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An interaction between DNA ligase I and proliferating cell nuclear antigen: Implications for Okazaki fragment synthesis and joining

(DNA repair/DNA replication)

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ABSTRACT **Although three human genes encoding DNA ligases have been isolated, the molecular mechanisms by which these gene products specifically participate in different DNA transactions are not well understood. In this study, fractionation of a HeLa nuclear extract by DNA ligase I affinity chromatography resulted in the specific retention of a replication protein, proliferating cell nuclear antigen (PCNA), by the affinity resin. Subsequent experiments demonstrated that DNA ligase I and PCNA interact directly via the amino-terminal 118 aa of DNA ligase I, the same region of DNA ligase I that is required for localization of this enzyme at replication foci during S phase. PCNA, which forms a sliding clamp around duplex DNA, interacts with DNA pol δ and enables this enzyme to synthesize DNA processively. An interaction between DNA ligase I and PCNA that is topologically linked to DNA was detected. However, DNA ligase I inhibited PCNA-dependent DNA synthesis by DNA pol δ. These observations suggest that a ternary complex of DNA ligase I, PCNA and DNA pol δ does not form on a gapped DNA template. Consistent with this idea, the cell cycle inhibitor p21, which also interacts with PCNA and inhibits processive DNA synthesis by DNA pol δ, disrupts the DNA ligase I–PCNA complex. Thus, we propose that after Okazaki fragment DNA synthesis is completed by a PCNA–DNA pol δ complex, DNA pol δ is released, allowing DNA ligase I to bind to PCNA at the nick between adjacent Okazaki fragments and catalyze phosphodiester bond formation.**

DNA-joining events are required during the replication of the mammalian genome because of the discontinuous nature of lagging strand DNA synthesis. There is substantial biochemical evidence indicating that DNA ligase I, which is one of four DNA ligases in mammalian cells (1, 2), is the enzyme responsible for joining Okazaki fragments. The involvement of DNA ligase I in DNA replication was first suggested by studies demonstrating an elevated level of this enzyme activity in regenerating liver compared with normal liver (3). More recently, DNA ligase I has been identified as a component of a high molecular weight replication complex (4, 5) and has been shown to efficiently join Okazaki fragments in a DNA replication assay with other highly purified replication proteins (6, 7).

In agreement with this putative role in DNA replication, human DNA ligase I cDNA complements the conditional lethal phenotype of a *Saccharomyces cerevisiae cdc9* DNA ligase mutant (8), and *LIG1* homozygous null mouse embryonic stem cells are viable only when a full-length DNA ligase I cDNA is ectopically expressed (9). Moreover, a DNA ligase I mutant human cell line, 46BR, exhibits abnormal joining of Okazaki fragments (10–13). The replication defect in extracts from this cell line can be complemented by the addition of DNA ligase I but not by the addition of DNA ligase III or T4 DNA ligase (14).

DNA polymerases α and δ, replication protein A, proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), RNase H, FEN1, and DNA ligase I are required to reconstitute lagging strand DNA synthesis (7). Here we describe an interaction between DNA ligase I and PCNA that explains at the molecular level why DNA ligase I is uniquely able to join adjacent Okazaki fragments. We suggest that DNA ligase I is recruited to the nick between Okazaki fragments by binding to PCNA, which is left in this position after the completion of Okazaki fragment DNA synthesis by the PCNA–DNA pol δ complex. This interaction between PCNA and DNA ligase I may also be required for the completion of DNA repair pathways in which both these proteins participate.

MATERIALS AND METHODS

Materials. Plasmids encoding human PCNA and glutathione *S*-transferase (GST)-p21 and a polyclonal PCNA antibody (15) were gifts from Phang-Lang Chen. Human PCNA was purified from an *Escherichia coli* strain that expresses human *PCNA* cDNA from an inducible T7 promoter (16). Calf thymus DNA pol δ was provided by Cheng-Keat Tan and Antero So (17). Recombinant human DNA ligase I was purified from baculovirus-infected insect cells (18) and used to produce a specific rabbit antiserum as described (19). RF-C was purified from HeLa cytosolic extracts as described (20). A monoclonal antibody specific for PCNA (PC10) was from Santa Cruz Biotechnology. The DNA pol α antibody, DPN (21), and DNA pol ε monoclonal antibody (22) were provided by Stuart Linn. A monoclonal antibody specific for DNA pol δ (23) was provided by Marrietta Lee.

Preparation of Affi-Gel Affinity Resins. Recombinant human DNA ligase I (9 mg) and BSA (19 mg) were each dialyzed against 25 mM Hepes·KOH (pH 8.0), 0.2 M NaCl, and 20% glycerol (buffer A), and then incubated with Affi-Gel 10 beads (9 ml, Bio-Rad). After mixing by rotation for 6 h at 4°C, the beads were collected by centrifugation and the protein content of the supernatant was determined by the method of Bradford (24) using BSA as the standard. Greater than 90% of DNA ligase I was coupled to the beads whereas only about 50% of BSA was coupled to the beads. The remaining reactive groups

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Abbreviations: GST, glutathione *S*-transferase; PCNA, proliferating cell nuclear antigen; pol, polymerase; RF-C, replication factor C.

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on the beads were inactivated by incubation with 100 mM ethanolamine (pH 8.4) for 1 h at 4°C.

Fractionation of a HeLa Nuclear Extract by Affinity Chromatography. Nuclear and cytosolic extracts were prepared from a frozen pellet of HeLa S3 cells ($\approx 10^9$ cells) as described (4). The nuclear extract (20 mg) and the cytoplasmic/soluble extract (30 mg) were dialyzed against 50 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride, 0.4 μg/ml aprotinin, 0.5 μg/ml chymostatin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin (buffer B). DNA ligase I beads (5 ml of a 50% slurry) and BSA beads (5 ml of a 50% slurry) were each preequilibrated with buffer B and then incubated with 10 mg of nuclear extract for 2 h at 4°C. The beads were poured into a column and then washed extensively with buffer B. Bound proteins were eluted stepwise with buffer B containing 0.15, 0.3, and 1.0 M NaCl (12.5 ml of each buffer). Fractions (500 μl) were analyzed for protein by silver staining (Bio-Rad) after separation by SDS/PAGE (25).

Immunoblotting. Proteins were transferred electrophoretically from polyacrylamide gels to nitrocellulose membranes. Antigen–antibody complexes were detected by enhanced chemiluminescence (Pierce).

***In Vitro* Transcription and Translation.** Coupled *in vitro* transcription and translation reactions (50 μl) were performed as described (26). Labeled polypeptides were resuspended in 50 μl of 50 mM Hepes (pH 7.7), 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol, and 0.1% Nonidet P40 (buffer C).

Interaction of Recombinant PCNA with DNA ligase I– and BSA–Affinity Resins. DNA ligase I beads (20 μl) and BSA beads (20 μl) were resuspended in 240 μl of buffer C containing 2% dried milk and rocked at 4°C for 20 min. After the addition of purified recombinant PCNA (800 ng) in 240 μl of buffer C, incubation was continued for 2 h at 4°C. The beads were washed with and then resuspended in buffer C (20 μl). After the addition of an equal volume of SDS sample buffer, samples were heated at 100°C for 3 min before the separation of polypeptides by SDS/PAGE. PCNA was detected by immunoblotting.

Radiolabeling of Recombinant PCNA. Recombinant PCNA with a poly (histidine) sequence and kinase site at the amino terminus (pK-PCNA) was purified from *E. coli* BL21 (DE3) cells harboring a plasmid that expresses the recombinant PCNA from an inducible T7 promoter (27). Purified pK-PCNA was radiolabeled with [γ-^{32}P]ATP using cAMP-dependent protein kinase to a specific activity of 24 cpm/fmol as described (28).

Loading of Labeled PCNA onto Nicked DNA. Supercoiled plasmid DNA, pQE16 (Qiagen), was incubated with DNase I to generate nicked circular duplexes with approximately 1 nick per molecule (29). Nicked plasmid DNA (4 μg) was incubated with 29.5 pmol of trimeric ^{32}P-labeled PCNA and 2.18 units replication factor C (RF-C) in buffer D (20 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 40 μg/ml BSA, 5 mM DTT, 8 mM $MgCl_2$, 4% glycerol, and 4 mM ATP) for 10 min at 37°C. DNA-bound PCNA was separated from free PCNA by filtration through a 5-ml Bio-Gel A15 m (Bio-Rad) column (30). Fractions containing the DNA-bound PCNA molecules were pooled and stored in 200-μl aliquots at −80°C.

Formation of Labeled DNA Ligase I–Adenylate Complex. Recombinant DNA ligase I (98 pmol) was incubated with 290 μCi [α-^{32}P]ATP (10 mCi/ml, 3,000 Ci/mol, Amersham) in 60 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, and 50 mg/ml BSA for 15 min at 25°C (2, 19).

Interaction Between DNA-Bound PCNA and DNA Ligase I. DNA-bound PCNA (2 pmol PCNA trimer, 1 μg nicked plasmid DNA) was incubated with adenylated DNA ligase I (45 pmol) or in the absence of DNA ligase I in buffer D minus ATP (final volume, 225 μl) for 6 min at 37°C. In addition, adenylated DNA ligase I (45 pmol) was incubated with nicked plasmid DNA (1 μg) in buffer D minus ATP (final volume, 225 μl) for 6 min at 37°C. Reactions were stopped by placing them on ice. DNA–protein complexes were separated from unbound proteins by gel filtration as described above. In assays without DNA ligase I, the elution of labeled PCNA was monitored by liquid scintillation counting. Approximately 40% of labeled PCNA was recovered. In assays with labeled DNA ligase I, fractions from the gel filtration column were separated by denaturing gel electrophoresis and labeled proteins were detected and quantitated by PhosphorImager (Molecular Dynamics) analysis.

Pull-Down Assays with Glutathione Sepharose Beads. Glutathione Sepharose beads (30 μl) with either GST or GST fusion proteins as the ligand were equilibrated with and resuspended in buffer C before incubation, with rocking, for 30 min at room temperature with labeled *in vitro*-translated DNA ligase I in a final volume of 150 μl. After centrifugation, the supernatant was collected and the beads were washed with buffer C before incubation at 100°C for 3 min in the presence of SDS sample buffer (20 μl). After SDS/PAGE, proteins were detected by fluorography (26).

Immunoprecipitation Assay. Recombinant DNA ligase I (1 μg) and PCNA (0.5 μg) were preincubated in buffer C (50 μl final volume) for 20 min at room temperature before the addition of DNA ligase I antiserum diluted in 200 μl of buffer C containing 2% dried milk. After incubation at 4°C for 1 h, protein A Sepharose beads (30 μl, Pharmacia) were added and the incubation was continued with rocking for 1 h. After washing with buffer C, the beads were divided into three equal aliquots. The immunoprecipitates were incubated with either GST-p21 (1 μg) or GST (1 μg) for 20 min at room temperature. After centrifugation, the supernatants were collected and the beads were washed with buffer C. The supernatants were incubated with glutathione Sepharose beads (20 μl) with either GST-p21 or GST as the ligand as described above. Proteins bound specifically to the protein A or glutathione beads were separated by SDS/PAGE and detected by immunoblotting.

Purification of GST and GST Fusion Proteins. Human *PCNA* cDNA encoding amino acid residues 1–261 (full length) and residues 1–127 were subcloned in-frame into pGEX-3X (Pharmacia) to generate plasmids encoding GST-PCNA fusion proteins. In addition, a PCNA cDNA fragment was subcloned in-frame into pGEX-2T (Pharmacia) to produce a plasmid that directed the expression of a GST-PCNA fusion protein containing amino acid residues 127–261 of PCNA. After induction of plasmid-encoded GST and GST fusion proteins, cell lysates were prepared and protein expression was examined as described previously (26). Glutathione Sepharose beads with approximately equivalent amounts of either GST or GST fusion proteins as the ligand were produced (26). GST and GST-p21 proteins were eluted from the glutathione Sepharose beads with 10 mM reduced glutathione according to the manufacturer's protocol (Pharmacia).

DNA Polymerase Assay. DNA polymerase δ activity was assayed with poly(dA)–oligo(dT_{16}) (10:1 nucleotide ratio) as template-primer essentially as described previously (17, 31). Reaction mixtures (60 μl) that contained 0.2 units of DNA polymerase δ were incubated for 30 min at 37°C. PCNA and DNA ligase I were included in the reactions as described in the figure legend.

RESULTS

Interaction of PCNA with a DNA ligase I–Affinity Resin. We hypothesized that the unique involvement of DNA ligase I in DNA replication is mediated by specific interactions with other proteins participating in lagging strand DNA synthesis. To identify these proteins, we fractionated a HeLa cell nuclear extract by DNA ligase I–affinity chromatography. Initially we

considered DNA polymerases as potential DNA ligase I-interacting proteins because an interaction between DNA ligase I and DNA pol β had been described previously (32) and it remains to be determined whether DNA pol δ or DNA pol ε completes the synthesis of Okazaki fragments during chromosomal DNA replication (7). However, we failed to detect specific binding of DNA pol α, DNA pol δ, or DNA pol ε to the DNA ligase I–affinity resin (Fig. 1*A*).

In contrast, PCNA was detected by immunoblotting in the 150- and 300-mM NaCl eluates from the DNA ligase I column but not in eluates from the BSA column (Fig. 1*A*). The amount of PCNA in the pass-through fractions from the DNA ligase I column was significantly lower than that in the flow-through fractions from the BSA column (data not shown). By quantitating the amounts of PCNA that were loaded onto and eluted from the DNA ligase I column by immunoblotting, we estimate that greater than 50% of the PCNA molecules in the nuclear extract bound to the DNA ligase I column.

The binding of PCNA to the DNA ligase I–affinity resin could reflect an interaction between a PCNA-associated protein(s) and DNA ligase I. Alternatively, it could be a result of the binding of DNA ligase I and other proteins to nucleic acid. To address this issue, recombinant PCNA was incubated with the DNA ligase I and BSA resins. The specific binding of recombinant PCNA, either purified after overexpression in *E. coli* (Fig. 1*B*) or *in vitro*-translated (data not shown) to the DNA ligase I resin, demonstrates that PCNA and DNA ligase I interact directly. A complex containing DNA ligase I and PCNA has also been detected by gel filtration, sucrose density gradient sedimentation, and glutaraldehyde cross-linking after preincubation of DNA ligase I purified from baculovirus-infected insect cells and PCNA purified from *E. coli* (data not shown).

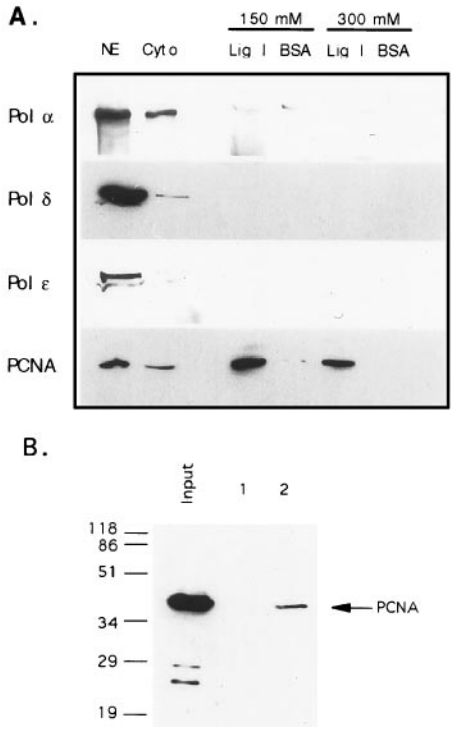

FIG. 1. Interaction of PCNA with a DNA ligase I–affinity resin. (*A*) A HeLa nuclear extract was applied to and eluted from DNA ligase I– and BSA–affinity columns as described in *Materials and Methods*. Proteins in the HeLa nuclear extract (NE, 30 μg), the cytoplasmic extract (Cyto, 30 μg), and in the peak fractions of the 150 and 300 mM NaCl eluates from the DNA ligase I column (Lig I) and the BSA column (BSA) were separated by SDS/PAGE, transferred to nitrocellulose membranes, and incubated with the indicated antibody. (*B*) Purified recombinant PCNA (800 ng) was incubated with: lane 1, BSA beads and lane 2, DNA ligase I beads as described in *Materials and Methods*. After separation of proteins that bound to the beads by denaturing gel electrophoresis, PCNA was detected by immunoblotting with the polyclonal PCNA antibody. A sample of recombinant PCNA (800 ng, Input) was included in the immunoblot. The positions of prestained molecular mass standards (Bio-Rad) are indicated on the left.

DNA Ligase I Interacts with PCNA Trimers That Are Topologically Linked to Duplex DNA. PCNA exists as a homotrimeric ring that interacts with the replication proteins RF-C and DNA pol δ (33, 34). RF-C loads PCNA onto DNA so that the DNA helix runs through the central hole of the trimer (30, 35, 36). The DNA-bound PCNA acts as a sliding clamp that tethers interacting DNA polymerases to the DNA template (33, 36, 37). In the previous section we demonstrated that DNA ligase I and PCNA can interact in the absence of DNA. In the context of lagging strand DNA synthesis (7, 38), it seems likely that this interaction will occur between DNA ligase I and PCNA that is topologically linked to DNA. To test this idea, ^{32}P-labeled PCNA was loaded onto nicked, circular duplex DNA (30) and the PCNA–DNA complexes were incubated with DNA ligase I that had been labeled by adenylation (39). In the absence of DNA ligase I, DNA-bound PCNA molecules were detected in fractions corresponding to the void volume of the Bio-Gel A15m gel filtration column (Fig. 2*A*, fractions 9–11). When DNA ligase I was preincubated with the DNA-bound PCNA before gel filtration, a similar amount of DNA-bound PCNA was recovered, indicating that DNA ligase I does not effect the stability of the PCNA–DNA complex under these conditions. However, a fraction of the DNA ligase I molecules (3% of input) reproducibly coeluted with DNA-bound PCNA in fractions corresponding to the void volume (Fig. 2*B*, fractions 9–11). Within these fractions we estimate that PCNA trimers and DNA ligase I are present in approximately equimolar amounts.

The presence of DNA ligase I in the high molecular fractions could be the result of DNA ligase I binding to either DNA-bound PCNA or the nicked duplex DNA. To distinguish between these possibilities, similar assays were carried out with an equivalent amount of the DNA substrate without topologically linked PCNA (Fig. 2*C*). No peak of DNA ligase I was detected in the void volume fractions (compare Fig. 2 *B* and *C*, fractions 9–12), demonstrating that DNA ligase I does not bind directly to the nicked duplex DNA under these reaction conditions. Thus, we conclude that the presence of DNA ligase I in the high molecular weight fractions is the result of an interaction between this enzyme and PCNA trimers that are topologically linked to DNA.

The Amino Terminus of DNA Ligase I Binds to PCNA. To map the region of DNA ligase I that interacts with PCNA, deletions of human DNA ligase I cDNA were made and, after expression of the resultant truncated polypeptides by coupled *in vitro* transcription and translation, we examined their reactivity with a GST-PCNA fusion protein. A truncated form of DNA ligase I consisting of the C-terminal 440 aa, which constitute the catalytic domain of this enzyme (19), does not interact with GST-PCNA or GST (Fig. 3). In contrast, full-length DNA ligase I (919 residues), a version of DNA ligase I with an internal deletion of residues 212–790 and amino-terminal fragments of DNA ligase I corresponding to residues 1–261 and 1–118, all bind to the glutathione Sepharose beads with GST-PCNA as the ligand but not to the glutathione Sepharose beads with GST as the ligand (Fig. 3). Thus, it appears that the amino-terminal 118 residues of DNA ligase I are necessary for the interaction with PCNA.

Because *in vitro*-translated DNA ligase I (Fig. 3) and purified, recombinant DNA ligase I (data not shown) bound specifically to glutathione beads with GST-PCNA as the ligand, we constructed and purified GST-PCNA fusion proteins containing different fragments of PCNA in an attempt to

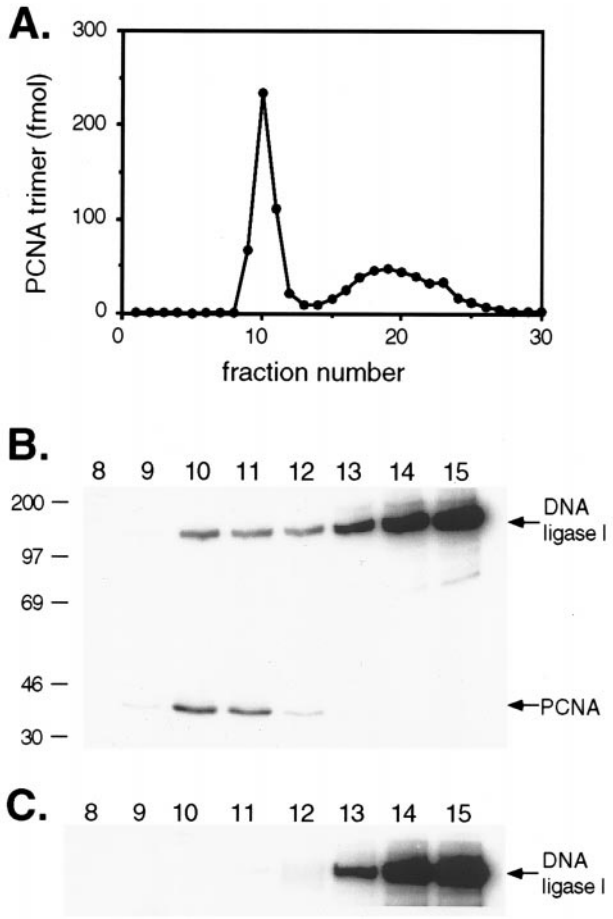

FIG. 2. Formation of DNA–protein complexes by PCNA and DNA ligase I. Nicked plasmid DNA, DNA bound-PCNA, and DNA ligase I were incubated and then fractionated by gel filtration as described in *Materials and Methods*. (*A*) Radiolabeled PCNA trimers (2 pmol trimer) that had been loaded onto nicked, circular DNA duplexes (1 μg). ^{32}P-labeled PCNA was detected by liquid scintillation counting. (*B*) Radiolabeled PCNA trimers (2 pmol trimer) bound to DNA incubated with ^{32}P-labeled DNA ligase I (44 pmol). (*C*) ^{32}P-labeled DNA ligase I (44 pmol) incubated with nicked, circular DNA duplexes (1 μg). After separation by denaturing gel electrophoresis, labeled proteins were detected and quantitated by PhosphorImager analysis. The positions of DNA ligase I and PCNA are indicated on the right.

map the region of PCNA that interacts with DNA ligase I. However, recombinant DNA ligase I did not bind to glutathione beads with GST-PCNA fusion proteins containing either the amino-terminal 127 or the carboxyl-terminal 135 residues of PCNA as the ligand (data not shown). Although the absence of binding may be a result of inappropriate folding of the PCNA fragments, it is also possible that DNA ligase I may bind either to the central region of PCNA, which is also known as the interdomain connector loop (33) and is disrupted in the truncated versions of PCNA, or to more than one region of PCNA, as is the case for both RF-C and DNA pol δ when they interact with PCNA (40).

Dissociation of the DNA Ligase I–PCNA Complex by the Cell Cycle Inhibitor, p21. Because the cell cycle inhibitor p21 binds to the interdomain connector loop of PCNA (41, 42), we examined whether DNA ligase I and p21 can bind to the same PCNA molecule by coincubating DNA ligase I and PCNA with either GST-p21 or GST fusion proteins attached to glutathione beads. As expected, PCNA bound to the GST-p21 beads but not to the GST beads (Fig. 4*A*). However, the binding of PCNA to p21 did not result in the specific retention of DNA ligase I by the GST-p21 beads (Fig. 4*A*). To exclude the possibility that only PCNA molecules not complexed with DNA ligase I bound

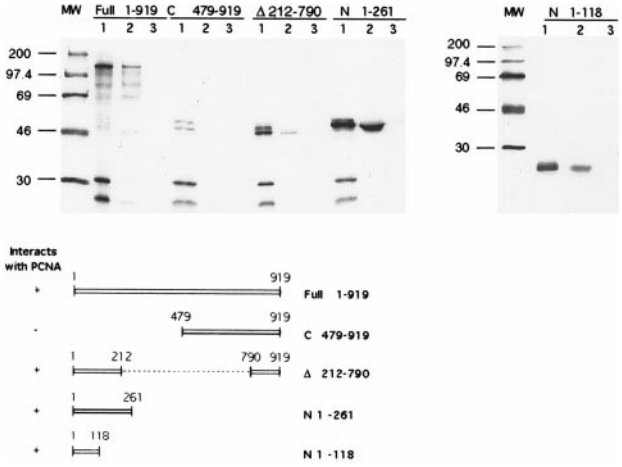

FIG. 3. Interaction of *in vitro*-translated DNA ligase I polypeptides with GST-PCNA. DNA ligase I and the deleted versions of DNA ligase I indicated were synthesized by coupled *in vitro* transcription and translation and then incubated with either GST-PCNA or GST beads as described in *Materials and Methods*. After separation by SDS/PAGE, labeled polypeptides were detected by fluorography. The positions of labeled molecular mass standards (Amersham) are indicated on the left. Lane 1, *in vitro*-translated polypeptide (one-tenth of input into binding reaction). Labeled polypeptides bound to: lane 2, GST-PCNA beads and lane 3, GST beads.

to the GST-p21 beads, the amount of PCNA remaining in the supernatants was quantitated by immunoblotting. Incubation with the GST-p21 beads depleted essentially all of the PCNA from the supernatant (Fig. 4*A*), implying that the interaction of p21 with PCNA disrupts the DNA ligase I–PCNA complex.

To address this issue more directly, the DNA ligase I–PCNA complex was immunoprecipitated with a DNA ligase I antiserum (Fig. 4*B*) and then incubated with either GST-p21 or GST. In agreement with the previous result, PCNA was released from the immunoprecipitated DNA ligase I–PCNA complex by incubation with GST-p21 but not with GST (Fig. 4*C*). Furthermore, we were able to specifically deplete a GST-p21–PCNA complex from the supernatant of the immunoprecipitates that had been incubated with GST-p21 by the addition of glutathione beads (Fig. 4*C*). These results are consistent with p21 disrupting the DNA ligase I–PCNA complex by binding to at least part of the region of PCNA involved in the interaction with DNA ligase I and forming a p21–PCNA complex.

The ability of PCNA to Function as a Processivity Factor for DNA Polymerase δ Is Inhibited by DNA Ligase I. To gain further insights into the functional consequences of the interaction between DNA ligase I and PCNA, we examined the effect of PCNA on DNA joining by DNA ligase I. Preincubation of differing amounts of PCNA with linear duplex substrates containing a single nick (43) before the addition of DNA ligase I had no significant effect on DNA joining. Similar results were obtained in assays with PCNA-bound circular DNA substrates containing a single nick (data not shown). These results can be explained if the interaction of DNA ligase I with DNA-bound PCNA tethers DNA ligase I to the DNA duplex but does not significantly enhance the ability of DNA ligase I to locate nicks within the DNA molecule.

Next we sought to determine whether DNA ligase I effects the ability of PCNA to act as a processivity factor for DNA pol δ (37). Preincubation of increasing amounts of DNA ligase I with PCNA inhibited PCNA-dependent DNA synthesis by DNA pol δ in a concentration-dependent manner, whereas the addition of DNA ligase I in the absence of PCNA resulted in a small stimulation of DNA synthesis by DNA pol δ (Fig. 5). The addition of 41.1 pmol of DNA ligase I to 4.6 pmol of

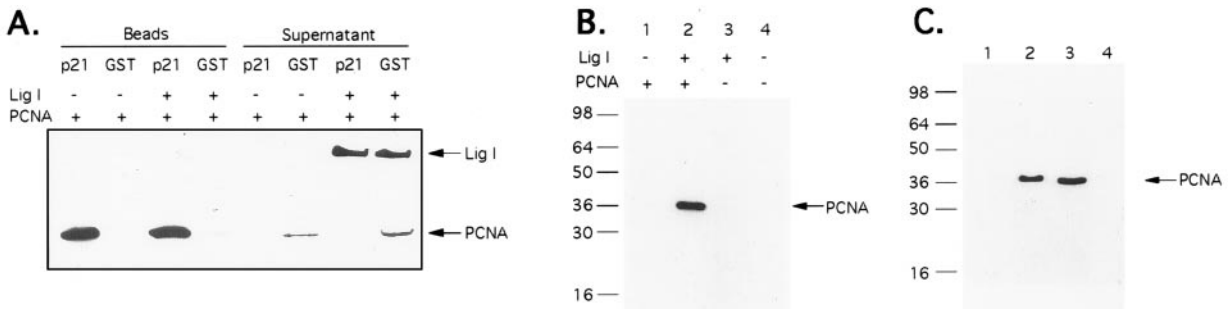

FIG. 4. Interaction of the DNA ligase I–PCNA complex with GST-p21 beads. (*A*) DNA ligase I (180 ng) and PCNA (60 ng) were preincubated before incubation with either GST or GST-p21 beads as described in *Materials and Methods*. Proteins specifically bound to the beads or remaining in the supernatant were detected by immunoblotting with the polyclonal DNA ligase I antiserum and the PCNA monoclonal antibody after denaturing gel electrophoresis. DNA ligase I and PCNA are indicated on the right. (*B*) DNA ligase (1 μg) and PCNA (0.5 μg) were preincubated as indicated and then immunoprecipitated with DNA ligase I antiserum as described in *Materials and Methods*. PCNA was detected in the immunoprecipitates by immunoblotting with the PCNA monoclonal antibody. (*C*) Equal amounts of the immunoprecipitated DNA ligase I–PCNA complex were incubated with GST (1 μg) or GST-p21 (1 μg). The protein A beads were removed by centrifugation, and the supernatants were incubated with glutathione beads. After separation of proteins bound to beads by SDS/PAGE, PCNA was detected by immunoblotting with the PCNA monoclonal antibody. Protein A beads incubated with: lane 1, GST-p21 and lane 2, GST. Glutathione beads incubated with: lane 3, GST-p21 supernatant and lane 4, GST supernatant. The positions of prestained molecular mass standards (Novex) are indicated on the left.

PCNA reduced DNA synthesis by DNA pol δ from 71.8 pmol to 18.8 pmol. In contrast, the same amount of DNA ligase I in the absence of PCNA increased DNA synthesis by DNA pol δ from 3.7 pmol to 16.6 pmol. After subtracting PCNA-independent DNA synthesis (3.7 pmol), we estimate that a 9-fold molar excess of DNA ligase I inhibits PCNA-dependent DNA synthesis by at least 75%. It is unlikely that this inhibition is a result of DNA joining by DNA ligase I because of the absence of both ATP and a 5′ phosphate on the oligo(dT) of the DNA substrate in these assays. However, these results are consistent with DNA ligase I and DNA pol δ binding to the same or overlapping regions of PCNA.

DISCUSSION

In this report we describe an interaction between DNA ligase I and PCNA that underlies the specific involvement of DNA ligase I in DNA replication. The association between DNA ligase I and PCNA was detected initially by fractionation of a HeLa nuclear extract by DNA ligase I–affinity chromatography. Subsequently, the direct nature of this interaction was demonstrated in experiments with purified recombinant proteins. These observations provide an explanation at the molecular level for previous biochemical and cell biology studies linking DNA ligase I with DNA replication that include identification of DNA ligase I as a component of a high molecular weight replication complex (4, 5), colocalization of DNA ligase I with replication factories (44), and the specific requirement for DNA ligase I when DNA replication is reconstituted with highly purified proteins (6).

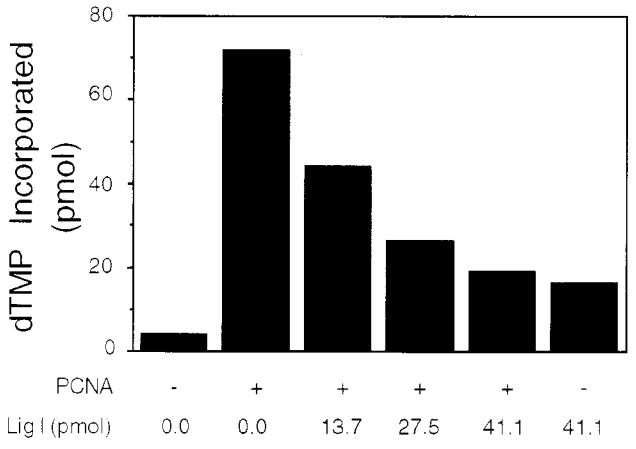

FIG. 5. Effect of DNA ligase I on PCNA-dependent DNA synthesis by DNA polymerase δ. PCNA (132 ng, 4.6 pmol) was preincubated with DNA ligase I as indicated for 30 min at 37°C in a final volume of 20 μl. BSA was added to the reactions so that the final protein concentration was the same in each assay. Preincubated proteins were added to reaction mixtures containing oligo(dT)/poly(dA), [^{3}H]TTP, and 0.2 units DNA pol δ in a final volume of 60 μl, and incubation was continued for 30 min at 37°C. Incorporation of [^{3}H]TMP was measured as described in *Materials and Methods*.

During DNA synthesis, PCNA functions as a trimeric sliding clamp, tethering DNA pol δ to the template and ensuring processive DNA synthesis (7). In this study we have shown that, under conditions where DNA ligase I does not bind to nicked duplex DNA, DNA ligase I is able to form a stable complex with PCNA molecules that are topologically linked to DNA. Thus, the interaction of DNA ligase I with PCNA results in the recruitment of DNA ligase I to DNA. However, PCNA does not stimulate DNA joining by DNA ligase I *in vitro*. This suggests that, although the binding of DNA ligase I to DNA-bound PCNA tethers DNA ligase I to DNA, this interaction alone does not result in DNA ligase I finding nicks in duplex DNA more efficiently.

At the *E. coli* replication fork, the β protein sliding clamp is left at the nick between Okazaki fragments while the DNA polymerase, having completed DNA synthesis, is released (45). If PCNA behaves in an identical manner during lagging strand synthesis in mammalian cells, the binding of DNA ligase I to the DNA-bound PCNA clamp during or after the completion of Okazaki fragment DNA synthesis would result in the recruitment of DNA ligase I to the nick between adjacent Okazaki fragments. Because the binding of p21 to PCNA disrupts the DNA ligase I–PCNA complex and both p21 (46) and DNA ligase I inhibit the ability of PCNA to act as a processivity factor for DNA pol δ (37), it appears that the binding of DNA ligase I and DNA pol δ to PCNA are mutually exclusive. Thus, we propose that the RNA–DNA oligonucleotides synthesized by the DNA pol α holoenzyme are extended up to the 5′ phosphate of the adjacent Okazaki fragment by a PCNA–DNA pol δ complex and then DNA pol δ is released. If PCNA is retained at the nick between adjacent Okazaki fragments by an as yet unknown mechanism, the subsequent binding of DNA ligase I to PCNA would position this enzyme so that it can join the Okazaki fragments together.

PCNA interacts with the N-terminal 118 aa of DNA ligase I. Although this region of DNA ligase I is dispensable for catalytic activity (19), it is essential for *in vivo* function (9). Further support for the idea that the N-terminal domain of

DNA ligase I is involved in critical interactions with other replication proteins has been provided by a recent study demonstrating that addition of the N-terminal domain but not the catalytic C-terminal domain of DNA ligase I to a cell-free extract inhibits T antigen-dependent replication of a plasmid containing the simian virus 40 replication origin (14). Interestingly, the amino-terminal domain of DNA ligase I is required for the localization of this enzyme at replication factories (44). Thus, the specific recruitment of DNA ligase I to replication factories during S phase may be mediated, at least in part, by an interaction with PCNA. Alternatively, PCNA binding and subnuclear localization to replication factories may be two separate functions that reside within the amino-terminal domain of DNA ligase I.

The DNA repair-deficient phenotype of the DNA ligase I mutant cell line 46BR indicates that DNA ligase I also functions in DNA repair (12, 13). Moreover, PCNA is required for DNA nucleotide excision repair (47) and DNA mismatch repair (48). Therefore, we suggest that the dynamic interaction between DNA ligase I and PCNA, which occurs in Okazaki fragment metabolism, may also be involved in the gap-filling and DNA-joining steps of these DNA excision repair pathways.

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