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Interplay between DNA polymerases β and λ in repair of oxidation DNA damage in chicken DT40 cells

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Abstract

DNA polymerase λ (Pol λ) is a DNA polymerase β (Pol β)-like enzyme with both DNA synthetic and 5'-deoxyribose-5'-phosphate lyase domains. Recent biochemical studies implicated Pol λ as a backup enzyme to Pol β in the mammalian base excision repair (BER) pathway. To examine the interrelationship between Pol λ and Pol β in BER of DNA damage in living cells, we disrupted the genes for both enzymes either singly or in combination in the chicken DT40 cell line and then characterized BER phenotypes. Disruption of the genes for both polymerases caused hypersensitivity to H₂O₂-induced cytotoxicity, whereas the effect of disruption of either polymerase alone was only modest. Similarly, BER capacity in cells after H₂O₂ exposure was lower in Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ cells than in Pol $\beta^{-/-}$, wild-type and Pol $\lambda^{-/-}$ cells, which were equivalent. These results suggest that these polymerases can complement for one another in counteracting oxidative DNA damage. Similar results were obtained in assays for *in vitro* BER capacity using cell extracts. With MMS-induced cytotoxicity, there was no significant effect on either survival or BER capacity from Pol λ gene disruption. A strong hypersensitivity and reduction in BER capacity was observed for Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ and Pol $\beta^{-/-}$ cells, suggesting that Pol β had a dominant role in counteracting alkylation DNA damage in this cell system.

Keywords

DNA polymerase λ ; DNA polymerase β ; oxidation DNA damage; DT40

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1. Introduction

DNA polymerase λ (Pol λ) is a recently identified eukaryotic member of the DNA Pol X family of DNA polymerases that also includes Pol β , Pol μ , Pol σ , and terminal deoxynucleotidyl-transferase (1). Sharing 54% amino acid sequence identity, Pol λ is the closest homologue of Pol β ; each enzyme possesses a 31-kDa polymerase and 8-kDa 5'-deoxyribose-5'-phosphate (dRP) lyase domain (1). Mammalian Pol β has been shown to play important roles in the base excision repair (BER) pathway through gap-filling DNA synthesis and removal of the abasic site sugar phosphate by its dRP lyase activity (2,3). Gap-filling DNA synthesis and dRP lyase activities of Pol λ have been demonstrated *in vitro*, suggesting its participation in BER (4,5). It also has been shown that Pol λ is able to participate in BER using mouse embryonic fibroblast (MEF) cell extracts and in a reconstructed BER system using several purified mammalian enzymes (5). In addition, it was found that Pol λ co-immunoprecipitated with one of the oxidized base DNA glycosylases, SMUG1, and co-localized to oxidative DNA lesions *in situ* (6) suggesting that Pol λ participates in BER of oxidative DNA damage in mammalian cells. While these studies had implicated Pol λ in BER, the interrelationship *in vivo* between Pol λ and Pol β in response to DNA damage is still unclear.

In the study described here, we evaluated the impact of Pol λ and Pol β gene disruption either singly or in combination on BER capacity and cell survival in isogenic chicken DT40 cell lines. We found that Pol β and Pol λ show additive effect in providing survival resistance and repair capacity after treatment with an oxidative stress-inducing agent. In contrast, our data suggests that Pol β alone plays a dominant role in the repair of alkylating agent-induced DNA damage.

2. Materials and Methods

2.1. Cell Culture

DT40 and isogenic DT40-derived cells were cultured as described previously (7).

2.2 Plasmid Constructs

A full-length cDNA fragment encoding for a chicken Pol β homologue (DDBJ/ EMBL/ GeneBank accession: on file) was isolated from a cDNA library prepared from the bursa of Fabricius of chicken. With primers designed from chicken Pol β , we isolated genomic clones of the Pol β locus by long-range PCR amplification. These were partially sequenced to determine the positions of exons and introns. The chicken Pol β disruption constructs Pol β -Bsr and Pol β -Puro were made by replacing approximately 4 kb of genomic sequence containing the sequence encoding amino acids 77-213 with blasticidin-resistance (BsrR) or puromycin-resistance (PuroR)-selection marker cassettes (8). The EST dkfz426_34f4r1 of the bursal EST database shows significant homology to the human Pol λ gene. The full-length cDNA sequence of chicken Pol λ was obtained by 5' and 3' RACE reactions using the SMART RACE cDNA amplification kit (BD Biosciences Clontech). Because the Pol λ genomic locus was relatively large (18 kb), the exon-intron structure was determined by long-range PCR using primers derived from the Pol λ cDNA sequence and was later confirmed by partial sequencing of its corresponding genomic locus. The chicken Pol λ disruption constructs pPol λ -Bsr and pPol λ -Puro were made by replacing 1.5 kb of genomic sequence containing amino acids 210-398 with BsrR or PuroR floxed selection marker cassettes (9). Both disruption constructs were linearized prior to electroporation.

2.3. Gene Targeting

The Pol λ and Pol β knockout constructs were transfected into the DT40 cell line with inducible Cre recombinase (MerCreMer) and v-myb [DT40 Cre1 (9)]. DNA transfection was performed as previously described (9) except that 700 V instead of 550 V was used for electroporation,

and 0.4 µg/ml puromycin (Sigma) and 15 µg/ml of blasticidin S (Sigma) were used for selection of drug-resistance colonies. *Clonogenic Assay* - Colony formation was assayed in medium containing methylcellulose as described previously (10).

2.4. Monitoring Intracellular NAD(P)H

BER capacity of living DT40-derived cells was determined by monitoring intracellular NAD(P)H levels using a real-time, colorimetric assay. NAD(P)H depletion served as a surrogate for NAD⁺ consumption from PARP1 activation, a requisite step in SSB repair (11). For cells continuously exposed to methyl methanesulfonate (MMS, Aldrich), NAD(P)H levels were monitored using a redox dye mixture consisting of XTT (Sigma) and 1-methoxy PMS (Sigma) as described previously (12). For H₂O₂ (Sigma) exposure, cells were first incubated with H₂O₂ for 30 min with the subsequent addition of complete medium, dye mixture, and catalase (Sigma, 36 units/ml) for the monitoring of intracellular NAD(P)H.

2.5. Substrate

The DNA substrate for the uracil-initiated BER assay was a 35-bp duplex DNA constructed by annealing two synthetic oligodeoxyribonucleotides (Oligos Etc, Inc.) to introduce a G:U base pair at position 15: 5'-GCCCTGCAGGTGCAUTCTAGAGGATCCCCGGGTAC-3' and 5'-GTACCCGGGGATCCTCTAGAGTCGACCTGCAGGGC-3' (Fig. 3A).

2.6. Cell Extract Preparation

Cell extracts were prepared as previously described (13). Briefly, cells were washed twice with PBS at 25°C, detached by scraping, collected by centrifugation, and resuspended in Buffer I [10 mM Tris-HCl, pH 7.8, 200 mM KCl, and protease inhibitor cocktail (Boehringer Mannheim)]. An equal volume of Buffer II (10 mM Tris-HCl, pH 7.8, 200 mM KCl, 2 mM EDTA, 40% glycerol, 0.2% Nonidet P-40, 2 mM DTT, and protease inhibitor cocktail) was added. The suspension was rotated for 1 hr at 4°C, and the resulting extract was clarified by centrifugation at 14,000 rpm at 4°C for use in the *in vitro* BER assays. The protein concentration of the extract was determined by Bio-Rad protein assay analysis using BSA as a standard.

2.7. In Vitro BER Assay Using Oligonucleotide Substrate

The BER incubation (final volume of 10 µl) was performed with a 35-bp oligonucleotide DNA substrate containing uracil at position 15. The duplex oligonucleotide substrate at a final concentration of 50 nM was incubated with 10 µg of DT40 whole cell extract prepared from wild-type, Pol β^{-/-}, Pol λ^{-/-}, and Pol β^{-/-}/Pol λ^{-/-} cells in 50 mM Tris-HCl, pH7.5, 5 mM MgCl₂, 20 mM NaCl, 0.5 mM DTT, and 4 mM ATP. The incubation was conducted in the presence of 20 µM each dATP, dGTP, and dTTP, and 2.3 µM [α-³²P]dCTP at 37°C for 2, 5, and 10 min. The reaction products were analyzed by 15% denatured polyacrylamide gel electrophoresis. The initial rate of the *in vitro* BER reaction was determined from a plot of phosphorimager radiolabel in ligated BER product against each time point. The relationship between activity and time for each extract was examined. The slope of the time curve was determined and compared among the cell lines.

3. Results and Discussion

Preparation of cell lines

To generate Pol β gene disruption constructs, approximately 4 kb of genomic DNA within the Pol β locus was replaced with either the BsrR or PuroR gene, as illustrated in Figure 1A. Targeted integration of these constructs was expected to delete amino acids 77-213. Targeting events were defined by the presence of an 8 kb band after Southern blot analysis of Hind III-digested genomic DNA hybridized to a probe (Fig. 1A). To isolate heterozygous Pol β^{+/-}

mutant clones, the Pol β -Bsr construct was transfected into DT40 cells and drug resistant clones were examined by Southern blot analysis (Fig. 1C). Subsequently, the Pol β -Puro construct was transfected into Pol $\beta^{+/-}$ clones to isolate Pol $\beta^{-/-}$ cells. Drug-resistance cassettes were excised by Cre/loxP recombination.

To generate Pol λ gene disruption constructs, approximately 1.5 kb of the Pol λ locus was replaced with either the PuroR or BsrR gene as shown in Figure 1A. Targeted integration of these constructs was expected to delete amino acids 210-398 which is part of the Proline-rich domain, whole the dRP lyase domain and part of the polymerase domain (Fig. 1B). Targeting events were defined by the presence of a 7 kb band after Southern blot analysis of Xba I-digested genomic DNA hybridized to an external probe (Fig. 1A, C). To isolate heterozygous Pol $\lambda^{+/-}$ clones, the pPol λ -Bsr construct was transfected into DT40 cells and drug resistant clones were examined by Southern blot analysis (Fig. 1A, C). Subsequently, the pPol λ -Puro construct was transfected into Pol $\lambda^{+/-}$ clones to isolate Pol $\lambda^{-/-}$ cells. Finally, drug-resistance cassettes were excised by tamoxifen-induction of MerCreMer recombinase. The Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ cell line was generated by targeting disruption of the Pol β gene in the Pol $\lambda^{-/-}$ cell line using pPol β -Bsr and pPol β -Puro in this order. Targeted integration of these constructs was expected to delete amino acids 77-213 of Pol β , spanning 8kDa dRP lyase domain and the N-terminal portion of the 31 kDa polymerase domain (Fig. 1B). The absence of these loci in the knockout cell lines were confirmed by locus specific PCR (data not shown).

Pol λ and Pol β are involved in repair of oxidative DNA damage

Reactive oxygen species (ROS) induce a variety of DNA lesions that are repaired by BER. Pol $\beta^{-/-}$ MEFs are only moderately hypersensitive to H_2O_2 exposure (14,15). The reason for this resistance to a form of ROS-induced damage is not clear, but could involve the existence of a backup BER polymerase such as Pol λ . To further address this uncertainty, we exposed wild-type and isogenic Pol $\beta^{-/-}$, Pol $\lambda^{-/-}$, and Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ cells to H_2O_2 and then monitored cell survival. While both Pol $\beta^{-/-}$ and Pol $\lambda^{-/-}$ cells exhibited slight hypersensitivity, Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ cells showed strong hypersensitivity to H_2O_2 (Fig. 2A). Next, we measure BER capacity *in vivo* after exposure to H_2O_2 . BER capacity was assayed through poly (ADP-ribose) polymerase (PARP) activation and intracellular NAD(P)H depletion following exposure (11); the extent of intracellular NAD(P)H depletion reflects the relative amount of single-stranded DNA breaks (SSBs) in the form of BER intermediates (12,16). We first compared SSBs accumulating in cell after 60 min exposure to increasing concentrations of H_2O_2 . Relative to wild-type and Pol $\lambda^{-/-}$ cells, Pol $\beta^{-/-}$ cells had a slightly higher level of SSBs (Fig. 2B). No difference in SSBs was detected between Pol $\lambda^{-/-}$ and wild-type cells. In contrast, the Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ cells showed strong accumulation of SSBs. Similar results were obtained with different periods of exposure from 0.5 to 2 hrs (Fig. 2C). These results, taken together, suggested that Pol β and Pol λ can complement for one another in protection of DT40 cells against the cytotoxic and genotoxic effects of H_2O_2 treatment.

BER capacity in cell extracts

To further examine roles of Pol β and Pol λ in DT40 cell BER, an *in vitro* BER assay was employed that involved an oligonucleotide substrate containing a damaged base (Fig. 3A). The BER capacities of the wild-type, Pol $\beta^{-/-}$, Pol $\lambda^{-/-}$, and Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ cell extracts (10 μ g) were then measured during the first 10 min of incubation. The rate of repair of the cell extracts varied in the order: wild-type (100%), Pol $\lambda^{-/-}$ (~75%) = Pol $\beta^{-/-}$ (~70%) > Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ (~20%) (Fig. 3C, D). This pattern of decrease in BER as a function of polymerase gene disruption was in accord with the pattern of response to H_2O_2 treatment. These polymerases appeared to complement on another.

Pol β has a dominant role in repair of alkylating agent-induced DNA damage

DNA lesions induced by MMS, such as N7-methylguanine and N3-methyladenine, are repaired mainly by Pol β -dependent BER in mammalian cells (17). In the DT40 cell system, we found that the survival of Pol $\lambda^{-/-}$ cells after MMS exposure was similar to that of wild-type cells. In contrast, the Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ and Pol $\beta^{-/-}$ cells were hypersensitive to MMS (Fig. 4A), and this was to a similar degree.

In the assay for *in vivo* BER capacity, both Pol $\beta^{-/-}$ and Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ cells showed a similar and strong accumulation of SSBs. In wild-type and Pol $\lambda^{-/-}$ cells, accumulation of SSBs was only modest (Fig. 4B, C). Thus, the presence of Pol λ did not significantly complement the repair deficiency in Pol $\beta^{-/-}$ cells. The results suggest that Pol λ is unlikely to be involved in a backup pathway for Pol β -dependent BER of alkylative DNA damage in DT40 cells.

Pol λ in H₂O₂-induced oxidative damage repair

The bias of Pol λ for oxidative damage repair versus alkylation damage repair observed here raises the possibility that its recruitment to sites of damage is more efficient in association with oxidative lesion DNA glycosylases, and a role of Pol λ in protection of DT40 cell against oxidative DNA damage induced stress is consistent with earlier results in mammalian cells (6). In that case, Pol λ was required for normal recruitment of an oxidative damage glycosylase to sites of oxidative damage (6). The possible interaction and cooperation between oxidative damage glycosylases and Pol λ should be interesting to evaluate in future studies.

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FIGURE 1. Disruption of the chicken *Pol λ* and *Pol β* genes

A, Physical maps of the chicken *Pol λ* and *Pol β* loci, knockout constructs and targeted loci. Solid boxes represent exons. After the targeted integration of the knockout constructs, floxed drug-resistance cassettes were excised out by Cre/loxP recombination. H: Hind III, X: Xba I. **B,** Structures of the chicken *Pol λ* and *Pol β* proteins. **C,** Southern blot analysis of wild-type and knockout clones using the probes shown in A. Xba I- and Hind III- digested genomic DNA were used to detect targeted disruption of *Pol λ* and *Pol β* loci, respectively.

FIGURE 2. Sensitivity of Pol β^{-/-}, Pol λ^{-/-}, and Pol β^{-/-}/Pol λ^{-/-} cells to H₂O₂

A, Survival curves of wild-type (wt) DT40 and DT40-derived cells exposed to H₂O₂ for 30 min. Mean and S.D. (bars) were from duplicate experiments. **B,** Intracellular NAD(P)H levels in DT40 and DT40-derived cells exposed to H₂O₂ at different concentrations for 30 min followed by 1 hr repair period. **C,** Wt and DT40-derived cells treated with 10 μM H₂O₂ for 30 min followed by up to 2 hrs repair period.

FIGURE 3. Kinetic analysis of BER using oligonucleotide duplex DNA substrate
 Incorporation of [α - 32 P]dCMP was measured as a function of incubation time using various DT40 cell extracts. The reaction conditions and product analysis are described under “EXPERIMENTAL PROCEDURES” A, A line diagram of a 35-bp oligonucleotide containing a uracil residue at position 15 is illustrated. B, Photographs of phosphorimager analysis illustrating the ligated BER products are shown. C, *In vitro* BER capacity of wild-type DT40 (open square), Pol $\beta^{-/-}$ (closed square), Pol $\lambda^{-/-}$ (open triangle), and Pol $\beta^{-/-}$ /Pol $\lambda^{-/-}$ (closed triangle) cell extracts was examined using the 35-bp duplex DNA substrate. Whole cell extract (10 μ g) was incubated with DNA (50 nM) and the repair reaction was performed as described under “EXPERIMENTAL PROCEDURES” at 37°C. Aliquots were withdrawn at different time intervals, and an equal volume of gel-loading buffer was added to terminate the reaction. The reaction products were separated by 15% denaturing polyacrylamide gel electrophoresis; the gels were dried and scanned by a phosphorimager. Ligated BER products (35-bp) were quantified by using ImageQuant software. The amount of ligated BER product formed in 10

min incubation with wild-type cell extract was set to 100% in each experiment. The experiments were repeated 3 times, and the average values of relative products formed (%) were plotted against incubation time (min). Initial rates were calculated by using a curve fit program for each time of incubation. *D*, The average initial rate of activity of each extract for the *in vitro* BER reaction is shown in a bar diagram.

FIGURE 4. Sensitivity of *Pol β*^{-/-}, *Pol λ*^{-/-}, and *Pol β*^{-/-}/*Pol λ*^{-/-} cells to MMS

A, Survival curves of wild-type (wt) DT40 and DT40-derived cells continuously exposed to MMS. Mean and S.D. (bars) were from duplicate experiments. B, Intracellular NAD(P)H levels in wt and DT40-derived cells continuously exposed to MMS at different concentrations for 3 hrs. C, Wt and DT40-derived cells treated with 500 μ M MMS for up to 4 hrs.