DNA Ligase I Selectively Affects DNA Synthesis by DNA Polymerases δ and ε Suggesting Differential Functions in DNA Replication and Repair*

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The joining of single-stranded breaks in double-stranded DNA is an essential step in many important processes such as DNA replication, DNA repair, and genetic recombination. Several data implicate a role for DNA ligase I in DNA replication, probably coordinated by the action of other enzymes and proteins. Since both DNA polymerases δ and ε show multiple functions in different DNA transactions, we investigated the effect of DNA ligase I on various DNA synthesis events catalyzed by these two essential DNA polymerases. DNA ligase I inhibited replication factor C-independent DNA synthesis by polymerase δ. Our results suggest that the inhibition may be due to DNA ligase I interaction with proliferating cell nuclear antigen (PCNA) and not to a direct interaction with the DNA polymerase δ itself. Strand displacement activity by DNA polymerase δ was also affected by DNA ligase I. The DNA polymerase δ holoenzyme (composed of DNA polymerase δ, PCNA, and replication factor C) was inhibited in the same way as the DNA polymerase δ core, strengthening the hypothesis of a PCNA interaction. Contrary to DNA polymerase δ, DNA synthesis by DNA polymerase ε was stimulated by DNA ligase I in a PCNA-dependent manner. We conclude that DNA ligase I displays different influences on the two multipotent DNA polymerases δ and ε through PCNA. This might be of importance in the selective involvement in DNA transactions such as DNA replication and various mechanisms of DNA repair.

DNA ligases play essential roles in important cellular pathways, such as DNA replication, DNA recombination, and DNA repair, by joining single- and double-stranded breaks in an ATP-dependent manner (1). Four DNA ligases (I, II, III, and IV), the functions of which are not yet completely understood, have been identified in mammalian cells (2). Human DNA ligase I is a monomer of 102 kDa (3) composed of two clearly distinct regions as follows: a highly conserved 78-kDa C-terminal domain containing the active site (4), and a 24-kDa N-terminal region that is not required for ligase activity but contains the nuclear localization signal and directs the enzyme to sites of DNA replication (5). Several of the following observations indicate an involvement of DNA ligase I in DNA replication: (i) DNA ligase I is responsible for a major part of DNA ligase activity in proliferating mammalian cells (6–9); (ii) cyto-staining experiments with antibodies against DNA ligase I showed that the enzyme co-localizes in the nucleus with DNA polymerase (pol)1 α (10); (iii) the enzyme co-purifies with a protein complex competent in in vitro SV40 DNA replication (11); (iv) a mutation in the DNA ligase I gene in the human 46BR cell line leads to a delay in the joining of the Okazaki fragments (12). These, together with several other observations (3, 13), imply an important role of DNA ligase I in DNA replication as well as in DNA repair (5, 14). Experiments by Macken-ey et al. (15) suggest that DNA ligase I, through its N-terminal region, interacts with other proteins.

The most important proteins in the DNA synthesis reaction are the pols. So far six pols have been identified in eukaryotic cells, called α, β, γ, δ, ε, and θ (reviewed in Refs. 16 and 17). Three of them (α, δ, and ε) have been shown to be essential in DNA replication (18–20). pol α is a complex consisting of four polypeptides as follows: a 180-kDa subunit that harbors the polymerase activity, a 70-kDa peptide of uncertain function, and two small subunits of 48 and 58 kDa, respectively, containing the primase activity. pol α is responsible for initiation of DNA replication; the primase synthesizes RNA primers, which are then elongated into DNA primers by the polymerase activity on both leading and lagging strands (21). It is moderately processive and dissociates from the DNA, facilitating a switch to a highly processive, proofreading pol such as pol δ. pol δ consists of at least two identified subunits with molecular masses of 125 and 50 kDa. The polymerase and the 3′ → 5′ exonuclease activities are both located on the large subunit (22, 23). pol δ exhibits very low activity on its own, but upon addition of the auxiliary factor proliferating cell nuclear antigen (PCNA) (reviewed in Refs. 24 and 25), the activity and the processivity are stimulated up to 100-fold (26, 27). PCNA is loaded onto DNA by a second auxiliary factor, replication factor C (RF-C) (reviewed in Ref. 28), in an ATP-dependent fashion. RF-C recognizes the 3′-end of the nascent strand, thereupon inducing the dissociation of pol α/primase and recruiting pol δ (29), which then performs processive leading strand synthesis. On the lagging strand, pol δ has been shown in vitro to extend the Okazaki fragments (13, 21). Whether this is also the case in vivo remains controversial, since pol ε has been proposed as the lagging strand pol (30–33). pol δ is only not implicated in DNA replication but also plays an important role in DNA repair (reviewed in Ref. 17) and V(D)J recombination (34). pol ε is enzymatically distinguishable from pol δ by its differing re-

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1 The abbreviations used are: pol, DNA polymerase; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; SV40, simian virus 40; ds, double-stranded; BSA, bovine serum albumin; DTT, dithi-othreitol; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.
sponse to PCNA. Whereas pol δ is processive only in the presence of PCNA, pol ε is very active even in the absence of PCNA. Previous studies have reported that pol ε is completely unrespon-sive to the addition of PCNA (20, 35); however, more detailed kinetic studies have shown that PCNA can also stimulate the processivity of pol ε and that pol ε interacts with PCNA, increasing its rate of nucleotide incorporation (36). In the presence of RF-C and ATP, pol ε, like pol δ, is able to form a stable complex with PCNA at the 3'-end of the primer, called pol ε holoenzyme (30, 37). Similar to pol δ, the pol ε also has a role in DNA repair (38, 39), and a function in the repair of double-stranded DNA breaks has been suggested (40).

In this report we show that pol δ and ε are affected in different ways by DNA ligase I. DNA elongation by pol δ is strongly inhibited by DNA ligase I, most likely through the already observed interaction of DNA ligase I with PCNA (41). DNA synthesis by pol ε on the other hand is stimulated by DNA ligase I. Here PCNA plays a dual role; at low concentrations pol ε stimulation by DNA ligase I is increased, whereas at high concentrations the stimulatory effect is canceled. All these effects are ATP-independent, that is they do not involve the DNA ligase I per se and consequently appear to be caused by protein-protein interactions between PCNA and DNA ligase I.

EXPERIMENTAL PROCEDURES

Chemicals—Radiolabeled nucleoside triphosphates were purchased from Amersham Pharmacia Biotech, and unlabeled dNTPs were from Boehringer Mannheim. All other reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland).

Nucleic Acids—The single-stranded M13 DNA was hybridized to the universal sequencing primer as outlined (42). The homopolymer poly(dA)1030 (Amersham Pharmacia Biotech) was mixed in the desired weight ratio with the oligomer oligo(dT)12–18 (Amersham Pharmacia Biotech) in 20 mM Tris-HCl (pH 8.0), containing 20 mM KCl and 1 mM EDTA, heated at 60 °C for 5 min with subsequent slow cooling to room temperature. Gapped double-stranded DNA was constructed as described (43).

Enzymes and Proteins—Recombinant human DNA ligase I was purified (15). Human PCNA was overexpressed in Escherichia coli BL21(DE3) harboring the expression plasmid pT7/PCNA as described (44). The phosphorylated PCNA was expressed in E. coli, purified, and phosphorylated in vitro using [γ-32P]ATP as described (45). Recombi-nant RF-C was purified from baculovirus infected HighFive cells (46). Pol δ and ε were purified from fetal calf thymus as described (47). One unit of enzyme activity corresponds to the incorporation of 1 nmol of total dTMP into acid-precipitable material in 60 min at 37 °C in a standard assay containing 500 ng of poly(dA)/oligo(dT)10:1 and 20 μM dTTP.

RF-C-independent pol δ Assay—A final volume of 25 μl contained the following: 50 mM Tris-HCl (pH 6.5), 6 mM MgCl2, 1 mM DTT, 250 μCi/ml [3H]dTTTPs (400 cpm/pmol), 500 ng of poly(dA)/oligo(dT)12, 120 ng of PCNA (unless differently mentioned) and pol δ to be titrated. The reactions were incubated at 37 °C for the indicated times and precipitated with 10% trichloroacetic acid, and the insoluble radioactive material was determined as described (48).

RF-C-independent pol ε Assay—A final volume of 25 μl contained the following: 75 mM Hepes-NaOH (pH 7.5), 1 mM DTT, 20% (v/v) glycerol, 250 μg/ml BSA, 10 mM MgCl2, 10 mM KCl, 20 μM [3H]dTTTPs (400 cpm/pmol), 500 ng of poly(dA)/oligo(dT), and pol ε to be titrated. Reactions were analyzed as described above for pol δ.

RF-C-dependent pol ε Assay—A final volume of 25 μl contained the following: 40 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM ATP, 5 mM DTT, 200 μg/ml BSA, 15 μCi [3H]dNTPs (300 cpm/pmol), 100 ng of singly primed M13 DNA, 120 ng of PCNA, 350 ng of single-stranded DNA-binding protein, 20 ng of RF-C, and 0.25 units of pol δ (or 0.16 units pol ε). The reactions were incubated for 60 min at 37 °C, stopped, and quantified as described (48).

RF-C-dependent PCNA Loading—A recombinant PCNA form that could be artificially phosphorylated in vitro at the N terminus by cAMP-dependent protein kinase was used (45). A final volume of 25 μl contained the following: 40 mM triethanolamine HCl (pH 7.5), 200 μg/ml BSA, 10 mM MgCl2, 1 mM ATP, 1 mM DTT, 50 ng of [γ-32P]phosphorylated PCNA, 50 ng of RF-C, 60 ng of gapped circular DNA (49), and DNA ligase I in the amount indicated in the figure. The samples were incubated for 3 min at 37 °C, followed by the addition of glutaraldehyde to the final concentration of 0.1% (v/v) and further incubation at 37 °C for 10 min. The samples were then adjusted with 2.5% Ficoll-400 and marker dyes, loaded on a 0.8% agarose gel, and electrophoresed in 45 mM Tris borate buffer (pH 8.3) containing 1 mM EDTA and 0.1% SDS. The gel was stained with 10% acetic acid, 12% methanol, dried, and exposed to x-ray film. PCNA loading onto DNA was quantified by PhosphorImager (Molecular Dynamics).

Strand Displacement DNA Synthesis—The steps of the DNA substrate synthesis are described (see Ref. 43). Gapped dsDNA was first digested with KpnI and PvuII in suitable buffer conditions for 1 h at 37°C. The gapped DNA was used for the experiment without further purification. For strand displacement DNA synthesis the following components were mixed in a final volume of 25 μl: 50 mM BisTris (pH 6.5), 6 mM MgCl2, 1 mM DTT, 250 μg/ml BSA, 120 ng of PCNA, 5 μCi of [α-32P]dATP (3000 Ci/mmol), 20 ng of gapped dsDNA, and 0.25 units pol δ. The mixture was incubated for 7 min at 37 °C to allow incorporation of the first two dATPs. Then dCTP, dGTP, and dTTP (30 μM each) and DNA ligase I (in amounts as described in the figure legend) were added. DNA synthesis was allowed to continue for 30 min at 37 °C. Reactions were terminated by heating for 10 min at 70 °C. After cooling to room temperature, reactions were treated with proteinase K (60 μg/ml) in the presence of 1% SDS (w/v) and 20 mM EDTA (pH 8.0) for 30 min at 37 °C. After addition of the same volume of 100% formamide and marker dyes, samples were loaded on an 8% polyacrylamide gel, containing 7 M urea (17 × 21 × 0.8 mm). The gel was run in 1× TBE buffer at 30 V/cm until the bromphenol blue dye reached the bottom. The gel was finally fixed in 10% acetic acid, 12% methanol, dried, and exposed to x-ray film.

RESULTS

Human DNA Ligase I Inhibits DNA Elongation by pol δ—Pol δ was titrated on poly(dA)900/oligo(dT)12–18, and all reactions were carried out under conditions under which less than one nucleotide was incorporated per 3'-OH primer present, so that statistically no more than one enzyme molecule per 3'-OH primer was bound. Under these conditions most primers initiated no synthesis at all, whereas DNA synthesis starting from the few primers that were bound was the result of only one stretch of processive activity. The length of extension of the utilized 3'-OH primers reflects the processivity of the enzymes (50). Oligo(dT)12–18 was hybridized to poly(dA)100 at a weight ratio of 1:10, the equivalent of a molar ratio of 1.9:1. DNA replication reactions were carried out with 500 ng of template DNA, corresponding to 8.9 pmol 3'-OH ends. 1, 2, 5, 10, 15, and 20 pmol of DNA ligase I, respectively, were first mixed with the required amount of PCNA (1.4 pmol) and then added to the reaction tube. Replication was allowed to proceed for 30 min at 37 °C. Fig. 1A shows that DNA ligase I affected DNA elongation by pol δ in two different ways. At DNA ligase I concentrations between 40 and 200 nM the amount of nucleotides incorporated in 30 min increased by about 10–20%. An intrinsic polymerase activity of the ligase sample used could be excluded (data not shown). At higher DNA ligase I concentrations (0.4–0.8 μM) DNA synthesis was clearly inhibited to an extent of 70–85%. At 50% inhibition the calculated ratio between DNA ligase I and PCNA trimer molecules was 10:1, whereas DNA ligase I molecules were not in excess over pol δ. That the inhibitory effect is not due to DNA ligase I binding to the 3'-OH groups is indirectly shown in the experiment illustrated in Fig. 10 (see below). A competition experiment with an excess of primer ends cannot be carried out because of the ability of pol δ to bind to the competitor DNA.

DNA Ligase I Inhibition of δ Processivity Is Due to Its Interaction with PCNA—Levin and co-workers (41) demonstrated a direct interaction of DNA ligase I with PCNA; furthermore, they found that pol α, δ, and ε did not directly interact with DNA ligase I. We therefore next determined whether the DNA ligase I effects observed in Fig. 1A could be attributed to an influence on pol δ or on PCNA. Fig. 1B shows that the inhibition by DNA ligase I could be overcome with high PCNA con-
DNA Ligase I Effect on DNA Polymerases $\delta$ and $\epsilon$

Fig. 1. A, DNA ligase I inhibits RF-C-independent DNA replication by pol $\delta$. The RF-C-independent assay was performed as described under “Experimental Procedures,” with 0.016 units of pol $\delta$ and 1.4 pmol of PCNA, 1, 2, 5, 10, 15, and 20 pmol of DNA ligase I, respectively, were included in the reaction from the beginning. Therefore, we next determined whether pol $\delta$ inhibition by DNA ligase I can be partially alleviated by excess of PCNA. The RF-C-independent assay was performed as described under “Experimental Procedures,” with 0.03 units of pol $\delta$, 1, 2, 5, 10, and 15 pmol of DNA ligase I were included in the reaction, together with either 1.4 pmol of PCNA (squares) or 15 pmol of PCNA (circles). 100% of activity corresponds to the nucleotide incorporation by pol $\delta$ in the absence of DNA ligase I.

concentrations. Addition of 15 pmol of PCNA, which corresponds to the highest DNA ligase I amount present, resulted in a partial reversion of the inhibitory effect. These results therefore suggest that DNA ligase I might inhibit in vitro DNA synthesis by trapping the PCNA molecules and consequently preventing them from interacting with pol $\delta$. pol $\delta$ has very poor processivity by itself; PCNA was established as an auxiliary factor of pol $\delta$ that dramatically increased the processivity of the latter (26, 27, 51). Gel analysis of the DNA synthesis products confirmed the loss of processivity of pol $\delta$ in the presence of DNA ligase I. Only a decrease of long products (>200 nucleotides), but not of short products (<200 nucleotides), was observed (data not shown).

The Inhibitory Effect of DNA Ligase I Does Not Directly Involve pol $\delta$—From the results shown in Fig. 1B an involvement of PCNA in the inhibitory effect by DNA ligase I can be postulated. Therefore, we next determined whether pol $\delta$ had also a role in this process by performing an experiment in which two of the three proteins, pol $\delta$, DNA ligase I, and PCNA, were first allowed to form a possible “complex,” before being exposed to the rest of the assay components. In the experiment illustrated in Fig. 2A, PCNA was incubated with DNA ligase I for 2 min at 37 °C, and then the rest of the reaction mixture containing pol $\delta$ and DNA substrate was added, and DNA synthesis was allowed to proceed for 30 min. As mentioned above, DNA ligase I traps the PCNA molecules, thus preventing interaction with the pol. The expected inhibitory effect (60%) was observed. In the second case (Fig. 2B) the pol was preincubated with PCNA (2 min at 37 °C) prior to the addition of DNA ligase I and DNA. No difference in the inhibition pattern was evident. Whether pol $\delta$ and PCNA can interact in the absence of DNA is still controversial. If this were the case, the results would speak for a competition between pol and DNA ligase for PCNA, whereby DNA ligase I would be the winner. On the other hand, it would be more probable that PCNA remains free to be trapped by DNA ligase I. In the third case (Fig. 2C), DNA ligase I and pol $\delta$ were preincubated, pol $\delta$ and DNA ligase I do not interact in vitro (data not shown and Ref. 41), and in fact DNA synthesis was affected to exactly the same extent as above (Fig. 2, A and B), confirming a lack of interaction between pol $\delta$ and DNA ligase I. From this experiment it can therefore be concluded that the inhibitory effect of DNA ligase I on pol $\delta$ activity might be due to DNA ligase I interaction with PCNA and does not involve the pol itself.

DNA Ligase I Does Not Affect a Sliding pol $\delta$ Clamp—In the experiments described so far, DNA ligase I was included in the reaction from the beginning. Therefore, the next question to answer was whether DNA ligase I had any effect on an active, replicating pol $\delta$PCNA complex. DNA ligase I was added to the in vitro reaction at different time points, and the amount of nucleotides incorporated after 2, 5, 10, 20, and 30 min was followed. Results in Fig. 3 show that inhibition of DNA synthesis occurs if DNA ligase I is added during the first 5 min of synthesis. The inhibitory effect is not immediate, but a delay is observed, which is directly proportional to the elapsed time between the beginning of synthesis and addition of DNA ligase I (data not shown). When DNA ligase I was added in the reaction after 10 or 20 min, no significant inhibition was observed. This suggests that the DNA ligase I molecules do not interfere with an actively moving PCNA-pol $\delta$ complex, the interaction to PCNA being limited to free trimers.

The Inhibitory Effect of DNA Ligase I on DNA Replication by pol $\delta$ Is More Pronounced with Low Amounts of Primer Termini—Next, we investigated the behavior of DNA ligase I in the presence of different ratios of primer versus template; pol $\delta$ was titrated using different template/primer ratios, varying between a weight ratio of 25:1 and 1:1. The amount of pol $\delta$ was chosen so that no more than 1 pol molecule bound per 3’-OH primer end. Table I illustrates the different templates used as well as the results obtained after addition of DNA ligase I. The maximal inhibition of activity is exerted on a 25:1 template/primer base weight ratio; an increase of the number of 3’-OH ends per poly(dA) molecule led to a decrease of the inhibitory effect. These results might be due to the fact that more 3’-OH ends support more active pol $\delta$PCNA complexes, which are then inaccessible to the DNA ligase I molecules.

DNA Ligase I Inhibits RF-C-dependent DNA Replication by pol $\delta$ on Primed M13 DNA—RF-C-dependent DNA elongation on singly primed M13 DNA was measured during 60 min in the presence of DNA ligase I (Fig. 4). When DNA ligase I was preincubated with PCNA for 2 min on ice, prior to addition to the rest of the reaction mix, a 60% inhibition of DNA replication was observed with 15 pmol of DNA ligase I (Fig. 4, squares), a situation reflecting what was observed in the RF-C independent DNA replication assay. When, on the contrary, the holoenzyme containing RF-C, PCNA, and pol $\delta$ was first allowed to assemble onto the DNA, DNA ligase I addition did not show any effect on DNA synthesis (Fig. 4, diamonds). This suggests that the holoenzyme, once assembled on DNA, is inaccessible to any external action by DNA ligase I. On the other hand, when an interaction between PCNA and DNA ligase I is allowed, DNA synthesis is inhibited as already ob-
served in the RF-C-independent assay (Fig. 1A).

**DNA Ligase I Stimulates RF-C-Dependent Loading of PCNA onto Gapped Circular DNA**—As a first step in the assembly of the holoenzyme, RF-C loads PCNA onto DNA in an ATP-dependent manner. Therefore, we wanted to investigate whether the presence of DNA ligase I could affect in some way the loading of PCNA. For this, we used a PCNA form that carried an artificial phosphorylation site for cAMP-dependent protein kinase at its N termini (45). The loading of the labeled PCNA onto DNA was allowed to proceed for 30 min at 37 °C and stopped as described under “Experimental Procedures.” B, 1.4 pmol of PCNA and 0.02 units of pol δ were incubated 2 min at 37 °C. Then DNA ligase I (in the amounts indicated in the figure) and the reaction mix containing the DNA were added. Reaction was carried out as above. C, pol δ and various amounts of DNA ligase I were first incubated for 2 min at 37 °C. After addition of the reaction mix containing 1.4 pmol of PCNA, the DNA synthesis took place as described above. D, combination of the results shown in A–C.

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**Fig. 2. The inhibitory effect of DNA ligase I does not directly involve pol δ.** A, DNA ligase I in the amounts indicated and 1.4 pmol of PCNA were first incubated together for 2 min at 37 °C. After addition of the reaction mix containing 0.02 units of pol δ and 500 ng of poly(dA/oligo(dT)), DNA synthesis was allowed to proceed for 30 min at 37 °C and stopped as described under “Experimental Procedures.” B, 1.4 pmol of PCNA and 0.02 units of pol δ were incubated 2 min at 37 °C. Then DNA ligase I (in the amounts indicated in the figure) and the reaction mix containing the DNA were added. Reaction was carried out as above. C, pol δ and various amounts of DNA ligase I were first incubated for 2 min at 37 °C. After addition of the reaction mix containing 1.4 pmol of PCNA, the DNA synthesis took place as described above. D, combination of the results shown in A–C.

I concentration (10 or 15 pmol) the loading was stimulated only 2.5 times. The precise mechanism by which the PCNA loading is stimulated remains to be investigated (see also “Discussion”). It is surprising, however, that the reaction is not inhibited, as would be expected in the case of an interaction of PCNA with DNA ligase I instead of RF-C. Perhaps the reason for the stimulation must be sought in an eventual effect of DNA ligase I on RF-C, for example by increasing the DNA binding capacity of RF-C.

**DNA Ligase I Inhibits Strand Displacement Activity by pol δ**—To measure the effect of DNA ligase I on strand displacement activity by pol δ (31), we used a linear, double-stranded DNA with a defined gap of 26 nucleotides (43). Its final structure is shown in Fig. 6A. The DNA template was first incubated
with the pol and [α-32P]dATP at 37 °C for 5 min, to allow incorporation of the first two nucleotides (pulse). This enables the detection of a 44-nucleotide fragment. After addition of the three missing nucleotides, synthesis was allowed to continue for 30 min at 37 °C. Gap filling DNA synthesis results in a 70-nucleotide fragment. Strand displacement activity of pol gives rise to longer products of up to 344 nucleotides in length.

When DNA ligase I was added to the strand displacement reaction, an accumulation of 70-base pair products occurred that led to a simultaneous decrease in longer products resulting from strand displacement (Fig. 6B). These results suggested that DNA ligase I, most likely through its interaction with PCNA, forces pol to stop at a 5'-junction so that ligation can be performed. Such a reaction might be important in certain DNA repair events (e.g. long-patch base excision repair, see also “Discussion”).

DNA Ligase I Effect on DNA Polymerases δ and ε

**Table I**
The inhibitory effect of DNA ligase I on DNA replication by pol δ is more pronounced at low amounts of primer termini.

| Poly(dA)/oligo(dT) (w/w ratio) | Maximum inhibition (with 20 pmol DNA ligase I) |
|-------------------------------|-----------------------------------------------|
| Poly(dA)/oligo(dT) (x ratio)  | %                                             |
| 25:1                          | 71                                            |
| 10:1                          | 63.5                                          |
| 5:1                           | 56.6                                          |
| 2:1                           | 40                                            |
| 1:1                           | 35                                            |

**Fig. 3.** DNA ligase I does not affect a sliding pol clamp. 15 pmol of DNA ligase I were added to 30 μl of reaction mix (containing 1.4 pmol of PCNA and 0.03 units of pol δ) at 0, 2, 5, 10, and 20 min. 5 μl were taken out at different time points and stopped as described under “Experimental Procedures.” No addition of DNA ligase 1, squares; addition after 1 min, diamonds; addition after 2 min, circles; addition after 5 min, triangles; addition after 10 min, cross; addition after 20 min, asterisks. The DNA synthesis after 30 min in the absence of DNA ligase I was taken as 100%.

**Fig. 4.** DNA ligase I inhibits RF-C-dependent replication by pol δ. The RF-C-dependent DNA replication on singly primed M13 DNA was carried out as described under “Experimental Procedures.” Various amounts of DNA ligase I (1, 2, 5, 10, and 15 pmol) were either preincubated for 2 min on ice with PCNA before addition of the missing components (squares) or added last after holoenzyme assembly (diamonds). DNA synthesis was allowed to proceed for 60 min at 37 °C. Quantification of nucleotide incorporation was done as described under “Experimental Procedures.”

**Fig. 5.** DNA ligase I stimulates the loading of PCNA onto DNA. A, autoradiography of the agarose gel analysis of RF-C-dependent loading of radiolabeled PCNA onto gapped circular DNA. The effect of various DNA ligase I amounts (1, 2, 5, 10, and 15 pmol) is shown. B, quantification of the radioactivity co-migrating with the DNA. Loading in the absence of DNA ligase I was designated as 1.
PCNA dependence suggests that pol ε has a different relationship to PCNA than pol δ. As shown in Fig. 7, pol ε activity was clearly affected by the presence of DNA ligase I. 10 pmol of DNA ligase I could stimulate nucleotide incorporation by about 100%. Product analysis on denaturing polyacrylamide gels showed that the synthesis of all products, independent of the length, was equally stimulated (data not shown). Interestingly, the stimulation could be influenced by adding PCNA. Low concentrations of PCNA (1.4 pmol were used in the experiment shown) increased the stimulation of pol ε by DNA ligase I by 20%. High PCNA concentrations (15 pmol) did not lead to higher stimulation but on the contrary suppressed the stimulatory effect. This could be due to a similar effect as observed for pol δ, where the DNA ligase I inhibitory effect was overcome by an excess of PCNA. In a similar way, PCNA could trap DNA ligase I, preventing it from acting on pol ε. Differently to what observed with pol δ, the stimulatory action showed no delay, as stimulation could be observed immediately after DNA ligase I addition (data not shown). All together, the results obtained with pol ε suggest that the action of DNA ligase I on both pols is clearly distinguishable and implies different mechanisms.

DNA Ligase I Cannot Discriminate between pol δ and pol ε—The final question we addressed was whether DNA ligase I has any preference for pol δ over pol ε or vice versa. For this, DNA synthesis was performed on poly(dA)/oligo(dT) in the presence of both pols δ and ε under conditions favorable for pol δ, that is at pH 6.5 and in the presence of PCNA. DNA ligase I was added at the beginning of the reaction. Fig. 10 shows the results of the cohabitation of the four proteins, pol δ, pol ε, PCNA, and DNA ligase I, in the same reaction tube. Diamonds and circles represent the DNA synthesis carried out by pol δ and pol ε, respectively, in the presence of DNA ligase I. The lower extent of pol δ inhibition is due to different experimental conditions. DNA ligase I and DNA were in fact preincubated prior to the addition of the other components. This observation shows indirectly that the inhibition by DNA ligase I is not due
to its interference with the DNA substrate but that on the contrary binding of DNA ligase I to the primer ends decreases the effect observed. Squares show the effect of DNA ligase I when both pols are simultaneously present in the reaction. The curve represented with triangles derives from the addition of the picomoles incorporated by pol $d$ and pol $e$ separately. Comparison of this curve with the one where both pols are present shows that DNA ligase I does not show any preference for one determinate pol. This could be the case of those processes where pol $e$ but not pol $d$ is required.

**DISCUSSION**

Studies on DNA ligase I suggest that it is involved in several important cellular pathways such as DNA replication, DNA repair, and DNA recombination (2). The intervention of DNA ligase I in these processes implies interactions with other proteins and enzymes. A class of enzymes that plays an essential role in various DNA transactions are the pols. Among them, two were of particular interest for us, pol $d$ and pol $e$. Both pols have been shown to play a role in DNA replication, nucleotide excision repair, base excision repair, and V(D)J recombination (reviewed in Ref. 17). In this study the effect of DNA ligase I on pol $d$ and $e$ DNA synthesis was investigated. Our results show that DNA ligase I can inhibit *in vitro* RF-C-independent DNA replication by pol $d$ (Fig. 1A). A similar effect on DNA replication has been shown by Mackenney et al. (15) where an excess of DNA ligase I inhibited *in vitro* SV40 DNA replication with HeLa cell extracts. In our experiment, carried out with purified components, the inhibition was twice as strong, suggesting pol $d$ and PCNA as possible targets for DNA ligase I. Competition experiments with PCNA showed that an excess of the trimer could decrease the degree of inhibition (Fig. 1B), implying that the effect observed may be due to DNA ligase I interaction with PCNA and not with the pol itself. Along this line, results from Levin et al. (41) give evidence for an interaction of the DNA ligase I with PCNA but not with pol $a$, $d$, or $e$. The inhibitory effect of DNA ligase I is also dependent on the amount of 3'-ends present in the reaction. The highest level of inhibition
(71%) was obtained with a poly(dA)/oligo(dt) ratio of 25:1, which corresponds to one primer per template molecule. An increase of the primer/template ratio led to a decrease in the inhibitory effect (Table I). More 3'-ends mean more active pol δPCNA clamps, synthesizing shorter patches of DNA. This suggested that an active synthesizing clamp was inaccessible to the DNA ligase I. Evidence for this hypothesis came from another experiment (Fig. 3), where the effect of DNA ligase I on an active pol δPCNA clamp was investigated. The results show that inhibition of DNA synthesis was achieved only if DNA ligase I was included in the reaction within the first 5 min; once this threshold was passed, the synthesizing complex became inaccessible for DNA ligase I. Moreover, the inhibitory effect was not immediate after DNA ligase I addition, again confirming the inaccessibility to an active pol δPCNA complex. Product analysis on denaturing SDS-polyacrylamide gels showed a decrease in the amount of long products, with a shift to a majority of short products. Therefore, DNA ligase I might directly influence DNA elongation by pol δ. pol δ, in contrast to pol α and ε, has been shown to carry out limited strand displacement synthesis on single-stranded DNA templates containing two primers (31) and on gapped dsDNA (56). By using a linear dsDNA template with a defined gap, we were able to demonstrate that DNA ligase I inhibits the strand displacement capacity of pol δ (Fig. 6). After the gap filling reaction up to the 5'-end of the downstream DNA fragment, pol δ is usually able to displace the DNA strand, giving rise to longer products (31). DNA ligase I inhibits the creation of such products, enhancing the pausing of the pol at the 5'-end of the synthesized fragment. Data with a DNA ligase I-defective human cell line implicated this enzyme in the processing of Okazaki fragments (12). In our case, DNA ligase I might recognize the single-stranded break formed by the 5'-end of the upstream product and the 3'-end of the downstream product coming together, a situation similar to those created by the synthesis of Okazaki fragments. Its presence on the DNA would destabilize the pol δ complex, thereby inhibiting strand displacement activity. However, the action of DNA ligase I on pol δ does not involve any ligase activity per se, since all the effects described so far do not require ATP (data not shown).

RF-C-dependent DNA replication is inhibited by DNA ligase I in a similar way (Fig. 4); 60% inhibition of DNA synthesis is reached with 15 pmol of DNA ligase I. But when DNA ligase I was added after the formation of the pol δ holoenzyme, no inhibition was observed. These results confirm what was observed before, namely that a sliding pol complex, once assembled and performing synthesis, cannot be affected by DNA ligase I. Although the inhibitory effect observed on pol δ may be due to an interaction with PCNA, the inhibition does not affect the loading of the trimer on DNA. On the contrary, the RF-C-dependent loading of PCNA is strongly stimulated upon addition of DNA ligase I (Fig. 5). It is very likely that the stimulation seen is not due to the stabilization of PCNA at the 3'-OH end by DNA ligase I, since (i) a half-life of 23 min for PCNA on DNA has been determined (45), whereas the proteins were fixed already after 3 min incubation (see “Experimental Procedures”); (ii) the increase in the amount of loaded PCNA is not proportional to the increase in DNA ligase I concentration (see Fig. 5). The distinct effects of DNA ligase I on the elongation on the one hand and on the loading on the other hand make sense, considering the fact that PCNA has been proposed as a communicator in the different DNA transactions (25). Stimulation of PCNA loading may be of physiological relevance in processes such as DNA repair, where PCNA would recruit the required proteins to the gaps or nicks. At the same time further DNA elongation by the pol would be stopped and DNA ligase I action could immediately take place.

The DNA ligase I effect observed on pol ε was strikingly different from what was observed with pol δ. In vitro RF-C-independent DNA synthesis was stimulated by 100% upon addition of DNA ligase I (Fig. 7). The stimulation was influenced by PCNA in a concentration-dependent way. High PCNA concentrations (1:1 ratio between PCNA and DNA ligase I) eliminated the stimulatory effect, whereas low concentrations increased the stimulation. A similar effect could also be observed in the case of pol δ, where at low DNA ligase I concentrations and in the presence of 1.4 pmol of PCNA, DNA synthesis stimulation was increased by 10–20%. The reason for such an effect is unknown but is not unique, as the human cell cycle-dependent kinase inhibitor p21(CIP1, WAF1) shows a similar behavior at low concentrations (57). In RF-C-dependent DNA replication, DNA ligase I presence did not affect the pol ε holoenzyme in its activity (Fig. 8), contrary to what observed with pol δ. Finally, the kinetics of pol ε stimulation on poly(dA)/oligo(dt) (Fig. 9) was different from the one of pol δ inhibition, since the stimulatory effect was immediate.

The distinct effects of DNA ligase I on pol δ and ε suggest divergent mechanisms affecting both pols. This might implicate a different relationship between the DNA ligase I and both pol δ and pol ε holoenzymes, probably due to distinct protein-protein interactions. The N terminus of DNA ligase I has no homology to other protein sequences; therefore, it is likely to be involved in interactions with other proteins, thereby endowing the DNA ligase I with the specificity required in different cellular pathways. The inhibition of pol δ by DNA ligase I is probably achieved through direct interaction with PCNA. Nevertheless, an involvement of pol δ in this interaction, probably through other proteins, cannot be excluded. The PCNA function in pol ε stimulation is less clear, since the roles of pol ε in vivo are not yet completely established. As a gap filling enzyme it is apparently involved in DNA transactions such as lagging strand synthesis, DNA repair (nucleotide excision repair and base excision repair (38, 58)), and DNA recombination (reviewed in Ref. 17). DNA ligase I, which is essential for joining the Okazaki fragments, could increase the affinity of pol ε for primer binding. A similar function was already suggested (36) for PCNA; our results go along with these observations, since the simultaneous action of DNA ligase I and PCNA led to a higher stimulation of pol ε.

We therefore speculate that in DNA replication the DNA ligase I would control pol δ DNA synthesis on the lagging strand, preventing any strand displacement when the pol encounters the downstream DNA strand. On the other hand, pol ε, which lacks strand displacement activity, could be recruited by DNA ligase I and PCNA to the primer ends. Stimulation of synthesis could be of special necessity in the case of DNA repair. In an alarm situation, the highly processive pol δ would be slowed, favoring either immediate ligation or stimulating pol ε for filling the gap. In summary, our data suggest that DNA ligase I might modulate different roles for pol δ and pol ε in DNA replication or DNA repair. PCNA as an intracellular communicator (25) could therein act as the selective partner for DNA ligase I.

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