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Bypass of a Psoralen DNA Interstrand Cross-Link by DNA Polymerases β, ι, and κ in Vitro

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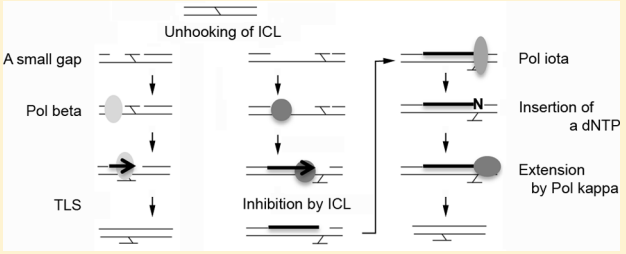
Supporting Information

ABSTRACT: Repair of DNA interstrand cross-links in mammalian cells involves several biochemically distinctive processes, including the release of one of the cross-linked strands and translesion DNA synthesis (TLS). In this report, we investigated the in vitro TLS activity of a psoralen DNA interstrand cross-link by three DNA repair polymerases, DNA polymerases β, κ, and ι. DNA polymerase β is capable of bypassing a psoralen cross-link with a low efficiency. Cell extracts prepared from DNA polymerase β knockout mouse embryonic fibroblasts showed a reduced bypass activity of the psoralen cross-link, and purified DNA polymerase β restored the bypass activity. In addition, DNA polymerase ι misincorporated thymine across the psoralen cross-link and DNA polymerase κ extended these mispaired primer ends, suggesting that DNA polymerase ι may serve as an inserter and DNA polymerase κ may play a role as an extender in the repair of psoralen DNA interstrand cross-links. The results demonstrated here indicate that multiple DNA polymerases could participate in TLS steps in mammalian DNA interstrand cross-link repair.



DNA interstrand cross-links (ICLs) are repaired by multiple pathways in mammalian cells[1] (Figure 1). Each pathway requires the release of one of the cross-linked strands (unhooking of a cross-link) followed by translesion DNA synthesis (TLS) of the unhooked (yet cross-linked) strand. Several DNA polymerases have been found to accomplish this TLS process, including DNA polymerases ν, κ, and ζ; however, recent studies indicate redundancy among TLS polymerases in ICL repair.[2,3]

DNA polymerase β participates in base excision repair (BER). Interestingly, DNA polymerase β-overexpressing cells are resistant to several chemotherapeutics, including cisplatin. Suppression of DNA polymerase β altered the cellular sensitivity to cisplatin, but not to oxaliplatin, indicating a role of DNA polymerase β in the repair of cisplatin ICLs.[4] In addition, DNA polymerase β bypasses 8-oxoguanine, UV photoproducts, and the cisplatin intrastrand cross-link (G^G) when the lesion is placed in a gap.[5] These reports suggest that DNA polymerase β is involved in a TLS step in ICL repair.

DNA polymerase κ inserts nucleotides opposite of a variety of DNA bases and has a unique property to bypass minor groove adducts that form at the N^2 position of guanine in vitro.[6,7] DNA polymerase κ can function during the gap filling step of nucleotide excision repair (NER), in place of DNA polymerase δ, under certain conditions.[8] Importantly, DNA polymerase κ bypasses N^2–N^2 guanine ICLs, which mimic mitomycin C ICLs, in vitro.[9] Also, the suppression of DNA polymerase κ results in sensitivity to mitomycin C, suggesting a role for DNA polymerase κ in the bypass of minor groove ICLs in human cells.[9]

DNA polymerase ι is a highly error prone DNA polymerase, especially opposite from pyrimidines.[10] DNA polymerase ι also has the ability to bypass various types of DNA adducts in vitro.[11] It has been demonstrated that the sequential action of DNA polymerase ι and DNA polymerase κ promotes the efficient bypass of some lesions, in which DNA polymerase ι incorporates the nucleotide opposite the lesion site and DNA polymerase κ performs the extension reaction.[12]

We investigated the in vitro TLS activity of DNA polymerases β, κ, and ι using a psoralen ICL as a template. DNA polymerase β incorporated a dNMP opposite a psoralen ICL and extended the primer to a much lesser extent. We also detected DNA polymerase β-dependent bypass activity of a psoralen ICL in cell extracts from mouse embryonic fibroblasts. DNA polymerases κ and ι did not demonstrate the bypass activity of a psoralen ICL; however, the sequential action of DNA polymerase ι and DNA polymerase κ promotes the

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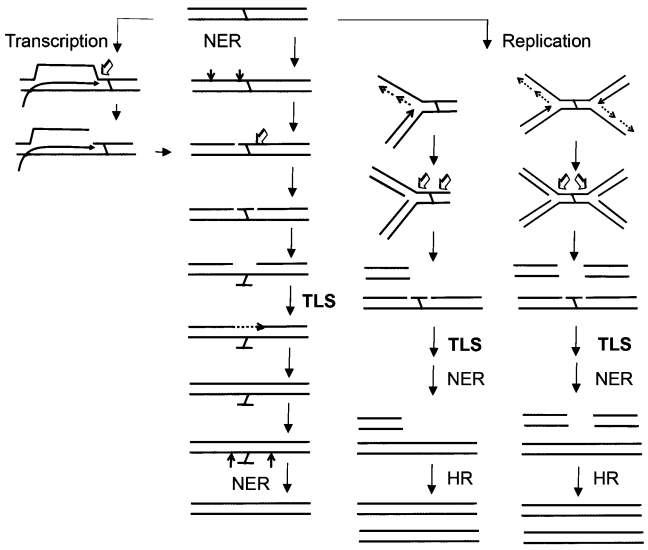

Figure 1. Proposed ICL repair pathways in humans. DNA interstrand cross-links (ICLs) are repaired by multiple pathways. In each pathway, translesion DNA synthesis (TLS) plays a critical role. (1) Nucleotide excision repair (NER) makes dual incisions at the 5′ side of an ICL and leaves a nick. A subsequent incision by a nuclease/exonuclease releases one of the cross-linked strands (unhooking of an ICL). A generated gap is filled by a TLS polymerase to restore duplex DNA. A second round of NER removes a lesion with the unhooked ICL. DNA repair synthesis and DNA ligation complete a repair process. (2) When RNA polymerase encounters an ICL during transcription, RNA polymerase stalls near the site of the ICL and generates a DNA–RNA–protein complex. This complex might recruit NER and/or nucleases to generate a nick 5′ to the ICL. Once the nick is introduced, the ICL will be repaired by the same process as the NER-mediated ICL repair. (3) During DNA replication, a replication fork is blocked near the site of the ICL and generates a "Y-shaped" structure. A structure-specific endonuclease such as XPF-ERCC1 generates a nick at one side of the ICL, and a second endonuclease makes a second incision at the other side of the ICL to unhook the ICL. These actions generate one broken end and a gap with an unhooked ICL. TLS fills the gap and restores duplex DNA. After NER removes a lesion with an unhooked ICL, homologous recombination (HR) repairs the broken end and restores a replication fork. A second scenario is initiated by a merging of two replication forks at an ICL. When two opposing replication forks meet at an ICL, an "X-shaped" structure is generated. Endonucleases generate incisions at the 5′ and 3′ sides of an ICL to unhook the ICL. These actions result in the formation of two broken ends and a gap with an unhooked ICL. TLS fills the gap and restores duplex DNA. After NER removes a lesion with an unhooked ICL, HR repairs the broken ends. Endonucleases and other repair factors involved and exact repair intermediate structures in each pathway are being investigated.

bypass of a psoralen ICL. Moreover, DNA polymerase ι misincorporated TMP opposite of a psoralen ICL with an efficiency similar to that of the incorporation of dAMP. Because psoralen modifies thymines to form an ICL, the misincorporation of TMP opposite a psoralen ICL results in a T-to-A transversion, which is the main point mutation induced by psoralen ICLs. These results suggest that DNA polymerases β, κ, and ι might be involved in TLS steps in mammalian ICL repair pathways.

EXPERIMENTAL PROCEDURES

Substrate Preparation. A 12-mer oligonucleotide (5′-GAAGCTACGAGC-3′) with a psoralen furan-side monoadduct at the T (100 pmol) was annealed to 100 pmol of a 53-mer oligonucleotide (5′-biotin-CCTGCTGCAGCCCAAGCTCGTAGCTTCTGACTGGCGCAGATCTGGCTCGAGGA-biotin-3′) (underlined portion complementary to the 12-mer). The partially duplexed DNA was exposed to UVA radiation (366 nm) for 10 min to convert the monoadduct to an ICL. The cross-linked substrate was purified from a 10% denaturing polyacrylamide gel and annealed to a 5′-^{32}P-labeled 22-mer primer (5′-TCCTCGAGCCAGATCTGCGCCA-3′). The substrate was then purified from a 6% nondenaturing polyacrylamide gel. Under these conditions, the cross-linked substrate is well separated from the non-cross-linked substrate. Typically, ~0.3% of the substrate was non-cross-linked.[13,14] For the undamaged substrate, a ^{32}P-labeled 22-mer primer was annealed to the 53-mer template (Figure 2).

The psoralen-modified 12-mer was a generous gift from J. Hearst. The 5′- and 3′-biotin-labeled 53-mer was purchased from OPERON, and the 22-mer was synthesized at the Molecular Biology Core at the University of Nebraska Medical Center.

In Vitro DNA Polymerase Assay. Reaction mixtures [10 mM HEPES-KOH (pH 7.9), 7.5% glycerol, 2.5 mM KCl, 5 mM $MgCl_2$, 0.1 mg/mL BSA, dNTPs (250 μM each), 2 nM substrate, and the indicated concentration of DNA polymerase in 20 μL] were incubated at 37 °C for the indicated time. The amount of DNA polymerase β or DNA polymerase κ used was determined to provide a similar primer extension efficiency on an undamaged template. The reactions were terminated by the addition of 25 mM EDTA, followed by phenol/chloroform extraction. The reaction products were isolated by ethanol precipitation and analyzed on a 10% denaturing polyacrylamide gel. The dried gel was exposed to a PhosphorImage screen; an image was obtained by scanning the screen with the Typhoon 9410 (GE Healthcare), and the products were quantified with ImageQuant. The amount of products longer than 34 nucleotides was used to calculate the percent bypass activity.

For DNA polymerase ι, a reaction was performed in iota buffer [40 mM Tris-HCl (pH 8.0), 0.15 mM $MnCl_2$, 0.2 mg/mL BSA, 2.5% glycerol, dNTPs (100 μM each), 60 pM substrate, and the indicated amount of DNA polymerase ι] at 37 °C for the indicated time. For the steady-state kinetic study, DNA polymerase ι (2 nM) was incubated with 200 pM substrate at 37 °C for 3 min in the presence of a range of concentrations (1, 3, 10, 30, and 100 μM) of each dNTP.

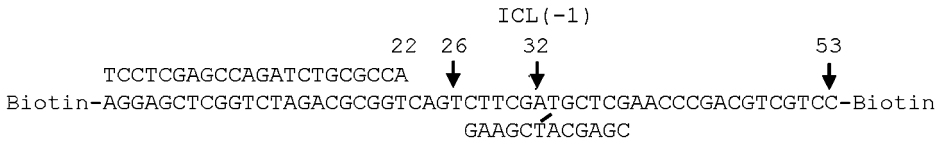

Figure 2. Defined substrate with a single psoralen ICL. A psoralen ICL is located in the middle of a 12-mer oligonucleotide annealed to a 53-nucleotide template. Biotin moieties are linked to the 5′ and 3′ ends of the 53-nucleotide template.

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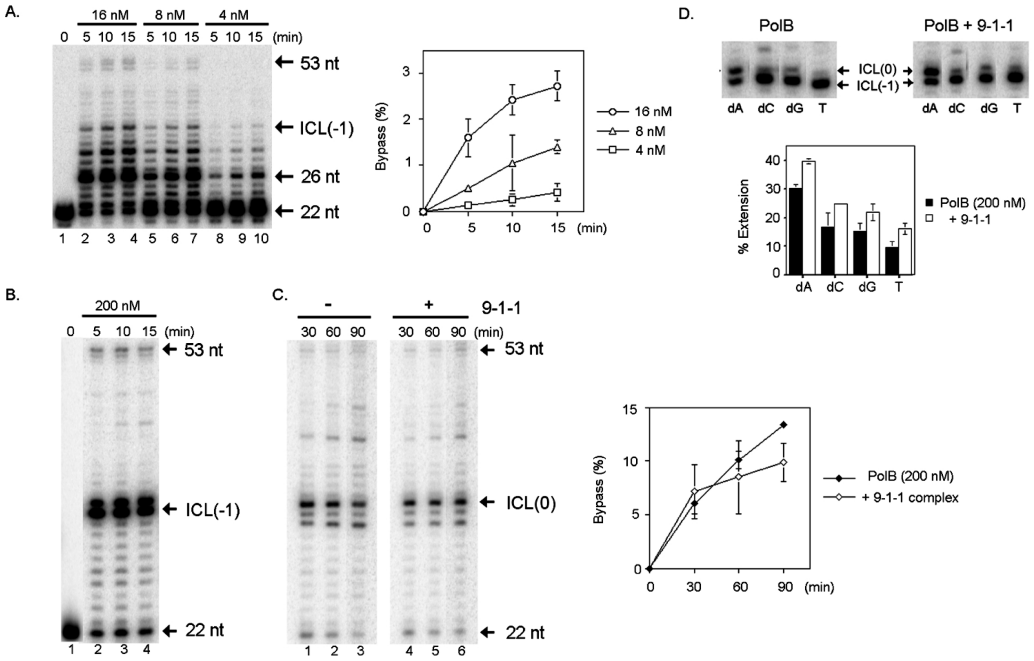

Figure 3. Bypass of an unhooked psoralen ICL by DNA polymerase β. (A) DNA polymerase β bypasses a psoralen ICL in vitro. DNA polymerase β was incubated with a defined substrate (2 nM) with a psoralen ICL in the presence of 250 μM dNTPs at 37 °C. The reaction mixtures were incubated for 5 min (lanes 2, 5, and 8), 10 min (lanes 3, 6, and 9), and 15 min (lanes 4, 7, and 10): lane 1, no polymerase; lanes 2−4, 16 nM DNA polymerase β; lanes 5−7, 8 nM DNA polymerase β; lanes 8−10, 4 nM DNA polymerase β. Twenty-two nucleotides, primer; ICL(−1), 32-nucleotide fragment (a termination product at one nucleotide before the ICL); and 53 nucleotides, a fully extended product. Fragments with more than 34 nucleotides were considered as bypass products. Bypass efficiency was determined and is plotted as a graph next to the gel. The error bars represent standard deviations obtained from three independent experiments. (B) DNA polymerase β incorporates nucleotides opposite a psoralen ICL. An increased concentration of DNA polymerase β (200 nM) was incubated with 2 nM substrate in the presence of 250 μM dNTPs at 37 °C for 5 min (lane 1), 10 min (lane 2), and 15 min (lane 3). Twenty-two nucleotides, primer; ICL(−1), 32-nucleotide fragment (a termination product at one nucleotide before the ICL). (C) A checkpoint clamp 9-1-1 complex has no effect on the ICL bypass activity of DNA polymerase β in vitro. The 9-1-1 complex (25 nM) was preincubated with 2 nM substrate on ice for 5 min. After DNA polymerase β (200 nM) had been added, the reaction mixture was incubated at 37 °C for 30 min (lanes 1 and 4), 60 min (lanes 2 and 5), and 90 min (lanes 3 and 6): lanes 1−3, without the 9-1-1 complex; lanes 4−6, with the 9-1-1 complex. Twenty-two nucleotides, primer; ICL(−1), 32-nucleotide fragment (a termination product at one nucleotide before the ICL); ICL(0), 33-nucleotide fragment (product of incorporation of a nucleotide opposite the ICL). Bypass efficiency was determined and is plotted as a graph next to the gel image. The error bars represent standard deviations obtained from three independent experiments. (D) Preferential incorporation of dAMP opposite a psoralen ICL by DNA polymerase β. The ICL(−1) substrate was prepared by extending the 22-nucleotide primer of the substrate to 32 nucleotides by Klenow fragment. The ICL(−1) substrate (2 nM) was incubated with DNA polymerase β in the presence of a single dNTP (1 mM) at 37 °C for 20 min: left panel, without the 9-1-1 complex; right panel, with the 9-1-1 complex (25 nM). The percentage of primer extension was determined in each lane and is plotted as a graph below the gel image. The error bars represent standard deviations from three independent experiments.

In Vitro TLS Assay with Cell Extracts. Nuclear extracts were prepared from mouse embryonic fibroblast (MEF) cells as described previously.[15,16] Nuclear extract (3 μg) from wild-type MEF cells or DNA polymerase β knockout MEF cells was incubated with 0.3 nM substrate in 20 μL of reaction buffer [12.5 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 250 μM dNTPs, 2 mM ATP, 0.1 mg/mL BSA, 5% glycerol, and the indicated amount of purified DNA polymerase] at 37 °C for 30 min. After phenol/chloroform extraction, the reaction products were isolated by ethanol precipitation and analyzed on a 10% denaturing polyacrylamide gel.

Cell Lines and Purified Proteins. MEF cells with wild-type DNA polymerase β and DNA polymerase β knockout[17] were generous gifts from J. Sweasy (Yale University, New Haven, CT). MEF cells were grown in DMEM medium with 10% FBS at 37 °C and 5% CO_2.

Human GST-tagged DNA polymerase ι was expressed in yeast and purified as described by Makarova et al.[18] Purified recombinant DNA polymerases β and κ[19] were generously provided by L. V. Gening (Institute of Molecular Genetics of the Russian Academy of Science), and purified human 9-1-1 complex[20] was a generous gift from L. Lindsey-Boltz and A. Sancar (University of North Carolina at Chapel Hill, Chapel Hill, NC).

RESULTS

Bypass of a Psoralen ICL by DNA Polymerase β in Vitro. One of the cross-linked strands is proposed to be released by various mechanisms (Figure 1). To restore duplex DNA for a subsequent repair process, bypass of the released fragment that is attached to the other strand is a prerequisite (Figure 1). However, an exact DNA structure that is utilized by TLS polymerases (how many nucleotides are attached after unhooking and how far the unhooked strand is located from a primer end) is not known. We prepared a 53-nucleotide template DNA with a 12-mer oligonucleotide attached via a psoralen ICL and placed a four-nucleotide gap between a primer end and a 5′ end of an unhooked strand (Figure 2). Similar types of substrate DNA have been used to study the TLS activity of an ICL.[9,13,14,21] A primer extension to the attached oligonucleotide by a DNA polymerase results in the

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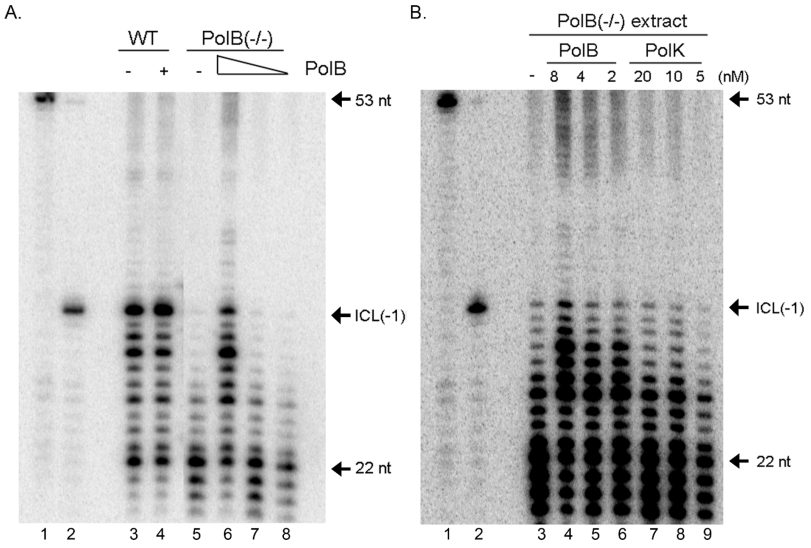

Figure 4. DNA polymerase β-dependent bypass of a psoralen ICL in mouse embryonic fibroblast cell extract in vitro. Only the products with fewer than 53 nucleotides were shown (see Figure 4 of the Supporting Information for the entire gel image). (A) In vitro bypass of a psoralen ICL is compromised in nuclear extract from DNA polymerase β-deleted MEF cells. Nuclear extract (3 μg) from wild-type (WT) or DNA polymerase β-deleted (PolB−/−) MEF cells was incubated with 2 nM substrate at 37 °C for 30 min. The level of bypass products [longer than ICL(−1)] was significantly lower for the reaction with nuclear extract from DNA polymerase β-deleted MEF cells (lane 5) than for those with extract from wild-type MEF cells (lane 3). The addition of purified human DNA polymerase β (lane 6−8) successfully restored the bypass activity of the ICL in the nuclear extract from DNA polymerase β-deleted MEF cells. The same amount of purified DNA polymerase β did not influence the bypass activity in nuclear extract from wild-type MEF cells (compare lanes 3 and 4): lane 1, 53-nucleotide marker; lane 2, 32-nucleotide marker; lane 3, WT extract; lanes 4, WT extract with purified PolB; lane 5, PolB−/− extract; lanes 6−9, PolB−/− extract with purified PolB. (B) DNA polymerase κ cannot substitute for DNA polymerase β for in vitro bypass of a psoralen ICL in nuclear extract. Purified human DNA polymerase κ failed to restore bypass activity in nuclear extract from DNA polymerase β-deleted MEF cells (lanes 7−9). DNA polymerase β that showed DNA polymerase activities (Figures 2 and 3 of the Supporting Information) similar to that of DNA polymerase κ complemented the defect in bypass activity in nuclear extract from DNA polymerase β-deleted MEF cells (lanes 4−6).

production of a 26-nucleotide fragment. An inhibition of a polymerase-mediated chain elongation by a psoralen ICL will generate a 32-nucleotide fragment [designated as ICL(−1)], and an incorporation of a dNMP opposite a psoralen ICL generates a 33-nucleotide fragment [ICL(0)]. Fragments with more than 34 nucleotides generated by a DNA polymerase are considered to be bypass products.

DNA polymerase β is found to play a role in ICL repair in mammalian cells. We examined the in vitro bypass of a psoralen ICL by DNA polymerase β (Figure 3). Purified human DNA polymerase β was incubated with a defined substrate with an unhooked psoralen ICL at 37 °C for the indicated time. We detected a low-efficiency bypass of the psoralen by DNA polymerase β, although a major product by DNA polymerase β was a 32-nucleotide length fragment showing that DNA polymerase β was stalled one nucleotide before the psoralen ICL (Figure 3A). An increased concentration of DNA polymerase β improved the bypass efficiency (Figure 3B). Interestingly, DNA polymerase β produced two major fragments with 32 and 33 nucleotides with a higher concentration (Figure 3B,C). These results demonstrated that DNA polymerase β is capable of incorporating a dNMP opposite the psoralen ICL. We conclude that DNA polymerase β is capable of bypassing a psoralen ICL in vitro.

Because DNA polymerase β incorporates a dNMP opposite a psoralen ICL, we examined whether one of the four nucleotides is preferentially incorporated opposite a psoralen ICL. The reaction products generated by *Escherichia coli* Klenow fragment were used as a template for this purpose [termed "ICL(−1) substrate"]. After the reaction with the Klenow fragment that stops one nucleotide before a psoralen ICL, the products were isolated and used to determine a specificity of incorporation of a nucleotide opposite the psoralen ICL. DNA polymerase β was incubated with the ICL(−1) substrate in the presence of only one dNTP. DNA polymerase β incorporated a nucleotide opposite a psoralen ICL in the following order: dAMP ≫ dCMP = dGMP ≫ TMP (Figure 3D). Because psoralen modifies thymines to form an ICL, DNA polymerase β incorporates the proper nucleotide dAMP opposite a psoralen ICL.

It was reported that accessory factors of DNA polymerases such as PCNA influence the efficacy of the bypass of DNA damage and the preference of incorporation of dNTP opposite DNA damage.[22,23] A checkpoint clamp, RAD9-RAD1-HUS1 (9-1-1) complex, stimulates the polymerase activity of DNA polymerase β in vitro.[24] We investigated the impact of the 9-1-1 complex on the bypass activity of a psoralen ICL by DNA polymerase β. The 9-1-1 complex stimulated the polymerase activity of DNA polymerase β on a nondamaged template under the conditions used, as reported in Figure 1 of the Supporting Information. The addition of the 9-1-1 complex to the DNA polymerase β-mediated TLS reaction mixture did not improve the bypass efficiency (Figure 3C, lanes 4−6, graph) or alter the specificity of the incorporation of a nucleotide opposite a psoralen ICL, although the complex stimulated the overall efficiency of dNTP incorporation (Figure 3D, graph).

To study a contribution of DNA polymerase β in a TLS step of ICL repair, we examined the in vitro bypass activity of a psoralen ICL using cell-free extracts (Figure 4). Nuclear extracts were prepared from wild-type mouse embryonic fibroblasts (MEFs) or DNA polymerase β−/− MEFs. The substrate DNA was incubated with nuclear extracts, and then

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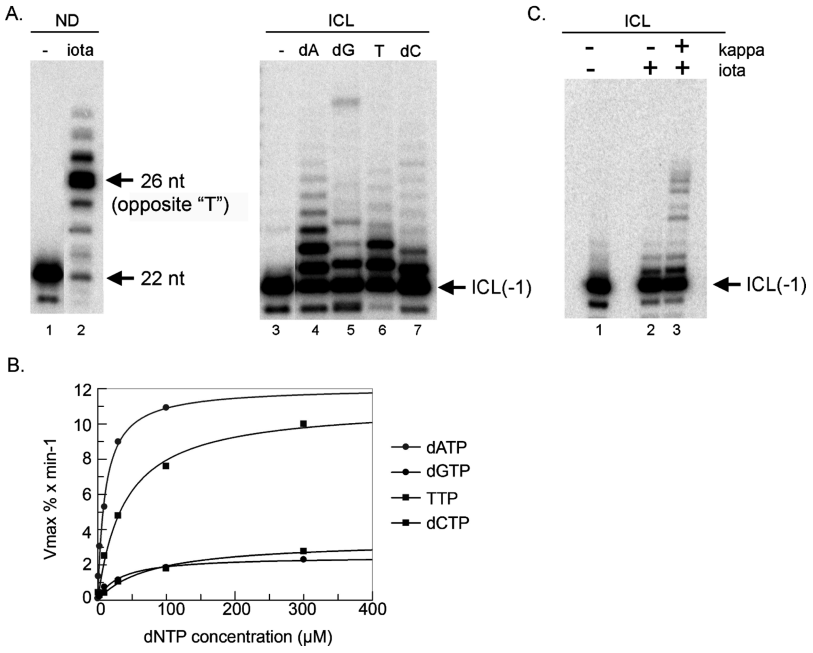

Figure 5. Potential role of DNA polymerase ι in the bypass of a psoralen ICL. (A) DNA polymerase ι preferentially incorporates TMP opposite a psoralen ICL in vitro. Purified GST-tagged DNA polymerase ι tends to stall at a template thymine (nondamaged) (lane 2). The ICL(−1) substrate (60 pM) was incubated with purified GST-tagged DNA polymerase ι in the presence of a single dNTP (100 μM) at 37 °C for 3 min. DNA polymerase ι incorporates TMP and dAMP opposite a psoralen ICL (lanes 6 and 4, respectively). (B) Steady-state kinetic experiments. TMP and dAMP were incorporated opposite a psoralen ICL by DNA polymerase ι. (C) Sequential action of DNA polymerases ι and κ in the bypass of a psoralen ICL. The ICL(−1) substrate (60 pM) was incubated with GST-tagged DNA polymerase ι at 37 °C for 15 min, and then DNA polymerase κ was added and incubated for an additional 15 min. DNA polymerase κ extended the DNA polymerase ι-generated mismatched primers (compare lanes 2 and 3).

bypass activity was examined. Wild-type nuclear extracts showed a bypass activity (Figure 4A, lane 3), while DNA polymerase β−/− nuclear extracts displayed a significantly reduced bypass activity (Figure 4A, lane 5). Furthermore, purified DNA polymerase β was able to restore the bypass activity in the DNA polymerase β−/− nuclear extracts (Figure 4A, lanes 6−8; Figure 4B, lanes 4−6). Importantly, purified DNA polymerase κ, which showed a polymerase activity similar to that of the DNA polymerase β used (Figures 2 and 3 of the Supporting Information), failed to recover the reduced bypass activity in the DNA polymerase β−/− nuclear extracts (Figure 4B, lanes 7−9). In addition to the products with more than 34 nucleotides, we also detected fragments with more than 53 nucleotides in the reaction mixture with extracts (Figure 4 of the Supporting Information). These products were generated after ligation of different sizes of extended primers that contained cross-links. Thus, they were not the products of TLS (Figure 4 of the Supporting Information). These results demonstrate the existence of DNA polymerase β-dependent TLS of a psoralen ICL in vitro.

Bypass of a Psoralen ICL by the Sequential Action of DNA Polymerases ι and κ. DNA polymerase ι is a highly error prone DNA polymerase with a low processivity.[10,25] Interestingly, DNA polymerase ι tends to stall after incorporating dGMP opposite thymine on a template (Figure 5A, lane 2).[18,25] Those biochemical properties prompted us to examine whether DNA polymerase ι can insert a nucleotide opposite a psoralen ICL. Indeed, DNA polymerase ι incorporated a nucleotide opposite a psoralen ICL on the ICL(−1) substrate (Figure 5A, lanes 4−7). We further investigated the specificity of the incorporation of a nucleotide opposite a psoralen ICL by steady-state kinetics experiments. In contrast to the preferential incorporation of dAMP opposite a psoralen ICL by DNA polymerase β (Figure 3D), DNA polymerase ι efficiently incorporated dAMP and TMP opposite a psoralen ICL (Figure 5B and Table 1). This result is very surprising because a signature property of DNA polymerase ι is the preferential misincorporation of dGMP opposite a thymine on a template. The data might indicate a structural change induced by a psoralen modification on thymine, which alters a specificity of incorporation opposite a psoralen-modified thymine.

Table 1. Kinetic Measurement of the Incorporation of a Nucleotide opposite Psoralen ICL by DNA Polymerase ι

dNTP	V_{max} (fmol/min; mean ± SD)	K_m (μM; mean ± SD)	V_{max}/K_m	f_{inc} [a]
dATP	12.1 ± 0.5	10.9 ± 1.5	1.1	
dGTP	2.5 ± 0.1	30.1 ± 5.4	0.08	7.3×10^{-2}
dCTP	3.4 ± 0.3	75.5 ± 16.4	0.04	3.6×10^{-2}
TTP	11.1 ± 0.4	39.4 ± 4.8	0.28	0.25

[a] $f_{inc} = (V_{max}/K_m)_{incorrect}/(V_{max}/K_m)_{correct}$.

DNA polymerase κ was shown to bypass an N^2−N^2 guanine minor groove ICL efficiently.[9] The N^2−N^2 guanine ICL was especially part of a two-nucleotide fragment attached to a template. DNA polymerase κ did not bypass a psoralen ICL in our substrate (Figure 3B of the Supporting Information), which is part of the 12 nucleotides attached on a template, in agreement with the notion that longer nucleotides attached to a template are inhibitory for the bypass of the N−N^2 guanine minor groove ICL. Having the data that DNA polymerase ι incorporates a nucleotide opposite a psoralen ICL and the

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known extension activity from a mismatched primer end by DNA polymerase κ,[26] we examined the impact of a sequential action of DNA polymerases ι and κ (Figure 5C). DNA polymerase ι was incubated with the ICL(−1) substrate in the presence of four dNTPs at 37 °C for 15 min. Then, DNA polymerase κ was added to the reaction mixture and incubated for an additional 15 min at 37 °C. DNA polymerase κ extended a primer end opposite a psoralen generated by DNA polymerase ι (Figure 5C, lanes 2 and 3). DNA polymerase κ does not incorporate a nucleotide opposite a psoralen ICL at the concentration used in these reactions (Figure 3B of the Supporting Information). These results showed that a sequential action of DNA polymerases ι and κ bypasses a psoralen ICL.

DISCUSSION

DNA interstrand cross-links (ICLs) are repaired by multiple pathways, DNA replication-coupled and DNA replication-independent pathways.[1] The repair factors required and the detailed mechanisms for these pathways are different among species.[27,28] However, the following two principles of the repair are very similar. A release (or separation) of one of the cross-linked strands is the prerequisite step for all of the pathways reported. A gap generated opposite the released cross-linked strand that is still attached to the complementary strand should be filled by a TLS DNA polymerase to be further processed to complete the repair. DNA replication-coupled ICL repair has been studied using *Xenopus* egg extracts.[29,30] It has been demonstrated that REV7 mediates a TLS reaction to bypass a cisplatin ICL in the replication-coupled ICL repair.[30] Genetic and biochemical studies indicate the involvement of various TLS polymerases, including DNA polymerases η, κ, and ζ in DNA replication-independent ICL repair pathways in mammalian cells.[2,3] In addition, DNA polymerase ν is found to play a role in human ICL repair.[14,31] In this report, we provided biochemical evidence that DNA polymerases β, κ, and ι may participate in ICL repair in mammalian cells. When a gap is generated at the 5′ side of an ICL during the processing of the ICL, DNA polymerase β might have a better chance to access the gap and bypass the ICL (Figures 3 and 4). Once DNA polymerases stalled one nucleotide before an ICL, DNA polymerase ι might incorporate a nucleotide and DNA polymerase κ may extend a primer end generated by DNA polymerase ι for TLS steps in ICL repair.

Our results showed that DNA polymerase β incorporates a proper nucleotide, dAMP, opposite a psoralen ICL, which are modified thymines at a 5′-TA-3′ site (Figure 3D). It is interesting to note that DNA polymerase β shows a similar preference for the incorporation opposite UV photoproducts, cyclobutane pyrimidine dimer (CPD) and the (6-4) photoproduct at a 5′-TT-3′ site. DNA polymerase β incorporates the proper dAMP opposite these UV photoproducts, and it also incorporates dCMP to a lesser extent.[32] DNA polymerase β also incorporates the proper dAMP opposite another modified thymine, thymine glycol,[33] while it misincorporates TMP[34]opopposite the modified guanine, cisplatin GpG intrastrand cross-link.[35,36] Because the psoralen ICL, UV photoproducts, and thymine glycol are modified at the 5 and 6 positions of thymines, the base pairing capacity of the modified thymine with adenine may not be influenced. Therefore, DNA polymerase β that has a relatively high fidelity incorporates dAMP opposite these lesions. The bypass efficiency of a DNA lesion by a given DNA polymerase will be determined by the base pairing potential of the lesion and proper fitting of the base pairing in the active pocket of the polymerase. DNA polymerase β might be suitable for bypass of thymine-derived DNA damage.

In sharp contrast to nondamaged thymine in the template, DNA polymerase ι prefers to insert dAMP and TMP and seems to reject dGMP opposite a psoralen ICL (Figure 5B). The mechanistic aspects of these incorporations opposite a psoralen ICL are not clear. It was proposed that the active site of DNA polymerase ι was narrow so an incorporation of purines using Watson−Crick base pairing is not efficient and DNA polymerase ι might use noncanonical, Hoogsteen, or wobble base pairings.[34,37,38] Therefore, one possible mechanism of incorporation of a nucleotide opposite a psoralen ICL by DNA polymerase ι is the formation of a Hoogsteen base pair between an incoming nucleotide and a template psoralen ICL. We used 7-deaza-dATP to test this possibility. Thymine on a template forms a Hoogsteen base pair with an incoming dATP with the *syn* configuration through N6 and N7. The 7-deaza-dATP cannot form a Hoogsteen base pair with a thymine. DNA polymerase ι incorporated 7-deaza-dAMP opposite a psoralen ICL as efficiently as dAMP (Figure 5 of the Supporting Information). The results indicate that DNA polymerase ι does not use a Hoogsteen base pair and probably uses wobble base pairing to incorporate dAMP opposite a psoralen ICL.

Because the 9-1-1 complex stimulates DNA polymerase β,[24] we also examined whether the complex has any impact on DNA polymerases κ and ι. The 9-1-1 complex slightly stimulated DNA polymerase ι (Figure 6A of the Supporting Information, lanes 1 and 2), but not DNA polymerase κ (data not shown). DNA polymerase ι did not alter the specificity of the incorporation of a nucleotide opposite a psoralen ICL in the presence of the 9-1-1 complex (Figure 6B of the Supporting Information). Interestingly, DNA polymerase ι incorporated dAMP and dCMP better opposite a nonmodified thymine or cytosine on a template in the presence of the 9-1-1 complex (Figure 6C,D of the Supporting Information). We do not know the significance of these phenomena. However, the results might suggest that the 9-1-1 complex changes the efficiency of the incorporation of a nucleotide by DNA polymerase ι, depending on the structure of a base on a template.

Unlike DNA polymerases β and ι, DNA polymerase κ does not insert a nucleotide opposite a psoralen ICL, which is a part of a 12-mer oligonucleotide under the conditions we used. In addition to the bypass activities of minor groove adducts, DNA polymerase κ is capable of extending a primer from mispaired ends, suggesting its role as an extender in a two-step TLS of DNA damage.[26] It was shown that the sequential action of DNA polymerases ι and κ allows efficient and error-free bypass of a minor groove adduct, γ-hydroxy-1,N^2-propano-2′-deoxyguanosine.[12] DNA polymerase ι incorporates a correct nucleotide dCMP opposite the lesion, and DNA polymerase κ conducts an efficient extension of the primer generated. Analogous to this mechanism, we demonstrated that DNA polymerase κ extends mispaired primers generated by the incorporation of nucleotides opposite a psoralen ICL. Each dNTP was incorporated opposite a psoralen ICL by an exonuclease-deficient Klenow fragment (exo-), and the mispaired primers were used as a template. DNA polymerase κ was able to extend all four primers (Figure 7 of the Supporting Information). Our results might indicate that DNA polymerase κ plays two separate roles in TLS of ICLs,

bypassing ICLs and extending a primer generated by other DNA polymerases.

Psoralen ICLs are known to induce T-to-A transversions at 5′-TA-3′ sites in mammalian cells.[39−42] These data demonstrate that a misincorporation of TMP opposite a psoralen ICL causes a T-to-A transversion. Our results showed that DNA polymerase ι preferentially incorporates dAMP and TMP opposite a psoralen ICL, while DNA polymerases β and κ incorporate dAMP opposite a psoralen ICL (Figure 5A,B and Table 1). DNA polymerase ι might be a corresponding polymerase to induce psoralen-induced T-to-A transversions. It is important to investigate whether inactivation of DNA polymerase ι in mammals alters the mutational specificity induced by psoralen ICLs. "Psoralen plus UVA treatment" (PUVA treatment) has been a major therapy for psoriasis. Patients, in many cases, require repeated PUVA treatment for many years. The patients undergoing PUVA treatment have a risk of developing squamous cell carcinoma. While the exact mechanism of the formation of squamous cell carcinoma is not known, psoralen-induced mutagenesis should significantly contribute to the development. The development of a specific inhibitor of DNA polymerase ι might help reduce the risk of developing squamous cell carcinoma in PUVA treatment.

DNA interstrand cross-links are repaired by multiple pathways in mammalian cells. An intermediate structure, in which a TLS polymerase is involved, is unclear. Our model substrate DNA contains a four-nucleotide gap between a primer end and the 5′ end of the cross-linked 12-mer through a psoralen. The primer is degraded significantly with the DNA polymerase β−/− extracts, and the addition of purified DNA polymerase β averts these degradations (Figure 4). These results indicate that our substrate with a four-nucleotide gap was not recognized and utilized by other DNA polymerases under the conditions used. In contrast, DNA polymerase β prefers a substrate with a gap;[43] therefore, our substrate is suitable for investigating the role of DNA polymerase β in TLS of ICLs. It has been reported that DNA polymerase κ increases the efficiency of the bypass of N^2−N^2 guanine ICLs by shortening the size of the attached oligonucelotide.[9] When the ICL is in a two-nucleotide fragment, DNA polymerase κ polymerizes this template with a similar efficiency to polymerize the nondamaged template. These results emphasize a significant contribution of the DNA structure of a template to bypass the ICLs. The identification of an intermediate structure for a TLS step in each ICL repair pathway should help in our understanding of which DNA polymerase is involved in the TLS step.

■ ASSOCIATED CONTENT

Supporting Information

Stimulation of DNA polymerase β by the 9-1-1 complex (Figure 1), DNA polymerase activity of DNA polymerase β (Figure 2), DNA polymerase activity of DNA polymerase κ (Figure 3), analysis of reaction products with more than 53 nucleotides generated by nuclear extracts (Figure 4), DNA polymerase ι that incorporates 7-deaza-dATP as efficiently as dATP opposite a psoralen ICL (Figure 5), impact of the 9-1-1 complex on DNA polymerase ι (Figure 6), and DNA polymerase κ extends mispaired primer ends opposite a psoralen ICL (Figure 7). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

ICLs, DNA interstrand cross-links; TLS, translesion DNA synthesis; PolB, DNA polymerase β; PolK, DNA polymerase κ.

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