β dimer, indicating that δ puts some of its binding energy into performing work on the dimer, which it does not need to do on the monomer, such as cracking open the dimer interface (27). In support of this, the recent crystal structure analysis of δ-β complex indicates that the interface of β is distorted by δ (28). The structure analysis of δ in complex with a $\beta_{monomer}$, compared with the structure of the β dimer alone, also indicates that β undergoes significant conformational changes upon being freed of the constraint of forming a ring (*i.e.* upon breaking one interface of the β dimer). The $\beta_{monomer}$, no longer needing to bend into the shape of a half-circle, relaxes to a shallower crescent shape (28). The structural differences between the β dimer and the $\beta_{monomer}$ (*i.e.* strained closed circle *versus* relaxed open form of β) suggests that the strain in the β dimer may build up between the globular domains of β. This "spring tension" is apparently relieved upon cracking the dimer interface, with the result that the cracked interface is parted by 14–16 angstroms (28).

Ability of γ to unload β from DNA suggests that γ may assist δ in the ring opening step. However, β unloading by γ is so much less efficient than by δ that γ is likely to act somewhat differently. For example, γ may increase the spring tension between β domains, which would aid δ in destabilizing the β ring and would also cause the β ring to open further than that which occurs when only δ acts upon β. Alternatively, the β ring may undergo rapid conformation changes between the open

[3] The SPR results were performed using $\beta_{monomer}$. γ complex subunits may display different affinity for $\beta_{monomer}$ relative to β dimer, as demonstrated earlier by the 50-fold tighter binding of δ to $\beta_{monomer}$ over β dimer.

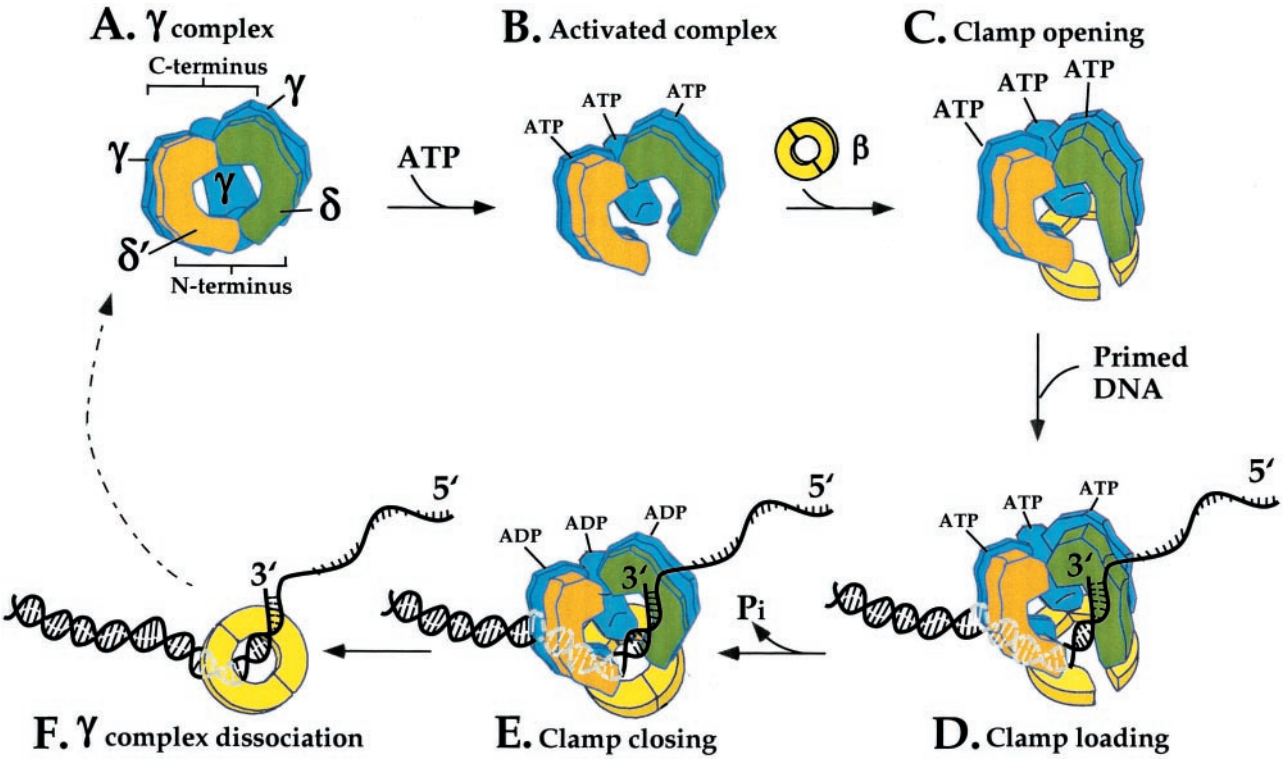

FIG. 8. **Clamp loading mechanism.** The $\gamma_3\delta\delta'$ subunits are arranged as a circular pentamer as indicated in *diagram A*. This "closed form" of γ complex does not bind β tightly because of steric hindrance of δ'. In *diagram B*, ATP binding to the γ subunits activates the clamp loader by inducing conformational changes that open up the N-terminal region of the pentamer, allowing N-terminal domains to bind β. In *diagram C*, the activated complex binds to β via contacts to N-terminal domains of δ, γ, and possibly δ'. The δ subunit cracks the interface of the β ring. *Diagram D* shows positioning of DNA into the open ring, which triggers ATP hydrolysis and ring closing indicated in *diagram E*. After hydrolysis, γ complex dissociates leaving the β ring on the DNA (*diagram F*).

and closed form, but rarely stay open long enough, or wide enough, to fall off DNA. The γ may stabilize β in the open form, thereby increasing its dissociation rate from DNA.

A Model for γ Complex Action—The major observations of this report, coupled with previous findings, form an internally consistent model of γ complex action and are also consistent with the model derived from crystal structure analysis (18). The crystal structure of δ', the first of the $\gamma\delta\delta'$ subunits to be solved, reveals a three domain protein (47). The N-terminal and middle domains belong to the AAA+ class of proteins (ATP-associated activity) (48). The δ' AAA+ sequence is also homologous to γ (32). Thus, γ has long been expected to have a similar chain fold as δ' (47), and this has proved to be the case upon solution of the $\gamma\delta\delta'$ crystal structure (18). The surprise was the structure of δ, which shows no significant homology to δ' or γ. The crystal structure analysis of the δ-β complex (28) and $\gamma\delta\delta'$ complex (18) shows that, even though δ is not homologous to δ' (and γ), δ comprises three domains with the same chain folding topology as δ' (and γ). Therefore the schematic of $\gamma\delta\delta'$ complex in Fig. 8 shows each of these subunits as comprising three domains.

The crystal structure of $\gamma_3\delta\delta'$ shows that the subunits are arranged as a circular pentamer, connected mainly by packing among their C-terminal domains. The structure appears to be of the ATP activated state, as the N-terminal domains of δ and γ are bent out from underneath the complex producing a more open form (*i.e.* in Fig. 8*B*). The $\gamma_3\delta\delta'$ pentamer diagrammed in Fig. 8*A* is the inactive, or hypothetical closed form of γ complex, lacking ATP in which the N-terminal domains pack together. In the absence of ATP, γ complex lacks tight interaction with β, and the diagram illustrates this as being caused by the proximity of the N-terminal domains to one another, sequestering their β interacting elements (as hypothesized in Ref. 18). This sequestration is consistent with the observation herein that δ' prevents clamp unloading by δ.

The binding of ATP by γ induces a conformation change in γ, and in γ complex (29). This change is likely the conformation of $\gamma_3\delta\delta'$ observed in the crystal structure (28) and is illustrated in Fig. 8*B* as an opening of the pentamer at the N-terminal domains, pulling δ and γ away from δ'. This change essentially activates γ complex for interaction with β, as determined, biochemically (26). As indicated by modeling δ-β into $\gamma_3\delta\delta'$, the β ring appears to touch the N-terminal surfaces of $\gamma_3\delta\delta'$ in the activated, or open, conformation (18), as illustrated in *diagram C* of Fig. 8. This report documents interaction of γ, and possibly δ', with β, although these contacts are weaker than the δ-β contact. The δ-β contact alters the structure of the β ring at the interface (28), which likely underlies ability of δ to unload β from DNA. Hence, β is shown as open in Fig. 8*C*.

The γ complex has very low affinity for DNA in the absence of β, whether ATP is present or not (29). However, in the presence of β, and using ATPγS, γ complex·β binds DNA quite tightly (29). Further, the ring remains open provided that ATPγS is used, indicating that the ATP-activated γ complex can open β and bind DNA, as illustrated in Fig. 8*D*. The β subunit suppresses the γ complex ATPase activity in the absence of DNA (25), but upon interacting with DNA, especially primed DNA (49), hydrolysis ensues and the ring closes (Fig. 8*E*). Following this, the ADP·γ complex dissociates from the β·DNA complex, and dissociation of ADP appears to be the rate-limiting step in the clamp loading cycle (50).

The action of δ', in preventing γ and δ from unloading β, is presumably mimicking the action of δ' in the closed, or inactive,

state of γ complex. The results of this report show that ATP largely reverses the ability of δ′ to block γ-mediated β unloading, and γδ-mediated β unloading. These results therefore imply that δ′ may be playing a role somewhat akin to an anvil in which γ and δ are the hammers. In the closed state of γ complex, the γ and δ hammers are closed down on δ′. However, ATP lifts the hammers (powered by γ), allowing interaction with β, and ring opening. Binding to DNA leads to hydrolysis of ATP, which releases the hammers and, upon striking the δ′ anvil, the connection to β is severed, allowing β to close around the DNA.

The behavior of the γδ mixture in the β unloading assay is most peculiar and may actually reveal more about δ′ than about either γ or δ. We demonstrate here that γ inhibits δ in β unloading. This, in and of itself, is not too surprising. The γ and δ subunits interact, and γ may simply occlude δ from binding β. The curious thing is that ATP does not reverse the block of γ on δ action in unloading β. However, ATP reverses the blocking action of δ′ on γ, and ATP activates the γδδ′ complex for β unloading (presumably pulling γ and δ away from δ′). One interpretation of these observations is that δ′ is required to observe an effect of ATP on these mixed subunit complexes, even though ATP binds γ and not δ′. For example, the proper ATP-induced conformational change in γ may require δ′ to be associated with γ.

The effects of ATP on γδ′ unloading of β and γδδ′ unloading of β are similar. In the absence of ATP, δ′ inhibits the weak β unloading activity of γ. However, in the presence of ATP, this inhibition of γ-mediated β unloading by δ′ is abolished, and β unloading occurs at a similar rate as in the absence of δ′ (*i.e.* Fig. 6). Likewise, ATP is needed for γδδ′ to unload β (*i.e.* Fig. 7). However, ATP has no effect on γδ activity in β unloading in the absence of δ′ (*i.e.* Fig. 4). Hence, δ′ appears to be needed for the conformation change that allows access of β to δ and γ.

Predictions for Eukaryotic Replication—The eukaryotic clamp loader, RFC, consists of five subunits, each of which are homologous to one another and to *E. coli* γ and δ′ (51, 52). It therefore seems likely that they form a circular pentamer like $\gamma_3\delta_1\delta'_1$. A circular pentamer structure is also consistent with at least some of the images collected in an electron microscope analysis of RFC (53). Studies on human RFC indicate that at least two of its subunits (p140 and p40), and possibly others, interact with the PCNA ring (54–56), similar to observations in this report of multiple contacts between β and subunits of γ complex. The human RFC p140 and yeast RFC-1 subunit, like δ, are the only subunit in their respective clamp loader complex that lack the SRC motif. Thus, yRFC-1 and the human p140 RFC subunit may be functionally analogous to δ in having the major role in binding the PCNA ring and distorting an interface of the sliding clamp. A complex of human p36·p37·p40 RFC subunits is capable of ATP-independent unloading of PCNA from DNA, but it is much less efficient at clamp unloading compared with RFC in the presence of ATP (55). However, the unloading activity of the p36·p37·p40 complex is 1000-fold lower than the complete RFC (55). Therefore, in light of the current study, it is possible that these subunits are more like the γ trimer, capable of unloading the ring only when provided at high concentration. Further, like γ, each of the subunits of the p36·p37·p40 complex contain both the SRC motif and a P-loop (as do the homologous yeast RFC2, -3, and -4 subunits). Like γ complex, RFC interaction with PCNA is strengthened by ATP (24), and clamp unloading also requires ATP (45). Therefore, we propose that one RFC subunit acts like δ′, to modulate interaction of other RFC subunits with PCNA, dependent on ATP. A distinguishing characteristic of δ′ is the presence of the SRC motif, and a mutated P-loop. Thus, the RFC functional homolog to δ′ is likely the h-p38 RFC subunit (yRFC-5), which contains the SRC motif but has a mutated P-loop. These several similarities underscore the high degree to which replication mechanisms have been conserved in prokaryotes and eukaryotes.

Acknowledgments—We are grateful to Drs. David Jeruzalmi and John Kuriyan for information prior to publication of crystal structure analysis of δ-β and γδδ′ complex.

REFERENCES

1. Stukenberg, P. T., Studwell-Vaughan, P. S., and O'Donnell, M. (1991) *J. Biol. Chem.* **266,** 11328–11334
2. Hingorani, M. M., and O'Donnell, M. (2000) *Curr. Biol.* **10,** R25–R29
3. Kelman, Z., and O'Donnell, M. (1995) *Annu. Rev. Biochem.* **64,** 171–200
4. Baker, T. A., and Bell, S. P. (1998) *Cell* **92,** 295–305
5. Kong, X. P., Onrust, R., O'Donnell, M., and Kuriyan, J. (1992) *Cell* **69,** 425–437
6. Hughes, A. J., Jr., Bryan, S. K., Chen, H., Moses, R. E., and McHenry, C. S. (1991) *J. Biol. Chem.* **266,** 4568–4573
7. Bonner, C. A., Stukenberg, P. T., Rajagopalan, M., Eritja, R., O'Donnell, M., McEntee, K., Echols, H., and Goodman, M. F. (1992) *J. Biol. Chem.* **267,** 11431–11438
8. Tang, M., Shen, X., Frank, E. G., O'Donnell, M., Woodgate, R., and Goodman, M. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8919–8924
9. Wagner, J., Gruz, P., Kim, S. R., Yamada, M., Matsui, K., Fuchs, R. P., and Nohmi, T. (1999) *Mol. Cell* **4,** 281–286
10. Lopez de Saro, F. J., and O'Donnell, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8376–8380
11. Stillman, B. (1994) *Cell* **78,** 725–728
12. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) *Cell* **79,** 1233–1243
13. Gulbis, J. M., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. (1996) *Cell* **87,** 297–306
14. Kelman, Z., and Hurwitz, J. (1998) *Trends Biochem. Sci* **23,** 236–238
15. Maki, S., and Kornberg, A. (1988) *J. Biol. Chem.* **263,** 6555–6560
16. Onrust, R., and O'Donnell, M. (1993) *J. Biol. Chem.* **268,** 11766–11772
17. Onrust, R., Finkelstein, J., Turner, J., Naktinis, V., and O'Donnell, M. (1995) *J. Biol. Chem.* **270,** 13366–13377
18. Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. (2001) *Cell* **106,** 429–441
19. Tsuchihashi, Z., and Kornberg, A. (1989) *J. Biol. Chem.* **264,** 17790–17795
20. Flower, A. M., and McHenry, C. S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3713–3717
21. Pritchard, A. E., Dallmann, H. G., and McHenry, C. S. (1996) *J. Biol. Chem.* **271,** 10291–10298
22. Yuzhakov, A., Kelman, Z., and O'Donnell, M. (1999) *Cell* **96,** 153–163
23. Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) *Cell* **84,** 643–650
24. Gerik, K. J., Gary, S. L., and Burgers, P. M. (1997) *J. Biol. Chem.* **272,** 1256–1262
25. Turner, J., Hingorani, M. M., Kelman, Z., and O'Donnell, M. (1999) *EMBO J.* **18,** 771–783
26. Naktinis, V., Onrust, R., Fang, L., and O'Donnell, M. (1995) *J. Biol. Chem.* **270,** 13358–13365
27. Stewart, J., Hingorani, M. M., Kelman, Z., and O'Donnell, M. (2001) *J. Biol. Chem.* **276,** 19182–19189
28. Jeruzalmi, D., Yurieva, O., Zhao, Y., Young, M., Stewart, J., Hingorani, M., O'Donnell, M., and Kuriyan, J. (2001) *Cell* **106,** 417–428
29. Hingorani, M. M., and O'Donnell, M. (1998) *J. Biol. Chem.* **273,** 24550–24563
30. Bertram, J. G., Bloom, L. B., Turner, J., O'Donnell, M., Beechem, J. M., and Goodman, M. F. (1998) *J. Biol. Chem.* **273,** 24564–24574
31. Studwell, P. S., and O'Donnell, M. (1990) *J. Biol. Chem.* **265,** 1171–1178
32. Dong, Z., Onrust, R., Skangalis, M., and O'Donnell, M. (1993) *J. Biol. Chem.* **268,** 11758–11765
33. Xiao, H., Crombie, R., Dong, Z., Onrust, R., and O'Donnell, M. (1993) *J. Biol. Chem.* **268,** 11773–11778
34. Studwell-Vaughan, P. S., and O'Donnell, M. (1993) *J. Biol. Chem.* **268,** 11785–11791
35. Leu, F. P., Hingorani, M. M., Turner, J., and O'Donnell, M. (2000) *J. Biol. Chem.* **275,** 34609–34618
36. Kelman, Z., Naktinis, V., and O'Donnell, M. (1995) *Methods Enzymol.* **262,** 430–442
37. Stukenberg, P. T., Turner, J., and O'Donnell, M. (1994) *Cell* **78,** 877–887
38. Rowens, L., and Kornberg, A. (1978) *J. Biol. Chem.* **253,** 758–764
39. Dallmann, H. G., and McHenry, C. S. (1995) *J. Biol. Chem.* **270,** 29563–29569
40. Pritchard, A. E., Dallmann, H. G., Glover, B. P., and McHenry, C. S. (2000) *EMBO J.* **19,** 6536–6545
41. ODonnell, M., and Studwell, P. S. (1990) *J. Biol. Chem.* **265,** 1179–1187
42. Xiao, H., Dong, Z., and O'Donnell, M. (1993) *J. Biol. Chem.* **268,** 11779–11784
43. Glover, B. P., and McHenry, C. S. (2000) *J. Biol. Chem.* **275,** 3017–3020
44. Glover, B. P., and McHenry, C. S. (1998) *J. Biol. Chem.* **273,** 23476–23484
45. Yao, N., Turner, J., Kelman, Z., Stukenberg, P. T., Dean, F., Shechter, D., Pan, Z. Q., Hurwitz, J., and O'Donnell, M. (1996) *Genes Cells* **1,** 101–113
46. Kelman, Z., Yuzhakov, A., Andjelkovic, J., and O'Donnell, M. (1998) *EMBO J.* **17,** 2436–2449
47. Guenther, B., Onrust, R., Sali, A., O'Donnell, M., and Kuriyan, J. (1997) *Cell* **91,** 335–345
48. Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999) *Genome Res.* **9,** 27–43

49. Onrust, R., Stukenberg, P. T., and O'Donnell, M. (1991) *J. Biol. Chem.* **266,** 21681–21686
50. Ason, B., Bertram, J. G., Hingorani, M. M., Beechem, J. M., O'Donnell, M., Goodman, M. F., and Bloom, L. B. (2000) *J. Biol. Chem.* **275,** 3006–3015
51. O'Donnell, M., Onrust, R., Dean, F. B., Chen, M., and Hurwitz, J. (1993) *Nucleic Acids Res.* **21,** 1–3
52. Cullmann, G., Fien, K., Kobayashi, R., and Stillman, B. (1995) *Mol. Cell. Biol.* **15,** 4661–4671
53. Shiomi, Y., Usukura, J., Masamura, Y., Takeyasu, K., Nakayama, Y., Obuse, C., Yoshikawa, H., and Tsurimoto, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 14127–14132
54. Uhlmann, F., Cai, J., Gibbs, E., O'Donnell, M., and Hurwitz, J. (1997) *J. Biol. Chem.* **272,** 10058–10064
55. Cai, J., Gibbs, E., Uhlmann, F., Phillips, B., Yao, N., O'Donnell, M., and Hurwitz, J. (1997) *J. Biol. Chem.* **272,** 18974–18981
56. McAlear, M. A., Tuffo, K. M., and Holm, C. (1996) *Genetics* **142,** 65–78

DNA: REPLICATION REPAIR AND RECOMBINATION:

Interplay of Clamp Loader Subunits in Opening the β Sliding Clamp of *Escherichia coli* DNA Polymerase III Holoenzyme

Frank P. Leu and Mike O'Donnell

J. Biol. Chem. 2001, 276:47185-47194.
doi: 10.1074/jbc.M106780200 originally published online September 25, 2001