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Formation of DNA-Protein Crosslink at Oxidized Abasic Site Mediated by Human DNA Polymerase Iota and Mitochondrial DNA Polymerase Gamma

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Human genomic DNA is continuously attacked by oxygen radicals originated from cellular metabolic processes and numerous environmental carcinogens. 2-deoxyribonolactone (dL) is a major type of oxidized abasic (AP) lesion implicated in DNA strand scission, mutagenesis, and formation of covalent DNA-protein crosslink (DPC) with DNA polymerase (Pol) β. We show here that human DNA polymerase (Pol) ι and mitochondrial Polγ give rise to stable DNA-protein crosslink (DPC) formation that is specifically mediated by dL lesion. Polι mediates DPC formation at the incised dL residue by its 5'-deoxyribose-5-phosphate (dRP) lyase activity, while Polγ crosslinks with dL thorough its intrinsic dRP lyase and AP lyase activities. Reactivity in forming dL-mediated DPC was significantly higher with Polγ than with Polι. DPC formation by Polγ, however, can be reduced by an accessory factor of Polγ holoenzyme that may attenuate deleterious effects of crosslink adducts on mitochondrial DNA. Comparative kinetic analysis of DPC formation showed that the rate of DPC formation with either Polι or Polγ was lower than that with Polβ. These results revealed that the activity of catalytic lyase in DNA polymerases determine the efficiency of DPC formation with dL damages. Irreversible crosslink formation of such DNA polymerases by dL lesions may result in a prolonged strand scission and a suicide of DNA repair proteins, both of which could pose a threat to the genetic and structural integrity of DNA.

Key Words: Oxidative DNA damage, DNA repair, DNA polymerase, DNA-protein cross-link

INTRODUCTION

Oxidative damages to genomic DNA are highly associated with aging process (Stadtman and Berlett, 1998) and numerous human disorders including cancer and neurodegenerative diseases (Halliwell et al., 1992; Richter et al., 1995). Reactive oxygen species, generated as byproducts of aerobic metabolism or inflammatory responses, are the primary source of oxidative DNA damages (Friedberg, 2003; Lindahl, 1993). Environmental factors that induce oxidative DNA lesions include known cancer risk factors such as UV, ionizing radiation, and numerous chemical oxidants absorbed by inhalation and ingestion (David et al., 2007; Friedberg, 2003; Luch A, 2005).

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The major defense mechanism conserved in most living organisms to avert the deleterious effects of oxidative DNA damages is the base excision DNA repair (BER) (Fan and Wilson, 2005; Krokan et al., 2000). Common intermediate of BER is the baseless DNA residue, abasic (AP) site, which is one of the most common DNA lesions in mammalian cells (Demple and Sung, 2005; Nakamura and Swenberg, 1999). Repair of AP sites is carried out by a series of coordinated reactions involving several BER enzymes (Sung and Demple, 2006a; Mol et al., 2000). Among BER enzymes, DNA polymerase beta (Polβ) has been known to play a key role in removing 5'-deoxyribose-5-phosphate (dRP) abasic residues through its dRP lyase activity (Matsumoto & Kim, 1995). However, C1'-oxidized form of AP site, 2-deoxyribolactone, cannot be processed by such enzymatic activity of Polβ. Instead, Polβ has been shown to form a stable covalent DNA-protein crosslink (DPC) at the dL lesion (DeMott et al., 2002; Demple and DeMott, 2002).

2-Deoxyribolactone (dL) is the earliest-identified X-ray damage in DNA and has been reported to be generated by

exposure to various genotoxic agents including chromium carcinogens, heterocyclic N-oxides of the tirapazamine family, and neocarzinostatin of the ene-diyne family of antibiotics (Chowdhury et al., 2007; Kappen and Goldberg, 1992; Pratviel et al., 1991; Sigman et al., 1993). Recent reports suggested that an alternative BER pathway may be evolved to avoid DPC formation mediated by dL damages (Liu et al., 2008; Sung et al., 2005). However, specific biological processes for repairing dL lesion or dL-mediated DPC formation associated with DNA repair enzymes are still remains unclear.

In the present study, we have identified novel mechanisms of DPC formation with dL lesions, which involve DNA polymerases other than Polβ. The only DNA polymerase in mitochondria, Pol gamma (Polγ), showed reactivity to crosslink with DNA at the dL lesion through its DNA repair activities that normally act as AP lyase and dRP layse during BER of mitochondrial DNA (Longley et al., 1998). In addition, the major translesion DNA polymerase, Pol iota (Polι), was found to form DPC at the dL lesion. Kinetic analysis of dL-mediated DPC formation showed that the efficiencies of dL crosslink formation with each polymerase differ significantly, suggesting distinct reaction mechanisms may involve in DPC formation with this oxidized DNA damage.

MATERIALS AND METHODS

1. Materials

All reagents were from Sigma/Aldrich (St. Louis, MO). Radionucleotides were from PerkinElmer Lifer Sciences (Wellesley, MA). DNA oligonucleotides containing a site-specific dL precursor or uracil residue were obtained from Dr. M. M. Greenberg (Johns Hopkins University, USA). *E. coli* endonuclease IV (EndoIV), purified human Polβ, Polγ, and p55 (PolγB) were provided by Dr. B. Demple (Harvard University, USA). Purified Polι was obtained from Dr. S. H. Wilson (National Institute of Environmental Health, USA). *E. coli* uracil-DNA glycosylase (Ung) was purified as described previously (Sung and Mosbaugh, 2003). *E. coli* DNA polymerase I (Klenow fragment) deficient in 3' to 5' exonuclease activity and T4 polynucleotide kinase were obtained from New England BioLabs (Beverly, MA).

2. Preparation of DNA substrates containing a site-specific DNA lesion

Duplex 31-mer DNA substrate was prepared by using DNA oligonucleotide containing a site-specific dL precursor residue (indicated by X in the 30-mer 5'-GTCACGTGCTG-CAXACGACGTGCTGAGCCT), as described previously (Sung et al., 2005). DNA substrates used in crosslinking reactions for dRP layse activity was 3'-end labeled by incorporation of [α-^{32}P]dCTP using the exonuclease-free Klenow fragment of DNA polymerase I. DNA substrates for AP lyase assay was 5'-end labeled by incubation of [γ-^{32}P]ATP and T4 polynucleotide kinase, as described previously (Sung and Dempel, 2006b). To generate a site-specific dL residue in DNA substrates, 0.5 pmol of radio-labeled duplex DNA containing a dL precursor residue were diluted with water to a volume of 100 μl, transferred to a glass tube, and subjected to photolysis in a Photochemical Reactor (RMR-600 from Rayonet Corp., Branford, CT) at 350 nm, 4500 microwatts/cm^2 for 2 h. The efficiency of the photo-conversion was typically >90% when monitored by dL-specific cleavage using hot-alkali treatment and subsequent analysis of DNA by denaturing polyacrylamide gel electrophoresis. To generate 5'-incised dL DNA substrates, duplex dL-DNA oligonucleotides were incubated with 10 nM EndoIV for 10 min at 30℃. In cases of producing a regular AP site in DNA, the oligonucleotide duplex containing a site-specific uracil residue, instead of dL precursor, was treated with 20 nM Ung for 30 min at 30℃.

3. Analysis of crosslinking reactions

Standard crosslinking reactions with dL-containing DNA substrates with various DNA polymerases and the analysis of resulting DPC products were performed as described previously (Sung and Demple, 2006b). Briefly, standard crosslinking reactions contained 50 mM Hepes-KOH (pH 7.5), 20 mM NaCl, 0.5 mM DTT, 2 mM EDTA, 5% (v/v) glycerol, 0.1 mg/ml bovine serum albumin (BSA), 1 nM EndoIV, 1~10 nM 3'-^{32}P-labeled 31-mer DNA substrates, and protein concentrations as indicated in the figure legends. Following incubation at 30℃ for the specified times,

reactions were terminated by addition of SDS-PAGE loading buffer and heating at 100℃ for 5 min. DPCs and free DNA were resolved by 10% SDS-PAGE, and ^{32}P radioactivity associated with DPCs was visualized and quantified using a Fuji BAS2000 Phosphorimager and ImageQuant software (Amersham Biosciences, Piscataway, NJ).

RESULTS

Once generated in duplex DNA, dL in mammalian cells is recognized and cleaved by AP endonuclease 1 efficiently, leading to the formation of a single-strand break with a 5'-incised dL residue in DNA (Xu et al., 2003). We have previously shown that the DPC formation with such oxidized AP lesions mainly occur by Polβ during the repair attempt via its dRP layse activity, which normally involved in the efficient repair of regular dRP abasic residues (Sung and Demple, 2006a). As the counterpart of Polβ, Polγ has been implicated in the repair of AP sites in mitochondria (Longley et al., 1998). Another Y-family DNA polymerase, Polι, has been also described to associate with weak dRP lyase activity (Bebenek et al., 2001). Thus, we examined whether human Polγ or Polι forms crosslink with DNA at the 5'-incised dL lesion by dRP lyase activity associated with these DNA polymerases. Upon incubation of purified human Polγ or Polι with 5'-dL in a 31-mer duplex oligonucleotide, distinct DPC products were produced for both proteins, corresponding to DNA bands that migrated much slower than the free DNA on protein denaturing gel (Fig. 1A). Since the crosslinking reaction was performed in the presence of a high concentration (0.1 mg/ml) of BSA to ensure that any nonspecific interaction of cellular proteins with DNA was abolished, this result indicated that the polymerases attached to the DNA by covalent crosslinking. Quantitative analysis indicated that such DPC formation by Polι and Polγ was increased in a concentration-dependent manner, although Polι was required at significantly higher concentrations than Polγ to yield a similar level of DPCs (Fig. 1B).

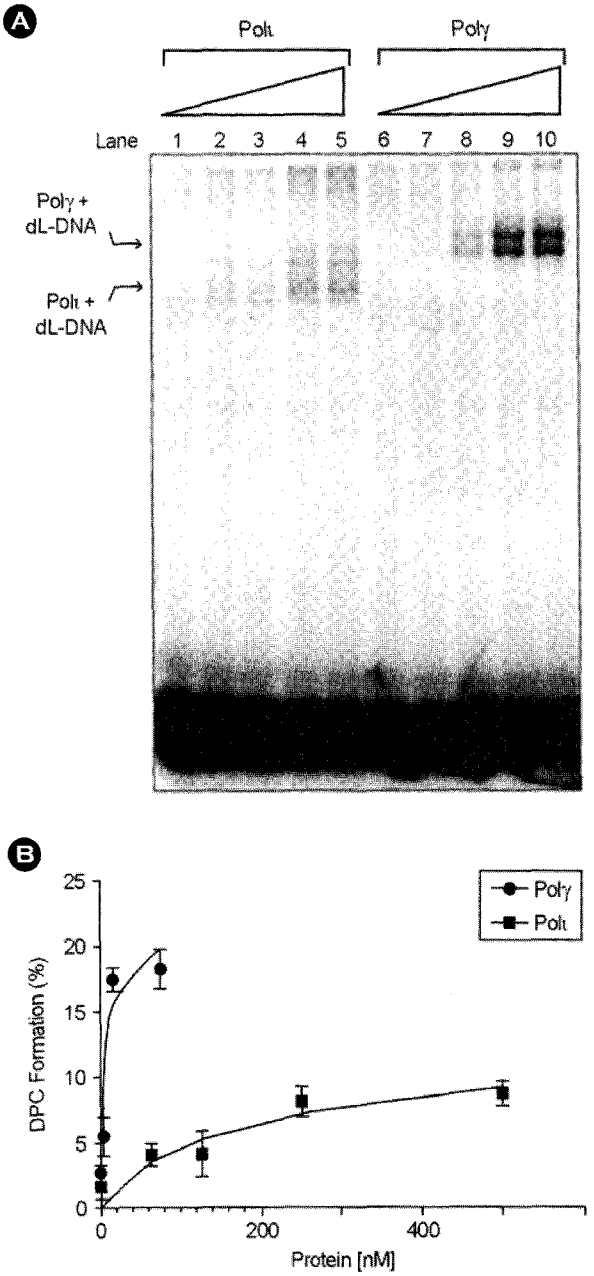

Fig. 1. Formation of DNA-protein crosslink (DPC) with dL lesion by Polι and Polγ. **(A)** A duplex 3'-^{32}P-labled DNA substrate containing a 5'-incised dL residue was incubated for 4 h at 30℃ with 0, 62.5, 125, 250, and 500 nM Polι (lanes 1~5) or 0, 1, 5, 20, and 100 nM Polγ (lanes 6~10). Reaction products were analyzed by 10% SDS-PAGE and visualized using a Phosphorimager. **(B)** Quantification of DPC formation as shown in (A). The percentages of DPC formation determined as ([DPC]/[total DNA]) × 100, are indicated for Polι (■) and Polγ (●). Mean values and S.D. of three experiments are indicated.

Polγ is known as a multi-functional DNA polymerase in mitochondria and its role in BER has been attributed to the removal of AP site from DNA through both dRP lyase and AP lyase activities (Copeland and Longley, 2003). To test whether AP lyase activity of Polγ also involves in the formation of DPC with dL lesion, we subjected 5'-^{32}P-labled

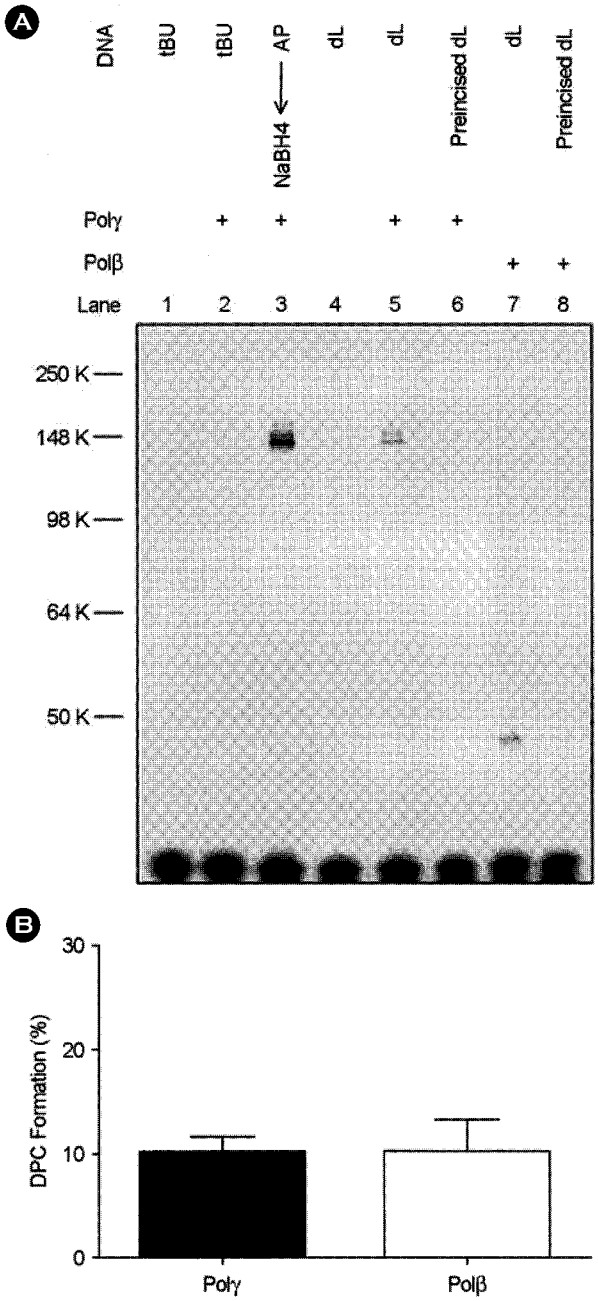

Fig. 2. Formation of dL-mediated crosslink with Polγ mediated by AP lyase activity. **(A)** Duplex 5'-^{32}P-labled 31-mer DNA substrates containing a site-specific dL precursor, tBU (lanes 1 and 2), regular AP site (lane 3), dL (lanes 4, 5, and 7) or incised dL (lanes 6 and 8) was incubated with either 100 nM Polγ (lanes 2, 3, 5, and 6) or 100 nM Polβ (lanes 7 and 8) for 4 h at 30℃, where indicated. Reaction with the regular AP site (lane 3) was then treated with 500 mM $NaBH_4$. Reactions were analyzed by 10% SDS-PAGE and visualized using a Phosphorimager. **(B)** Quantification of DPC formation with dL containing DNA by Polγ (■) or Polβ (□), as shown in (A). Mean values and S.D. of three experiments are indicated.

duplex DNA containing a dL residue for the detection of crosslinking that may occur at the 3'-site of dL lesion (Fig. 2A). Analysis of the reaction containing Polγ revealed formation of distinct DPC species with dL (Fig. 2A, lane 5), similar to the DPC produced with the regular AP site upon chemical-induced trapping of Polγ (Fig. 2A, lane 3). Such DPC products were not observed with DNA substrates containing an unconverted precursor residue (tBU) (Fig. 2A, lane 2). These results demonstrated that Polγ indeed involved in DPC formation specifically with the dL lesion. This DPC product, however, was not detected with the DNA substrates containing a pre-incised dL lesion (Fig. 2A, lane 6). Similar results were also observed in the reactions with Polβ (Fig. 2A, lane 7~8), suggesting that Polγ can mediate DPC formation through its AP lyase activity with the dL on uncleaved DNA. Comparative quantification of DPCs indicated that AP lyase activity of Polγ is effective to react with dL, as shown for Polβ, and may generate significant level of DPC formation during its aberrant access to dL damages (Fig. 2B).

To determine the rates of DPC formation mediated by Polβ, Polγ and Polι, the crosslinking reactions including each protein were carried out for various incubation times. Each DNA polymerase was observed to form the corresponding DPC products in a time-dependent manner (Fig. 3). The apparent rate constant of DPC formation with incised dL DNA was calculated from the amount of crosslink product produced per hour and the concentration of each protein by applying second-order reaction kinetics, where k_{app} = [DPC product] / ([dL DNA] × [Protein] (Table 1). The k_{app} value for Polβ was 9-fold higher than k_{app} for Polι, but only 2-fold higher than that of Polγ (Table 1). Basic amino acids, such as Lys and Arg residues, are the most likely to react with C1' of dL to form DPCs (DeMott et al., 2002; Matsumoto and Kim, 1995), but Polβ does not contain more Lys and Arg than do the Polι or Polγ. The catalytic activity of dRP lyase associated with Polβ reacts most efficiently with dL and mediates rapid DPC formation, whereas weak enzymatic activity of Polι on dL leads the slow DPC formation. On the other hand, it should be noted that Polγ forms DPC with dL at a relatively higher rate through its dRP lyase activity, implying biological significances of such DPC formation in mitochondria.

PolγB (p55), the accessory subunit of Polγ, has been

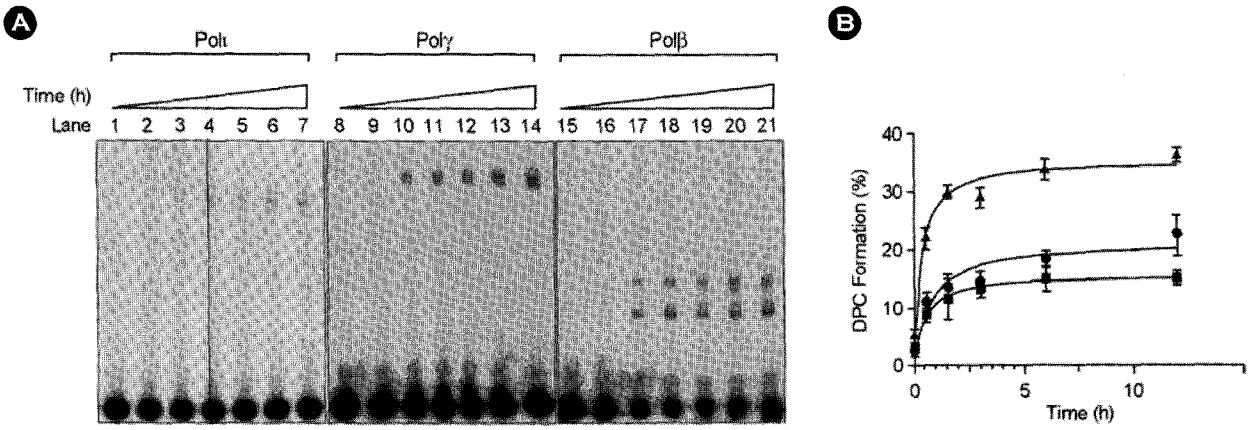

Fig. 3. Time-dependent increase in DNA-protein crosslink formation between dL and DNA polymerases. **(A)** Standard cross-linking reactions were conducted in the presence of 400 nM Polι (lanes 1~7), 100 nM Polγ (lanes 8~14), or 100 nM Polβ (lanes 15~21) for 0, 0.5, 1.5, 3, 6, 12 h at 30℃. Samples were analyzed by 10% SDS-PAGE and visualized using a Phosphorimager. **(B)** Quantification of DPC formation as shown in (A). The percentages of DPC formation are indicated for each protein; Polι (■), Polγ (●), and Polβ (▲). Mean values and S.D. of three experiments are indicated.

Table 1. Apparent rate constants of DNA-protein crosslink (DPC) formation of 2-deoxyribonolactone (k_{app}) with DNA polymerases

Protein[a]	Protein	dL DNA	DPC product[b]	k_{app}[c]	Relative k_{app}[d]	Arg + Lys[e]
	nM	*nM*	*nM*	$nM^{-1} \cdot h^{-1}$		
Pol ι	400	10	0.29	7.3×10^{4}	1.0	85
Pol γ	100	10	0.36	3.6×10^{5}	5.0	140
Pol β	100	10	0.68	6.8×10^{5}	9.4	55

[a] The molecular weight and amino acid composition (Arg + Lys) of proteins were taken from the GenBank™ data base: human Polι (accessi on number: Q9UNA4), human Polγ (P54098), and human Polβ (P06746).
[b] The hourly yield (*i.e.* concentration) of DPC products. The values were calculated from the data obtained at 0, 0.5, 1.5, 3, 6, and 12 h for each DNA polymerase.
[c] k_{app} = [DPC product]/([dL DNA] × [Protein]).
[d] Ratios of k_{app} relative to that of Polι.
[e] The number of Arg and Lys residues per molecule

described to increase affinity of Polγ to DNA in mitochondria (Farge et al, 2007; Lim et al., 1999). However, the presence of p55 in the crosslinking reactions with Polγ did not enhance DPC formation, but rather inhibited crosslinking activity of Polγ in a dose dependent manner (Fig. 4). Such inhibitory effect of p55 was not observed for the DPC formation with Polβ, suggesting that specific interaction of p55 with mitochondrial Polγ is required to reduce the level of dL-mediated DPC formation.

DISCUSSION

When cellular DNA is exposed to various environmental agents involving radical formation, various DNA lesions may be formed including dL as a significant product. Until recently, the cytototoxic consequences of this oxidized AP site were assigned mainly to the DNA strand breakage at dL sites and signaling pathways to cell death. Several studies have now revealed distinct genotoxic potential of the dL lesion. First, the dL lesion constitutes a strong block for DNA replication polymerases including mitochondrial Polγ (Berthet et al., 2001; Liu et al., 2008). Mutagenic effects may also result from incorrect nucleotide insertion opposite dL lesions by bypass DNA polymerases, as shown in bacteria and budding yeast (Faure et al., 2004; Kow et al., 2005; Kroeger et al., 2004). Of note is the observation that dL forms a DPC by crosslinking with DNA repair proteins dependent on their catalytic lyase activity, which normally play roles in the repair of regular AP sites (Kroeger et al., 2003; DeMott et al., 2002). In this case, catalytic functions of the protein should be necessary for efficient reaction with dL. Consistent with this notion, we have found here

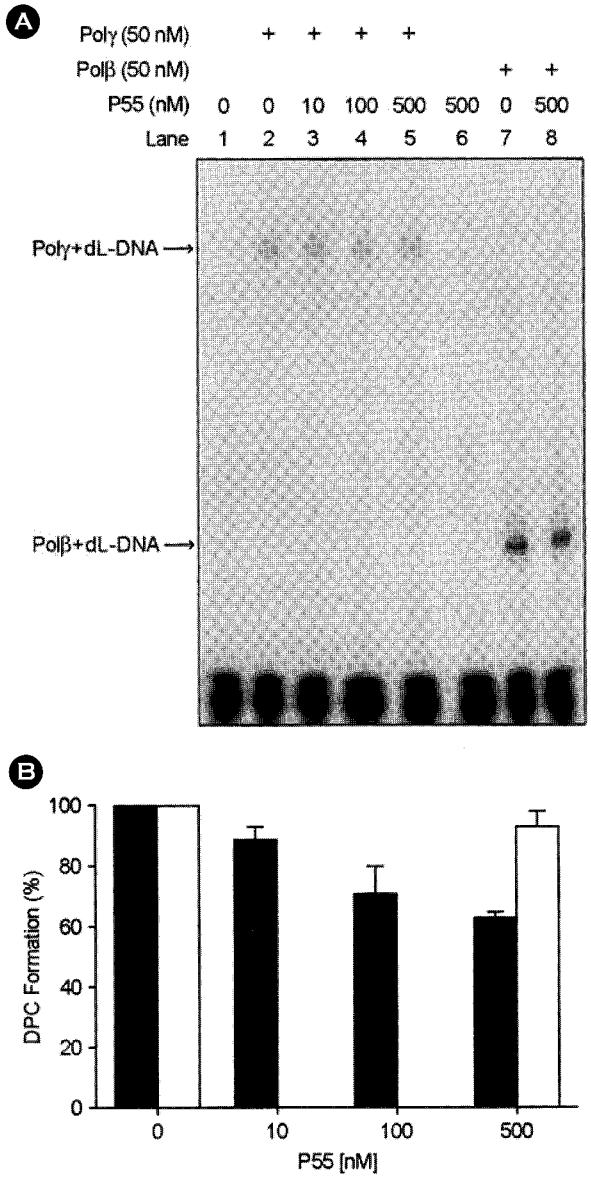

Fig. 4. Effects of p55 on the formation of dL-mediated DNA-protein crosslink by Polγ. **(A)** A duplex 3'-^{32}P-labled DNA substrate containing a 5'-incised dL residue was incubated with 50 nM of Polγ (lanes 2~5) or Polβ (lanes 7 and 8) for 4 h at 30℃ in the presence of various amounts of p55, as indicated. Reactions were analyzed by 10% SDS-PAGE and visualized using a Phosphorimager. **(B)** Quantification of DPC formation as shown in (A). The percentages of DPC formation are indicated for Polγ (■) and Polβ (□). Mean values and S.D. of three experiments are indicated.

that dRP layse and AP layse activities of Polγ, or dRP lyase associated with Polι, involve in the formation of DPC at dL lesion (Fig. 1 and 2). DPC formation with Polι may inhibit recruitment of this DNA polymerase to the DNA damage sites, where Polι bypasses and continues DNA replication, thereby increasing more strand breaks of genomic DNA. Generation of dL damages and consequent trapping of Polγ to dL on mitochondrial DNA may become an especially difficult problem, since the Polγ is the only DNA polymerase in mitochondria (Copeland and Longley, 2003).

We have previously demonstrated that the formation of DPC with dL in nucleus can be prevented by repair of dL via long-patch BER, which is an alternative BER pathway mediated by flap endonuclease1 (FEN1) (Sung et al., 2005). Recently, Liu et al. (2008) showed FEN1 localization in mitochondria, suggesting that FEN1 may participate in long-patch BER of mitochondrial dL damages. However, the presence of FEN1 in mitochondria does not necessarily ensure that most dL lesions are repaired by long-patch BER mechanisms (Szczesny et al., 2008). Other possible ways to avoid dL-mediated DPC formation may include a participation of other protein components that inhibit the reactivity of Polγ to dL lesions. Indeed, the major subunit of Polγ holoenzyme, p55, appeared to reduce the level of DPC formation with Polγ (Fig. 4). Our results suggest that, at least in mitochondria, DPC formation at dL lesions may be attenuated by specific protein-protein interactions between Polγ and other DNA replication components including p55.

DNA-protein crosslinks have been reported for various chemical and physical agents such as aldehydes, nitrous acid, antineoplastic drugs, arsenite, chromate, UV light, and ionizing radiation, which are mutagenic and known carcinogens (Costa, 1991; Miller and Costa, 1990; Paustenbach et al., 1996; Schuessler & Jung, 1989). Although the bulky nature of DPC and their clinical significance have been well documented, no studies have been performed to identify which enzymatic activity is specifically or preferentially involved in the formation of DPC. In this study, we have utilized site-specific incorporation of a dL lesion in DNA and labeled these DNA substrates differentially to determine which lyase activity is involved in DPC formation at a defined dL lesion. Interestingly, Polγ was found to form DPC with dL by both dRP lyase and AP lyase activities (Fig. 1 and 2). Until now, dL-mediated DPCs in mammalian cells have been believed to be mainly produced by Polβ since it possesses major dRP lyase activity that preferentially reacts with dL (DeMott et al., 2002). However, our data suggest that other cellular components involving AP lyase

reactions may also yield DPC adducts with dL damages. Previous studies indicated that non-enzymatic attack at C1' of deoxyribose in a lyase reaction may occur with basic macromolecules such as polyamines or histones (Matsumoto and Kim, 1995).

The comparison of the reaction efficiency (k_{app}) in forming DPCs has revealed that Polβ reacts with dL more rapidly than do the other DNA polymerases (Table 1). Comparing the value of k_{app} for DPC formation by different proteins can be useful in understanding mechanistic aspects of these reactions (Nakano et al., 2003). However, this parameter may not necessarily reflect the biological relevance of a particular type of DPC. The only DNA polymerase in mitochondria, Polγ, may be recruited effectively into a lesion site in a coordinated manner through interaction with other repair proteins, leading to a significant amount of DPCs. On the other hand, replication blocks induced by dL lesions may recruit bypass DNA polymerase, such as Polι, resulting in a high local concentration of this protein at the damage site. The presence of Polγ or Polι, in this scenario, may cause more production of DPCs by its specific reactivity with dL lesion, leading to an increased cytotoxicity. Further understanding of the biological significance of DPC formation, described in the present study, will provide useful information in evaluating the cytotoxic effects mediated by dL lesion and its associated clinical disorders.

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