Published in final edited form as:

DNA Repair (Amst). 2009 August 6; 8(8): 912–919. doi:10.1016/j.dnarep.2009.05.002.

The DNA binding domain of human DNA ligase I interacts with both nicked DNA and the DNA sliding clamps, PCNA and hRad9-hRad1-hHus1

Wei Song^{1,#}, John M. Pascal², Tom Ellenberger³, and Alan E. Tomkinson^{1,*}

¹Radiation Oncology Research Laboratory and Marlene & Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore

²Department of Biochemistry and Molecular Biology, Thomas Jefferson University; Philadelphia

³Department of Biochemistry and Molecular Biophysics, Washington U. School of Medicine, St. Louis

Abstract

The participation of the DNA ligase (hLigI) encoded by the human *LIG1* gene in DNA replication and repair is mediated by an interaction with proliferating cell nuclear antigen (PCNA), a homotrimeric DNA sliding clamp. Interestingly, the catalytic fragment of hLigI encircles a DNA nick forming a ring that is similar in size and shape to the PCNA ring. Here we show that the DNA binding domain (DBD) within the hLigI catalytic fragment interacts with both PCNA and the heterotrimeric cell-cycle checkpoint clamp, hRad9-hRad1-hHus1 (9-1-1). The DBD preferentially binds to trimeric PCNA and the hRad1 subunit of 9-1-1. Unlike the majority of PCNA interacting proteins, the DBD does not interact with the interdomain connector loop region of PCNA but instead appears to interact with regions adjacent to the intersubunit interfaces within the PCNA trimer. Notably, the DBD not only binds specifically to DNA nicks but also mediates the formation of DNA protein complexes with PCNA. Based on these results, we suggest that the interface between the DBD and PCNA acts as a pivot facilitating the transition of the hLigI catalytic region fragment from an extended conformation to a ring structure when it engages a DNA nick.

INTRODUCTION

Among the multiple DNA ligases in human cells, DNA ligase I (hLigI) plays the major role in DNA replication. This enzyme is recruited to DNA replication foci through an interaction with proliferating cell nuclear antigen (PCNA), a homotrimeric ring protein that encircles and slides along DNA [1,2]. As with many other PCNA-interacting proteins, hLigI contains a conserved PCNA Interacting Peptide (PIP) box [2,3], which is located at its amino terminus

© 2009 Elsevier B.V. All rights reserved.

*Corresponding author; Alan Tomkinson, Radiation Oncology Research Laboratory, Bressler Research Building 7-025, 655 West Baltimore Street, University of Maryland School of Medicine, Baltimore, MD21201, atomkinson@som.umaryland.edu, Tel. No. 410-706-2365

#Present address; Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

(Fig. 1). Inactivation of the hLigI PIP box markedly reduces stable binding with PCNA *in vitro* and also abolishes the targeting of hLigI to replication foci and the ability of hLigI to correct the defects in Okazaki fragment joining and long patch base excision repair of DNA ligase I-deficient human cells [2,4].

By encircling and sliding along DNA, PCNA acts as a DNA polymerase processivity factor [5]. PCNA that is topologically linked to nicked circular DNA also tethers hLigI to DNA but does not significantly stimulate DNA joining [1]. With nicked linear DNA, a modest increase in joining by hLigI occurs in reactions containing high concentrations of PCNA [6]. Interestingly, this stimulatory effect is abolished when the DNA ends are blocked, suggesting that it is dependent upon PCNA encircling the DNA [6]. The recent atomic resolution structure of hLigI complexed with nicked DNA revealed that the catalytic fragment of hLigI contains three domains, DNA binding (DBD), adenylation (AdD) and OB-fold (OBD) (Fig. 1), each of which contacts the DNA forming a ring structure around the DNA nick [7]. Since hLigI has an elongated asymmetric shape in the absence of DNA [8], it appears that the enzyme undergoes a large conformational change when it engages nicked DNA. Intriguingly, the size and shape of the ring formed by the hLigI catalytic fragment, which lacks the N-terminal PIP box, is similar to the homotrimeric PCNA ring [5,7], suggesting that there may be additional contacts between adjacent surfaces of the protein rings formed on DNA. In accord with this idea, we identified a physical interaction between the DBD of hLigI and PCNA [9].

In addition to PCNA, hLigI also interacts with hRad9-hRad1-hHus1, a heterotrimeric DNA sliding clamp that is involved in cell cycle checkpoints activated by either DNA replication abnormalities or DNA damage [10-12]. The hRad9-hRad1-hHus1 clamp complex is loaded onto DNA by hRad17-RFC in response to replication blockage and DNA damage to initiate a signaling cascade [13-18]. Unlike PCNA, the PIP box of hLigI does not contribute significantly to the interaction with hRad9-hRad1-hHus1 [10]. Furthermore, hRad9-hRad1-hHus1 is more effective at stimulating hLigI activity than PCNA but this stimulatory effect is not dependent upon hRad9-hRad1-hHus1 encircling DNA [10-12]. The heterotrimeric PCNA of *Sulfolobus solfataricus* also stimulates its cognate DNA ligase [9,19] suggesting that this may be a conserved feature of interactions between heterotrimeric DNA sliding clamps and DNA ligases. Unlike the *Sulfolobus* DNA ligase, which preferentially interacts with one subunit of the PCNA heterotrimer via its DBD [9,19], there are no significant differences in the binding of full-length hLigI to each of the subunits of the hRad9-hRad1-hHus1 clamp complex [10].

In this study, we have further characterized the interaction of hLigI DBD with the PCNA and hRad9-hRad1-hHus1 DNA sliding clamps. Notably, hLigI DBD preferentially interacts with the hRad1 subunit of the hRad9-hRad1-hHus1 complex. In addition, hLigI DBD preferentially interacts with trimeric versus monomeric PCNA and appears to bind to regions adjacent to the subunit/subunit interfaces within the trimeric PCNA ring. Finally, we show that hLigI DBD preferentially binds to nicked DNA and can simultaneously contact both DNA and PCNA, forming a DNA-protein complex.

MATERIALS AND METHODS

Protein purification

Recombinant hLigI was overexpressed in and purified from *E. coli* as described previously [10]. Fragments of hLigI (Fig.1), N-terminal (residues 1-232), DNA binding domain (DBD, residues 233-534), catalytic core (Cat, residues 533-919), and N-terminal deletion fragment (Δ N232, residues 233-919) were expressed as His₆-tagged proteins in *E. coli* and purified by affinity chromatography followed by gel filtration and ion exchange chromatography [7].

PCNA was expressed as a glutathione S transferase (GST) fusion protein in *E. coli* and purified from cell extracts by affinity chromatography using glutathione-Sepharose beads (Amersham Biosciences) as described previously [1]. Untagged versions of wild-type PCNA and the mutant Y114A, which is defective in forming the homotrimeric ring structure [20], were overexpressed in *E. coli* and purified from cell extracts by ion exchange chromatography followed by gel filtration and ion exchange chromatography. Recombinant PCNA with a 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 [21]. After expression in *E. coli*, GST-hRad9, GST-hRad1 and GST-hHus1 fusion proteins were purified from cell extracts by affinity chromatography using glutathione-Sepharose beads [10].

Antibodies

A monoclonal antibody against hPCNA was purchased from Santa Cruz Biotechnology. A polyclonal antibody against hLigI has been described previously [1,22,23].

Site-directed mutagenesis

pET3a-hPCNA and pET28a-hLigI were mutated by site-directed mutagenesis using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instruction. The mutations altered the coding sequence so that alanine residues replace the targeted residues at corresponding positions. Changes in the nucleotide sequence were verified by endonuclease digestion and DNA sequencing.

In vitro transcription and translation

Coupled *in vitro* transcription and translation reactions were performed using the TNT Quick Coupled Transcription/Translation system (Promega). The plasmids for the *in vitro* transcription and translation of hLigI cDNA have been described previously [24]. Labeled *in vitro* translated polypeptides were partially purified by ammonium sulfate precipitation.

Glutathione-Sepharose pull-down assays

To prepare glutathione beads for pull down assays, 5 µg of purified GST or GST-fusion protein was incubated with a 20 µl slurry of glutathione-Sepharose beads equilibrated in buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 0.1% Igepal CA-630, and 1 µg/µl of bovine serum albumin) for 30 min at 4 °C with constant agitation and then washed with buffer A.

For reactions with *in vitro* translated polypeptides, beads were resuspended in 500 µl of buffer A containing a labeled *in vitro* translated hLigI polypeptide and then incubated at 4 °C for 1 hr with constant agitation. Next the beads were collected by centrifugation and then washed extensively in binding buffer prior to being resuspended in 20 µl of SDS-PAGE sample buffer. After separation by SDS-PAGE, labeled polypeptides were visualized by phosphorImager analysis (Amersham Biosciences).

For reactions with purified proteins, beads were resuspended in 250 µl of binding buffer and incubated with either hLigI (full-length protein and fragments thereof) or hFEN-1, at 4 °C for 1 hr with constant agitation. The glutathione beads were collected by centrifugation and washed extensively in binding buffer prior to resuspension in 20 µl of SDS-PAGE sample buffer. After separation by SDS-PAGE, proteins were detected by immunoblotting with the appropriate antibodies.

Nickel-agarose pull down assays

Purified his-tagged hLigI (full-length protein and fragments thereof, 5 µg) were bound to 20 µl nickel-agarose beads (Qiagen Inc., Valencia, CA) according to the manufacturer's procedures. Beads resuspended in 250 µl of buffer B (50 mM potassium phosphate, pH 7.5, 100 mM NaCl, 1 mM β-mercaptoethanol, 0.1% Igepal CA-630, 20 mM imidazole, 10% glycerol and 1 mg/ml BSA) were incubated with purified PCNA (1 µg) at 4°C for 1 hr with constant agitation. After collection by centrifugation, beads were washed extensively in buffer B prior to being resuspended in 20 µl of SDS-PAGE sample buffer. After separation by SDS-PAGE, proteins were detected by immunoblotting with PCNA antibodies.

Gel filtration analysis

Purified PCNA (9 µg) and hLigI DBD (3.3 µg) were incubated either alone or together for 15 min at RT in buffer C (50 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM DTT, 0.1 M EDTA, 0.1% Igepal CA-630, 10% glycerol, 1 mM β-mercaptoethanol, 1mM phenylmethylsulphonyl fluoride) prior to loading onto a 24 ml Superose 12 column (GE Healthcare) that had been equilibrated in the same buffer. Proteins in eluted fractions were detected by immunoblotting using the appropriate antibodies.

Peptide competition assay

A peptide corresponding to the first 23 amino acids of hLigI and a version of this peptide in which the phenylalanine residues at positions 8 and 9 were replaced by alanine residues, were synthesized by the peptide synthesis facility at The University of Texas Health Science Center at San Antonio, TX. Purified full-length hLigI or the DBD fragment (35 pmol) were incubated with hLigI antibody (5 µl) in 200 µl of buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 0.1% Igepal CA-630, and 1 µg/µl of bovine serum albumin) for 1 h at 4°C [1]. Protein A-agarose beads (20 µl) were added and the incubation was continued for 1 hour. After collection by centrifugation, the agarose beads were washed extensively with buffer A. Purified human PCNA (35 pmol) was pre-incubated with either the wild-type or mutant hLigI peptide for 10 minutes at 4°C in 200 µl of buffer A with 5% bovine serum albumin (BSA) before addition to the protein A-agarose beads. After incubation for 30 min at 4°C, the agarose beads were washed extensively with buffer A. PCNA bound to the protein A-agarose beads was detected by immunoblotting.

Kinase assay

For each kinase assay, full-length hLigI and various fragments or BSA were incubated with 50 pmol of pK-PCNA, 2 units of protein kinase A (Sigma), 20 µM ATP and 10 µCi [γ -³²P] ATP in PKA buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂) for 2 hrs at 37°C. After separation by SDS-PAGE (10% polyacrylamide gel), labeled proteins were detected in the dried gel by phosphorimager analysis.

Electrophoretic mobility shift assay (EMSA)

The following oligonucleotides 5' CGC CAG GGT TTT CCC AGT CAC GAC ddC 3' (oligo #1) and a 5' ³²P end-labeled version of 5' GTA AAA CGA CGG CCA GTG 3' (oligo #2) were annealed with a complementary 43-mer oligonucleotide to generate a linear duplex substrate with a central nick that is non-ligatable because of the 3' dideoxy C residue. Intact hLigI and various fragments were incubated with the labeled DNA substrate in the presence or absence of PCNA in reactions (10 µl) containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM MgCl₂, 100 µM ATP and 0.25 mg/ml bovine serum albumin and the indicated concentration of NaCl. After incubation for 15 minutes at 37°C, reactions were terminated by the addition of 10 µl of 30% glycerol, 0.25% Bromophenol blue, 0.25% Xylene cyanol. DNA and DNA - protein complexes were separated by electrophoresis through non-denaturing polyacrylamide

gel. The gel was dried prior to the detection of labelled DNA and DNA-protein complexes by phosphorImager analysis.

To determine the effect of the structure of the nick termini on complex formation with hLigI, we carried out EMSAs with the following DNA substrates in addition to the one described above; oligo #1 and a version of oligo #2 with an unlabelled 5' phosphate group were annealed with a 5' ³²P end-labelled complementary 43-mer oligonucleotide to generate a linear substrate with a central nick that is non-ligatable because of the 3' dideoxy C residue; a version of oligo #1 in which the 3' ddC is replaced by a 3' dC residue and oligo #2 without a 5' phosphate group were annealed with a 5' ³²P end-labelled complementary 43-mer oligonucleotide to generate a linear substrate with a central nick that is non-ligatable because the 5' dG residue does not have a phosphate group; oligo #1 and a version of oligo #2 without a 5' phosphate group were annealed with a 5' ³²P end-labelled complementary 43-mer oligonucleotide to generate a linear substrate with a central nick that is non-ligatable because of the 3' dideoxy C residue and the 5' dG residue that does not have phosphate group; the 43-mer 5' CGC CAG GGT TTT CCC AGT CAC GAC CGT AAA ACG ACG GCC AGT G 3' was annealed with a 5' ³²P end-labelled complementary 43-mer oligonucleotide to generate a linear duplex substrate without a nick.

RESULTS

Previously we have shown that full-length hLigI does not exhibit preferential binding to any one of the three subunits of the human heterotrimeric 9-1-1 clamp and that disruption of the hLigI PIP box does not have a significant effect on the interaction with the hRad9-hRad1-hHus1 complex [10]. Since the specific interaction of the *Sulfolobus* DNA ligase (ssLig) with one subunit of the heterotrimeric *Sulfolobus* PCNA (ssPCNA) is mediated by the DBD of the ssLig [9,19], we asked whether the DBD of hLigI is involved in the interaction with the human 9-1-1 complex. Similar to full-length hLigI, the catalytic fragment (Δ N232) bound with similar efficiency to each of the subunits of the 9-1-1 complex (Fig. 2A, compare lanes 4, 7 and 10). Moreover, there was no significant difference in the binding of full-length hLigI (Fig. 2B, lane 7) and the catalytic fragment of hLigI (Fig. 2A, lane 10) to GST-Rad1 beads, indicating that the non-catalytic N-terminal region of hLigI does not make a major contribution to the interaction between hLigI and the subunits of the 9-1-1 complex.

In contrast to full-length hLigI and its catalytic fragment (Fig. 2A), the hLigI DBD exhibited differential binding to the subunits of the 9-1-1 complex. Notably, the binding efficiency of hLigI DBD to hRad1 is markedly greater than that of the catalytic fragment (Fig. 2A, compare lanes 10 and 11) whereas the binding of the DBD to either hRad9 (Fig. 2A, compare lanes 4 and 5) or hHus1 is much less than that of the catalytic fragment (Fig. 2A, compare lanes 4 and 5 and lanes 7 and 8). There is no detectable interaction between hRad1 and the catalytic core of hLigI containing the AdD and ODB (Fig. 2A, lane 12 and Fig. 2B, lane 9), indicating that the catalytic core does not compete with the DBD for binding to hRad1. Similar results were obtained with hRad9 (Fig. 2A, lane 6) and hHus1 (Fig. 2A, lane 9). Based on these results, we suggest that the presence of the catalytic core domains, AdD and ODB, reduce the ability of hLigI DBD to adopt the most favorable conformation for binding to hRad1 and favor conformations in which the interaction is weaker but occurs with each of the subunits of the 9-1-1 complex to a similar extent. As expected, hLigI DBD is more efficiently retained by GST-hRad1 beads than full-length hLigI (Fig. 2B, lanes 7 and 8). In contrast, full-length hLigI is more efficiently retained by GST-PCNA beads than hLigI DBD (Fig. 2B, lanes 4 and 5), presumably because of the predominant contribution of the hLigI PIP box to the interaction with PCNA.

In previous studies, we showed that PCNA bound to nickel beads liganded by hLigI DBD but not hLigI catalytic core (AdD and ODB) [9]. To confirm that the PIP box and DBD of hLigI

bind independently to PCNA, we performed pull down experiments with a GST-PCNA fusion protein and fragments of hLigI labeled by coupled *in vitro* transcription and translation (Fig. 3A). As expected, hLigI and fragments of hLigI that contain either the PIP box (residues 1-118) or the DBD (residues 109-479) were specifically retained by the GST-PCNA beads (Fig. 3A). Since disruption of the hLigI PIP markedly reduces the stable binding of hLigI to PCNA [4], we asked whether the complex formed between the hLigI DBD and PCNA was stable enough to be detected by gel filtration. When PCNA was fractionated in the absence of DBD, the protein peak eluted in fraction 31 (Fig. 3B, upper panel) corresponding to an estimated molecular mass of 96 kDa. This is consistent with PCNA behaving as homotrimer. In contrast, the peak of purified hLigI DBD eluted in fraction 35 (Fig. 3B, bottom panel), corresponding to an estimated molecular of 26 kDa. Thus, it appears that the DBD of hLigI, which has a molecular weight of 33,200 behaves as a monomer under these conditions. When PCNA and hLigI were pre-incubated prior to gel filtration, there was a significant change in the elution profile of hLigI DBD but not PCNA (Fig. 3B, middle panel). Notably, the elution profile of hLigI DBD was almost coincident with that of PCNA with the peak hLigI DBD eluting in fraction 31. Based on these results we conclude that hLigI DBD forms a stable complex with homotrimeric PCNA.

The absence of an obvious PIP box-like sequence within hLigI DBD suggests that the DBD may contact PCNA via regions other than the interdomain connector loop, where PIP box motifs bind to PCNA [3,5]. If this is the case, then the addition of a peptide that contains the hLigI PIP box should have no effect on the interaction between hLigI DBD and PCNA. As expected [1], the hLigI PIP box peptide but not a mutant version, in which the key adjacent phenylalanine residues were replaced by alanine residues, reduced complex formation between full-length hLigI and PCNA that is primarily mediated by the PIP box/interdomain connector loop interaction (Fig. 4A, right panel and Fig. 4B). In contrast, neither the wild-type nor the mutant peptide had any significant effect on complex formation between hLigI DBD and PCNA (Fig. 4A, left panel and Fig. 4B). Using the atomic resolution structure of hLigI [7] as a guide, we have attempted to identify residues within the DBD domain that play a key role in the interaction with PCNA. Replacement of several amino acid pairs (K422 K428, L392 M393 L394, G363 Q365, I426 I427 and L430 F431) located on surface exposed loops of the DBD with alanine had no noticeable effect on binding to PCNA. Only the substitution of residues K422 K423 K428 with alanine reduced PCNA binding, but these changes also reduced DNA binding and hLigI catalytic activity suggesting that they negatively impact the overall structure and functions of the DBD rather than selectively disrupting the interaction with PCNA (data not shown).

Since the inter-subunit interfaces within the PCNA homotrimer involve contacts between the N-terminal domain of one subunit and the C-terminal domain of the adjacent subunit [5], hLigI DBD may interact with regions adjacent to the subunit/subunit interface and/or specific conformations of the PCNA subunits resulting from formation of the homotrimeric ring. To test this idea, we constructed and purified a mutant version of PCNA, Y114A, that does not form the homotrimer ring [20]. In accord with the published study [20], purified PCNA Y114A behaved as a monomer during gel filtration (data not shown). Compared with intact hLigI, the DBD exhibited about a 3.5-fold preference for binding to trimeric PCNA (Fig. 5A). To provide more direct evidence that hLigI DBD interacts with regions adjacent to the subunit/subunit interfaces of the PCNA monomers within the homotrimeric ring, we examined the effect of intact hLigI and various fragments on phosphorylation of a recombinant version of PCNA with an added N-terminal PKA site (Fig. 5B). Pre-incubation of PCNA with hLigI DBD inhibited phosphorylation of the PKA site (Fig. 5C, lanes 2 and 3, and Fig. 5D) albeit to a lesser extent than full-length hLigI (Fig. 5C, lane 6 and Fig. 5D) whereas hLigI catalytic core did not (Fig. 4C, lanes 4 and 5, and Fig. 5D).

In addition to interacting with PCNA, the DBD is the predominant DNA binding activity within hLigI [7]. This prompted us to examine the formation of DNA-protein complexes by hLigI in the absence and presence of PCNA. In initial studies, we examined the binding of intact hLigI and various fragments to DNA substrates with different nick structures (Fig. 6A) using an electrophoretic mobility shift assay. Full-length hLigI (Fig. 6B, lanes 5-8) and a truncated derivative lacking the non-catalytic N-terminal region of hLigI (Fig. 6B, lanes 9-12) exhibited preferential binding to a DNA nick with 5' phosphate and 3' dideoxy termini. There was detectable, albeit reduced binding to DNA nicks with 3' and 5' HO termini whereas binding to DNA molecules containing nicks with 3' dideoxy and 5'HO termini was equal to or less than intact DNA duplexes (Fig. 6B). The hLigI DBD also bound preferentially to DNA nicks with 5' phosphate and 3' dideoxy termini (Fig. 6B, lanes 13-16) although larger amounts of the hLigI DBD, about 5-fold, were required to generate similar levels of DNA-protein complex. Under these reaction conditions, the hLigI catalytic core did not form DNA-protein complexes but, when the NaCl concentration was reduced from 100 to 40 mM, the hLigI catalytic core did bind specifically to DNA nicks with 5' phosphate and 3' dideoxy termini (data not shown).

Because PCNA is a sliding clamp and lacks robust DNA binding activity, we did not observe DNA-protein complexes when the linear nicked DNA substrate with 5' phosphate and 3' dideoxy termini was incubated with PCNA (Fig. 6C, lanes 14-16). However, when increasing amounts of PCNA were incubated with intact hLigI and the nicked DNA substrate, a formation of the novel DNA-protein complex, which presumably contains both hLigI and PCNA, was observed (Fig. 6C, lanes 3-5). As expected, both this DNA-protein complex and the other complex formed by hLigI alone with nicked DNA (Fig. 6B, lane 5) were supershifted by hLigI antibody (data not shown). Addition of a PCNA antibody did not change the mobility of the DNA-protein complexes indicating that either PCNA is not present in the DNA-protein complexes or the antibody does not recognize PCNA within the DNA protein-complexes. In similar experiments with a version of hLigI lacking a functional PIP box (Fig 6C, lanes 6-9), the novel DNA-protein complex observed with wild-type hLigI and PCNA was not detected (Fig. 6C, compare lanes 3-5 with lanes 7-9). However, co-incubation of PCNA with a version of hLigI lacking the non-catalytic N-terminal region that includes the PIP box resulted in the formation of a novel DNA-protein complex. These results indicate that the two different modes of interaction between hLigI and PCNA involving the PIP box and DBD also occur when hLigI is complexed with nicked DNA. In fact, it appears that the presence of the non-catalytic N-terminal region with an inactivated PIP box inhibits the interaction between PCNA and hLigI DBD.

DISCUSSION

Protein-protein interaction involving homotrimeric PCNA plays a key role in the co-ordination of DNA synthesis, processing and ligation steps that occur during DNA replication and several DNA repair pathways. The majority of these interactions occur between the interdomain connector loop of PCNA and a conserved PIP box within the PCNA partner protein [3,25, 26]. Previous studies have shown that the participation of hLigI in DNA replication and repair is dependent upon its N-terminal PIP box of hLigI [1,2,4]. However, more recent studies have provided evidence that the DBD of hLigI also interacts with PCNA and may be involved in an interaction with the heterotrimeric cell cycle checkpoint clamp, hRad9-hRad1-hHus1 [10]. In this study we have further characterized the interaction of the hLigI DBD with both the PCNA and hRad9-hRad1-hHus1 clamps.

The trimeric composition of DNA sliding clamps suggest that up to three different protein partners can bind simultaneously to the same clamp, thereby co-ordinating their activities. This appears to be the case for the heterotrimeric PCNA clamp of *Sulfolobus* with each subunit preferentially interacting with either the DNA polymerase, FEN-1 or DNA ligase of *Sulfolobus*

[19]. Notably, it is the DBD of the *Sulfolobus* DNA ligase that mediates the interaction with the clamp [9]. Here we have shown that, in the interactions between hLigI and hRad9-hRad1-hHus1, it is also the hLigI DBD that appears to be the major contributor to the interaction interface and the DBD preferentially binds to the hRad1 subunit. Thus, it appears that the role of the DBD in the interaction between DNA ligases and heterotrimeric DNA clamps is conserved. Unlike the hLigI DBD alone, full length hLigI does not preferentially bind to hRad1 but instead exhibits similar binding to each of the subunits of the hRad9-hRad1-hHus1 complex. It is possible that the interaction of hLigI is restricted to the hRad1 subunit *in vivo* because the hRad9 and hHus1 subunits are involved in interactions with other partner proteins such as FEN-1. Although the interaction with hRad9-hRad1-hHus1 robustly stimulates DNA joining by hLigI, the mechanism of stimulation is not defined but does not require that the heterotrimeric clamp encircles DNA [9-11,19,27].

In addition to the PIP box, the DBD of hLigI also interacts with PCNA [10]. Here we have shown that hLigI DBD and PCNA form a stable complex and that hLigI DBD preferentially interacts with the trimeric ring form of PCNA. This interaction appears to involve the regions adjacent to the interface between the subunits within trimeric PCNA. Interestingly, the same regions of PCNA contribute to the interaction with the clamp loader RFC [28]. Although hLigI also physically and functionally interacts with RFC [24,29], it seems likely that the PCNA interaction interfaces contacted by hLigI and RFC will be mutually exclusive suggesting that the interplay among RFC, PCNA and hLigI will involve exchange of protein partners rather than ternary complex formation.

There are differences in the mechanisms by which hLigI interacts with homotrimeric and heterotrimeric DNA sliding clamps. For PCNA, it appears that asymmetric, elongated hLigI initially contacts PCNA via the PIP box [1,8]. We envision that this initial tethering at the PIP box/IDCL interface facilitates the subsequent engagement of the hLigI DBD, which is linked to the PIP box by a flexible, unstructured region, with PCNA. In contrast, the initial interaction of hLigI with the human 9-1-1 complex is mediated by the DBD of hLigI, presumably via the hRad1 subunit of 9-1-1. In addition we have found that both intact hLigI and the DBD stably interact with DNA nicks with 3' dideoxy and 5' phosphate termini. Notably, PCNA appears to form distinct complexes with hLigI complexed with nicked DNA depending on whether the interaction occurs via the PIP box or the DBD domain. Based on these and other results, we suggest that the interaction between hLigI and PCNA that is topologically linked to DNA initially occurs via the hLigI PIP box. This docking facilitates a subsequent interaction between the hLigI DBD and PCNA. Based on the SAXS results with the *Sulfolobus* heterotrimeric PCNA and DNA ligase [9], it appears likely that the hLigI catalytic domain remains in an extended conformation even after the hLigI DBD has engaged either the PCNA or 9-1-1 clamp. We suggest that, when a nick is encountered, the interface between the hLigI DBD and PCNA acts as a pivot, enabling the hLigI catalytic fragment to productively engage the nick by transitioning from the extended conformation to the ring form [7].

ACKNOWLEDGEMENTS

This work was supported by the Structural Biology of DNA Repair Program Project Grant (P01 CA092584) and other grants awarded to A.E.T (GM47251) and T.E. (GM52504) from the National Institutes of Health.

REFERENCES

- [1]. Levin DS, Bai W, Yao N, O'Donnell M, Tomkinson AE. An interaction between DNA ligase I and proliferating cell nuclear antigen: implications for Okazaki fragment synthesis and joining. *Proc Natl Acad Sci U S A* 1997;94:12863–12868. [PubMed: 9371766]
- [2]. Montecucco A, Rossi R, Levin DS, Gary R, Park MS, Motycka TA, Ciarrocchi G, Villa A, Biamonti G, Tomkinson AE. DNA ligase I is recruited to sites of DNA replication by an interaction with

- proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories. *Embo J* 1998;17:3786–3795. [PubMed: 9649448]
- [3]. Warbrick E. PCNA binding through a conserved motif. *Bioessays* 1998;20:195–199. [PubMed: 9631646]
 - [4]. Levin DS, McKenna AE, Motycka TA, Matsumoto Y, Tomkinson AE. Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair. *Curr Biol* 2000;10:919–922. [PubMed: 10959839]
 - [5]. Krishna TS, Kong XP, Gary S, Burgers PM, Kuriyan J. Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. *Cell* 1994;79:1233–1243. [PubMed: 8001157]
 - [6]. Tom S, Henriksen LA, Park MS, Bambara RA. DNA ligase I and proliferating cell nuclear antigen form a functional complex. *J Biol Chem* 2001;276:24817–24825. [PubMed: 11331287]
 - [7]. Pascal JM, O'Brien PJ, Tomkinson AE, Ellenberger T. Human DNA ligase I completely encircles and partially unwinds nicked DNA. *Nature* 2004;432:473–478. [PubMed: 15565146]
 - [8]. Tomkinson AE, Lasko DD, Daly G, Lindahl T. Mammalian DNA ligases. Catalytic domain and size of DNA ligase I. *J Biol Chem* 1990;265:12611–12617. [PubMed: 1695631]
 - [9]. Pascal JM, Tsodikov OV, Hura GL, Song W, Cotner EA, Classen S, Tomkinson AE, Tainer JA, Ellenberger T. A Flexible Interface between DNA Ligase and PCNA Supports Conformational Switching and Efficient Ligation of DNA. *Mol Cell* 2006;24:279–291. [PubMed: 17052461]
 - [10]. Song W, Levin DS, Varkey J, Post S, Bermudez VP, Hurwitz J, Tomkinson AE. A conserved physical and functional interaction between the cell cycle checkpoint clamp loader and DNA ligase I of eukaryotes. *J Biol Chem* 2007;282:2271–22730.
 - [11]. Smirnova E, Toueille M, Markkanen E, Hubscher U. The human checkpoint sensor and alternative DNA clamp Rad9-Rad1-Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery. *Biochem J* 2005;389:13–17. [PubMed: 15871698]
 - [12]. Wang W, Lindsey-Boltz LA, Sancar A, Bambara RA. Mechanism of stimulation of human DNA ligase I by the Rad9-Rad1-Hus1 checkpoint complex. *J Biol Chem* 2006;281:20865–20872. [PubMed: 16731526]
 - [13]. Ellison V, Stillman B. Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5' recessed DNA. *PLoS Biol* 2003;1:E33. [PubMed: 14624239]
 - [14]. Majka J, Binz SK, Wold MS, Burgers PM. Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions. *J Biol Chem* 2006;281:27855–27861. [PubMed: 16864589]
 - [15]. Majka J, Burgers PM. Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint. *Proc Natl Acad Sci U S A* 2003;100:2249–2254. [PubMed: 12604797]
 - [16]. Majka J, Niedziela-Majka A, Burgers PM. The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint. *Mol Cell* 2006;24:891–901. [PubMed: 17189191]
 - [17]. Zou L, Cortez D, Elledge SJ. Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. *Genes Dev* 2002;16:198–208. [PubMed: 11799063]
 - [18]. Zou L, Liu D, Elledge SJ. Replication protein A-mediated recruitment and activation of Rad17 complexes. *Proc Natl Acad Sci U S A* 2003;100:13827–13832. [PubMed: 14605214]
 - [19]. Dionne I, Nookala RK, Jackson SP, Doherty AJ, Bell SD. A heterotrimeric PCNA in the hyperthermophilic archaeon *Sulfolobus solfataricus*. *Mol Cell* 2003;11:275–282. [PubMed: 12535540]
 - [20]. Jonsson ZO, Podust VN, Podust LM, Hubscher U. Tyrosine 114 is essential for the trimeric structure and the functional activities of human proliferating cell nuclear antigen. *Embo J* 1995;14:5745–5751. [PubMed: 8521831]
 - [21]. Kelman Z, Naktinis V, O'Donnell M. Radiolabeling of proteins for biochemical studies. *Methods Enzymol* 1995;262:430–442. [PubMed: 8594367]
 - [22]. Lindsey-Boltz LA, Bermudez VP, Hurwitz J, Sancar A. Purification and characterization of human DNA damage checkpoint Rad complexes. *Proc Natl Acad Sci U S A* 2001;98:11236–11241. [PubMed: 11572977]
 - [23]. Uhlmann F, Gibbs E, Cai J, O'Donnell M, Hurwitz J. Identification of regions within the four small subunits of human replication factor C required for complex formation and DNA replication. *J Biol Chem* 1997;272:10065–10071. [PubMed: 9092550]

- [24]. Levin DS, Vijayakumar S, Liu X, Bermudez VP, Hurwitz J, Tomkinson AE. A conserved interaction between the replicative clamp loader and DNA ligase in eukaryotes: implications for Okazaki fragment joining. *J Biol Chem* 2004;279:55196–55201. [PubMed: 15502161]
- [25]. Maga G, Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J Cell Sci* 2003;116:3051–3060. [PubMed: 12829735]
- [26]. Majka J, Burgers PM. The PCNA-RFC families of DNA clamps and clamp loaders. *Prog Nucleic Acid Res Mol Biol* 2004;78:227–260. [PubMed: 15210332]
- [27]. Rossi ML, Purohit V, Brandt PD, Bambara RA. Lagging strand replication proteins in genome stability and DNA repair. *Chem Rev* 2006;106:453–473. [PubMed: 16464014]
- [28]. Bowman GD, O'Donnell M, Kuriyan J. Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex. *Nature* 2004;429:724–730. [PubMed: 15201901]
- [29]. Vijayakumar S, Chapados BR, Schmidt KH, Kolodner RD, Tainer JA, Tomkinson AE. The C-terminal domain of yeast PCNA is required for physical and functional interactions with Cdc9 DNA ligase. *Nucleic Acids Res* 2007;35:1624–1637. [PubMed: 17308348]

Figure 1. Schematic representation of the domain structure of full-length hLigI and truncated fragments

The positions of the PCNA Interacting Peptide (PIP) box within the non-catalytic N terminal domain (residues 1-233), and the DNA binding domain (DBD), the adenylation domain (AdD) and the OB-fold domain (OBD) are indicated. The catalytic fragment ($\Delta N232$) is generated by removal of the non-catalytic N terminal domain.

Figure 2. The DBD of hLigI interacts with both the PCNA and the 9-1-1 clamp complexes

A. Purified fragments of hLigI (10 pmol of each) corresponding to residues 233-919 (Δ N232), DNA binding domain (residues 233-534, DBD) and catalytic domain (residues 533-919, Cat), were incubated with glutathione beads liganded by hRad9 (GST-Rad9, lanes 4-6), hHus1 (GST-Hus1, lanes 7-9) or hRad1 (GST-Rad1, lanes 10-12) as described in Materials and Methods. Lane 1, 5% of input of Δ N232 fragment (5% Δ N232 input); lane 2, 5% of input of DBD fragment (5% DBD input); lane 3, 5% of input of Cat core (5% Cat Input). **B.** Purified full-length hLigI (FL) and the DNA binding domain (DBD) and catalytic core fragments (Cat) (10 pmol of each), were incubated with glutathione beads liganded by PCNA (GST-PCNA, lanes 4-6), hRad1 (GST-Rad1, lanes 7-9) or GST (GST, lanes 10-12), as described in Materials and Methods. Lane 1, 10% of input of full-length hLigI (10% FL Input); lane 2, 10% of input of DBD (DBD Input); Lane 3, 10% Catalytic core fragment input (Cat Input). The binding of intact hLigI and fragments of hLigI to the beads was detected by immunoblotting.

Figure 3. Interaction of the non-catalytic N-terminal domain and the DBD domain of hLigI with PCNA; analysis of the DBD/PCNA complex by gel filtration

A. Full-length hLigI (FL, lanes 1-3) and fragments corresponding to residues 1-118 (lanes 4-6), 109-479 (lanes 7-9) and 479-792 (lanes 10-12) were labeled by *in vitro* transcription and translation and then incubated with glutathione beads liganded by either GST (GST, lanes 2, 5, 8 and 11) or PCNA fused to GST (GST-PCNA, lanes 3, 6, 9 and 12) as described in Materials and Methods. Lane 1, 10% of input of hLigI (10% DNA LigI FL Input); lane 4, 10% of input of fragment 1-118 (10% DNA LigI 1-118 Input); lane 7, 10% of input of fragment 109-479 (10% DNA LigI 109-479 Input); lane 10, 10% of input of fragment 479-792 (10% DNA LigI 479-792 Input). Labeled fragments of hLigI were detected by phosphorImaging analysis (Molecular Dynamics). **B.** PCNA (9 µg, 100 pmol trimer) was loaded onto a Superose 12 gel filtration column (Upper panel). PCNA (9 µg, 100 pmol trimer) was pre-incubated with hLigI DBD (3.3 µg, 100 pmol) prior to loading onto a Superose 12 gel filtration column (middle panel). hLigI DBD (3.3 µg, 100 pmol) was loaded onto a Superose 12 gel filtration column (lower panel). The presence of PCNA and hLigI in the eluted fractions were detected by immunoblotting. Samples corresponding to 25% of the protein loaded onto the gel filtration column are shown (25% Input).

Figure 4. The interaction between the hLigI DBD and PCNA does not involve the IDCL of PCNA
A. PCNA (35 pmol trimer) was pre-incubated with a peptide corresponding to the first 23 residues of hLigI (wt, lane 2), a version of this polypeptide in which the phenylalanine residues at the position 8 and 9 were replaced by alanine residues (mt, lane 3) or without added peptide (-, lane 1). The reactions were then incubated with either full length hLigI (DNA Lig I, 35 pmol, right panel) or hLigI DBD (DBD, 35 pmol, left panel) that were linked to protein A-agarose beads via hLigI antibody. The binding of PCNA to the beads was detected by immunoblotting. **B.** The results of the peptide competition assay are shown graphically. PCNA binding, which was first normalized to the amount of either full length hLigI or hLigI DBD bound to the beads, is expressed as a ratio of that observed without peptide (black bars), wild-type peptide (gray bars), mutant peptide (white bars).

Figure 5. The DBD of hLigI preferentially interacts with the trimeric form of PCNA, binding at or near the subunit-subunit interface within the trimer

A. Purified wild type trimeric PCNA (10 pmol trimer) and a mutant version Y114A that is predominantly monomeric were incubated with nickel-agarose beads (Ni²⁺) liganded by; lanes 3 and 4, no protein; lanes 5 and 6, his-tagged full-length hLigI (10 pmol, His-wtFL); lanes 7 and 8, his-tagged hLigI DBD (His-DBD, 10 pmol) (lane 7, 8) as described in Materials and Methods. Lane 1, 10% of PCNA input (10% PCNA Input); lane 2, 10% input of Y114A input (10% Y114A Input). PCNA binding to the beads was detected by immunoblotting. **B.**

Diagrammatic representation of a version of PCNA with an N-terminal protein kinase A site (pK-PCNA). The positions of the PKA site and the IDCL within the PCNA trimer are shown. **C.** pK-PCNA (50 pmol trimer) was pre-incubated with; lane 1, no addition; lane 2, hLigI DBD (50 pmol); lane 3, hLigI DBD (150 pmol); lane 4, hLigI cat core (50 pmol); lane 5, hLigI cat core (150 pmol); lane 6, full-length hLigI (50 pmol); lane 7, BSA (150 pmol) prior to incubation with protein kinase A and [³²P]ATP as described in Materials and Methods. Lane M, molecular mass standard. After separation by SDS-PAGE, proteins were stained with Coomassie blue (upper panel) and labeled PCNA was detected by phosphorimaging analysis (lower panel). **D.** The results of 3 independent experiments are shown graphically. Lane 1, 1:1 molar ratio of PCNA and DBD; lane 2, 1:3 molar ratio of PCNA and DBD; Lane 3, 1:1 molar ratio of PCNA and Cat core; lane 4, 1:3 molar ratio of PCNA and Cat core; lane 5, 1:1 molar ratio of PCNA and full-length hLigI; lane 6, 1:3 molar ratio of PCNA and BSA.