Mechanism of Stimulation of Human DNA Ligase I by the Rad9-Rad1-Hus1 Checkpoint Complex*

Received for publication, March 10, 2006, and in revised form, May 18, 2006 Published, JBC Papers in Press, May 25, 2006, DOI 10.1074/jbc.M602289200

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Accumulating evidence suggests that the Rad9-Rad1-Hus1 (9-1-1) checkpoint complex, known to be a sensor of DNA damage, is also a component of DNA repair systems. Recent results show that 9-1-1 interacts with several base excision repair proteins. It binds the DNA glycosylase MutY homolog, and stimulates DNA polymerase β, flap endonuclease 1, and DNA ligase I. 9-1-1 resembles proliferating cell nuclear antigen (PCNA), which stimulates some of these same repair enzymes, and is loaded onto DNA in a similar manner. The complex of 9-1-1 with DNA ligase I can be immunoprecipitated from human cells. Moreover, UV irradiation stimulates 9-1-1 to interact with DNA ligase I can be immunoprecipitated from human cells. Examining the nature of 9-1-1 interaction with DNA ligase I, we show that there is a similar degree of stimulation on ligation substrates with different structures, and that there is specificity for DNA ligase I. 9-1-1 improves the binding of DNA ligase I to nicked double strand DNA. Furthermore, although high concentrations of casein kinase II strongly inhibits DNA ligase I activity, it does not affect the ability of 9-1-1 to stimulate. This suggests that 9-1-1 is also an activator of DNA ligase I during DNA damage.

Proliferating cell nuclear antigen (PCNA)2 is a toroidal sliding clamp that serves as a platform for the sequential actions of proteins involved in eukaryotic DNA replication and repair. DNA damage produces a response that inhibits DNA replication while allowing DNA repair. It includes induction of p21, a protein that mediates growth arrest. A domain of p21 binds and inactivates PCNA (1, 2). Expression of this domain can cause cells to arrest in G1 phase, suggesting that inhibition of PCNA is part of the damage response (3–5). PCNA binds and stimulates proteins with dual roles in DNA replication and repair, e.g. the flap endonuclease FEN1 and DNA ligase I (6). In an effort to identify whether there is a substitute platform for DNA repair if PCNA were inactivated, we considered the Rad9-Rad1-Hus1 (9-1-1) checkpoint complex.

Rad9-Rad1-Hus1 (9-1-1) bears a strong structural, but not sequence, resemblance to PCNA. It is loaded to encircle double-stranded DNA in vitro by a variant of RFC, the PCNA clamp loader, called Rad17-RFC (7–9). Rad17-RFC can recruit 9-1-1 onto chromatin, and this action is independent of the phosphorylation of Rad17, which can be phosphorylated by ATM and ATR after DNA damage in vivo (10, 11). These studies show that 9-1-1 can load on chromatin, recognize DNA damage, and then activate the checkpoint pathway, suggesting that 9-1-1 is a damage sensor (12).

Other results point to a direct role of 9-1-1 as a mediator and possible platform for DNA repair. 9-1-1 stimulates or associates with enzymes involved in nearly every step of the long patch base excision repair (LP-BER) pathway (13). In this pathway, damaged bases are removed by glycosylases, the abasic site is cleaved by an abasic nuclease, and a short patch of synthesis is carried out by DNA polymerase β, δ, or ε, displacing a 5′ flap that is removed by FEN1. The nick produced by this reaction is sealed by DNA ligase I (14). The DNA glycosylase MutY homolog in yeast co-immunoprecipitated with the yeast 9-1-1 homolog (15). The activity and reaction specificity of DNA polymerase β was altered by 9-1-1 (16). 9-1-1 bound and greatly stimulated FEN1 (17, 18). The binding sites for PCNA and 9-1-1 on FEN1 were identified and found to be distinct (16). Finally, 9-1-1 was shown to bind DNA ligase I and stimulate ligation (19).

DNA ligase I is toroidal and can encircle a nicked DNA substrate (20). DNA ligase I activity is high in proliferating cells (21–23), and the level correlates with the rate of cell proliferation (24). In addition, retarded joining of Okazaki fragments has been observed in 46BR cells, deficient in DNA ligase I (25). These facts suggest that DNA ligase I participates in Okazaki fragment maturation. In reconstituted replication systems, DNA ligase I joins Okazaki fragments (26–28). DNA ligase I was shown to function in BER and nucleotide excision repair (29, 30). Results in vitro also suggest that DNA ligase I participates in these pathways (31–34).

Recently it was reported that purified 9-1-1 binds to and stimulates DNA ligase I (19). Here we address the specificity of the stimulation and show that the complex of DNA ligase I and

* This work was supported by National Institutes of Health Grants GM024441 (to R. A. B.) and GM322833 (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: PCNA, proliferating cell nuclear antigen; 9-1-1, Rad9-Rad1-Hus1 checkpoint complex; FEN1, flap endonuclease 1; CK2, casein kinase II; BER, base excision repair; LP, long patch; IR, ionizing radiation; RFC, replication factor C.
9-1-1 can be immunoprecipitated from cell extracts. We also address whether the 9-1-1 needs to be loaded onto the DNA for stimulation.

**EXPERIMENTAL PROCEDURES**

**Substrate Preparation**—All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). ATP was from Roche Molecular Biochemicals. φX174 single-stranded DNA was from New England Biolabs. Micro Bio-Spin columns (Bio-Lab) were used for purification of labeled DNA. T4 ligase and CK2 were from Roche Molecular Biochemicals. All the other reagents were the best available commercial grade.

Labeling and purifying of substrates were performed as described (35). Oligonucleotide sequences are listed in Table 1. Each substrate consisted of a downstream primer (D), a template (T), and an upstream primer (U).

**Enzyme Expression and Purification**—Human DNA ligase I was cloned into the T7 expression plasmid pET-15b (Novagen). The final expression vector pHIS-hLIG1 was grown in *Escherichia coli* BL21 strain. Recombinant human DNA ligase I was purified as previously described (36). Rad9-Rad1-Hus1 was expressed and purified as previously described (37).

**Enzyme Assays**—Reactions were performed in 30 mM HEPES (pH 7.5), 40 mM KCl, 4 mM MgCl₂, 5% glycerol, and 0.1 mg/ml bovine serum albumin with 0.1 mM ATP. Enzyme stocks were diluted in 30 mM HEPES (pH 7.5), 5% glycerol, 40 mM KCl, and 0.1 mg/ml bovine serum albumin. Each reaction contained 10 fmol of substrate in a 20-μl reaction and amounts of the enzymes as indicated in the figure legends. All the reactions were incubated at 37 °C for 10 min, and the products were resolved on 15% polyacrylamide, 7M urea denaturing gels. The extent of ligation was quantitated using a PhosphorImager (GE Healthcare) and ImageQuant version 1.2 software from GE Healthcare. All enzyme assays were performed at least in triplicate, and representative assays are shown.

**Electrophoretic Mobility Shift Assay**—Enzyme/DNA mixtures were incubated at 37 °C for 10 min in binding buffer (30 mM HEPES, pH 7.5, 5% glycerol, 40 mM KCl, 0.1 mg/ml bovine serum albumin with 0.1 mM ATP). Samples were then run on a 12% polyacrylamide gel in 0.5 × TBE buffer (44.5 mM Tris-borate, pH 8.3, 1 mM Na₂EDTA). The gel was analyzed by PhosphorImager.

**Immunoprecipitation**—TK6 cells were incubated at 37 °C for 1 h and then harvested after treated by 9 gray γ-irradiation or 50 J/m² UV. Nuclear extracts of TK6 cells were prepared by standard methods (38). Protein G beads (Roche Diagnostics) were equilibrated with buffer C + 0.01% Nonidet P-40 prior to addition of antibody. Five μl of human Rad9 monoclonal antibody (Calbiochem, San Diego) and hemagglutinin monoclonal antibody (Cell Signaling Technology, Inc.) were added to the protein G beads and incubated at 4 °C for 2 h. The beads were washed twice with buffer C + 0.01% Nonidet P-40. 5 mg of nuclear extract was mixed with protein G beads and incubated overnight at 4 °C. The beads were washed three times with buffer C + 0.01% Nonidet P-40 and then with buffer A + 0.01% Nonidet P-40. The beads were then collected by centrifugation. Sample buffer was added and then the samples were run on SDS-PAGE, and analyzed by DNA ligase I antibody (10H5, GeneTex, Inc., San Antonio, TX) and Rad9 antibody, respectively.

**Pulldown Assays**—Because human DNA ligase I was tagged by histidine and human Rad1 was tagged by FLAG, 20 μl of histidine or FLAG antibody-conjugated beads were used in these experiments. Histidine antibody-conjugated agarose beads (Novus Biologicals, Inc.) and FLAG antibody-conjugated agarose beads (Sigma) were equilibrated with buffer C + 0.1% Nonidet P-40. One pmol of purified 9-1-1 or DNA ligase I was incubated at 4 °C for 1 h with FLAG antibody-conjugated agarose beads and histidine antibody-conjugated agarose beads, respectively. After being washed twice with buffer C + 0.1% Nonidet P-40, FLAG beads or histidine beads were further incubated with 2 pmol of purified DNA ligase I or 9-1-1 at 4 °C for 1 h. Beads were washed three times with buffer C + 0.1% Nonidet P-40 and once with buffer A + 0.1% Nonidet P-40. Finally beads were suspended in SDS sample buffer. After SDS-PAGE, samples were analyzed by DNA ligase I monoclonal antibody (10H5, GeneTex, Inc.) and Rad1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**RESULTS**

9-1-1 Interaction with DNA Ligase I Is Promoted by DNA Damage—PCNA has been shown to interact directly with both FEN1 and DNA ligase I. This interaction is presumed to medi-

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**TABLE 1**

Oligonucleotide sequences (5’−3’)

| template | Downstream primers | Upstream primers |
|----------|--------------------|-------------------|
| D1 | CAT ACC GCC CCT TCT CTG GTT CAT GAT | U1 | TCT GCC AGT ACG TGC AAT TGG AGA AGA GCC |
| D2 | TAT ACC GCC CCT TCT CTG GTT CAT GAT | U2 | TCC CCA GTC ACC CCT CTG CTG CTG CTG CTG CTG CTG CTG CTG CTG |
| D3 | CTG CTG CTG GTA AAA CGA CGG CCA GTG | U3 | CGA CCG TGC CAG CCT AAA AC |
| D4 | ACT TGC CCG TGC CAC CAT CCC GCC ACC TCC TGC | U4 | CAT ACC GCC CCT TCT CTG GTT CAT GAT |
| D5 | dCT TGC CCG TGC CAC CAT CCC GCC ACC TCC TGC | U5 | |
| D6 | ACT GGG AAA CAT ACC GCT TCT CTG GTT CAT GAT GAA |

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* d in downstream primer D5 represents 1’-2’-dideoxyribose (dSpacer).
9-1-1 Stimulates DNA Ligase I without an Apparent Change in Mechanism—To assess the mechanism of stimulation of human DNA ligase I by 9-1-1, we first confirmed the robust stimulation reported on a linear nicked substrate (19) (Fig. 2, lanes 7–12). We then asked whether 9-1-1 altered the specificity of ligation for different types of substrates. We considered three possibilities. First, the substrate equilibrates into many structures only a few of which are ligatable. For this we employed a substrate with overlapping primers containing tandem CTG triplet repeats (Fig. 3A, lanes 1–6). The substrate can equilibrate into numerous bubble, flap, double flap, and fold-back flap structures (43, 44). Only a few of the many possible structures have bubble configurations that produce a nick susceptible to ligation. Second, we tested a substrate with a nick in which the 5′ position was an abasic site (Fig. 2A, lanes 13–18). Third, the substrate had a nick with a mismatched 5′ nucleotide (Fig. 2B, lanes 1–6). In all cases, 9-1-1 stimulated ligase I activity to the same extent (Fig. 2, A and B). These results suggest that the stimulation is general, and not capable of altering substrate specificity.

To further assess specificity we compared the effect of 9-1-1 on T4 ligase and DNA ligase I (Fig. 3 and data not shown). Under our assay conditions, 9-1-1 enhanced DNA ligase I activity ~6-fold and had no effect on T4 ligase (Fig. 3, lanes 4–8 and data not shown). This suggests a stimulation specificity for DNA ligase I. It is also consistent with the interpretation that 9-1-1 does not alter the structure of the substrate to make it more efficient for ligation.
larly, it was found that CK2 phosphorylates the base excision repair protein XRCC1, suggesting that CK2 has a direct role in DNA repair (47). 9-1-1 stimulates ligation as expected in the absence of CK2 (Fig. 6A, lanes 4 and 5). In the presence of CK2, there is reduced ligation activity overall, but an enhancement by 9-1-1 is still observed (Fig. 6A, lanes 9 and 10). Moreover, the proportion of stimulation by 9-1-1 was approximately the same. Comparing lanes 3 to 8 in Fig. 6A, we found that DNA ligase I activity itself decreased by CK2. To clarify the effect of CK2 on ligation, we titrated the kinase included in the reaction. As shown in Fig. 6B, we observed that increased CK2 was progressively more inhibitory. DNA ligase I activity was almost completely abolished in the highest concentration of CK2 (lane 7, 2 milliunits/20 μl). In our previous studies, CK2 was shown to

FIGURE 2. 9-1-1 stimulates DNA ligase I on different substrates. The proportion of ligation product was calculated according to the formula below: (density of ligated DNA band - background)/(density of unligated DNA band - background) + (density of ligated DNA band - background). We set the proportion of the lane without enzyme as 0, the proportion of the lane with DNA ligase I alone as 1. A, left, for lanes 1–6, an equilibrating CTG repeat substrate (D3:T2:U2) was used. For lanes 7–12, a conventional nick substrate (D4:T3:U3) was used. For lanes 13–18, 1', 2'-dideoxyribose (dSpacer) nick substrate (D5:T3:U3) was used. Lanes 1, 7, and 13 had substrates only. Lanes 2, 8, and 14 had substrate and 500 fmol of 9-1-1. Lanes 3, 9, and 15 had substrate and 2 fmol of DNA ligase I. Lanes 4, 10, and 16 had substrate, 2 fmol of DNA ligase I, and 100 fmol of 9-1-1. Lanes 5, 11, and 17 had substrate, 2 fmol of DNA ligase I, and 250 fmol of 9-1-1. Lanes 6, 12, and 18 had substrate, 2 fmol of DNA ligase I and 500 fmol of 9-1-1. The substrates were labeled at the 5'-end of the downstream primer. The reactions were performed at 37 °C for 10 min. Right, graph of -fold stimulation. Regular represents conventional nick substrate (D4:T3:U3). Triple repeat represents equilibrating CTG repeat substrate (D3:T2:U2). D spacer represents 1', 2'-dideoxyribose (dSpacer) nick substrate (D5:T3:U3). B, left, for lanes 1–6, mismatch substrate (D2:T1:U1) was used. For lanes 7–12, conventional nick substrate (D1:T1:U1) was used. Lanes 1 and 7 had substrate only. Lanes 2 and 8 had substrate and 500 fmol of 9-1-1. Lanes 3 and 9 had substrate and 2 fmol of DNA ligase I. Lanes 4 and 10 had substrate, 2 fmol of DNA ligase I, and 100 fmol of 9-1-1. Lanes 5 and 11 had substrate, 2 fmol of DNA ligase I, and 250 fmol of 9-1-1. Lanes 6 and 12 had substrate, 2 fmol of DNA ligase I, and 500 fmol of 9-1-1. The substrates were labeled at the 5'-end of the downstream primers. The reactions were performed at 37 °C for 10 min. Right, graph of -fold stimulation. Regular represents conventional nick substrate (D4:T3:U3). Mismatch represents mismatch substrate (D2:T1:U1). nt, nucleotide.

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FIGURE 3. 9-1-1 stimulates DNA ligase I but not T4 ligase. In the experiments, the 5'-end of the downstream primers of substrates were radiolabeled using [γ-32P]ATP. Each reaction contained 10 fmol of substrate (D1:T1:U1). Reactions were incubated at 37 °C for 10 min. Lane 1 contained just substrate. Lane 2 contained substrate and 500 fmol of 9-1-1 complex. Lanes 3–8 contained substrate, 2 fmol of DNA ligase I, and 0, 25, 50, 100, 250, and 500 fmol of 9-1-1, respectively. The addition of 9-1-1 did not increase the amount of ligation product generated by T4 ligase (data not shown). The proportion of product was calculated as described in the legend of Fig. 2. nt, nucleotide.

Our present observations support the proposed direct role of 9-1-1 in DNA repair. We previously proposed that 9-1-1 is a damage-specific substitute for PCNA, in acting as a platform and coordination factor for DNA repair proteins (17). This possibility is consistent with the proposed movement of 9-1-1 to damage sites to serve its role as a damage sensor (49). It is also consistent with its structural and functional resemblance to PCNA. This hypothesis also offers an explanation for how repair functions could be maintained during a damage response in which p21 is induced and halts DNA replication by inactivation of PCNA (1, 4, 5, 50). Moreover, recent results show that 9-1-1 interacts with a variety of components of LP-BER, and stimulates DNA polymerase β, FEN1, and DNA ligase I (15–19), indicative of direct participation in DNA repair.

DISCUSSION

We previously proposed that 9-1-1 is a damage-specific substitute for PCNA, in acting as a platform and coordination factor for DNA repair proteins (17). This possibility is consistent with the proposed movement of 9-1-1 to damage sites to serve its role as a damage sensor (49). It is also consistent with its structural and functional resemblance to PCNA. This hypothesis also offers an explanation for how repair functions could be maintained during a damage response in which p21 is induced and halts DNA replication by inactivation of PCNA (1, 4, 5, 50). Moreover, recent results show that 9-1-1 interacts with a variety of components of LP-BER, and stimulates DNA polymerase β, FEN1, and DNA ligase I (15–19), indicative of direct participation in DNA repair.

Our present observations support the proposed direct role of 9-1-1 with respect to DNA ligase I activity (Fig. 7, lanes 10–12). The degree of stimulation on the linear and circular substrates was nearly the same. We conclude that stimulation does not require 9-1-1 loading and encirclement of the DNA.
9-1-1 in DNA repair. We show that 9-1-1 and DNA ligase I co-immunoprecipitate from cell extracts. Significantly, UV irradiation of the cells doubles the amount of 9-1-1/DNA ligase I interaction. Interestingly, IR treatment does not increase the amount of the complex. The damage caused by IR generates double strand breaks (12, 51). Mutants that are defective in double strand break repair, such as in RAD51, RAD52, Ku etc., are sensitive to IR. In double strand break repair, DNA ligase I is thought to participate in homologous recombination, and DNA ligase IV plays a key role in non-homologous end joining (12).

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FIGURE 6. CK2 inhibits DNA ligase I but allows 9-1-1 stimulation. Each reaction contained 10 fmol of substrate (D1: T1: U1). A, the addition of CK2 decreased the total amount of ligation products but did not affect stimulation of DNA ligase I by the 9-1-1 complex. Levels of 250 and 500 fmol of 9-1-1 were used in lanes 4, 9 and 5, 10, respectively. Lanes 1–5 were in the absence of CK2. Lanes 6–10 were in the presence of 1 million of CK2. B, CK2 inhibits DNA ligase I activity. Lanes 2–7 contained 5 fmol of DNA ligase I and 0, 0.05, 0.1, 0.5, 1, and 2 milliunits of CK2, respectively. For each figure, the proportion of product was calculated as described in the legend of Fig. 2. nt, nucleotide.

FIGURE 7. 9-1-1 stimulates DNA ligase I on either a linear nicked substrate or a closed circular substrate. Each reaction contained 10 fmol of substrate and was performed at 37 °C for 10 min as described under “Experimental Procedures.” Left, 9-1-1 stimulation on linear substrate (D1: T1: U1). Lane 1, substrate only. Lane 2, substrate and 500 fmol of 9-1-1 complex. Lanes 3–6, substrate, 2 fmol of DNA ligase I, and 0, 100, 250, and 500 fmol of 9-1-1 complex, respectively. Right, 9-1-1 stimulation on a closed circular substrate (D1: dX174:U1). Lane 7, substrate only. Lane 8, substrate and 500 fmol of 9-1-1 complex. Lanes 9–12, substrate, 2 fmol of DNA ligase I, and 0, 100, 250, and 500 fmol of 9-1-1 complex, respectively. Substrate and ligation product sizes were 30 and 59 nucleotides, respectively, as indicated. The proportion of product was calculated as described in the legend of Fig. 2. nt, nucleotide.

However, in human cells, 99% of double strand break repair is performed by non-homologous end joining (52). This may be why the DNA ligase I and 9-1-1 interaction does not increase after IR treatment. However, nucleotide excision repair is the main pathway for repair of UV damage. DNA ligase I acts on sealing the nick formed at the last stage of excision repair (12).

Both FEN1 and DNA ligase I bind PCNA and respond with higher catalytic activity (39, 40, 42, 53). There is evidence that the PCNA tethers these proteins to their substrates. Results obtained here suggest that 9-1-1 also promotes binding of DNA ligase I to its nicked DNA substrate. Besides the conventional nicked substrate, DNA ligase I has activity on substrates that have mismatches at the nicked site, have modified nucleotides, or equilibrate to a mixture of forms only some of which can be ligated. Our results show that the stimulation is similar on all of these substrate types. This suggests that the stimulation does not involve a structural change in the ligase that alters substrate specificity. One likely mechanism is that there is a general improvement in the affinity for DNA. Another possibility is that the ligase exists in several conformations, and the 9-1-1 promotes the most active conformation. This latter interpretation is consistent with the higher affinity of DNA ligase I for its substrate seen in gel shift assays. This latter behavior is also observed for PCNA stimulation of both FEN1 and DNA ligase I, suggesting a similarity in the stimulation mechanisms (42, 53).

9-1-1 was found to promote the conversion of a flap substrate to a ligated product when both FEN1 and ligase were present in the reaction. We have previously demonstrated that PCNA promotes this sequential reaction, supporting the role of PCNA in the final steps of Okazaki fragment processing (50). The similar result with 9-1-1 is consistent with the proposed role of 9-1-1 in the coordination of steps in long patch-base excision repair and other repair reactions involving FEN1 and DNA ligase I.

Why would it be necessary to have two types of clamps for FEN1 and DNA ligase I? As discussed earlier, the cellular response to sufficient DNA damage is to stop DNA replication but allow some level of DNA repair. This process involves the induction of p21, which has a C-terminal domain designed to bind and inactivate PCNA (1). If PCNA activity were reduced, it would be advantageous to have an alternative clamp for DNA repair proteins. Although 9-1-1 stimulates FEN1 and DNA ligase I activities, it does not stimulate DNA polymerase δ or promote highly processive synthesis like PCNA (16, 17). In this way it displays specificity for stimulation of DNA repair but not DNA replication. The recent observation that FEN1 has unique binding sites for PCNA and 9-1-1 is consistent with this interpretation (18). This supports the conclusion that FEN1 activity is promoted independently by the two clamps. The additional
observation that acetylation of FEN1 prevents only 9-1-1 from stimulation, suggests that independent sites have evolved so that the actions of FEN1 in DNA replication and repair can be regulated independently (18).

Treatment with high levels of CK2 strongly suppresses DNA ligase I activity. In vivo, Ser-66, which is part of the consensus site for phosphorylation by CK2, is phosphorylated during the G2/M phase (54). DNA ligase I activity is low in G2/M phase. We show that 9-1-1 still stimulates DNA ligase I activity even in the presence of CK2, although CK2 abrogates DNA ligase I stimulation by PCNA, indicating that 9-1-1 can stimulate DNA ligase I even after phosphorylation at some sites (42). Phosphorylation of Ser-66 is coincident with dissociation of PCNA from DNA ligase I (54). We suggest that during S phase, PCNA supports DNA replication by PCNA, indicating that 9-1-1 can stimulate DNA ligase I even after phosphorylation at some sites (42). Phosphorylation of Ser-66 is coincident with dissociation of PCNA from DNA ligase I (54). We suggest that during S phase, PCNA supports DNA replication and repair. During G2/M phase, PCNA cannot enhance DNA ligase I activity because of the phosphorylation status of the two proteins. Instead, 9-1-1 supports DNA ligase I in repair.

A striking difference between stimulation by PCNA and 9-1-1 is that PCNA must be loaded to encircle the DNA before it can stimulate FEN1 or DNA ligase I (42). However, 9-1-1 stimulates with apparently the same efficiency whether it is able to load onto the DNA or not. We suggest several reasons for this observation. Possibly, encirclement of the DNA is required for 9-1-1 checkpoint function. However, stimulation of ligation by 9-1-1 may derive from a protein-protein interaction that does not involve encirclement. By this view the toroidal structure of 9-1-1 is misleading in that it has evolved for only one of the two roles of the complex (55). Another possibility is that ligation in vivo is normally stimulated by a 9-1-1 molecule loaded onto the DNA. However, the stimulatory interaction site is still available when the 9-1-1 is free in solution. Therefore, when carrying out the experiment with pure proteins, we can still observe stimulation. However, in vivo, only the loading process raises the local concentration of the 9-1-1 at the damage site enough for it to function properly. This interpretation is consistent with the need for a substantial and similar molar excess of either PCNA or 9-1-1 for stimulation of either DNA ligase I or FEN1 on linear substrates (17, 42, 53). In each case the molar excess would be needed to stimulate the effective concentration normally achieved by loading onto a circular or closed substrate.

Overall, our results support the hypothesis that the 9-1-1 checkpoint complex has a dual role in serving as a damage sensor and a platform for DNA repair functions. This interpretation is consistent with its similarity to PCNA in both structure and loading mechanism and its ability to bind and stimulate DNA repair proteins. However, it is also clearly different from PCNA in its specificity for FEN1 and DNA ligase I, but not DNA polymerase δ. There is now evidence that it can stimulate both FEN1 and DNA ligase I without encirclement of the DNA. Clearly more biochemical and cell biological analyses will be required to sort out the role of the 9-1-1 in DNA repair.

Acknowledgments—We thank Marie Rossi for critical reading and suggestions, and Dr. Masamitsu Homma for kindly providing the TK6 cells. We are grateful to Dr. Peter Keng for critical advice about cell treatment. We also thank other members at the Bambara Laboratory for valuable discussions and suggestions.

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