Mechanism Underlying Replication Protein A Stimulation of DNA Ligase I*

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Replication protein A (RPA) is a heterotrimeric single-stranded DNA-binding protein that participates in multiple DNA transactions that include replication and repair. Base excision repair is a central DNA repair pathway, responsible for the removal of damaged bases. We have shown previously that RPA was able to stimulate long patch base excision repair reconstituted in vitro. Herein we show that human RPA stimulates the activity of the base excision repair component human DNA ligase I by approximately 15-fold. Other analyzed single-stranded binding proteins would not substitute, attesting to the specificity of the stimulation. Conversely, RPA was unable to stimulate the functionally homologous ATP-dependent ligase from T4 bacteriophage. Kinetic analyses suggest that catalysis of ligation is enhanced by RPA, as a 4-fold increase in $k_{cat}$ is observed, whereas $K_{m}$ is not significantly changed. Substrate competition experiments further support the conclusion that RPA does not alter the specificity or rate of substrate binding by DNA ligase I. Additionally, RPA is unable to significantly enhance ligation on substrates containing an unannealed 3'-upstream primer terminus, suggesting that RPA does not stabilize the nick site to enhance ligase recognition. Furthermore when DNA ligase I is pre-bound to the substrate and limited to a single turnover, RPA is still able to stimulate ligation. Overall, the results support a mechanism of stimulation that involves increasing the rate of catalysis of ligation.

Cells have evolved DNA repair mechanisms to protect the integrity and correct the informational content of the genome. Base excision repair (BER), the most frequently employed form of DNA repair, is responsible for the removal of bases that have become oxidized, alkylated, or deaminated (1). Currently, it is estimated that there may be between 10,000 and 50,000 damaged sites/cell/day (1–5). The BER process is initiated by the actions of a damage-specific DNA N-glycosylase, responsible for both the recognition and the removal of altered bases (6, 7). Removal results in the generation of an apurinic/apyrimidinic (AP) site, which is a substrate for an AP endonuclease. The predominant AP endonuclease in mammalian cells, Ape1 (also called HAP1/REF1/APE), is a multifunctional enzyme that is able to cleave 5' to an abasic site (8–13). The cleavage generates a 3'-OH terminus capable of supporting DNA polymerization. However, it also leaves a baseless residue at the 5' terminus, which must then be removed. BER can then proceed via two pathways, designated short patch or long patch repair (7, 14–20). In short patch repair, DNA polymerase $\beta$ is responsible for the addition of a single nucleotide as well as the cleavage of the 5'-deoxyribose phosphate residue using an intrinsic deoxyribosephosphate lyase activity (21–23). Finally, a DNA ligase can seal the nick to complete repair. This results in the replacement of only the damaged nucleotide.

Alternatively, in vivo, a portion of the AP sites become oxidized prior to repair (18, 19, 24). The deoxyribosephosphate lyase activity of polymerase $\beta$ is now unable to remove the chemically altered abasic sugar. Instead, a DNA polymerase (B, $\delta$, or $\epsilon$) will then displace the baseless sugar along with several additional nucleotides forming a single-stranded flap (25). The flap is a substrate for the flap endonuclease I (FEN1) (26, 27). FEN1 cleaves at the base of the flap generating a nick, which can then be sealed by DNA ligase I (28, 29). Long patch repair results in the removal and replacement of between 2 and 8 nucleotides. Interestingly, many of the proteins involved in long patch BER are also components of the DNA replication machinery, providing a mechanistic link between the BER and DNA replication complexes (30–35).

A minimal repair complex containing FEN1, polymerase $\beta$, $\delta$, or $\epsilon$, and DNA ligase I is necessary to complete the long patch repair reaction in vitro (19, 24, 33, 36). In addition to the minimal complex, a variety of other accessory proteins influence the efficiency of the reaction, suggesting their participation in vivo. Both proliferating cell nuclear antigen (PCNA) and RPA, two proteins that are essential for DNA replication, are also participants in DNA repair, including BER (37–40). PCNA has been shown to directly interact with both FEN1 and DNA ligase I and stimulate their activities (41–46). Both stimulation mechanisms have been investigated thoroughly and shown to be mediated through an increase in enzyme-substrate binding (45, 46). More efficient binding increases the overall efficiency of both the cleavage and ligation reactions. The results portray PCNA as a targeting and assembly component of DNA replication and repair protein complexes.

The roles of RPA in both DNA replication and nucleotide excision repair are well established (47, 48). For example, during DNA replication, RPA participates in the recognition and unwinding of the origin, binds to the single-stranded DNA to prevent secondary structure formation, and stimulates polymerase $\alpha$ through direct protein-protein interactions (49–52). Considerable evidence links RPA to BER, but the contributions

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The abbreviations used are: BER, base excision repair; RPA, replication protein A; AP, apurinic/apyrimidinic; PCNA, proliferating cell nuclear antigen; FEN1, flap endonuclease I; SSB, single-stranded binding protein.

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of the binding protein have not been determined. RPA has been shown to interact directly with the major nuclear DNA glycosylase, the uracil-DNA glycosylase (53, 54). Recent crystal structure data specifically localize the interaction between RPA and uracil-DNA glycosylase to the C-terminal region of the RPA 32 subunit (54). This interaction might target RPA binding specifically to sites of base damage. Yet, RPA does not alter the enzymatic activity of the glycosylase. In another observation, yeast that contain a defective RFA1 gene (encoding the yeast homolog to the large subunit of RPA) are sensitive to methyl methane sulfonate, a DNA damaging agent that produces lesions repaired by BER (55). Although not defining a mechanism, these results strongly suggest that RPA is a component of the BER complex.

Interestingly, when the final steps of long patch BER were reconstituted in vitro using only FEN1, calf polymerase ε, and DNA ligase I, the addition of RPA produced a manifolds stimulation of product formation (37). Furthermore, RPA has been shown to influence the rate of long patch repair using mammalian cell extracts in cooperation with PCNA (38). Again, the actual function of RPA in the process is unclear.

In some studies of reconstituted BER reactions in vitro, stimulation by RPA has not been observed (36, 56). This suggests that the concentrations of reaction components are critical determinants of the amount of stimulation. Very likely RPA stimulates a reaction component that limits the overall rate of BER. If that component is present in a sufficiently high concentration in vitro, the stimulatory effects may be masked.

An approach to determine the role of RPA in BER is to examine its effect upon individual steps in the reaction. A final step in both BER and the joining of nascent DNA fragments in DNA replication is the ligation reaction. Of the four known DNA ligases in mammalian cells, DNA ligase I has been linked to BER (57–59). DNA ligase I was also utilized for the in vitro reconstitution of long patch BER. Then RPA stimulation was observed (37). The quantity of DNA ligase used in our in vitro BER reconstitution reactions was not saturating, allowing for a reaction component that stimulates DNA ligase to increase overall product formation.

DNA ligases catalyze esterification of the 3′-OH and a 5′-phosphate termini of a nick in double-stranded DNA. Mammalian DNA ligases require a divalent cation and ATP for catalysis. The reaction mechanism for ligation has been elucidated previously, and involves at least three sequential steps (60–65). In the first, DNA ligase interacts with ATP to generate a ligase-adenylate covalent intermediate with the concomitant release of pyrophosphate (PPi). The ligase-adenylate can then bind to the 5′-phosphate terminus and transfer the AMP to form a DNA-AMP intermediate. Finally, the ligase catalyzes the reaction between the DNA-AMP and the 3′-OH terminus to join the two repaired strands together and releases the AMP. Crystallographic studies of the eukaryotic ATP-dependent DNA ligase from Chlorella virus have shed some light on the complicated chemistry of the ligation reaction. They show that the ATP-dependent ligases undergo significant conformational changes associated with the chemical steps of the ligation reaction mechanism (66). We show here for the first time that RPA is able to stimulate DNA ligase I and the mechanism underlying stimulation involves an increase in the rate of the chemical step of ligation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) or by Genosys Biotechnologies (The Woodlands, TX). Radionucleotides (γ-32P]ATP (3000 Ci/mmole) were obtained from PerkinElmer Life Sciences. The T4 polynucleotide kinase and the T4 DNA ligase were obtained from Roche Molecular Biochemicals.

**RPA Stimulates DNA Ligase I**

RPA stimulates DNA ligase I activity by promoting the efficient joining of nascent DNA fragments in DNA replication. Previous studies have demonstrated that RPA is recruited to sites of repair through a specific interaction with uracil DNA glycosylase (55). Subsequently, RPA was shown to enhance the efficiency of long patch BER (37, 38). Experiments analyzing the effects of RPA upon the individual enzymes involved in long patch BER focus here on DNA ligase I.

Fig. 1A shows a titration of RPA into reactions containing human DNA ligase I at either 0.02 nM (left panel) or 0.06 nM (right panel) concentrations. The reactions were performed in substrate excess so that the amount of product formation is...
indicative of the reaction rate. Upon addition of RPA to the ligation reactions, an increase in the amount of ligation product was observed (lanes 3–6 and 8–11). Fig. 1B is the graphical analysis of the results from three independent experiments performed as shown in Fig. 1A. The graph depicts the -fold stimulation of ligation product formation as a function of RPA concentration for both tested ligase concentrations. There was a consistent increase in ligation rate, and thus -fold stimulation, with increasing concentrations of RPA over the tested range. Furthermore, the stimulation was independent of DNA ligase concentration within the tested range. RPA-mediated stimulation of ligation rate was maximally 15-fold compared with reactions lacking RPA. It was necessary to include a molar excess of RPA in these and in subsequent experiments. This

| Downstream primers | Upstream primers | Templates |
|--------------------|-----------------|------------|
| D1 (18-mer) | U1 (25-mer) | T1 (44-mer) |
| D2 (42-mer) | U2 (31-mer) | T2 (73-mer) |
| D3 (44-mer) | U3 (25-mer) | T3 (54-mer) |
| D4 (28-mer) | U4 (25-mer) |

Oligonucleotide sequences (5'–3')

- **D1**: GTAAAACGACGGCCAGTG
- **D2**: UACCCCCGTATGGATCCACAAATGCCAGCTGACCGCCACCTTTGGAGC
- **D3**: CGTGACCGGCAGCAAAATGCCAGCTGACCGCCACCTTTGGAGC
- **D4**: CGACCTGGCCAGCCTTTGGAC

- **U1**: CGCCAGGGTTTTCCAGCTCGAGCC
- **U2**: TCCAGCCTAACAATTTGCCGAATGGACCGG
- **U3**: CGACGTGCCAGCCTTTGGAC
- **U4**: CGACGTGCCAGCCTTTGGAC

- **T1**: GCACCTGGCCAGCCTTTGGAC
- **T2**: TTTGCTAAACCAATTGGCGCAATGGACCGG
- **T3**: TTGCATTACGTCGAAGCTTGCTTGACCCATACGGGGGTACCGGTCCATTGCGCCAATTGGTTAGCTGGAGG

- U is defined as deoxyuridine.

### Table I

Oligonucleotide sequences (5'–3')

| Downstream primers | Upstream primers | Templates |
|--------------------|-----------------|------------|
| D1 (18-mer) | U1 (25-mer) | T1 (44-mer) |
| D2 (42-mer) | U2 (31-mer) | T2 (73-mer) |
| D3 (44-mer) | U3 (25-mer) | T3 (54-mer) |
| D4 (28-mer) | U4 (25-mer) |

Oligonucleotide sequences (5'–3')

- **D1**: GTAAAACGACGGCCAGTG
- **D2**: UACCCCCGTATGGATCCACAAATGCCAGCTGACCGCCACCTTTGGAGC
- **D3**: CGTGACCGGCAGCAAAATGCCAGCTGACCGCCACCTTTGGAGC
- **D4**: CGACCTGGCCAGCCTTTGGAC

- **U1**: CGCCAGGGTTTTCCAGCTCGAGCC
- **U2**: TCCAGCCTAACAATTTGCCGAATGGACCGG
- **U3**: CGACGTGCCAGCCTTTGGAC
- **U4**: CGACGTGCCAGCCTTTGGAC

- **T1**: GCACCTGGCCAGCCTTTGGAC
- **T2**: TTTGCTAAACCAATTGGCGCAATGGACCGG
- **T3**: TTGCATTACGTCGAAGCTTGCTTGACCCATACGGGGGTACCGGTCCATTGCGCCAATTGGTTAGCTGGAGG

- U is defined as deoxyuridine.
was because the annealing reactions performed to generate the nicked substrate contain an excess of single-stranded DNA that is capable of binding to and sequestering the RPA. The presence of single-stranded DNA at the levels used here has negligible effects upon ligation activity (data not shown). The substrate utilized was identical to the one used in a previous analysis of BER with RPA (37).

**RPA Does Not Stimulate T4 Ligase**—To clarify the specificity of the functional interaction between RPA and DNA ligase I, we determined whether RPA could stimulate another ATP-dependent ligase. RPA was titrated into a reaction containing the bacteriophage T4 ligase (Fig. 2). Stimulation of T4 ligase would suggest that the stimulation mechanism is independent of the ligase structure. For example, it could have involved an alteration in substrate structure that improves access of the ligase to the nick. Specificity for DNA ligase I would imply that the mechanism involves a direct improvement of ligase function. Titration of RPA into the T4 ligase reactions (lanes 1–9) shows that there is no enhancement of the accumulation of ligation product as compared with the reactions containing human DNA ligase I (lanes 10–18). Additionally, we demonstrated stimulation of DNA ligase I on a different substrate (both in sequence and length) than tested in Fig. 1 to ensure that RPA directed stimulation was not based upon a unique interaction with a specific substrate.

**SSB Does Not Enhance the Activity of DNA Ligase I**—To determine whether other single-stranded binding proteins are also able to stimulate DNA ligase I activity, we titrated *E. coli* SSB into reactions containing DNA ligase I. The conversion of radiolabeled substrate to ligation product in the absence of added binding proteins, the presence of RPA, or the presence of increasing amounts of SSB is shown (Fig. 3). Increasing the concentration of SSB in the reaction had no significant effect upon ligation activity. Quantitation of these results shows that 16% of the substrate was converted to product during a 5-min reaction containing 0.06 nmol ligase I (lane 2). Upon the addition of 500 fmol of RPA (25 nM) to the reaction, ~60% of the substrate was converted to product (lane 3). However, with increasing levels of SSB, we did not see a corresponding increase in ligation efficiency (lanes 4–9). Therefore, the stimulation of DNA ligase I by RPA appears to be protein-specific.

**RPA Enhancement of Ligation Rates over Time**—To further analyze the mechanism of stimulation, we determined the amount of ligation during repeated cycling of the DNA ligase from substrate to substrate. To determine this, the reaction was performed using fixed RPA and ligase concentrations, an excess of nicked substrate DNA, and monitored over time. All reactions contained 0.05 nmol DNA ligase I, and those done in the presence of RPA contained 12.5 nM RPA. Enhancement of product formation is seen over the entire time course in the presence of RPA.

Fig. 4 is a graphical representation of the quantitation of the percentage of substrate converted to ligated product over the entire time course of 30 min. In the presence of RPA, the ligation rate is linear with respect to time until ~10 min when the reaction slows with respect to product formation. Because the reaction without RPA is linear to 30 min, this suggests that the cause of the saturation is that all available substrate has been converted to product. Because the maximum conversion of substrate to product is between 60 and 70%, approximately one third of the labeled strand must not be available for ligation, possibly because it is not appropriately annealed. However, because the reaction lacking RPA converted only 19% of the substrate to product over the 30-min time course, we performed additional analyses to ensure that in the absence of RPA, excess DNA ligase I was able to convert all ligatable substrate to product (data not shown). The linear portions of the graph show that the approximate 6-fold stimulation occurs over multiple reaction cycles, indicating that RPA can repeatedly stimulate the same DNA ligase I protein. The result is consistent
Addition of Double-stranded DNA Does Not Alter Ligation Efficiency—One possible explanation for RPA-mediated stimulation is that it prevents DNA ligase I from binding to the ends of the substrate DNA. On a nicked double-stranded oligonucleotide substrate, the ligase could potentially be sequestered at ends or other nonproductive binding sites. This would effectively lower the concentration of ligase available to react at nicks. The addition of RPA to the reaction would then release the ligases from any nonproductive interactions, increasing the amount available for catalysis.

To examine this possibility, we titrated intact double-stranded oligomeric DNA into reactions containing either DNA ligase I alone or DNA ligase I and RPA. If RPA stimulates ligation in the above manner, we would expect to have found that increasing concentrations of non-nicked double-stranded DNA in the reactions lacking RPA would sequester the ligase and strongly inhibit sealing of the nicks. This inhibition would increase with the concentration of double-stranded DNA in the reactions. However, in the reactions containing RPA, we expected that there would be little effect of added DNA, as RPA would prevent the ligase from being sequestered. Therefore, in comparing the two, the overall effect would be an increase in the -fold stimulation of ligation, as the competitor DNA concentration was increased.

Fig. 5 shows a graph of the quantitation of three independent experiments in which increasing concentrations of double-stranded DNA were added to ligation reactions. In the absence of both competitor DNA and RPA, ~4% of the substrate was converted to product. When RPA was added to this reaction, ~21% of the substrate was converted to product. Increasing the concentration of double-stranded DNA had no inhibitory effect on ligation in reactions lacking RPA. The double-stranded DNA is equimolar with the substrate DNA at a concentration of 0.4 nM. However, only at the highest concentrations of double-stranded competitor did we observe any decrease in ligation efficiency. In contrast, the ligation efficiency in the reactions containing RPA is decreased throughout the range of competitor DNA concentration. Interestingly, this has an outcome opposite to that predicted by a nonspecific binding hypothesis, i.e., the amount of RPA-directed stimulation actually decreases with added competitor DNA. A reasonable explanation is that the competitor DNA sequesters the RPA rather than the DNA ligase. In view of these results, it is unlikely that RPA stimulation involves displacement of DNA ligase I from nonproductive interactions at the ends or other sites on the substrate DNA.

RPA Alters the $k_{cat}$ of the DNA Ligase I Ligation Reaction—To further elucidate the mechanism underlying RPA stimulation of ligation, we analyzed the ligation reaction using Michaelis-Menten kinetics. RPA may be able to stimulate ligation by affecting binding, catalysis, or a combination of both. If RPA were able to influence ligase binding to the substrate, we would expect this to be reflected by a decrease in the $k_{cat}$ (Michaelis constant). Alternatively, if RPA directly improves catalytic efficiency, we should detect a change in the $k_{cat}$, the direct measure of the catalytic production of product. Table II shows the resulting values determined from these experiments using a Lineweaver-Burk plot. The addition of RPA to the reaction generates approximately a 4-fold increase in $k_{cat}$ while not significantly altering the $K_m$. This result supports the interpretation that the primary effect of RPA is upon catalysis of the ligation reaction rather than binding of the DNA ligase to the substrate DNA.

RPA Does Not Significantly Enhance Ligation on an Unannealed 3′ Terminus—Although the kinetics experiment indicates that binding of ligase to a nicked substrate was not enhanced by RPA, we examined one potential binding-related stimulation mechanism more directly. RPA might present the nicked substrate to the DNA ligase in a conformation more suitable for binding and ligation. For example, the RPA might suppress terminal breathing at the nick, so that at any moment all of the substrate is in a form most favorable for ligase binding. We tested this concept by creating a structural alteration that simulated breathing. The test substrate had a mismatched nucleotide on the upstream primer at the position on the 3′ terminus. It has been shown previously that DNA ligase I has a decreased efficiency of ligation on substrates containing a mismatch on the 3′ terminus (67). If RPA-mediated stimulation of DNA ligase involves a stabilization of nick structure, we anticipated an increased stimulation of the mismatched substrate compared to a substrate of the same sequence and length lacking the 3′-mismatch.

Fig. 6 shows a titration of RPA into ligation reactions containing either a conventional nick (lanes 2–7) or a mismatched
3’ terminus on the upstream primer (lanes 9–13). Quantitation of results shows that, in the absence of RPA, on the conventional nick substrate, DNA ligase I converted ~5% of the substrate to product. Upon addition of RPA to the reaction, the amount of substrate converted to product increased to ~44%, resulting in an 8-fold stimulation of product formation at the highest level of RPA (lane 6). However, on a substrate containing a mismatched 3’ terminus, in the absence of RPA, only 0.8% of the substrate was converted to product (lane 8). Upon addition of RPA to the reaction, only 2.3% of the substrate was converted to product, resulting in only a 2.8-fold stimulation of product formation at the highest level of RPA (lane 13). Therefore, RPA was not only unable to compensate for alterations at the structure of the nick, but also unable to stimulate ligation as efficiently as when a conventional nick-flap substrate is used.

**RPA Stimulates DNA Ligase I under Single-turnover Conditions**—We next analyzed the ligation reaction under conditions in which each enzyme was limited to a single-turnover. This has the potential to determine whether the stimulation mechanism involves cycling of DNA ligase from one substrate to the next versus an improvement of the rate of catalysis on the substrate to which the ligase is already bound. The reaction mechanism for ligation has been studied extensively and is known to involve at least three distinct steps (60–62, 64, 65). In the first step, the ligase interacts with ATP to form an adeny-ligase complex. This complex can then interact with and bind to a nicked substrate. Once bound to the nick, the ligase can then complete the chemical steps of ligation, resulting in the generation of intact double-stranded DNA.

It is possible for RPA to affect any number of the steps of the ligation reaction mechanism. To ascertain the effect that RPA has upon the chemical steps of the reaction, we utilized ligase I that was pre-adenylated. Incorporation of [α-32P]ATP occurred only after pre-incubation of DNA ligase I with substrate, indicating that the ligase was purified in the adenylated state (data not shown). Thus, in the absence of added ATP, each ligase would be able to convert only a single nicked substrate to ligated double-stranded product. Furthermore, the adeny-ligase was pre-incubated with substrate in the absence of MgCl2. This allows for initial binding of the ligase to the DNA to occur

![Graphical representation of conversion of substrate to product (%) for ligation reactions in the presence of double-stranded DNA.](image)

**FIG. 5. Addition of double-stranded DNA does not affect ligase activity.** Graphical representation of conversion of substrate to product (%) for ligation reactions in the presence of double-stranded DNA. Double-stranded DNA comprising D3:T1 was incubated with nicked substrate comprising D4:U4:T3, DNA ligase I, and RPA. Reactions were incubated at 30 °C for 5 min. The 5’ end of the downstream primer of the nicked substrate was radiolabeled with γ-32P. Reactions of 20 μl containing 10 fmol of DNA substrate and 5 fmol of DNA ligase I were performed as described under “Experimental Procedures.” The reactions in the presence of RPA contained 0.250 pmol of RPA (12.5 nM). The dotted bars represent the reactions containing DNA ligase, and the solid bars represent the reactions containing both DNA ligase and RPA. The concentration of double-stranded DNA (nt) in the reaction is indicated on the x axis of the graph, and the conversion of substrate to product (%) is represented on the y axis.

| ds DNA (nM) | % Conversion |
|------------|-------------|
| 0          | 5           |
| 0.05       | 10          |
| 0.1        | 15          |
| 0.2        | 20          |
| 0.4        | 25          |
| 0.8        | 30          |

**FIG. 6. RPA does not significantly enhance ligation of substrates containing an unannealed 3’-upstream primer terminus.** Reactions of 20 μl containing 10 fmol of DNA substrate, 2 fmol of DNA ligase I (0.1 nM), and increasing amounts of RPA were performed as described under “Experimental Procedures.” Lanes 2–7 contained 0.100, 0.250, 0.500, 1.0, 3.0, and 5 pmol (150 nM) of RPA in addition to DNA ligase I. Lanes 9–13 contained 0.250, 0.500, 1.0, 3.0, and 5 pmol (150 nM) of RPA in addition to DNA ligase I. Reactions were incubated at 30 °C for 5 min. The 5’ end of the downstream primers of both substrates was radiolabeled with γ-32P. Schematic representations of the substrates are depicted above the figure. Product analysis of ligation reactions using substrate comprising D3:T1 (lanes 1–7) or substrate comprising D4:U4:T3 (lanes 8–13). Substrate and ligation product lengths are as indicated.

**Table II: Kinetic parameters for ligation by human DNA ligase I**

|          | km          | kcat          | kcat/Km        |
|----------|-------------|---------------|----------------|
| Ligase   | 3.4 ± 0.3 nM| 2.6 x 10^-3 s^-1| 7.65 x 10^-4   |
| Ligase + RPA | 4.9 ± 1.1  | 11.6 x 10^-3 s^-1| 23.67 x 10^-4  |

This allows for initial binding of the ligase to the DNA to occur.
prior to the initiation of the reaction. Because this assay allows only a single turnover, any effect that RPA has on the rate of product release would not be detected. If observed, an RPA-mediated stimulation would have had to be based upon an improvement of the catalysis of ligation by enzymes already bound to the substrate DNA.

Fig. 7A shows a time course of the generation of 43-nucleotide ligation product either in the absence (left panel) or the presence (right panel) of RPA as a function of time. Fig. 7B shows a graphical representation of the quantitation of the percentage (%) of ligation product formed as a function of time from Fig. 7A. The concentration of RPA utilized in this experiment was 12.5 nM. In the absence of RPA, 16 fmol of DNA ligase I was able to convert 11% of the substrate to product (4.4 fmol) in 10 min. While in the presence of RPA, ligase was able to convert 36% of the substrate to product (14.4 fmol). Upon addition of RPA to the reactions, the amount of product formation after 10 min was virtually equivalent to the amount of ligase in the assay. Therefore, by 10 min, every active ligase had ligated a single nicked substrate. This yielded a 3.3-fold enhancement of ligation under single-turnover conditions.

However, a more accurate measurement is obtained from the linear portion of the graph, at the 5-min time point. After 5 min, in the absence of RPA, 6% of the substrate was converted to product, which generates 2.4 fmol of product. When RPA was included, 31% of the substrate was converted to product, or 12.4 fmol of product was formed. This yielded a 5.2-fold stimulation of ligation. Therefore, in conjunction with the kinetic data suggesting that RPA affects catalysis by enhancing the reaction velocity, the single-turnover experiment further suggests a direct increase in the chemical step of ligation. These results focus on improved catalysis as the mechanism whereby RPA is able to mediate stimulation of human DNA ligase I.

**DISCUSSION**

Replication protein A is a multifunctional DNA-binding protein that participates in both DNA replication and repair. In addition to binding single-stranded DNA, RPA has been shown previously to influence the enzymatic activities of a variety of proteins critical for both these processes (47, 48, 68). We reported previously that the presence of RPA stimulates a reconstitution of long patch base excision repair using FEN1, DNA polymerase δ, and DNA ligase I (37). This result is consistent with an observed stimulation of long patch base excision repair in mammalian cell extracts by the addition of RPA (38). To identify the mechanism of stimulation, we examined the effect of RPA upon each of the enzymes individually that were employed in the reconstitution reaction. We found that RPA stimulated the sealing of nick structures catalyzed by DNA ligase I.

Maximum efficiency of long patch base excision repair reactions carried out both in cell extracts (38) and using purified proteins (36, 39, 56) requires PCNA. PCNA was originally characterized as the sliding clamp for DNA polymerase δ (69, 70). More recently, PCNA has been shown to stimulate other
enzymes involved in DNA replication and repair (45, 46). Specifically, it strongly augments the activity of FEN1 nuclease (45, 71). Analysis of the mechanism of stimulation demonstrated that PCNA decreases the $K_m$ with respect to substrate concentration but does not significantly affect the maximal velocity of the cleavage reaction, suggesting that the PCNA improves binding of the FEN1 to its substrate (45).

More recently we found that PCNA also stimulates nick-joining by human DNA ligase I (46). Previous work had shown that DNA ligase I and PCNA physically interact (43, 44, 58, 59). In our previous work, analysis of substrates that prevented PCNA from loading demonstrated that PCNA must encircle the nick site on the substrate to effect stimulation. Electrophoretic mobility shift assays indicate that PCNA improves binding of DNA ligase I to the nick site. Overall, these results strongly suggest that the basis of PCNA-directed stimulation of ligation is improved binding of the ligase to the nicked site mediated by the interaction with a PCNA molecule encircling the DNA at that site (46).

Experiments shown here, aimed at clarifying the mechanism of RPA stimulation of ligation, indicate that the RPA-mediated increase in ligation activity occurs by a fundamentally different mechanism than that employed by PCNA. Multiple control experiments using bacteriophage T4 ligase or E. coli SSB indicate that the stimulation is specific for DNA ligase I and RPA. Biochemical kinetic analysis of the RPA stimulation shows that the $k_{cat}$ but not the $K_m$ of the reaction with respect to the substrate concentration is altered. This result suggests that affinity of the DNA ligase for the nicked substrate is not improved by the presence of RPA. This conclusion was further supported by results showing that addition of double-stranded DNA lacking nicks does not effectively compete the DNA ligase away from the nicked substrate. Therefore, RPA could not be improving the binding specificity of DNA ligase for the nick site as compared with nonspecific binding sites on the double-stranded region or blunt-ended termini of the ligation substrate. Additionally, electrophoretic mobility analyses do not show an increase in ligase binding to a nick in the presence of RPA (data not shown). Finally, analyses using substrates containing an unannealed 3′-upstream primer terminus indicate that RPA is not able to suppress alternative structures at the nick site which are inhibitory to catalysis.

Because the reaction cycle of ligation consists of substrate binding, catalysis, and then dissociation to bind a new substrate, we focused on the catalysis and dissociation steps as potential points of stimulation. These steps can be readily distinguished if the reaction is limited to a single turnover. Fortunately the catalytic mechanism of DNA ligase I presents an opportunity to conveniently create this situation. The ATP-dependent ligation reaction involves formation of an adenylate intermediate that can be employed for catalysis in the absence of additional ATP. Under these circumstances each DNA ligase molecule can catalyze only one sealing reaction. Results show that RPA is still able to carry out stimulation, suggesting that improved catalysis is the basis of the increased rate. A reasonable speculation is that an interaction between DNA ligase and RPA promotes a conformation in the DNA ligase that is particularly favorable for the catalysis of nick sealing. Alternatively, RPA may be able to influence the rate at which conformational changes are occurring during the process of ligation. Because the reaction rapidly reaches a plateau, it is not informative to compare the exact percentage of stimulation under single-turnover conditions with that observed when the DNA ligase is cycling in the presence of excess ATP. For this reason, it remains formally possible that the dissociation step is also stimulated, augmenting the stimulation occurring at the catalytic step.

The demonstration that the mechanism of stimulation of ligation by PCNA is fundamentally different from that directed by RPA does not automatically exclude the possibility that the two stimulatory proteins might interfere with each others stimulatory effects. Alternatively, they could interact in a synergistic manner to facilitate ligation. In comparative titrations of PCNA, RPA, or both into ligation reactions, the stimulatory effects were approximately additive (data not shown). Although this observation does not prove that the two stimulatory proteins act independently, it is consistent with such a conclusion.

Stimulation of one protein by another is commonly observed in reconstitution of DNA replication and repair reactions in vitro. The ability of PCNA to stimulate DNA polymerases, FEN1, and DNA ligase I attests to the central role of the sliding clamp in maintaining the structural and functional integrity in the interactive protein complexes needed for these complex reactions. Our results suggest that RPA, already known to participate in DNA replication and repair, also mediates biologically relevant stimulation. Our previous observation of RPA stimulation of long path BER in vitro, together with the results reported here, suggest that RPA-directed stimulation of DNA ligase I is a distinct feature of the BER reaction in vivo. However, in some reconstituted BER reactions in vitro stimulation by RPA has not been observed (36, 56). Again, we suggest that the concentrations of reaction components when analyzed in vitro will influence the ability to quantify stimulation. The component levels utilized in our reconstitution assays allowed us to detect the effects of RPA. The relative concentration of DNA ligase compared with the other BER reaction components in vitro is likely to determine the degree of RPA-directed stimulation of both the repair process as well as DNA replication. Nevertheless, the very fact that RPA mediates a stimulation of catalysis by DNA ligase I supports the conclusion that these two proteins act in partnership in the cell.

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