DNA Ligase I and Proliferating Cell Nuclear Antigen Form a Functional Complex*

Received for publication, February 22, 2001, and in revised form, April 27, 2001
Published, JBC Papers in Press, April 30, 2001, DOI 10.1074/jbc.M101673200

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DNA ligase I is responsible for joining Okazaki fragments during DNA replication. An additional proposed role for DNA ligase I is sealing nicks generated during excision repair. Previous studies have shown that there is a physical interaction between DNA ligase I and proliferating cell nuclear antigen (PCNA), another important component of DNA replication and repair. The results shown here indicate that human PCNA enhances the reaction rate of human DNA ligase I up to 5-fold. The stimulation is specific to DNA ligase I because T4 DNA ligase is not affected. Electrophoretic mobility shift assays indicate that PCNA improves the binding of DNA ligase I to the ligation site. Increasing the DNA ligase I concentration leads to a reduction in PCNA stimulation, consistent with PCNA-directed improvement of DNA ligase I binding to its DNA substrate. Two experiments show that PCNA is required to encircle duplex DNA to enhance DNA ligase I activity. Biotin-streptavidin conjugations at the ends of a linear substrate inhibit PCNA stimulation. PCNA cannot enhance ligation on a circular substrate without the addition of replication factor C, which is the protein responsible for loading PCNA onto duplex DNA. These results show that PCNA is responsible for the stable association of DNA ligase I to nicked duplex DNA.

DNA metabolism requires the coordinated activity of a multitude of enzymes and enzyme complexes. Although the initiation of DNA replication and DNA repair are regulated through different mechanisms, the reactions performed to complete these pathways are similar. In particular, Okazaki fragment processing (1) and long patch base excision repair (2, 3) share many enzymes needed for completion of these pathways. These include flap endonuclease 1 (FEN1),1 proliferating cell nuclear antigen (PCNA), and DNA ligase I.

During lagging strand DNA synthesis, numerous initiator RNA primers must be removed. The resulting gaps are filled in and sealed by ligation to complete DNA synthesis. Two nucleases, Dna2 and FEN1, are responsible for excising the RNA primer (4–8). Both of these enzymes are unique structure-specific endonucleases. The preferred substrate contains a flap structure in which the RNA primer has been displaced to form a single-stranded tail (1, 9–13). The flap structure probably arises as a result of displacement synthesis from an upstream Okazaki fragment by a complex of DNA polymerase δ and its accessory factors, PCNA and replication factor C (RFC) (14). Dna2 is thought to cleave beyond the RNA segment within the tail, and the remaining displaced DNA is removed by FEN1 (5–7). Finally, the two fragments are joined through ligation by DNA ligase I (1, 15).

Long patch base excision repair utilizes several components common to Okazaki fragment processing to remove bases altered by ionizing radiation, oxidation, or alkylating agents (2, 3, 16–21). During the repair process, an abasic site is generated after removal of a damaged base by a DNA N-glycosylase. An apurinic/apyrimidinic endonuclease subsequently cleaves on the 5′-side of the abasic sugar to create a nick within the DNA. Similar to the removal of initiator RNA primers, synthesis by a DNA polymerase lifts the damaged residue and a few additional downstream nucleotides to form a flap. As during replication, this structure is removed endonucleolytically by FEN1 followed by ligation of the resulting nick by DNA ligase I (2, 3, 17, 19, 21). This entire process is stimulated in the presence of PCNA (22).

PCNA is a toroidal homotrimer that is assembled around double-stranded DNA to form a sliding clamp (23, 24). It has long been known to act as a processivity factor for DNA polymerases by tethering the polymerase to its template (25). However, PCNA also interacts with other replication proteins and appears to be responsible for recruiting these proteins to replication foci in vivo (26–28). The interaction of PCNA and FEN1 has been examined extensively (12, 13, 29–31). The FEN1 nuclease binds to the interdomain connecting loop region of PCNA (12, 29, 30, 32), and this association leads to a potent stimulation of FEN1 cleavage activity (12, 13, 29). The physical interaction between the PCNA toroid and FEN1 enhances the binding stability of FEN1 to cleavage sites (13). In this way, PCNA serves to clamp FEN1 to its substrate in much the same way as this protein clamps DNA polymerases to sites of DNA synthesis. The ability of PCNA to enhance cleavage by FEN1 leads to more efficient DNA replication and base excision repair. A physical interaction between PCNA and DNA ligase I has also been identified (27, 30, 33).

DNA ligases have essential roles in many important cellular pathways including DNA replication, recombination, and repair (34, 35). Of the four DNA ligases in mammalian cells, DNA ligase I has been linked to DNA replication (36) and base excision repair (37). This ligase has been identified as a component of a high molecular weight replication complex (38, 39). In addition, DNA ligase I has been shown to be responsible for a major part of ligation activity in proliferating cells (40–43).
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| Table I
| Oligonucleotide sequences (5’-3’) |
|---|
| Downstream primers |
| D₁ | (15-mer) | GTAAACGACCGCCAGTG |
| D₂ | (30-mer) | GCTCACAATTCACAAACACATACAGGCGG |
| D₃ | (37-mer) | ACTAAGCGCCGTGAAACGGGCGGAAATTCGAGGTCGTA |
| Upstream primers* |
| U₁ | (25-mer) | CCGCACGCTTTTCAGCGTCAGACC |
| U₂ | (25-mer) | CCGCACGCTTTTCAGCGTCAGACC |
| U₃ | (30-mer) | ATAGCTTTTCTCTGTGGAATTGTTATCC |
| U₄ | (35-mer) | CCGCTCGACTGTTGTAAGAAGCAGCAGGGATA |
| Templates* |
| T₁ | (44-mer) | GCACCGCGCGCGTTTTATCGGTGACTGGAAGACCTGCG |
| T₂ | (44-mer) | GCACCGCGCGCGTTTTATCGGTGACTGGAAGACCTGCG |
| T₃ | (61-mer) | TCGGCTCTGATATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTAT |
| T₄ | (76-mer) | GTACCGGCTCGAATTGGCGCCAGGTTCGTTATTACGCGT |

* The underlined nucleotide indicates a biotin modification.

Cytostaining experiments with antibodies against DNA ligase I revealed that the enzyme co-localizes in the nucleus with DNA polymerase α, implicating DNA ligase I in DNA replication (44). A DNA ligase I mutant human cell line, 46BR, exhibits abnormal joining of Okazaki fragments (45–48), but the replication defect in extracts from this cell line can be complemented by the addition of DNA ligase I (49). A recent study with the DNA ligase I mutant cell line 46BR.1G1 reveals that the interaction between PCNA and DNA ligase I is integral to coordinating the ligation steps that complete long patch base excision repair (37). These observations imply an important role for DNA ligase I in DNA replication and repair.

Human DNA ligase I is comprised of a C-terminal catalytic domain and a hydrophilic N-terminal domain. Although the N-terminal region is dispensable for catalytic activity in vitro (50, 51), this region is essential in vivo (52). DNA ligase I is regulated by phosphorylation at the N-terminal region of the protein (28). In addition, the nuclear localization site of the protein has been identified in the N-terminal domain (26, 53), and this region is also responsible for interaction with PCNA (27).

The physical interaction between PCNA and DNA ligase I has been characterized as a potential means by which DNA ligase I is recruited to a replication site. Because the interaction of PCNA with FEN1 improves the catalytic rate of the nuclease, we considered here whether the binding of PCNA to DNA ligase I is recruited to a replication site. Because the interaction between PCNA and DNA ligase I is integral to coordinating the ligation steps that complete long patch base excision repair (37).

Enzyme Assay—The reactions containing the indicated amounts of substrate, DNA ligase I or T4 DNA ligase, and PCNA were performed in reaction buffer (30 mM HEPES, pH 7.6, 40 mM KCl, 0.01% Nonidet P-40, 0.1 mg/ml bovine serum albumin, 8 mM MgCl₂, and 0.1 mM ATP). The reactions were incubated at 37 °C, terminated with 20 μl of formamide dye (90% formamide (v/v) with bromophenol blue and xylene cyanole), and heated to 95 °C for 5 min. After separation on a 15% polyacrylamide, 7 M urea denaturing gel, products were detected by PhosphorImager (Molecular Dynamics) analysis. For the biotin-streptavidin assay, the substrate was incubated with PCNA either before or after the addition of streptavidin. Conjugation of streptavidin (added in a 50-fold molar excess over substrate) to the biotinylated substrate was accomplished by placing the reactions at 4 °C for 10 min. These reactions contained 1 fmol of DNA ligase I. The reactions utilizing human RFC were performed in a buffer containing 30 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.01% Nonidet P-40, 0.1 mg/ml bovine serum albumin, and 0.1 mM ATP. All assays were performed at least in triplicate.

Electrophoretic Mobility Shift Assay—Reactions were performed in binding buffer (30 mM HEPES, pH 7.6, 40 mM KCl, 0.01% Nonidet P-40, 0.1 mg/ml bovine serum albumin, and 0.1 mM ATP) in a final reaction volume of 20 μl. After incubation at 4 °C for 15 min, products were separated on a 1% agarose, 0.5% polyacrylamide gel in 0.25% TBE (8.9 mM Tris base, 8.9 mM boric acid, and 0.2 mM EDTA, pH 8.0) and visualized by PhosphorImager (Molecular Dynamics) analysis.

RESULTS

PCNA Stimulates DNA Ligase I Activity—We first examined whether the presence of PCNA influences catalysis by DNA ligase I (Fig. 1A). Lane 1 only contains PCNA, and lane 2 only contains DNA ligase I. Titration of PCNA into the reactions (lanes 3–7) results in a progressive stimulation of product formation. Because the only proteins in these reactions are DNA ligase I and PCNA, the observed enhancement of ligation activity must derive from PCNA. There is an approximate 5-fold...
enhancement of ligation activity at the highest concentration of PCNA. The addition of an unrelated protein, E. coli single-stranded DNA-binding protein, to the DNA ligase I reaction did not result in any stimulation of ligation activity (data not shown). All experiments were performed in excess bovine serum albumin. The presence of this added protein did not affect DNA ligase I activity (data not shown).

It was also important to determine the specificity of the interaction between PCNA and DNA ligase I. Stimulation of other ligases would imply that the mechanism is nonspecific and does not depend on contacts between the two proteins. Therefore, PCNA was titrated into a T4 DNA ligase reaction (Fig. 1B). The results (lanes 3–7) show no additional accumulation of ligation product. This observation illustrates the specificity of the interaction between PCNA and DNA ligase I.

Fig. 2A shows a time course illustrating the activity of DNA ligase I in the absence and the presence of PCNA. A fixed concentration of PCNA was utilized as determined by the experiment shown in Fig. 1A. The presence of PCNA caused a substantial stimulation of ligation activity throughout the time course. Fig. 2B shows a second time course demonstrating stimulation of ligation with a substrate of different sequence and different length upstream and downstream primers than the substrate in Fig. 2A. In both cases, the rate of ligation was enhanced 3–4-fold.

Evidence that PCNA Enhances DNA Ligase I Binding to the Ligation Site—PCNA enhances the binding of various proteins to their corresponding substrates (24). Analysis of the interaction between PCNA and FEN1 reveals that PCNA enhances FEN1 binding stability, allowing for greater cleavage efficiency (13). Therefore, we considered the possibility that PCNA stimulates DNA ligase I by a similar mechanism. We examined the effect of PCNA on DNA ligase I interaction with its substrate using an electrophoretic mobility shift assay (Fig. 3). Incubation of the substrate with a high concentration of DNA ligase I clearly results in the formation of a DNA-ligase complex (lane 7). Lane 7 of Fig. 3 identifies the band corresponding to the DNA-ligase complex. Utilizing a lower concentration of DNA ligase I, the addition of progressively higher concentrations of PCNA increased the observed amount of the DNA-ligase complex (lanes 4–6). These results demonstrate that the binding of DNA ligase I to its substrate is enhanced by PCNA in a concentration-dependent manner. Furthermore, incubation of the substrate with a high level of PCNA alone failed to result in the formation of a protein complex with DNA (lane 2). These results suggest that greater ligation efficiency is the result of higher affinity binding of the ligase to DNA, which is achieved through an interaction with PCNA.

Role of Phosphorylation—DNA ligase I is a substrate for casein kinase II (51). The N-terminal region of DNA ligase I possesses several putative phosphorylation sites. This region contains seven casein kinase II consensus sites, and two of these sites (Ser\(^{66}\) and Ser\(^{144}\)) have properties that are optimal for casein kinase II phosphorylation (28). At the end of the S phase of the cell cycle, DNA ligase I is thought to be phosphorylated by casein kinase II (28). Although phosphorylation does not inactivate DNA ligase I for catalysis, it prevents interaction of DNA ligase I with the DNA replication apparatus (24). Therefore, we were interested in determining whether phosphorylation of DNA ligase I affects PCNA stimulation of ligation activity. In Fig. 4, the addition of PCNA to reactions with unphosphorylated DNA ligase I leads to enhanced formation of the product (lanes 3–4). Phosphorylation of DNA ligase I results in a slight reduction of catalytic activity (lane 6). This small reduction in activity is possibly a result of phosphorylation itself or of the presence of casein kinase II. Titration of PCNA into the reactions with phosphorylated DNA ligase I does not reveal any stimulation (lanes 7 and 8). These results demonstrate that phosphorylation of DNA ligase I prevents PCNA from stimulating ligation activity.

Increasing DNA Ligase I Concentration Reduces PCNA Stimulation—To further analyze the mechanism involved in PCNA stimulation of DNA ligase I, an enzyme titration was performed (Fig. 5). The percentage of PCNA stimulation decreases as the concentration of DNA ligase I is increased. For example, at 0.25 nM DNA ligase I, the addition of PCNA leads to a 1.8 ± 0.2-fold enhancement of product formation. This is a reduced percentage of stimulation compared with the 4.9 ± 0.4-fold enhancement at 0.05 nM DNA ligase I. Therefore, the presence of PCNA makes the DNA ligase I molecules act as if they were present at a higher concentration. This result indicates that PCNA increases the rate of binding of DNA ligase I to its oligonucleotide substrate or the rate of dissociation rather than the rate of catalysis. In view of the results showing that PCNA...
enhances ligase complex formation with DNA, the reduced stimulation is consistent with an enhanced rate of binding.

**L126D/I128E Mutant of PCNA Stimulates DNA Ligase I**—The L126D/I128E mutant of PCNA has a severe defect in FEN1 binding ability and a greatly reduced ability to stimulate nuclease activity (22). Presumably, the large reduction in FEN1 binding ability prevents PCNA from effectively stimulating catalysis by FEN1. These same mutations in the inter-domain connecting loop region of PCNA have minimal effects on DNA ligase I binding (30). Although these mutations do not


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Fig. 4. Phosphorylation of DNA ligase I by casein kinase II eliminates PCNA stimulation. Reactions of 20 μl containing 5 fmol of DNA substrate and 1 fmol of DNA ligase I were performed as described under “Experimental Procedures” (lanes 2–4 and 6–8). PCNA concentrations of 0.25 and 0.50 pmol were utilized as denoted by the triangles. The substrate is comprised of D-UA-T. The reactions were incubated at 37 °C for 10 min. Substrate and ligation product sizes are as indicated. The 5′-end of the downstream primer was radio-labeled with γ-32P. Lanes 2–4 represent an analysis of ligation activity with untreated DNA ligase I. Lanes 6–8 depict an analysis of ligation activity with DNA ligase I that has been treated with casein kinase II. Phosphorylation of DNA ligase I by casein kinase II was performed as described under “Experimental Procedures.” CKII, casein kinase II; LIG. I, DNA ligase I; nt, nucleotide.

Fig. 5. The level of PCNA stimulation decreases at high concentrations of DNA ligase I. The reactions were incubated at 37 °C for 5 min. Substrate and ligation product sizes are as indicated. The 5′-end of the downstream primer was radio-labeled with γ-32P. Reactions of 20 μl containing 5 fmol of DNA substrate and 1, 3, or 5 fmol of DNA ligase I were performed as described under “Experimental Procedures.” The reactions in the presence of PCNA contained 0.5 pmol of PCNA. The addition of PCNA leads to stimulation levels of 4.9 ± 0.4-fold (0.05 nm DNA ligase I), 2.3 ± 0.2-fold (0.15 nm DNA ligase I), and 1.8 ± 0.2-fold (0.25 nm DNA ligase I). The narrow standard deviations support the statistical relevance of the suppression of stimulation at high ligase concentrations. The substrate is comprised of D-UA-T. LIG. I, DNA ligase I; nt, nucleotide.

LIG. I (nM) - 0.05 0.15 0.25
PCNA + - + - + - +

-CKII + CKII

-60 nt

Lane 1 Lane 2 Lane 3 Lane 4 Lane 5 Lane 6 Lane 7 Lane 8

30 nt

To determine whether interdomain mutations affect catalysis of ligation, we measured the ability of the L126D/I128E mutant to increase DNA ligase I activity. In Fig. 6, DNA ligase I activity was monitored in the absence of PCNA and in the presence of either wild-type PCNA or the mutant PCNA. This analysis shows that both the wild-type and mutant PCNA can stimulate DNA ligase I activity to a similar degree. This observation supports the notion that FEN1 and DNA ligase I have partly or completely distinct binding sites on PCNA that mediate stimulation.

PCNA is Required to Encircle Duplex DNA to Interact Productively with DNA Ligase I—DNA ligase I binds to PCNA either in solution or when the PCNA molecule is topologically linked to DNA (33). Determining which mode of binding results in stimulation would clarify the mechanism by which the rate of reaction is increased. Hübscher and colleagues have shown that biotin-streptavidin conjugations at the ends of a double-stranded linear DNA can prevent PCNA loading (30). We employed this strategy to determine whether the association of PCNA and DNA ligase I in solution leads to an enhancement of ligation activity.

Fig. 7 shows the analysis of a substrate with biotin modifications at the 5′-end of the upstream primer and the 5′-end of the template. In this way, conjugation of streptavidin to the biotinylated ends makes this substrate inaccessible to PCNA loading from the ends of the substrate. In the absence of streptavidin, the addition of PCNA to the DNA ligase I reactions led to an enhancement of product formation. However, the conjugation of streptavidin to the substrate resulted in the absence of any significant stimulation upon the addition of PCNA. This result supports the conclusion that PCNA must encircle the substrate to effect stimulation of DNA ligase I. When PCNA was incubated with the substrate prior to the addition of streptavidin, there were slight enhancements in product formation at higher concentrations of PCNA. This result is suggestive that PCNA molecules were present on some of the substrate DNA molecules and remained trapped there upon conjugation of streptavidin to the substrate.

On a linear substrate, PCNA can enter the double-stranded region by sliding over the ends (13, 29, 30). However, on a circular substrate, PCNA requires RFC and ATP to encircle the DNA (54–58). In Fig. 8A, titration of PCNA into reactions with a linear substrate (lanes 3–7) leads to an enhancement of product formation. In Fig. 8B, titration of PCNA into the reactions with a circular substrate in the absence of RFC (lanes 3–7) does not result in any significant stimulation. The E. coli single-stranded DNA-binding protein was also added to minimize nonspecific interactions of either DNA ligase I or PCNA with the single-stranded regions of DNA. Although DNA ligase I interacts with PCNA in solution, this interaction does not lead to any stimulation. This result shows that PCNA is required to encircle duplex DNA to stimulate DNA ligase I activity.

RFC will open and reclose the PCNA trimeric ring around duplex DNA in an ATP-dependent fashion (54, 56–58). The requirement of the RFC-directed loading reaction on a circular substrate was demonstrated in Fig. 9. Lanes 1–4 are control lanes without any DNA ligase I. Lane 5 contains DNA ligase I only. The addition of PCNA (lane 6) does not result in any enhancement of product formation. The addition of RFC only (lane 7) also does not yield any stimulation. However, the addition of both PCNA and RFC in conjunction with DNA ligase I leads to the stimulation of product formation (lane 8). We interpret these results to mean that RFC-dependent encirclement of the substrate by PCNA is required for stimulation of DNA ligase I.
DISCUSSION

The toroidal PCNA molecule acts as a sliding clamp that facilitates the interaction of proteins with DNA in eukaryotic systems (59). Most of the numerous proteins that bind PCNA are involved in DNA transactions. PCNA has also been identified as the central component of a targeting mechanism by which proteins that metabolize DNA locate their substrates (27). DNA ligase I is required for DNA-joining reactions that are an essential part of DNA replication and repair pathways (37–49). Recent characterization of the binding interaction between PCNA and DNA ligase I (30, 33) led us to investigate the effect of this binding on the ligation reaction. We initially found that the presence of PCNA stimulates the DNA-joining reaction catalyzed by DNA ligase I. We further show that the stimulation reaction requires that PCNA encircle the nicked double-stranded DNA that serves as the ligation substrate. Additional evidence indicates that PCNA stimulates ligation by increasing the affinity and rate of binding of DNA ligase I to the nicked site on the substrate.

PCNA was found to stimulate DNA ligase I activity up to 2.5-fold. The L126D/I128E mutant of PCNA enhances DNA ligase I activity. Reactions of 140 μl containing 140 fmol of DNA substrate and 35 fmol of DNA ligase I were performed as described under “Experimental Procedures.” In the reactions with PCNA, 17.5 pmol of either wild-type or mutant PCNA was utilized. The reactions were incubated at 37 °C, and 20-μl aliquots were removed at 0, 1, 3, 5, 7, 10, and 15 min as indicated by the triangles. Substrate and ligation product sizes are as indicated. The 5'-end of the downstream primer was radiolabeled with γ-32P. The substrate is comprised of D3:U4:T4.

FIG. 6. The L126D/I128E mutant of PCNA enhances DNA ligase I activity. Reactions of 140 μl containing 140 fmol of DNA substrate and 35 fmol of DNA ligase I were performed as described under “Experimental Procedures.” In the reactions with PCNA, 17.5 pmol of either wild-type or mutant PCNA was utilized. The reactions were incubated at 37 °C, and 20-μl aliquots were removed at 0, 1, 3, 5, 7, 10, and 15 min as indicated by the triangles. Substrate and ligation product sizes are as indicated. The 5'-end of the downstream primer was radiolabeled with γ-32P. The substrate is comprised of D3:U4:T4. nt, nucleotide.

FIG. 7. The addition of streptavidin blocks PCNA loading onto the substrate, inhibiting stimulation of ligation. The substrate is comprised of D1:U2:T2. The 5'-end of the upstream primer and the 5'-end of the template were biotinylated. The 5'-end of the downstream primer was radiolabeled with γ-32P. Reactions of 20 μl containing 5 fmol of DNA substrate and 1 fmol of DNA ligase I were performed as described under “Experimental Procedures.” PCNA concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 pmol were utilized. Streptavidin was conjugated to the biotinylated 5'-ends according to “Experimental Procedures.” The reactions were incubated at 37 °C for 10 min. The conversion of substrate to product (%) was determined by quantitating the substrate and product on a denaturing polyacrylamide gel by PhosphorImager (Molecular Dynamics) analysis.

FIG. 8. PCNA does not stimulate DNA ligase I activity on a circular substrate. The reactions were incubated at 37 °C for 10 min. Substrate and ligation product sizes are as indicated. The 5'-end of the downstream primers were radiolabeled with γ-32P (as indicated by the asterisks). Reactions of 20 μl containing 5 fmol of DNA substrate were performed as described under “Experimental Procedures.” PCNA concentrations of 0.05, 0.1, 0.2, 0.3, and 0.4 pmol were utilized as denoted by the triangles. A, analysis of a linear substrate (D3:U4:T4). The reactions contained 0.8 fmol of DNA ligase I. B, analysis of a circular substrate (D2:U3:pBS(+)). E. coli single-stranded DNA-binding protein (0.25 pmol) was added to coat the single-stranded regions of the substrate. The reactions contained 0.2 fmol of DNA ligase I. LIG. I, DNA ligase I; nt, nucleotide.

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**Fig. 9. RFC is required to load PCNA onto a circular substrate to affect DNA ligase I activity.** The substrate is comprised of D$_2$U$_3$: pBS(+). The reactions were incubated at 37 °C for 10 min. Substrate and ligation product sizes are as indicated. The 5′-end of the downstream primer was radiolabeled with γ-$_{32}^c$P. Reactions of 20 μl containing 5 fmol of DNA substrate and 0.2 fmol of DNA ligase I were performed as described under “Experimental Procedures” (lanes 5–8). E. coli single-stranded DNA-binding protein (0.25 pmol) was added to coat the single-stranded regions of the substrate. The reactions in the presence of PCNA contained 50 fmol of PCNA, and the reactions in the presence of RFC contained 15 fmol of RFC. Lanes 1–4 represent control lanes without DNA ligase I. LIG. I, DNA ligase I; nt, nucleotide.

5-fold when the two purified proteins interacted *in vitro*. Initial reactions were performed using a linear double-stranded DNA with a nicked site as the substrate. It has been shown that the PCNA toroid can load onto such substrates by diffusion over the ends (13, 29, 30). Efficient entry requires a higher concentration of PCNA than needed for RFC-dependent loading. An enhanced rate of ligation was observed on substrates that differ in both sequence and length, suggesting that the stimulation is independent of structural features of substrates other than the requisite nick. We considered the possibility that PCNA alters the structure of the nicked site in a way that would facilitate the action of any DNA ligase. However, analysis of ligation reactions with T4 DNA ligase did not reveal any stimulatory effect. This supports the conclusion that the stimulation is related to the specific interaction between PCNA and DNA ligase I.

The interaction between PCNA and FEN1 has been well-characterized (12, 13, 29–31). PCNA can enhance nuclease activity by increasing the binding stability of FEN1 at its cleavage site (13). Because FEN1 and DNA ligase I operate sequentially in some of the same pathways (1–3), we anticipated that PCNA would stimulate DNA ligase I by a similar mechanism. An electrophoretic mobility shift assay shows that PCNA greatly increases the amount of DNA ligase I bound to DNA. A supershifted complex representing PCNA and DNA ligase I bound to the same DNA substrate is conspicuously absent. After facilitating the binding of DNA ligase I, PCNA must dissociate either before or during the electrophoresis step of the mobility shift assay. The DNA ligase I dissociation rate could be substantially lower than that of PCNA so that the ligase is retained during the movement of the complex on the gel. It is noteworthy that electrophoretic mobility shift assays showing enhancement of FEN1 binding by PCNA also have no supershifted complex (13).

Another interesting possibility is that PCNA induces a conformational change in DNA ligase I that enhances the binding stability of this enzyme to the ligation site. A recent study has shown that FEN1 undergoes a conformational change upon binding to a flap DNA substrate (60). If binding to PCNA can induce this conformational change, then the addition of PCNA may lead to the stable association of a larger population of FEN1 nuclease to the substrate. This would allow FEN1 to be retained on the substrate during electrophoresis even after PCNA dissociates. If DNA ligase I operates in a similar manner, the addition of PCNA would lead to a greater population of DNA ligase I molecules that are bound to the nicked duplex DNA. The stable association resulting from a conformational change would allow retention of DNA ligase I on the DNA in the absence of PCNA during electrophoresis.

Another important observation is that adding PCNA has the same effect on DNA ligase I binding and catalysis as increasing the concentration of ligase. In addition, at high concentrations of DNA ligase I, PCNA can no longer substantially improve the rate of the reaction. These results support the conclusion that the observed stimulation is the result of an increased rate of binding of DNA ligase I to the substrate DNA. Because the binding rate is maximized at high ligase concentrations, no further rate enhancement is possible.

Considering that the stimulatory effect appears to be a result of the physical interaction between PCNA and DNA ligase I, inhibiting this interaction should prevent stimulation. This was clearly illustrated when DNA ligase I was phosphorylated by casein kinase II. During the cell cycle, casein kinase II has been proposed to function in phosphorylating DNA ligase I to abolish the interaction of this protein with the replication machinery (24). Casein kinase II phosphorylates several residues within the N-terminal region of DNA ligase I (28). This region is not involved in catalysis but is required for binding to PCNA (27). Our experiments show that incubation of DNA ligase I with casein kinase II has little effect on the activity of the purified DNA ligase I. However, phosphorylation of DNA ligase I completely eliminates the stimulatory effect by PCNA.

Previous studies have shown that DNA ligase I can bind to PCNA in solution or PCNA that is topologically linked to DNA (33). However, functional interactions of other proteins with PCNA have always involved PCNA that is encircling duplex DNA (13, 23–25, 30, 61). Our results show no exception to this rule. Blocking the entrance of PCNA onto a linear substrate with biotin-streptavidin moieties effectively prevented stimulation. In addition, PCNA could not stimulate DNA ligase I activity on a circular substrate unless the PCNA molecules were loaded onto the substrate by the ATP-dependent action of RFC. Therefore, these observations support the requirement for PCNA to surround the duplex DNA to augment DNA ligase I activity.

Although both FEN1 and DNA ligase I are stimulated by PCNA, these two enzymes have different binding sites on PCNA (30). FEN1 association occurs through the interdomain connecting loop region of PCNA (12, 29, 30, 32), whereas DNA ligase I interacts with a hydrophobic pocket near the interdomain connecting loop (30). Our analysis of a PCNA mutant with a severe defect in FEN1 binding activity and a nuclease stimulation deficiency shows that this mutant is still capable of interacting with DNA ligase I to effectively stimulate ligation activity. Another group has shown that mutations in the interdomain connecting loop region of PCNA do not have significant effects on DNA ligase I binding (30). Evidently, these mutations also do not interfere with the stimulation of DNA ligase I activity. The results with this PCNA mutant further support the differentiation of PCNA interactions with FEN1 and DNA ligase I. FEN1 and DNA ligase I operate sequentially in DNA
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Fig. 10. A model of Okazaki fragment processing. RFC is responsible for loading PCNA onto the duplex DNA in an ATP-dependent manner. PCNA initially serves as a sliding clamp for DNA polymerase δ. This polymerase complex displaces a portion of the downstream Okazaki fragment. RPA binds to the single-stranded regions. Dna2 and FEN1 function to remove the initiator RNA and the displaced DNA. FEN1 binds to PCNA to perform cleavage. Subsequently, DNA ligase I binds to PCNA to seal the nick.

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Acknowledgments—We are grateful to the members of the Bambara laboratory for insightful discussions. We thank Dr. Hirohumi Terasaka for providing a human DNA ligase I expression plasmid (pHLigI) and Dr. Michael S. DeMott for the purification of recombinant human DNA ligase I. In addition, we thank Dr. Vladimir N. Podust for kindly providing purified human replication factor C.

This is the first report of PCNA stimulation of DNA ligase I activity. Although several groups have characterized the binding interaction between PCNA and DNA ligase I (27, 30, 33, 37), no stimulatory effect has previously been observed to result from this interaction. Observations have included the absence of any effect (33) and an inhibition of ligation (30). Differences in experimental conditions, including the concentrations of proteins and the types of buffers employed, may explain the discrepancies that have been reported.

In summary, we present evidence that human PCNA stimulates the activity of DNA ligase I, and the stimulatory effect is a result of PCNA stabilization of DNA ligase I binding to the ligation site on its oligonucleotide substrate. Stimulation occurs on a variety of ligation substrates, providing that these substrates have topologically-linked PCNA. Accordingly, the mechanism of stimulation can be explained by a binding interaction between a PCNA molecule encircling the duplex DNA and a DNA ligase I molecule at the ligation site as depicted in our proposed model.
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