Integrative Biosciences 9: 79-85, 2005

Integrative
Biosciences
http://www.ibiosci.or.kr

Formation of DNA-protein Cross-links Mediated by C1'-oxidized Abasic Lesion in Mouse Embryonic Fibroblast Cell-free Extracts

Jung-Suk Sung* and In Kook Park

Department of Biology, College of Sciences, Dongguk University, Seoul 100-715, Korea

Abstract: Oxidized abasic residues arise as a major class of DNA damage by a variety of agents involving free radical attack and oxidation of deoxyribose sugar components. 2-deoxyribonolactone (dL) is a C1'-oxidized abasic lesion implicated in DNA strand scission, mutagenesis, and covalent DNA-protein cross-link (DPC). We show here that mammalian cell-free extract give rise to stable DPC formation that is specifically mediated by dL residue. When a duplex DNA containing dL at the site-specific position was incubated with cell-free extracts of Pol β-proficient and -deficient mouse embryonic fibroblast cells, the formation of major dL-mediated DPC was dependent on the presence of DNA polymerase (Pol) β. Formation of dL-specific DPC was also observed with histones and FEN1 nuclease, although the reactivity in forming dL-mediated DPC was significantly higher with Pol β than with histones or FEN1. DNA repair assay with a defined DPC revealed that the dL lesion once cross-linked with Pol β was resistant to nucleotide excision repair activity of cell-free extract. Analysis of nucleotide excision repair utilizing a model DNA substrate containing a (6-4) photoproduct suggested that excision process for DPC was inhibited because of DNA single-strand incision at 5' of the lesion. Consequently DPC mediated by dL lesion may not be readily repaired by DNA excision repair pathway but instead function as unusual DNA damage causing a prolonged DNA strand break and trapping of the major base excision repair enzyme.

Key words: DNA damage, BER, NER, DNA-protein cross-link, Pol β

Cellular DNA is under continuous assault by DNA damaging agents of both endogenous and environmental origins. The outcome of DNA damages is generally adverse, contributing to degenerative processes such as aging and cancer (Friedberg, 2003; Hoeijmakers, 2001; Lindahl, 1993). Loss of a nucleic acid base leaving an abasic (AP) site is the most frequent damaging event, and may occur by spontaneous hydrolysis of *N*-glycosylic bond or as a consequence of the removal of inappropriate base by DNA glycosylase (Atamna et al., 2000; Krokan et al., 1997). In either case, the resulting AP sites are repaired by base excision DNA repair (BER) pathway. In mammalian cells, the major AP endonuclease, Ape1 (also called Apex, HAP1, or Ref-1), initiates BER by hydrolyzing the 5'-phosphodiester bond of the AP site to generate a repair intermediate that has a single strand break bracketed by 3'-hydroxyl and 5'-deoxyribose-5-phosphate (5'-dRP) termini (Dianov et al., 2003). Further repair process is achieved via at least two distinct BER pathways that involve different subsets of enzymes and result in replacement of one (short-patch BER) or more (long-patch BER) nucleotides (Sattler et al., 2003; Sung et al., 2001; Fortini et al., 1998).

Oxidative damage to DNA, mediated by free-radicals and reactive oxygen species, produces structurally distinct AP sites that modulate biochemical reactivity of the lesion to the BER enzymes (Demple and DeMott, 2002; Hwang et al., 1999). These lesions include 2-deoxyribonolactone (dL), a C1'-oxidized AP site, which is introduced into DNA by numerous genotoxic agents including UV and γ-irradiation, organometallic oxidants, chemical nuclease, and the antitumor agent neocarzinostatin chromophore (Hwang et al., 1999; Pratviel et al., 1991; Sigman et al., 1993). Very little was known about biological consequences of dL until the method to generate a site-specific dL lesion in DNA became available (Hwang et al., 1999; Jourdan et al., 1999). Several investigations on the repair of dL by DNA glycosylases with associated AP lyase activity revealed the

*To whom correspondence should be addressed.
Tel: 82-2-2260-3322, Fax: 82-2-2275-8294
E-mail: sungjs@dongguk.edu

formation of covalent DNA-protein cross-link (DPC) with dL dependent on the key lyase active site residue (Hwang et al., 1999; Kroeger et al., 2003). In contrast, mammalian AP endonuclease Ape1 was shown to incise dL residue rather efficiently leaving a 5'-terminal dL (5'-dL) residue (Xu et al., 2003). However, the reaction of the next BER enzyme, DNA polymerase β (Pol β), with oligonucleotide DNA containing an Ape1-cleaved dL residue resulted in covalent trapping of the polymerase to the DNA, possibly via a stable amide bond (DeMott et al., 2002).

Although *in vitro* study using purified enzymes has provided useful information about chemical mechanisms involved in dL-mediated DPC formation, cellular repair process of dL or its DPC product is still unknown. In the present study, we have utilized cell-free extracts from wild-type and Pol β null mouse embryonic fibroblasts (MEF) to monitor which cellular proteins involve in dL-mediated DPC formation. Furthermore, a possible repair of dL-mediated DPC by nucleotide excision repair (NER) pathway has been explored.

MATERIALS AND METHODS

Materials

All reagents were from Sigma/Aldrich. Radionuclides were obtained from PerkinElmer Life Science. Recombinant Pol β, FEN1 nuclease, and Ape1 were purified as described previously (Masuda et al., 1998; Prasad et al., 2000; Tom et al., 2000). All other enzymes were obtained from New England BioLabs. Histone proteins, H2A, H2B, H3, and H4, were purchased from Roche. Goat anti-Pol β polyclonal antibody was from Santa Cruz Biotechnology. Pol β-proficient (MB16tsA, clone 1B5) or -deficient (MB19tsA, clone 2B2) SV-40 immortalized mouse embryonic fibroblast (MEF) cell lines were obtained from American Type Culture Collection.

Preparation of DNA substrates

Oligonucleotide 30-mer (5'-GTCACGTGCTGCAXACG-ACGTGCTGAGCCT-3') containing a site-specific precursor residue (X; 1'-*t*-butylcarbonyl-uridylate) for dL lesion was provided by Dr. Demple of Harvard University. The set of oligonucleotides used in the construction of 136-mer DNA duplex containing a (6-4) photoproduct was described in the previous study (Reardon and Sancar, 2003), and provided by Dr. Reardon of University of North Carolina. Using standard methods (Ausubel et al., 1997), the DNA substrates were labeled at the 5'-end and hybridized to a complementary strand or hybridized first and labeled at the 3'-end. ^{32}P-labeled plasmid pGEM DNA substrate were constructed as described previously (Sung and Mosbaugh, 2003), except that ^{32}P-label was located 12 nucleotide downstream of target dL residue. To generate a site-specific 5'-dL lesion in DNA substrates, 5-10 pmol of duplex DNA containing a precursor residue was subjected to photolysis reaction at UV 350 nm, and then incised by Ape1, as described by DeMott et al. (2002). The efficiency of the photo-conversion was typically >90% monitored by dL-specific DNA cleavage by hot-alkali treatment and following analysis of DNA by denaturing polyacrylamide gel electrophoresis.

Preparation of cell-free extracts

MEF cell-free extracts were prepared from confluent cells as described previously (Bennett et al., 2001), dialyzed extensively against 20 mM HEPES-KOH (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM PMSF, 10% (v/v) glycerol, and 1x protease inhibitor cocktail (Sigma). The protein concentration of cell-free extracts was determined using Bio-Rad Protein Assay reagent. The *POLB* genotype of the two cell lines was ascertained and the levels of Pol β in cell-free extracts were determined by Western blot analysis as described previously (Chen et al., 1998).

Analysis of dL-mediated DPC formation

Standard DNA-protein cross-linking reactions contained 50 mM HEPES-KOH (pH 7.5), 20 mM NaCl, 0.5 mM dithiothreitol, 2 mM EDTA, 5% (v/v) glycerol, 0.1 mg/ml BSA, 20 nM of 3'-end ^{32}P-labeled DNA substrate containing a 5'-dL, and the protein concentrations as indicated in the Figure Legend. Following incubation at 30°C for the specified times, reactions were terminated by the addition of SDS-PAGE loading buffer and heating at 100°C for 5 min. DPC and free DNA were resolved by 8% SDS-PAGE and ^{32}P radioactivity associated with DNA-protein cross-links was quantified using PhosphorImager and ImageQuant program (Molecular Dynamics).

DNA repair assay

Standard DNA repair reaction mixtures contained 100 mM HEPES-KOH (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 2 mM ATP, 0.5 mM β-NAD, 20 μM dNTP, 5 mM phosphocreatine, 200 units/ml phosphocreatine kinase, 10 μg/ml of the appropriate DNA substrate, and the indicated amounts of cell-free extracts. For the DPC repair assay, plasmid DPC substrate was prepared by reaction of ^{32}P-pGEM DNA containing a dL lesion with excess Pol β protein. Following incubation at 30°C for 1 h, the reaction products were analyzed by 8% SDS-PAGE to determine the release of DNA oligonucleotide cross-linked with the protein. NER assay was conducted by using either unbroken or nicked 136-mer DNA substrates containing a (6-4) photoproduct for 30°C for 1 h, and the reaction DNA products were resolved by 8 M urea/10% denaturing polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

The selective generation of dL via a photosensitive precursor enables analysis of the repair of this key oxidative lesion (Hwang et al., 1999). Using this approach, we determined the profile of covalent DPCs mediated by dL residues with MEF cell-free extracts. Upon incubation of 5'-dL containing DNA with MEF cell extracts, several new DNA bands that migrated much slower than free DNA were observed (Fig. 1A). Theses species were not produced on the DNA that was not treated with UV and still contained a precursor residue (Fig. 1A, lane 1), indicating that the formation of these products was specifically mediated by dL lesion. Because the analysis was performed under the protein denaturing condition, any noncovalent interaction between DNA and protein was abolished. Thus, these features were consistent with formation of DNA-protein complex in which dL residue was bonded to the proteins via covalent linkage. A major cross-linked product corresponding to the M_r 45,000 complex appeared to increase in a time-dependent manner in Pol β-proficient cell extracts (Fig. 1A, lanes 3-7),

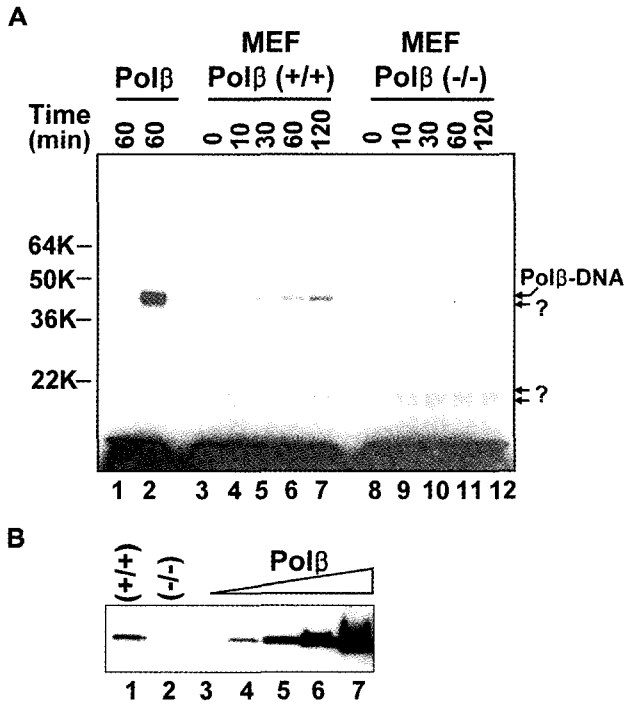

Fig. 1. Spontaneous cross-link formation between dL and MEF cell-free extract proteins. A, Standard cross-linking assay was performed with 10 μg of MEF Pol β-proficient (lanes 3-7) or -deficient (lanes 8-12) cell-free extracts for 0, 10, 30, 60, and 120 min. As controls, DNA containing an unconverted precursor residue (lane 1) or dL residue (lane 2) was incubated with 20 nM purified Pol β for 1 h. Samples were analyzed by SDS-PAGE and PhosphorImager. The band positions of the M_r markers are indicated in the left. B, Western blot analysis of 10 μg of either MEF Pol β (+/+) or Pol β (-/-) cell-free extracts are shown (lane 1 and 2, respectively), along with 0, 0.13, 0.5, 2, 8 pmol of Pol β standards (lanes 3-7).

while the complex showed the same mobility as the DPC product formed with purified Pol β (Fig. 1A, lane 2). However, the reaction with Pol β-deficient cell extract did not produce the corresponding DPC product (Fig. 1A, lanes 8-12), indicating that Pol β was the major protein involved in the formation of dL-specific DPC. Since Pol β constitutes a major activity in removal of 5'-dRP (regular AP lesion) in mammalian cells (Matsumoto and Kim, 1995; Sobol et al., 1996; Wong and Demple, 2004), our result agrees with the preposition that DPC formation at 5'-dL lesion mainly occurs via dRP lyase activity of Pol β. In both cell types, however, minor DPC products also appeared as additional bands with relatively lower M_r of ~20,000. The amount of Pol β was determined to be 0.2 pmol in 10 μg of wild-type cell-free extract by Western blot analysis (Fig. 1B). The level of DPC produced by Pol β in the cell-free extract was 8-fold lower compared to that generated by the same amount of purified Pol β (Fig. 1A, lanes 2 and 6). Therefore, our results suggest that the competition between Pol β and other cellular proteins may occur in forming DPC at the dL lesion.

Nonenzymatic attack at C1' of deoxyribose through a lyase mechanism may also be promoted by basic cellular macromolecules such as polyamines or histones, but with much slower rate than that achieved by Pol β (Matsumoto and Kim, 1995). Consistent with this notion, we have observed covalent cross-links between 5'-dL and purified H2A, H2B, H3, and H4 proteins (Fig. 2A). However, the level of DPC formed with histone proteins was significantly lower compared to that formed by Pol β (Fig. 2B). DPCs with all major histones are known to arise by a number of carcinogenic chemicals (aldehydes, nitrous acid, and metal compounds such as arsenite, nickel, and chromate), although the mechanistic link between DPCs and carcinogenesis is not fully elucidated (Kurtz and Lloyd, 2003). In these cases, histone proteins appeared to be predominant in forming DPCs through nonspecific interactions with DNA (Nackerdien et al., 1991; Nakano et al., 2003; Quievryn and Zhitkovich, 2000). Accordingly, the dL-mediated DPC with Pol β presents an unusual type of DPC that involves the enzymatic specificity leading to spontaneous formation of covalent cross-link.

Two scenarios are possible on the biological fates of dL lesion, since its unmodified form of abasic residue undergoes DNA repair either by short- or long-patch BER pathway. In short-patch BER pathway, as shown in Fig. 1, an abortive attempt of Pol β to excise dL residue results in a stable cross-link to the lesion. In an alternative long-patch BER pathway, 5'-dL may be excised as part of a flap oligonucleotide by FEN1 nuclease activity. FEN1 is also a basic protein that constitutes lysine rich surface (Hosfield et al., 1998), which can potentially form the cross-link with dL lesion. Thus, we examined whether FEN1 is capable of

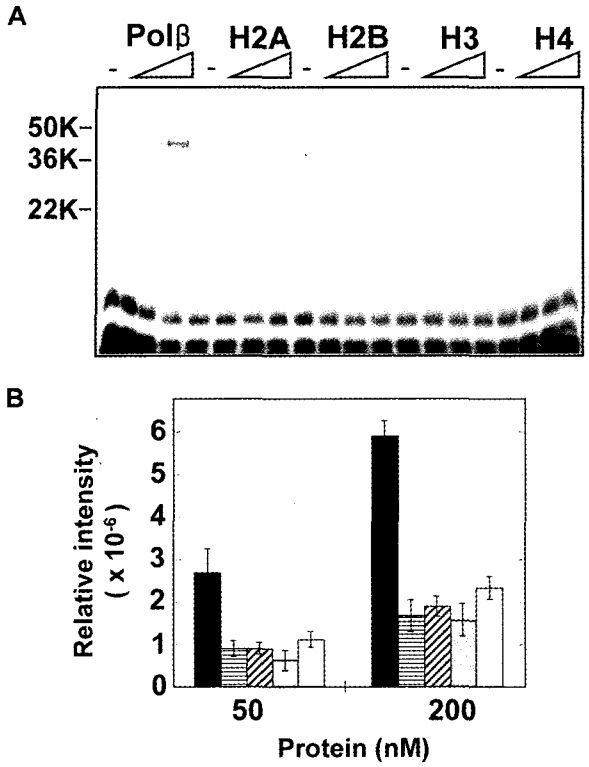

Fig. 2. Comparative analysis of dL-mediated DPC formation with Pol β and major histones. A, Standard cross-linking assay was performed in the presence of 0, 10, 50, and 200 nM of each Pol β, H2A, H2B, H3, and H4 at 30°C for 30 min. Samples were analyzed by SDS-PAGE and PhosphorImager. The band positions of the M_r markers are indicated in the left. B, The amounts of DPC between the protein and dL-DNA were quantified in PhosphorImager. The relative intensities of each DPC bands were determined by ImageQuant program and plotted as a function of the amount of each proteins as following: black bar, Pol β; horizontally striped bar, H2A; diagonally striped bar, H2B; grey bar, H3; white bar, H4.

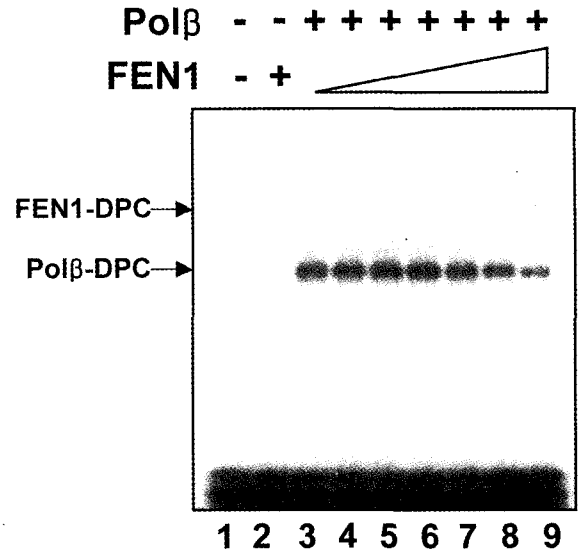

Fig. 3. Higher specificity of Pol β over FEN1 in cross-linking to dL residue. Standard cross-linking assay using 3'-^{32}P-labeled 31-mer duplex DNA containing a 5'-dL residue was conducted in with (lanes 3-9) or without (lanes 1 and 2) 20 nM Pol β, or in the presence of 2 μM FEN1 (lane 2). For the competition, the reactions containing Pol β were supplemented with 0, 1, 10, 50, 100, 500 nM, and 2 μM FEN1 (lanes 3-9, respectively). Following incubation at 30°C for 30 min, each reaction products were analyzed by SDS-PAGE and PhosphorImager.

producing dL-mediated DPC. Comparative analysis of DPC formation between Pol β and FEN1 showed that a large amount of FEN1 was required to form DPC, whereas an equimolar Pol β relative to dL-DNA concentration effectively produced DPC product (Fig. 3). The DPC formation of dL-DNA with Pol β was not significantly affected by excess FEN1 (Fig. 3, lanes 3-9). These results confirm the specificity of Pol β in forming dL-mediated DPC and suggest that FEN1 may act on dL-DNA without being covalently trapped by the lesion. It is not unreasonable to expect efficient processing of the flap containing a 5'-dL residue by FEN1, because FEN1 activity appears to tolerate variety of flap 5'-modifications (Bornarth et al., 1999). Consistent with this notion, a previous study demonstrated FEN1 dependent long-patch BER of a reduced AP site which was resistant to processing by Pol β dRP lyase activity, suggesting that the long-patch BER may be involved in cellular repair of the DNA lesions that cannot be processed by short-patch BER (Klungland and Lindahl, 1997). It would therefore be interesting to determine whether the dL lesion has any roles in 'switching over' of the BER pathway from short-patch to long-patch BER mode.

We next investigated DNA repair of dL-mediated DPC in cell-free extracts by utilizing circular plasmid DNA substrates to allow involvement of NER enzymes that require substantially longer DNA for efficient damage recognition (Reardon and Sancar, 2003). The reaction of the DNA substrate containing a pre-formed DPC with repair proficient cell-free extracts did not produce any discrete DNA products that could reflect the release of DPC within a short oligonucleotide (Fig. 4, lanes 2-5). On the other hand, co-treatment of the DPC substrate with restriction endonuclease, *Hind*III, generated a short DNA fragment cross-linked with Pol β, providing an indicative DNA species of the efficient repair of DPC (Fig. 4, lanes 7-10). This result thereby suggests that the DPC mediated by 5'-dL residue displays an unusual bulky DNA adduct that may be insensitive to general DNA excision repair mechanisms. Proteolytic digestion of Pol β formed in DPC did not appear to trigger the repair of the resulting cross-link between dL and small peptides (data not shown), indicating that the defect in repairing DPC may not be due to the steric hindrance associated with the protein in DPC.

One interesting study showed that the bacterial NER system can incise DNA containing an AP lyase trapped by chemical reduction in an 'unbroken' DNA strand (Minko

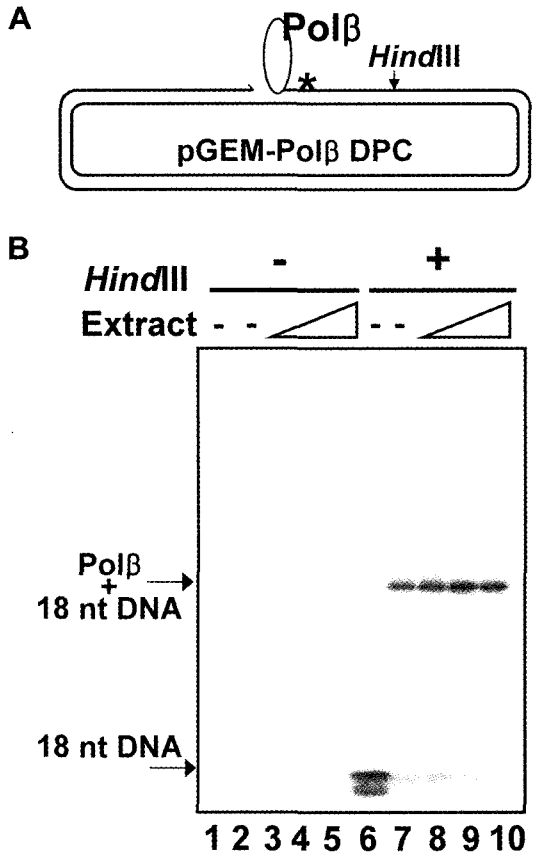

Fig. 4. Inefficient repair of dL-mediated DPC in cell-free extracts. A, Diagram of the plasmid DPC substrate containing a defined cross-link between Pol β and 5'-dL. The location of the ^{32}P-labeled nucleotide is indicated by an asterisk. B, DNA repair assay was performed using the plasmid DPC substrate with 0, 0.1, 1, 10 μg of wild-type MEF cell-free extracts in the absence (lanes 2-5) or the presence (lanes 7-10) of 10 units of *Hind*III. After incubation at 30°C for 1 h, reaction mixtures were analyzed by SDS-PAGE and PhosphorImager. As controls, the plasmid DNA containing no protein cross-link was untreated (lane 1) or treated (lane 6) similarly with *Hind*III.

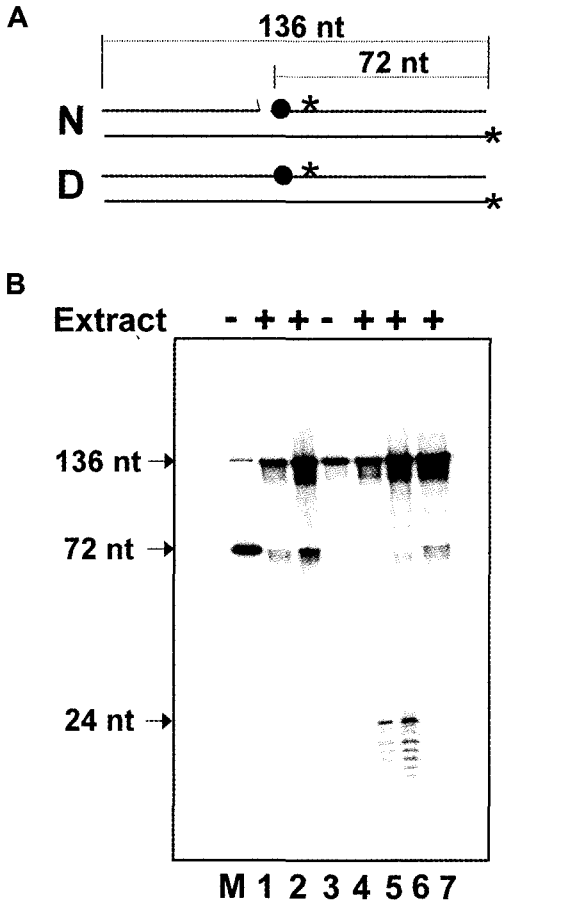

Fig. 5. DNA damage adduct formed at the nick of DNA is resistant to repair by NER. A, Schematic representation of the substrates used in NER assay is shown for nicked (N) or unbroken (D) 136-mer duplex DNA that contains a site-specific (6-4) photoproduct (●). The locations of the ^{32}P-labeled nucleotides are indicated by asterisks. B, DNA repair assay was performed with 10 nM DNA substrate, N (lanes 1-3) and D (lanes 4-6), or both N and D (lane 7), by incubation with 10 μg of wild-type MEF cell-free extracts. Reaction DNA products were isolated, analyzed by sequencing gel electrophoresis, and visualized by PhosphorImager. Numbers to the left indicate positions of DNA size markers (lane M).

et al., 2002). Thus, we designed the experiment to examine whether the 5'-nick at the DNA produced by ApeI incision prior to the DPC formation caused the NER-insensitive nature of dL-mediated DPC. As a model NER substrate, we utilized a 136-mer oligonucleotide duplex containing a site-specific (6-4) photoproduct and ^{32}P-labels located in both DNA strands (Fig. 5). NER of this lesion has been well characterized by previous studies (Petit and Sancar, 1999; Reardon and Sancar, 2003), but whether the 'nick' in DNA at 5' to the target adduct affects the repair activity of NER has not been addressed. In the reaction of DNA substrate with cell-free extract, the lesion was efficiently repaired from 'unbroken' DNA, accompanied by the release of short DNA fragments (Fig. 5, lanes 5 and 6). This result also confirmed that cell-free extracts used in the present study was proficient for DNA excision repair. However, the 'nick' DNA substrate constituted with the same sequence context and target residue, except the presence of 5'-nick at the lesion, was not readily repaired by cell-free extract (Fig. 5, lanes 2 and 3). Moreover, the repair of 'unbroken' DNA was significantly inhibited by the addition of 'nick' DNA to the same repair reaction (Fig. 5, lane 7). This result suggests that the nicks on the repair resistant DNA exclude NER enzymes from effective targets on the intact DNA, probably via abortive substrate recognitions by NER enzymes.

In addition to the mutagenic nature of dL lesion (Faure et al., 2004; Greenberg et al., 2004), its ability to covalently cross-link to the cellular protein would make this oxidized abasic site a potentially toxic oxidative DNA damage lesion. In particular, irreversible inhibition of Pol β at dL residue may have a dual biological effect: DPC formation with a

prolonged strand scission and the suicide of the major BER enzyme. Both could pose a threat to the genetic and structural integrity of DNA, hence initiating a carcinogenic process. Whether unidentified cellular processes involve in the repair of this type of DNA damage is still remained to be elucidated.

ACKNOWLEDGMENTS

We thank Drs. Bruce Demple and Joyce Reardon for generous gifts of DNA oligonucleotides. This work was supported by Dongguk University Research Fund (2004).

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[Received February 3, 2005; accepted May 12, 2005]