Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair

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DNA ligase I belongs to a family of proteins that bind to proliferating cell nuclear antigen (PCNA) via a conserved 8-amino-acid motif [1]. Here we examine the biological significance of this interaction. Inactivation of the PCNA-binding site of DNA ligase I had no effect on its catalytic activity or its interaction with DNA polymerase β. In contrast, the loss of PCNA binding severely compromised the ability of DNA ligase I to join Okazaki fragments. Thus, the interaction between PCNA and DNA ligase I is not only critical for the subnuclear targeting of the ligase, but also for coordination of the molecular transactions that occur during lagging-strand synthesis. A functional PCNA-binding site was also required for the ligase to complement hypersensitivity of the DNA ligase I mutant cell line 46BR.1G1 to monofunctional alkylating agents, indicating that a cytotoxic lesion is repaired by a PCNA-dependent DNA repair pathway. Extracts from 46BR.1G1 cells were defective in long-patch, but not short-patch, base-excision repair (BER). Our results show that the interaction between PCNA and DNA ligase I has a key role in long-patch BER and provide the first evidence for the biological significance of this repair mechanism.

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Results and discussion

We have suggested that interaction of DNA ligase I with PCNA provides a molecular explanation for the unique involvement of this ligase in DNA replication [2]. As the PCNA-binding motif of DNA ligase I also mediates recruitment of the ligase to replication foci [3], PCNA binding could be required for subnuclear targeting to sites of DNA replication but not for the catalytic reactions at the replication fork. We first investigated whether inactivation of

Figure 1
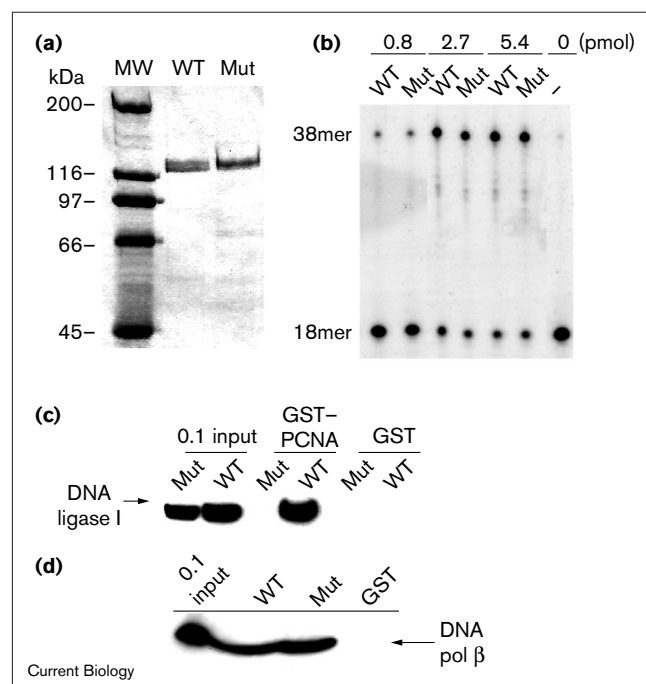
Effect of amino-acid substitutions that inactivate the PCNA-binding site of DNA ligase I on its catalytic and Pol β-binding activities. **(a)** After separation by SDS–PAGE, the wild-type (WT) and mutant (Mut) DNA ligase I purified from baculovirus-infected insect cells were detected by Coomassie blue. Molecular mass standards (in kDa) are on the left. **(b)** DNA joining by recombinant DNA ligase I. WT and mutant DNA ligase I were incubated with a labeled nicked oligonucleotide substrate (see Supplementary material). After separation by denaturing gel electrophoresis, labeled oligonucleotides were detected by autoradiography. The positions of the substrate (18mer) and ligated product (38mer) are indicated on the left. **(c)** Binding of DNA ligase I to PCNA. WT and mutant DNA ligase were incubated with glutathione beads bound by either GST–PCNA or GST (see Supplementary material). Ligase bound to the beads was detected by immunoblotting. **(d)** Binding of DNA ligase I to Pol β. Glutathione beads with the amino-terminal 118 amino acids of DNA ligase I (WT or mutant) fused to GST, or GST alone as the ligand, were incubated with Pol β (see Supplementary material). Pol β bound to the beads was detected by immunoblotting. The lane labeled 0.1 input contained one-tenth of the protein in the binding reactions.

PCNA binding had an effect on other biochemical properties of DNA ligase I. Using site-directed mutagenesis, the adjacent phenylalanine residues in the conserved PCNA-binding motif of human DNA ligase I (amino acids 8 and 9) were replaced by alanines. After subcloning into baculovirus expression vectors, wild-type and mutant DNA
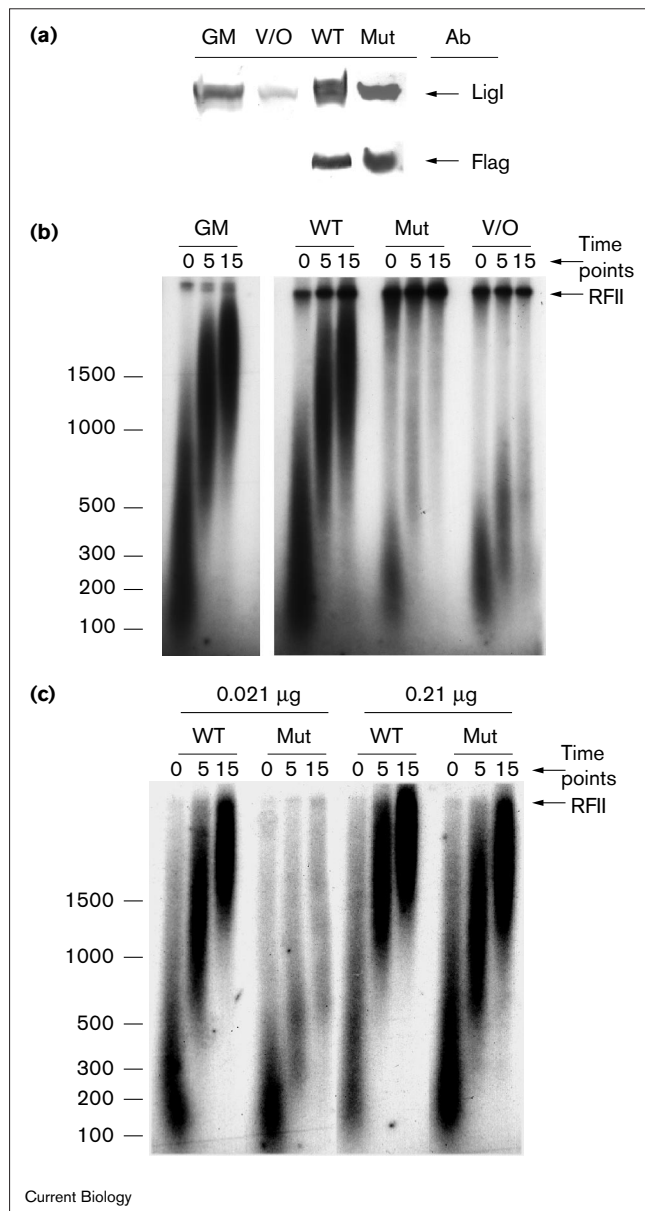
Expression of endogenous and tagged DNA ligase I in 46BR.1G1 cells and the effect of amino-acid substitutions that inactivate the PCNA-binding site of DNA ligase I on Okazaki fragment processing. Whole-cell extracts were prepared from the control cell line GM00847 (GM) and from derivatives of 46BR.1G1 stably transfected with empty expression vector (V/O), plasmid expressing Flag-tagged WT DNA ligase I, or plasmid expressing Flag-tagged mutant DNA ligase I (Mut) (see Supplementary material). **(a)** Endogenous and Flag-tagged DNA ligase I were detected in extracts (60 µg) by immunoblotting with anti-DNA ligase I and anti-Flag antibodies. **(b)** Analysis of DNA replication intermediates by pulse-chase labeling. Aliquots from DNA replication assays with the indicated extracts (360 µg) (see Supplementary material) were collected at the times indicated. **(c)** Aliquots from pulse-chase DNA replication assays supplemented with purified DNA ligase I (WT or Mut) were collected at the times indicated. The position of replicative form II DNA (RFII) is indicated. Positions of molecular mass standards (in nucleotides) are shown. After separation by alkaline agarose gel electrophoresis, labeled DNA replication intermediates were detected by autoradiography.

comparable amounts of the wild-type and mutant ligase. The level of endogenous ligase in 46BR.1G1 cells is about half that in control GM00847 cells, whereas the levels of tagged DNA ligase I in transfected 46BR.1G1 cells are about five times those of the endogenous protein.

A unique characteristic of 46BR.1G1 cells is the abnormal processing of Okazaki fragments [4,5], which can be revealed by pulse-labeling DNA replication intermediates in the SV40 *ori*-dependent DNA replication assay (Figure 2b) [5]. As expected, the replication intermediates produced by extracts from the 46BR.1G1 derivative expressing wild-type DNA ligase I were essentially identical to those produced by a control cell extract (Figure 2b). In contrast, extracts from 46BR.1G1 expressing the mutant DNA ligase I still exhibited the defect in lagging-strand DNA synthesis (Figure 2b). Addition of 21 nanograms of wild-type DNA ligase I to 46BR.1G1 extracts restored the pattern of replication intermediates to that produced by a control extract, whereas addition of an equivalent amount of mutant DNA ligase I had no effect (Figure 2c). When a 10-fold larger amount of purified ligase protein was added to the 46BR.1G1 extract, both wild-type and mutant DNA ligase I corrected the replication defect, indicating that high levels of DNA ligase protein can suppress the requirement for PCNA binding (Figure 2c). This is the first direct evidence that the interaction between DNA ligase I and PCNA coordinates the synthesis and ligation of Okazaki fragments. The possibility remains, however, that recruitment of DNA ligase I to replication foci via PCNA binding [3] is also important for cellular DNA replication.

As 46BR.1G1 cells are hypersensitive to killing by methyl methanesulfonate (MMS) [6], we compared the ability of wild-type and mutant DNA ligase I to correct this phenotype. Expression of wild-type DNA ligase I complemented the sensitivity to DNA damage (Figure 3),

ligase I were purified to near homogeneity from infected insect cells (Figure 1a). The alanine substitutions abolished PCNA binding ([3] and Figure 1c), but did not alter DNA joining activity (Figure 1b) or inactivate binding to DNA polymerase β (Pol β) (Figure 1d). Nor did the amino-acid changes abolish the ability of DNA ligase I to complement the temperature-sensitive phenotype of the *Escherichia coli lig* strain (see Supplementary material).

To elucidate the role of PCNA binding in the various DNA transactions involving DNA ligase I, we transfected DNA ligase I-deficient 46BR.1G1 cells with cDNAs encoding Flag-tagged versions of either wild-type DNA ligase I or the mutant enzyme that does not interact with PCNA. Figure 2a shows immunoblots of extracts from two stable transfected derivatives of 46BR.1G1 that express

Figure 3
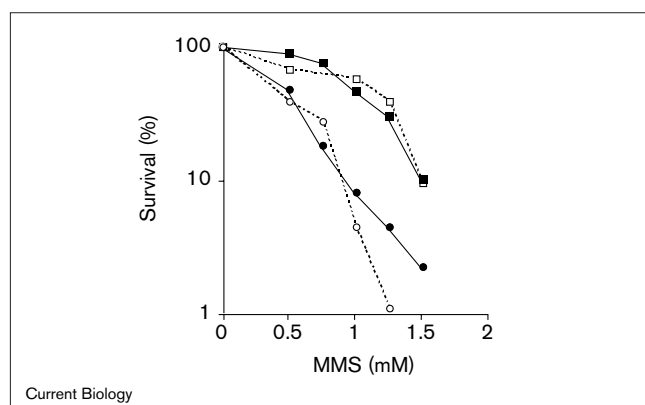
Effect of inactivation of the PCNA-binding site of DNA ligase I on the complementation of the MMS sensitivity of 46BR.1G1 cells. The control cell line GM00847 (open squares) and 46BR.1G1 stably transfected with the empty expression vector (open circles), or WT DNA ligase I cDNA (filled squares), or mutant DNA ligase I cDNA (filled circles) were incubated with MMS (see Supplementary material).

whereas the mutant DNA ligase I had no significant effect (Figure 3). These results show that participation of DNA ligase I in repair of cytotoxic DNA damage induced by MMS is mediated through its interaction with PCNA. It is generally accepted that the major cytotoxic DNA lesion introduced by MMS, 3-methyl adenine, is repaired by BER. Two subpathways of BER can be distinguished on the basis of the length of repair DNA synthesis and the requirement for PCNA [7]; the relative importance of these subpathways for the repair of different base lesions *in vivo* has not been established. As DNA ligase I has been linked with both short-patch BER (one-nucleotide repair) [8] and long-patch BER (repair tract of 2–11 nucleotides) [9], we compared the abilities of 46BR.1G1 extracts to catalyze different BER subpathways. To measure short-patch BER, we used a linear DNA duplex with a single uracil:guanine pair. The linear nature of this substrate makes it refractory to repair by PCNA-dependent BER [10]. In these assays, the 46BR.1G1 extract had essentially the same activity as a control extract both in terms of the amount of ligated product and the patch size, which was predominantly one nucleotide (Figure 4a). Similar results were obtained in assays with extracts from cells expressing either form of DNA ligase I (Figure 4a).

To measure long-patch BER, we used a circular DNA substrate with a single synthetic abasic (AP) site that cannot be repaired by short-patch BER (see Supplementary material). In these assays, the 46BR.1G1 extract generated significantly less repaired product than the control extract (Figure 4b). The addition of purified wild-type DNA ligase I protein to the 46BR.1G1 extract corrected the defect in AP site repair but had no effect on the repair reaction catalyzed by the control cell extract (Figure 4c).

Figure 4
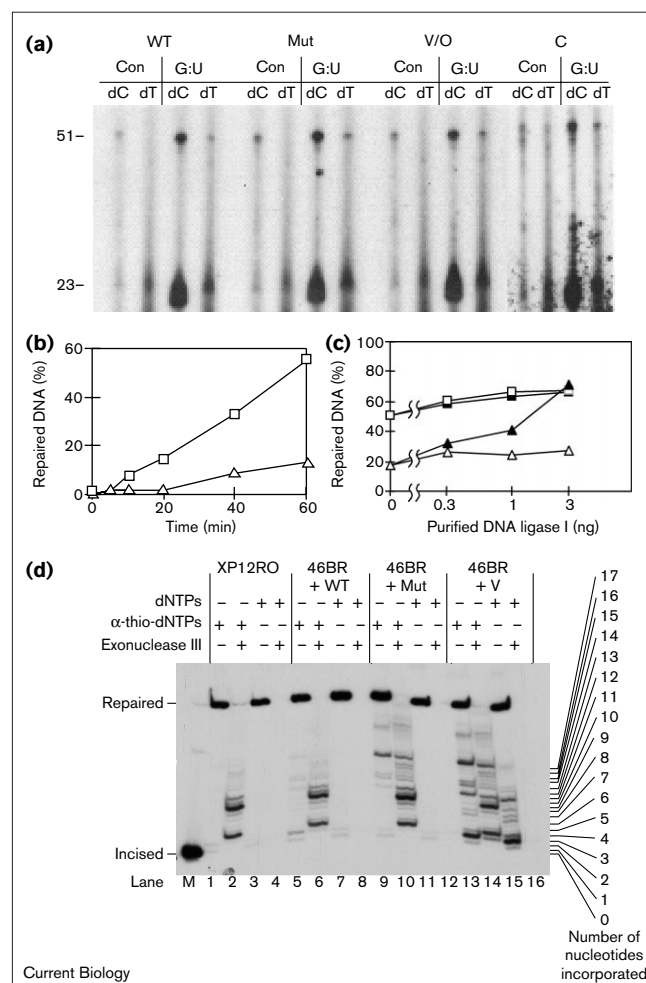
Effect of inactivation of the PCNA-binding site of DNA ligase I on short-patch and long-patch BER. **(a)** Extracts (10 μg) from the control cell line XP12RO (C) and from 46BR.1G1 stably transfected with the empty expression vector (V/O), or expressing WT DNA ligase I (WT), or mutant DNA ligase I (Mut), were assayed for short-patch BER activity using a linear oligonucleotide duplex with (G:U) or without (Con) a single uracil residue (see Supplementary material). Repair reactions contained either [α-32P]dCTP or [α-32P]TTP to detect single or multiple nucleotide incorporation events, respectively. The positions of labeled 23mer reaction intermediates and 51mer ligated products are indicated. **(b)** Extracts (2 μg) from either XP12RO (open squares) or 46BR.1G1 cells (open triangles) were assayed for long-patch BER activity using a labeled circular substrate containing a single synthetic AP site (see Supplementary material). **(c)** Long-patch BER reactions catalyzed by extracts from 46BR.1G1 cells were supplemented with either WT (closed triangles) or mutant (open triangles) purified DNA ligase I. In similar assays, control XP12RO extracts were supplemented with either WT (closed squares) or mutant (open squares) purified DNA ligase I. **(d)** Assays to determine the length of repair DNA synthesis in long-patch BER reactions catalyzed by extracts (5 μg) from control cells (XP12RO, lanes 1–4) and 46BR.1G1 expressing WT DNA ligase I (46BR + WT, lanes 5–8); mutant DNA ligase I (46BR + Mut, lanes 9–12) or containing the empty expression vector (46BR + V, lanes 13–16) (see Supplementary material). The positions of the incised product (lane M) and the repaired product are indicated on the left. DNA repair synthesis events of 0–17 nucleotides are indicated on the right.

In contrast, addition of equal amounts of the mutant form of DNA ligase I had no effect on the efficiency of AP site repair in either 46BR.1G1 or control extract (Figure 4c).

To characterize the defect in long-patch BER, we examined the effect of DNA ligase I deficiency on DNA repair synthesis. Under these reaction conditions (see Supplementary material), the repair reaction catalyzed by the control extract was essentially complete (Figure 4d, lanes 1,2), with most of the repair events having repair synthesis tracts of either two or seven nucleotides (Figure 4d, lane 2). In contrast, incomplete DNA repair events with abnormally long repair synthesis tracts, up to 17 nucleotides, were detected in assays with 46BR.1G1 extracts (Figure 4d, lane 15). This effect was more pronounced in reactions with α-thio-dNTPs (Figure 4d, lanes 13,14). As expected, extracts from 46BR.1G1 cells expressing tagged wild-type DNA ligase I produced the same pattern of repair tracts as the control extract (Figure 4d, lanes 5–8). Extracts from 46BR.1G1 cells expressing mutant DNA ligase I did not show a marked defect in the overall repair reaction (Figure 4d, lane 11). The presence of repair tracts greater than seven nucleotides, however, indicated that the abnormality in DNA repair synthesis was only partially corrected (Figure 4d, lanes 9,10). We conclude that the interaction between PCNA and DNA ligase I has a key role in the coordination of the DNA synthesis and ligation steps that complete long-patch BER. Taken together with the cell survival studies, our observations provide the first strong evidence that long-patch BER is an important DNA repair mechanism *in vivo* and is not functionally redundant with short-patch BER.

Extracts from the cell line EM9, which is deficient in DNA ligase III [11] and also hypersensitive to MMS [12], are defective in short-patch but not long-patch BER [13]. Thus, it appears that short-patch BER is completed by the DNA ligase IIIα–Xrcc1 complex, whereas long-patch BER is completed by DNA ligase I in a PCNA-dependent reaction. The simplest explanation of the alkylation-sensitive phenotypes shown by cell lines deficient in DNA ligase III or I is that these enzymes are involved in functionally distinct BER subpathways that repair different MMS-induced cytotoxic lesions. Alternatively, either DNA ligases I or III might function in DNA repair pathways, other than BER, that repair cytotoxic DNA lesions induced by alkylating agents.

Supplementary material
Supplementary material including additional methodological detail and a figure showing the complementation of the temperature-sensitive phenotype of the *E. coli lig* strain is available at http://current-biology.com/supmat/supmatin.htm.

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Supplementary material

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Figure S1
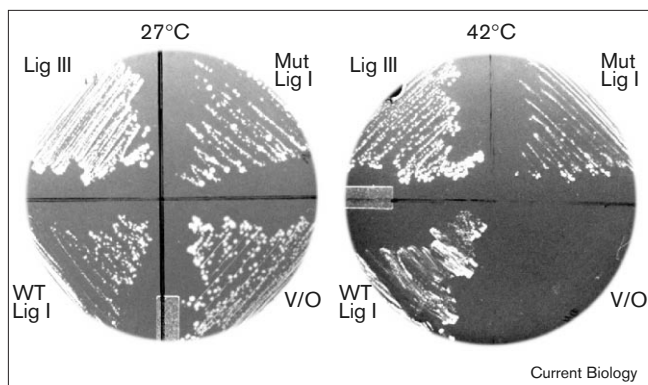
The effect of amino-acid substitutions that inactivate the PCNA-binding site of DNA ligase I on the complementation of the temperature-sensitive phenotype of an *E. coli lig* mutant. The temperature-sensitive *E. coli* strain AK76 *lig* ts7 was transformed with plasmids expressing the following proteins; empty expression vector (V/O); human DNA ligase IIIβ (Lig III); wild type DNA ligase I (WT Lig I); mutant DNA ligase I (Mut Lig I). Cultures were streaked out onto LB-AMP plates containing 2 mM isopropyl-β-D-thiogalactoside and then incubated overnight at the indicated temperatures.

Supplementary materials and methods

Materials
The SV40-immortalized DNA ligase I mutant human fibroblast cell line 46BR.1G1 has been described previously [S1]. SV40-immortalized fibroblast cell lines established from a normal individual, GM00847, and from an individual with xeroderma pigmentosum, XP12RO, were obtained from Roger Schultz. Purified antibodies against human Pol β and Pol β were supplied by Sam Wilson. A recombinant baculovirus encoding SV40 T antigen was a gift from Bruce Stillman. T antigen was purified to near homogeneity from extracts of infected insect cells by a series of column chromatography steps, including phosphocellulose, Resource Q and S (Pharmacia), hydroxylapatite, and gel filtration.

Site-directed mutagenesis of DNA ligase I cDNA and construction of DNA ligase I expression vectors
Human DNA ligase I cDNA was mutated by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The mutations altered the coding sequence such that alanine residues replaced the phenylalanines at positions 8 and 9 within the DNA ligase I ORF [S2,S3]. After verification of the nucleotide sequence by DNA sequencing, wild-type and mutant DNA ligase I cDNAs were subcloned into the pFastBac expression vector (Gibco BRL). Following infection of Sf9 cells, DNA ligase I was purified as described previously [S4]. The mutant form of DNA ligase I protein behaved identically to the wild-type enzyme during purification.

DNA joining assay
DNA joining assays were performed using an oligonucleotide substrate containing a single defined nick [S5]. DNA ligase I was incubated with

^{32}P-labeled DNA substrate in 60 mM Tris-HCl pH 8.0, 10 mM MgCl$_2$, 5 mM DTT, 1 mM ATP and 50 μg/ml BSA for 20 min at 25°C. Reactions were terminated by the addition of stop solution (40 mM EDTA pH 8.0, and 80% formamide). Labeled oligonucleotides were resolved by denaturing gel electrophoresis and detected by autoradiography.

Pull-down assays
Glutathione beads with either glutathione-*S*-transferase (GST) or GST–PCNA fusion protein as the ligand were incubated with purified DNA ligase I as described [S6]. After washing, proteins bound to the beads were separated by SDS–PAGE. DNA ligase I was detected by immunoblotting with human DNA ligase I antiserum. The binding of Pol β to glutathione beads with either GST or GST fused to the amino-terminal 118 amino acids of DNA ligase I (either wild type or with the alanine substitutions as described above), was detected in similar assays [S6].

Construction of E. coli *expression vectors and complementation of* E. coli lig *mutants*
Wild-type and mutant DNA ligase I cDNAs were subcloned into pQE32 (Qiagen) and then transformed into the temperature sensitive *E. coli* strain AK76 *lig* ts7. Transformants were plated out and grown at the permissive (27°C) or non-permissive (42°C) temperature. Similar experiments were carried out with a pQE plasmid expressing DNA ligase IIIβ cDNA, which complements the temperature-sensitive phenotype [S7].

Isolation of stable derivatives of 46BR.1G1 expressing Flag-tagged DNA ligase I
After the addition of an in-frame Flag tag (Kodak) to the 5′ end of the DNA ligase I ORF, wild-type and mutant DNA ligase I cDNAs were subcloned into the mammalian expression vector pRC/RSV (Invitrogen). 46BR.1G1 cells were transfected with either the DNA ligase I expression constructs or the empty vector using the FuGene transfection reagent (Roche) according to the manufacturer's directions. After selection for resistance to geneticin, the level of DNA ligase I protein in clones was determined by immunoblotting with DNA ligase I antiserum [S6] and an anti-Flag antibody (Sigma).

Cell survival assays
SV40-immortalized fibroblasts (4×10^4 cells) were plated in triplicate in DMEM supplemented with 10% fetal bovine serum. Methyl methanesulfonate (MMS) was added to the medium for 1 h. The drug was removed by extensive washing with PBS, before the addition of fresh media. After 5 days, surviving cells were counted using an improved Neubauer chamber.

Preparation of cell-free extracts
Cell-free extracts for replication and repair assays were prepared as described previously [S8]. Briefly, 10^8–10^9 cells were washed in ice-cold isotonic buffer (20 mM HEPES pH 7.8, 1 mM MgCl$_2$, 5 mM KCl, 250 mM sucrose, 1 mM DTT, 0.1 mM PMSF). Cells were then washed in hypotonic buffer (isotonic buffer lacking sucrose), and then resuspended in hypotonic buffer at 10^8 cells/ml. Following a 45 min incubation on ice, the cells were lysed with 10 strokes from a tight fitting Dounce homogenizer. After incubation on ice for 60 mins, the insoluble debris was removed by centrifugation and the supernatant was aliquoted, flash-frozen and stored at −80°C. Protein concentrations were measured by the method of Bradford using BSA as the standard [S9].

Cell-free DNA replication assay

To monitor replicative DNA synthesis catalyzed by cell-free extracts, DNA replication intermediates were pulse labeled with [α^{32}P]dATP followed by the addition of dATP in DNA replication assays (75 µl) as described previously [S10]. Briefly, 360 µg cell extract was incubated for 20 min at 37°C with 6 µg/ml plasmid DNA containing the SV40 *ori* in 30 mM HEPES pH 7.5, 7 mM MgCl$_2$, 0.5 mM DTT, 3 mM ATP, 40 mM phospho-creatine, 25 µg/ml creatine kinase. For assays containing added purified DNA ligase I, the enzyme was added to the cell extract before the addition of the above buffer, and incubated for 12 min at 37°C. SV40 large T antigen was then added and incubated for 30 min at 37°C. Next, pulse labeling was begun by adding CTP, GTP and UTP to 200 µM each, dCTP, dGTP and TTP to 100 µM each, and 40 µCi/ml [α^{32}P]-dATP. After a 20 sec pulse, dATP was added to a final concentration of 5 mM. Aliquots were collected 0, 5 and 15 min after the cold dATP chase. Reactions were terminated by the addition of SDS to 2% and EDTA to 20 mM. Proteinase K was added, and the mixtures were incubated for 1 h at 37°C. The reactions were then extracted with phenol-chloroform, and the DNA was ethanol precipitated. After separation by alkaline agarose gel electrophoresis, labeled DNA replication intermediates were detected by autoradiography. No labeled DNA replication intermediates were detected in the absence of added T antigen (data not shown).

Base-excision repair (BER) assays

To measure the PCNA-independent single-nucleotide insertion (short patch) subpathway of BER, we utilized a linear DNA oligonucleotide substrate containing a single uracil residue [S11]. Reactions (50 µl), which contained 10 µg of extract, were performed as described previously [S12]. To detect repair patches longer than one nucleotide, [α-^{32}P]TTP was substituted for [α-^{32}P]dCTP. After separation by denaturing gel electrophoresis, labeled oligonucleotides were detected by autoradiography.

To measure the PCNA-dependent long-patch subpathway of BER, we utilized a ^{32}P-prelabeled circular DNA duplex substrate containing a single synthetic abasic (AP) site that cannot be repaired by short-patch BER because the 5′ terminus generated by cleavage of the modified AP site is refractory to removal by Pol β [S13–S15]. For the time-course experiments, repair reactions (20 µl) contained 2 µg of the cell-free extract. For titration of purified DNA ligase I, the repair reactions were supplemented with the indicated amounts of purified DNA ligase I, either wild-type or mutant protein, and incubated for 1 h. After recovery from the reactions, the DNA substrates were digested with *Hinf*I and AP endonuclease for 2 h. Reaction products were separated by denaturing gel electrophoresis and quantified by PhosphorImager analysis. Repair of the AP site yielded a labeled 46mer that is resistant to cleavage by AP endonuclease.

To analyze repair patch size, reactions containing 5 µg of extract were incubated for 2 h in the presence of 40 µM each of the four α-thio-dNTPs instead of the regular dNTPs. After recovery from the reactions, the DNA substrates were digested with *Hae*III, *Pst*I and AP endonuclease for 2 h, followed by 1 h digestion with 8 units of *E. coli* exonuclease III [S13]. The lengths of DNA repair synthesis tracts correspond to the size of exonuclease-generated fragments composed of α-thionucleotides. Reaction products were separated by denaturing gel electrophoresis and visualized by PhosphorImager analysis.

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