Exchangeability of Mammalian DNA Ligases between Base Excision Repair Pathways

Kate M. Sleeth,[‡,§] Robert L. Robson,[§] and Grigory L. Dianov*[,‡]

Radiation and Genome Stability Unit, Medical Research Council, Harwell, Oxfordshire OX11 0RD, United Kingdom, and Microbiology Division, School of Animal and Microbial Sciences, The University of Reading, Reading RG6 6AJ, United Kingdom

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ABSTRACT: In mammalian cells, DNA ligase IIIα and DNA ligase I participate in the short- and long-patch base excision repair pathways, respectively. Using an in vitro repair assay employing DNA ligase-depleted cell extracts and DNA substrates containing a single lesion repaired either through short-patch (regular abasic site) or long-patch (reduced abasic site) base excision repair pathways, we addressed the question whether DNA ligases are specific to each pathway or if they are exchangeable. We find that immunodepletion of DNA ligase I did not affect the short-patch repair pathway but blocked long-patch repair, suggesting that DNA ligase IIIα is not able to substitute DNA ligase I during long-patch repair. In contrast, immunodepletion of DNA ligase IIIα did not significantly affect either pathway. Moreover, repair of normal abasic sites in wild-type and X-ray cross-complementing gene 1 (XRCC1)−DNA ligase IIIα-immunodepleted cell extracts involved similar proportions of short- and long-patch repair events. This suggests that DNA ligase I was able to efficiently substitute the XRCC1−DNA ligase IIIα complex during short-patch repair.

Base excision repair (BER)[1] is initiated by damage-specific DNA glycosylases that remove damaged bases, followed by endonucleases (APE1 in mammalian cells) that cleave a phosphodiester bond next to an apurinic/apyrimidinic site (AP site), leaving a 3′-hydroxyl group and a 5′-deoxyribose phosphate group flanking the strand break (*1*). Further repair can be accomplished via two pathways that involve different subsets of enzymes and result in the replacement of one (short-patch pathway) or more (long-patch pathway) nucleotides (*2*). The penultimate steps of both the short- and long-patch BER pathways result in the formation of a nicked site, which must be sealed by a DNA ligase for repair to be completed. In mammalian cells, two DNA ligases have been implicated in performing this role: DNA ligase I and DNA ligase IIIα (*3*). DNA ligase III exists in the nucleus as two isoforms, denoted DNA ligase IIIα and DNA ligase III*β*, which arise through differential splicing of RNA transcripts (*4*). DNA ligase IIIα is ubiquitously expressed, whereas DNA ligase III*β* is found only in testes and is therefore believed to be involved in homologous recombination during meiosis (*3*). DNA ligase IIIα was originally purified in a complex with X-ray cross-complementing gene 1 (XRCC1) protein (*5*), and this interaction was later demonstrated to be important for cell survival after treatment with DNA-damaging agents (*6*). Since XRCC1 null cells are characteristically deficient in short-patch BER, it has been suggested that the XRCC1−DNA ligase IIIα complex plays an important role in this sub-pathway (*7*).

DNA ligase I is conserved in all eukaryotes and plays an essential role in DNA replication (*8*). In addition to its replicative role, DNA ligase I has also been implicated in nucleotide excision repair (*9*) and BER (*10*, *11*). Human cell lines containing mutated and partially inactive DNA ligase I exhibit hypersensitivity to such DNA damaging agents as ionizing radiation and alkylating agents, suggesting a role for DNA ligase I in BER (*12*). In vitro studies also demonstrated that the interaction between proliferating cell nuclear antigen (PCNA) and DNA ligase I plays a key role in the long-patch BER pathway (*13*). Further support for DNA ligase I being involved in long-patch repair derives from the stimulation of its catalytic efficiency by replication protein A (RPA) (*14*), which has been shown to specifically stimulate long-patch BER in both mammalian cell extracts (*15*) and a reconstituted system (*16*).

Although assignment of DNA ligases to specific BER pathways is well-agreed, very little is known about their ability to substitute for each other. In this study, we generated cell extracts immunodepleted of the XRCC1−DNA ligase IIIα complex or of DNA ligase I and investigated their ability to repair damaged DNA via short- and long-patch BER pathways.

* To whom correspondence should be addressed: Radiation and Genome Stability Unit, Medical Research Council, Harwell, Oxfordshire OX11 0RD, U.K. Telephone: 44-1235-824-563. Fax: 44-1235-834-776. E-mail: g.dianov@har.mrc.ac.uk.

‡ Medical Research Council.

§ The University of Reading.

[1] Abbreviations: BER, base excision repair; AP sites, apurinic/apyrimidinic sites, abasic sites; WCE, whole cell extract; Pol *β*, DNA polymerase *β*; APE1, human AP endonuclease; PARP-1, poly(ADP-ribose) polymerase 1; XRCC1, X-ray cross-complementing gene 1; PCNA, proliferating cell nuclear antigen.

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EXPERIMENTAL PROCEDURES

Materials. Synthetic oligodeoxyribonucleotides purified by high-performance liquid chromatography were obtained from MWG (8-oxoguanine) or Oswel (uracil). (γ-^{32}P)ATP (3000 Ci/mmol) was purchased from NEN Life Science Products. DNA ligase III (ab587), DNA ligase I (ab615), and PCNA and actin (ac-15) antibodies were purchased from Abcam, Cambridge, U.K. PCNA antibody was purchased from Santa Cruz, CA. Antibodies against human XRCC1, human FEN1, rat Pol β, and human APE1 were raised in rabbit and affinity-purified as described (*17*). Uracil−DNA glycosylase was purified as previously described (*17*) and 8-oxoguanine−DNA glycosylase (mOGG1) was a gift from Dr. D. Zharkov.

DNA Substrates. The oligonucleotide 5′-ATATACCGCG-(8-oxo)GCCGATCAAGCTTATT-3′ (30 pmol) was 5′-end-labeled with 100 μCi (33 pmol) of (γ-^{32}P)ATP and used for the construction of substrates containing single 8-oxoguanine in closed-circular double-stranded DNA as previously described (*18*). The oligonucleotide 5′-CTAGAGGATC-CCCATATACCGCG-3′ (15 pmol) was 5′-end-labeled with 50 μCi (16.5 pmol) of (γ-^{32}P)ATP. The labeled oligonucleotide was mixed with an equimolar amount of oligonucleotide 5′-TGCATGCCTGCAGGTCGACU-3′ (where U stands for uracil), annealed to a complementary 36-mer strand, and ligated by incubation with 25 units of T4 DNA ligase in rapid ligation buffer (Promega). The 40-mer labeled ligation product was then purified by 20% denaturing polyacrylamide gel, 5′-end-labeled with 50 μCi (16.5 pmol) of (γ-^{32}P)ATP, and used for the construction of double-labeled substrates containing a single uracil in closed-circular double-stranded DNA as previously described (*18*). The AP-site-containing substrates were prepared by incubating 2 nmol of 8-oxoguanine-containing substrate with 25 nmol of 8-oxoguanine−DNA glycosylase or 2 nmol of uracil-containing substrate with 1 pmol of uracil−DNA glycosylase for 20 min at 37 °C in buffer containing 50 mM Hepes-KOH at pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, and 1.5 mM DTT, followed by phenol-chloroform extraction and filtration through a Sepharose G-25 spin column (Amersham Biosciences). Because of the instability of the AP-containing DNA, the substrates were prepared just before performing the BER reactions. To prepare the reduced AP site, AP-containing substrate was treated with 0.1 M sodium borohydride for 30 min on ice and then filtered through a Sephadex G-25 spin column.

BER Reactions. Repair reactions of single-lesion closed-circular DNA substrates with whole-cell extracts (WCEs) were carried out in 50 μL of reaction mixture as described (*19*). Reactions were initiated by the addition of the indicated amount of WCEs and incubated for the indicated time at 37 °C. The reactions were stopped by addition of 2 μL of 0.5 M EDTA, 2 μL of 10% SDS, and 2 μL of proteinase K. Substrate DNA was purified from the reaction mixture by phenol-chloroform extraction and filtered through a Sepharose G-25 spin column equilibrated with 10 mM Tris-HCl at pH 8.0. Filtrates were spin-dried, dissolved in 20 μL of appropriate restriction buffers supplied by the manufacturer, and treated with 10−40 units of the indicated restriction endonuclease(s) for 1 h at 37 °C. An equal volume of gel-loading buffer was then added (95% formamide, 20 mM EDTA, 0.02% bromphenol blue, and 0.02% xylene cyanole).

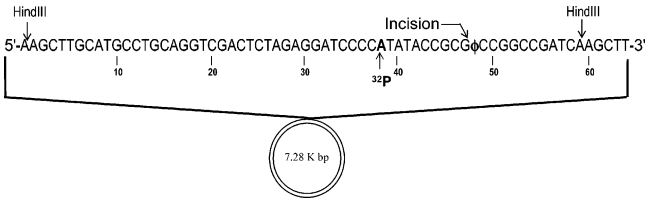

FIGURE 1: Schematic presentation of the AP site (ϕ) containing substrate (an AP site-containing strand of a double-stranded closed-circular substrate is shown). The sites of cleavage by restriction enzyme *Hind*III and the position of the ^{32}P-labeled nucleotide are shown. The site of incision by AP endonuclease is indicated (incision).

After incubation at 90 °C for 2−5 min, the reaction products were separated by electrophoresis in a 10% polyacrylamide gel containing 7 M urea in 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA at pH 8.0. Gels were exposed to PhosphorImager screens, and Quantity One software (Bio-Rad) was used for quantitation of the reaction products. The accomplished repair was calculated as the percentage of radioactivity in the 59-mer divided by the total amount of radioactivity (47-mer + 48-mer + 59-mer fragments).

Cells and Extracts. An XRCC1 null cell line (EM-C11) and its parental strain of normal Chinese hamster ovary cells (CHO-9) were kindly provided by Prof. Margaret Zdzienicka. Cells were maintained in Dulbecco's modified Eagle's medium and HAMS F12 1:1, supplemented with 10% fetal bovine serum, 1% glutamine, and 1% each of penicillin and streptomycin. HeLa cell pellets were purchased from Paragon. WCEs were prepared by the method of Tanaka (*20*) as modified by Vodenicharov (*21*) and dialyzed overnight against buffer containing 25 mM Hepes at pH 7.9, 0.1 M KCl, 12 mM $MgCl_2$, 17% glycerol, 1 mM EDTA, and 1 mM DTT. Extracts were aliquoted and stored at −80 °C.

Immunodepletion of DNA Ligase I or DNA Ligase III/XRCC1 Complex from WCEs. A total of 100 μL of WCEs (5 mg/mL in 25 mM Hepes at pH 7.9, 0.1 M KCl, 12 mM $MgCl_2$, 17% glycerol, 1 mM EDTA, and 2 mM DTT) was mixed with either DNA ligase I or XRCC1 antibodies (0.1 μg) and incubated at 4 °C for 2 h. A total of 50 μL of a 50% slurry of Protein A/G agarose (Santa Cruz) beads in the same buffer was added, and incubation was continued for a further 2 h. The beads were removed by centrifugation, and protein concentration of the immunodepleted WCEs was determined by the Bradford protein assay (Bio-Rad). Immunodepletion was verified by Western Blot.

Western Blots. Western blots were performed by standard procedure as recommended by the vendor (Novex, San Diego, CA). Blots were visualized using the ECL plus system (Amersham, Little Chalfont, U.K.).

All experiments were repeated at least 3 times, and representative gels are shown.

RESULTS

BER in WCEs Immunodepleted of XRCC1−DNA Ligase IIIα or DNA Ligase I. Two different substrates containing normal or reduced AP sites that are repaired via short- (*22*) or long-patch (*23*) BER pathways, respectively, have been used in this study. A BER assay using prelabeled single-lesion closed-circular DNA substrates (Figure 1) has been previously described in detail (*19*, *24*). In brief, after

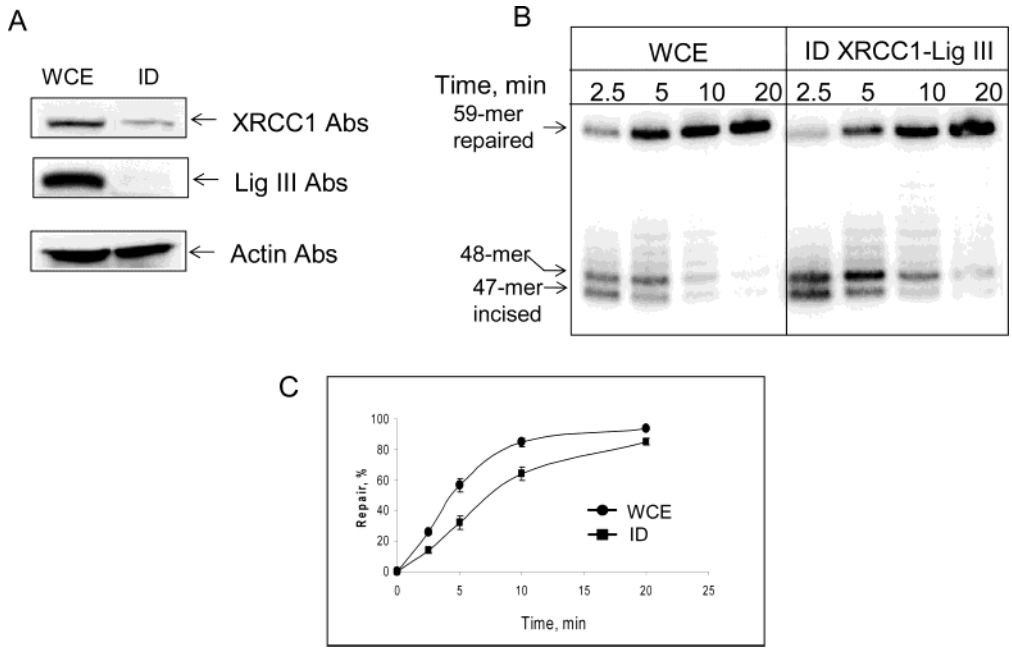

FIGURE 2: Repair of normal AP site-containing substrates by wild type and XRCC1−DNA ligase IIIα-deficient HeLa extracts. (A) XRCC1 and DNA ligase III levels in HeLa (WCE) and XRCC1−DNA ligase III-immunodepleted HeLa (ID) cell extracts. A total of 30 μg of the extract protein was subjected to electrophoresis through 10% polyacrylamide gel, transferred onto PVDF membrane, and immunobloted against indicated antibodies. (B) Time-dependent repair of normal AP sites by HeLa (WCE) and XRCC1−DNA-ligase-III-immunodepleted HeLa cell extracts (ID XRCC1−Lig III). Reactions containing 50 ng of substrate DNA and 12.5 μg of cell extract protein were incubated at 37 °C for the indicated time prior to isolation of the substrate DNA followed by digestion with *Hind*III. Reaction products were analyzed by electrophoresis in a 10% denaturing polyacrylamide gel. A phosphorimage of a representative gel is shown. (C) Graphical representation of three independent experiments.

incubation with WCE substrate, DNA is purified, cleaved with *Hind*III restriction endonuclease to release the labeled fragment, and analyzed by electrophoresis. During incubation with WCEs, AP endonuclease cleaves the AP site generating a 47-mer incision product. Then, DNA polymerase β adds one nucleotide to the 3′ end of the nicked DNA, generating the 48-mer labeled product while removing a 5′-sugar phosphate. However, processing of reduced AP sites through the long-patch BER pathway additionally requires DNA polymerase δ, PCNA, and flap endonuclease 1 (FEN1) (*25*) and will generate further extension of the 48-mer product. Finally, DNA ligase seals the DNA ends, which is monitored by accumulation of the 59-mer repaired product (left panel of Figure 2B). Any deficiency in DNA ligation would result in accumulation of the 48-mer product with the complementary decrease of the 59-mer repaired product. In cell extracts, DNA ligase IIIα is always present in a tight complex with XRCC1 (*26*); therefore, we used XRCC1 antibodies to generate XRCC1−DNA ligase IIIα-deficient cell extracts (Figure 2A). When the repair activity of this cell extract was compared to that of the wild type, we observed a small yet reproducible delay in repair of the substrate containing a normal AP site (parts B and C of Figure 2) and only a slight delay in repair of the reduced AP site-containing substrate (parts A and B of Figure 3). To address the question of which ligase functions in XRCC1−DNA ligase IIIα-deficient cell extracts, we used EM-C11 cells, which are deficient in XRCC1 and have a 3−5-fold reduced level of DNA ligase IIIα (*7*). We immunodepleted DNA ligase I from this extract and tested its ability to perform a repair on a normal AP site-containing DNA substrate. We found a 2−3-fold reduction in the repair activity after removal of DNA ligase I from EM-C11, suggesting that DNA ligase I is responsible for the majority of DNA ligation activity when the amount of XRCC1−DNA ligase IIIα is limited (Figure 4).

Repair in XRCC1−DNA Ligase III-Deficient Cell Extracts Is Accomplished through the Short-Patch Pathway. Normal AP sites are mostly processed through short-patch BER (*22*). As mentioned above, removal of the XRCC1−DNA ligase IIIα complex, the major DNA ligase activity involved in the short-patch BER pathway, did not markedly affect the repair of normal AP sites. Therefore, it was interesting to find out whether a substitute, DNA ligase I, which normally functions in long-patch BER, operates within the short-patch repair pathway or if repair was switched in favor of long-patch BER. To address this question, we constructed a double-labeled substrate DNA that allows relative usage of the BER pathways to be measured. Short-patch repair, which results only in removal and replacement of one nucleotide, should not excise the labeled cytosine located 3′ downstream to the AP site (Figure 5A). However, if repair involves excision of more than one nucleotide (long-patch BER), this should lead to excision of the labeled cytosine. Since another labeled nucleotide (thymidine) 5′ upstream to the AP site remains unaffected by repair, the relative usage of repair pathways may be calculated as the relative proportion of radioactivity in *Hind*III−*Xba*I (Figure 5C, 24-mer) and *Xba*I−*Hind*III (Figure 5C, 35-mer) fragments after the repair of substrate DNA in WCEs compared to the relative proportions in unrepaired substrate. Using this approach we found that, in agreement with previously published data (*27*), approximately 30% of the AP sites were repaired via the long-patch pathway (Figure 5B, WCE). Similarly, approximately 30% of the AP sites were repaired through long-patch BER in the XRCC1−DNA ligase IIIα-immunodepleted WCE (Figure 5B, ID XRCC1−Lig III). These data indicate that

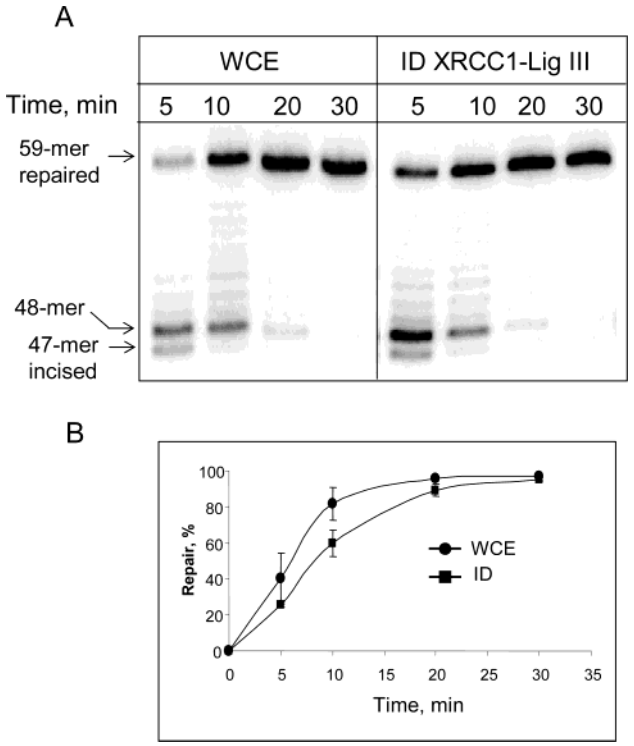

FIGURE 3: Time-dependent repair of reduced AP sites by HeLa (WCE) and XRCC1−DNA ligase IIIα-immunodepleted HeLa cell extracts (ID-XRCC1−Lig III). (A) Reactions containing 50 ng of substrate DNA and 12.5 μg of cell extract protein were incubated at 37 °C for the indicated time prior to isolation of the substrate DNA followed by digestion with *Hind*III. Reaction products were analyzed by electrophoresis in a 10% denaturing polyacrylamide gel. A phosphorimage of a representative gel is shown. (B) Graphical representation of three independent experiments.

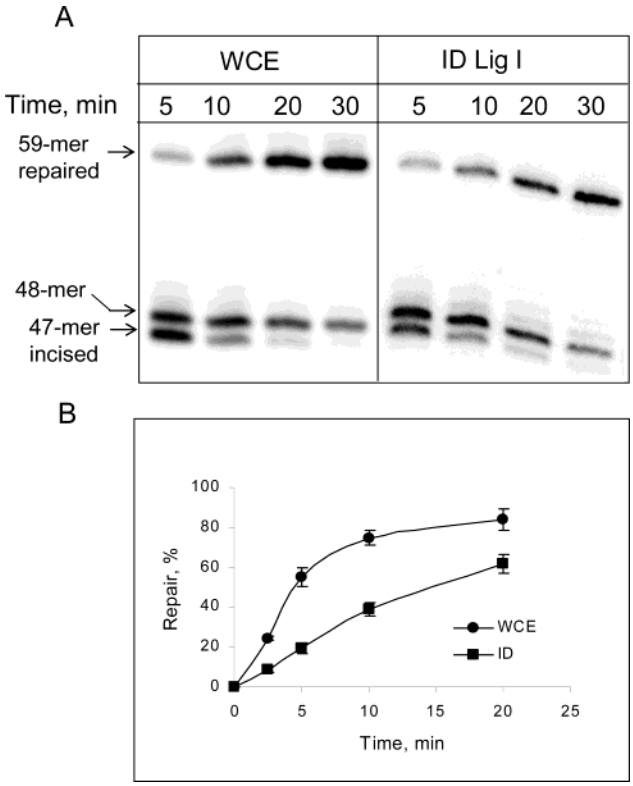

FIGURE 4: Time-dependent repair of normal AP sites by EM-C11 (WCE) and DNA ligase I-immunodepleted EM-C11 cell extracts (ID). (A) Reactions containing 50 ng of substrate DNA and 12.5 μg of cell extract protein were incubated at 37 °C for the indicated time prior to isolation of the substrate DNA followed by digestion with *Hind*III. Reaction products were analyzed by electrophoresis in a 10% denaturing polyacrylamide gel. A phosphorimage of a representative gel is shown. (B) Graphical representation of three independent experiments.

DNA ligase I was able to substitute for the XRCC1−DNA ligase IIIα complex during short-patch BER.

BER in WCEs Immunodepleted of XRCC1−DNA Ligase I. To address the question of whether XRCC1−DNA ligase IIIα is able to substitute for DNA ligase I, we generated a DNA ligase I-deficient cell extract using DNA ligase I antibodies. We demonstrated that, although the extract was nearly completely depleted in DNA ligase I, other BER proteins required for repair of both normal and reduced AP sites (PCNA, FEN1, APE1, and Pol β) were only slightly affected (Figure 6A). We next analyzed the repair activity of DNA ligase I-deficient extracts. We observed that repair of the normal AP site-containing substrate in these extracts was only slightly decreased (parts B and C of Figure 6). However, the extent of repair of the reduced AP site-containing substrate in the same extracts was reduced approximately 3-fold, and the 48-mer product characteristic of deficient ligation had accumulated (parts A and B of Figure 7). Residual repair activity is most likely due to incomplete reduction of the AP sites by sodium borohydride, because about 20−30% of the reduced AP sites were sensitive to alkaline treatment (data not shown). We thus conclude that the XRCC1−DNA ligase IIIα complex is unable to efficiently substitute for DNA ligase I during long-patch BER.

DISCUSSION

Both short- and long-patch BER pathways involved in the repair of AP sites are initiated by AP endonuclease, which generates a strand break 5′ to the AP site. Strand breaks are readily recognized by poly(ADP-ribose) polymerase-1 (PARP-1). The function of PARP-1 in BER is unknown; however, it is well-established that the PARP-1 homodimer binds to nicked DNA. Binding of PARP to a strand break activates its catalytic activity, which is to covalently link nuclear proteins, including PARP itself, with poly(ADP-ribose) synthesized from NAD^+. Poly(ADP-ribosyl)ation stimulates the dissociation of PARP-1 from DNA, allowing access to other BER enzymes (*28*). After PARP-1 dissociates, Pol β adds one nucleotide to the 3′ end of the nick, while removing the 5′-sugar phosphate (*29*). DNA ligase seals the DNA ends to accomplish short-patch BER. Although the role of PARP-1 turnover on nicked DNA is not clear, it was found recently that both XRCC1 and DNA ligase IIIα but not DNA ligase I (*30*, *31*) interact with poly(ADP-ribosyl)ated PARP-1. These data suggest that the apparent superiority of the XRCC1−DNA ligase IIIα complex over DNA ligase I during short-patch repair may be supported by its interaction with PARP-1. Our finding that removal of DNA ligase I from cell extracts did not affect the repair rate through the short-patch pathway (Figure 6) and supports the idea of a major role for the XRCC1−DNA ligase IIIα complex in this pathway as previously reported (*7*, *32*). Interestingly, immunodepletion of the XRCC1−DNA ligase IIIα complex did not change either the DNA repair activity of the immunodepleted cell extract or the repair pathway involved. Repair of normal AP sites still proceeded through the short-

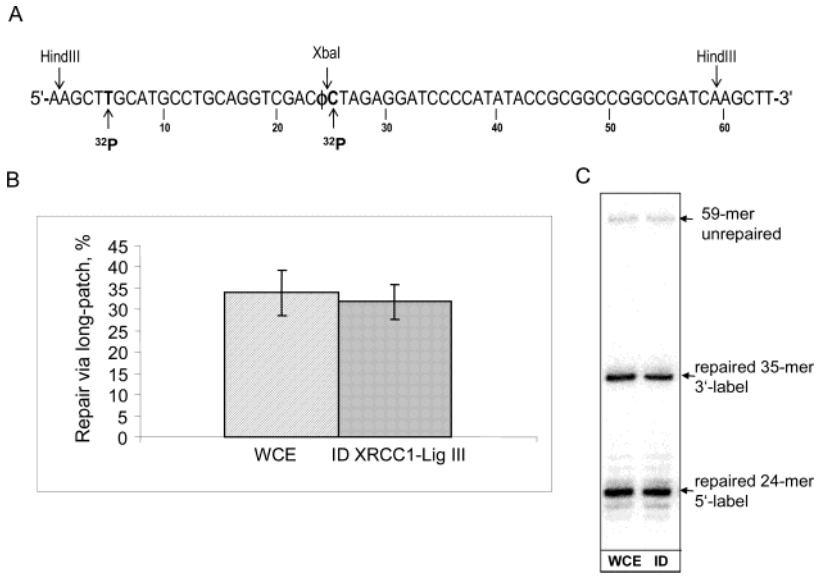

FIGURE 5: Repair of normal AP site-containing substrates by DNA ligase III-immunodepleted cell extracts proceeds via short-patch BER. (A) Schematic presentation of the double-labeled AP-site-containing substrate (an AP site-containing strand of double-stranded closed-circular substrate is shown). The AP site is indicated by ϕ. The cleavage sites for the restriction enzymes *Hind*III and *Xba*I and the positions of ^{32}P-labeled nucleotides are shown. (B) Graphical representation of three independent experiments showing the percentage repair of an abasic site via long-patch BER by HeLa (WCE) or XRCC1−DNA ligase IIIα-immunodepleted HeLa cell extracts (ID XRCC1−Lig III). Reactions containing 50 ng of substrate DNA and 12.5 μg of cell extract protein were incubated at 37 °C for 30 min prior to isolation of the substrate DNA followed by digestion with *Hind*III and *Xba*I. Reaction products were analyzed by electrophoresis in a 10% denaturing polyacrylamide gel, and the proportion of long-patch involvement was calculated as a ratio of thymidine-labeled *Hind*III−*Xba*I fragment (**T**) to cytidine-labeled *Hind*III−*Xba*I fragment (**C**). (C) Phosphorimage of a representative gel is shown.

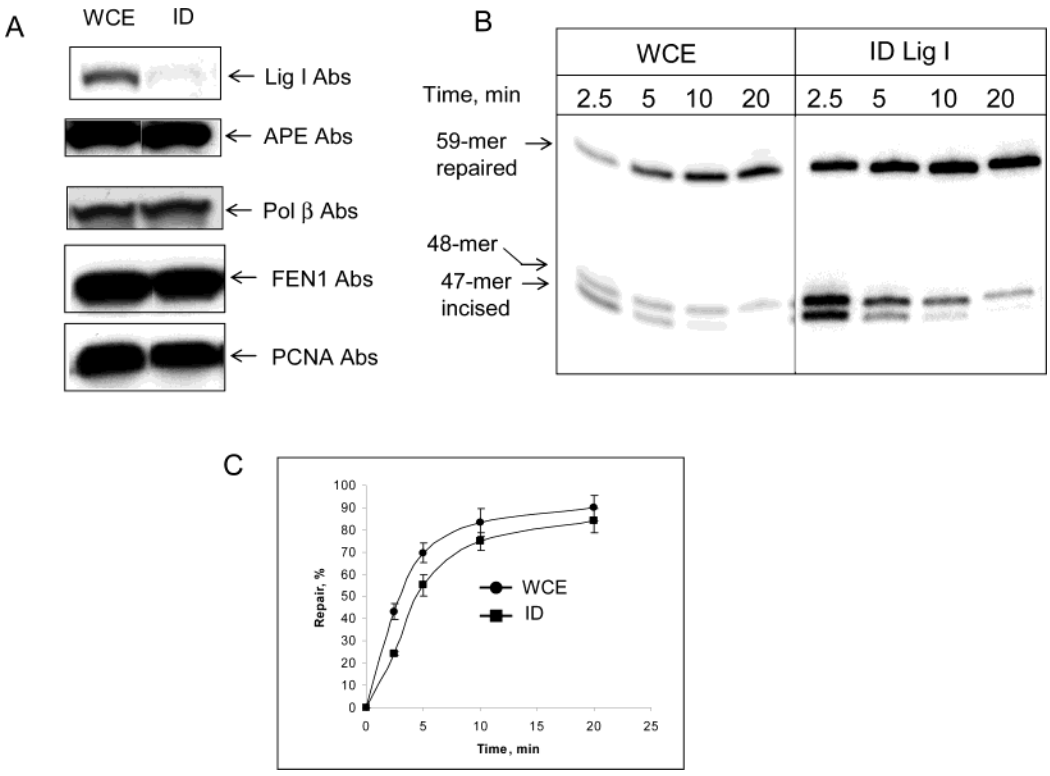

FIGURE 6: Repair of normal AP site-containing substrates by wild-type and DNA ligase I-deficient hamster cell extracts. (A) DNA ligase I, APE, PCNA, FEN-1, and Pol β levels in CHO-9 (WCE) and DNA ligase I-immunodepleted CHO-9 (ID) cell extracts. A total of 30 μg of the extract protein was subjected to electrophoresis through 10% polyacrylamide gel, transferred onto PVDF membrane, and immunobloted against indicated antibodies. (B) Time-dependent repair of normal AP sites by CHO-9 (WCE) and DNA ligase I-immunodepleted CHO-9 cell extracts (ID XRCC1−Lig I). Reactions containing 50 ng of substrate DNA and 12.5 μg of cell extract protein were incubated at 37 °C for the indicated time prior to isolation of the substrate DNA followed by digestion with *Hind*III. Reaction products were analyzed by electrophoresis in a 10% denaturing polyacrylamide gel. A phosphorimage of a representative gel is shown. (C) Graphical representation of three independent experiments.

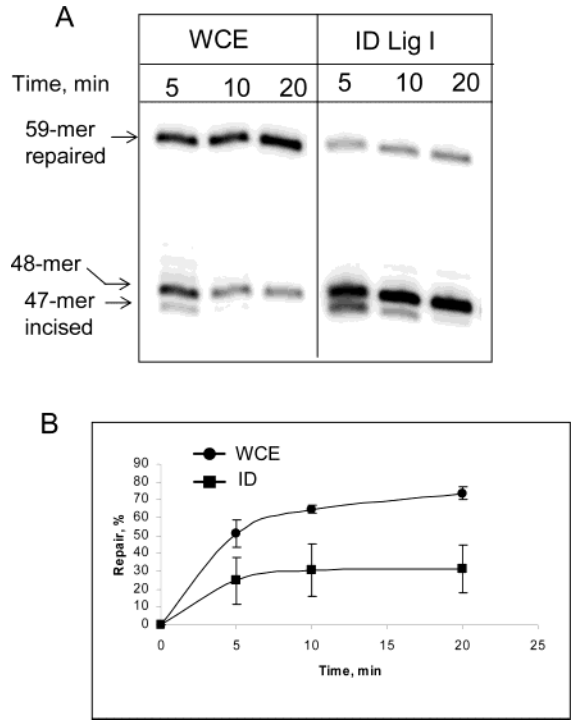

FIGURE 7: Time-dependent repair of reduced AP sites by CHO-9 (WCE) and DNA ligase I-immunodepleted CHO-9 cell extracts (ID). (A) Reactions containing 50 ng of substrate DNA and 12.5 μg of cell extract protein were incubated at 37 °C for the indicated time prior to isolation of the substrate DNA followed by digestion with *Hind*III. Reaction products were analyzed by electrophoresis in a 10% denaturing polyacrylamide gel. A phosphorimage of a representative gel is shown. (B) Graphical representation of three independent experiments.

patch pathway (Figure 3), suggesting that, in cell extracts, DNA ligase I is able to efficiently substitute for the XRCC1−DNA ligase IIIα complex during short-patch BER. Mechanistically, this function may be supported by the interaction of DNA ligase I with Pol β (*10*, *33*). Our data on the ability of DNA ligase I to operate efficiently in short-patch BER in the absence of the XRCC1−DNA ligase IIIα complex are supported by a recent finding by Tebbs et al. that transgenic mice with a 10-fold reduced amount of XRCC1−DNA ligase IIIα complex did not show any abnormalities in development or sensitivity to DNA alkylation (*34*).

We also found that repair of reduced AP sites through the long-patch pathway was significantly reduced in the DNA ligase I-immunodepleted cell extracts (Figure 7) but only slightly diminished in the XRCC1−DNA ligase IIIα-immunodepleted cell extracts (Figure 3). This suggests that DNA ligation during long-patch BER is mainly accomplished by DNA ligase I and that the XRCC1−DNA ligase IIIα complex is unable to replace it. Indeed, cultivated cells derived from a patient with reduced DNA ligase I activity are sensitive to DNA alkylating agents, confirming the inability of the XRCC1−DNA ligase IIIα complex to compensate for DNA ligase I (*12*).

The biological role of such "one way" restricted functionality (complete exchangeability of DNA ligase III and I in short-patch repair yet an exclusive role of DNA ligase I in long-patch repair) is unclear. Long-patch repair in addition to APE1, PARP-1, and Pol β also involves Pol δ/ϵ, flap endonuclease, and PCNA (*23*). Both XRCC1 (*35*) and DNA ligase I (*13*) interact with PCNA, and the DNA ligase I−PCNA interaction is critical for its function in long-patch BER and DNA replication (*13*). This is an important functional interaction because it can mechanistically exclude DNA ligases other than DNA ligase I from PCNA-dependent DNA replication.

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